IntechOpen

Current Topics in Tropical Medicine

Edited by Alfonso J. Rodriguez-Morales

WEB OF SCIENCE™

CURRENT TOPICS IN TROPICAL MEDICINE

Edited by **Alfonso J. Rodriguez-Morales**

Current Topics in Tropical Medicine
http://dx.doi.org/10.5772/1335
Edited by Alfonso J. Rodriguez-Morales

Contributors

Benjamin Koudou, José A. Oteo, Aránzazu Portillo, John Meade, Denise C Cornelius, Antonio Soriano Arandes, Frederic Gómez Bertomeu, Hippolite Amadi, Regina De Figueiredo, Erum Khan, Rumina Hasan, Jaishri Mehraj, Sajid Mahmood, Ruben Bueno-Mari, Ricardo Jiménez Peydró, MÃ¡rcia Marinho, Ute Inegbenebor, Valter Ferreira De Andrade-Neto, Oscar Bruna-Romero, Dulcilene Mayrink De Oliveira, Amidou Samie, Ali ElBakri, Kevin Leary, Licia Bordi, Ikram Guizani, Akila Fathallah Mili, Fatma Saghrouni, Zeineb BenSaid, Moncef BenSaid, Lourdes Lledo, Consuelo Giménez-Pardo, Josue De Moraes, Khaled Khatab, Ying Bai, Michael Kosoy, Ivan Marcipar, Claudia Marina Lagier, Louis-Albert Tchuem Tchuenté, Carlos Muskus, Andres Florez, Stanley Watowich, Cristiane Tefé-Silva, Eleuza R. Machado, Lúcia H. Faccioli, Simone Gusmão Ramos, Jere McBride, Lucy Ndip, David H. Walker, Roland Ndip, Yasuyoshi Mori, Norihiro Tomita, Hidetoshi Kanda, Tsugunori Notomi, Aline S. De Aluja, Julio Morales Soto, Edda Sciutto Conde, Asep K. Supriatna, Nursanti Anggriani, Adisak Bhumiratana, Prapassorn Pechgit, Apiradee Intarapuk, Danai Pinyoowong, Surachart Koyadun, Jinrapa Pothikasikorn, Alfonso J. Rodriguez-Morales, Diana Castañeda-Hernandez

Notice

First published in Croatia, 2012 by INTECH d.o.o.
eBook (PDF) Published by IN TECH d.o.o.
Place and year of publication of eBook (PDF): Rijeka, 2019.
IntechOpen is the global imprint of IN TECH d.o.o.
Printed in Croatia

Legal deposit, Croatia: National and University Library in Zagreb

Additional hard and PDF copies can be obtained from orders@intechopen.com

Current Topics in Tropical Medicine
Edited by Alfonso J. Rodriguez-Morales
p. cm.
ISBN 978-953-51-0274-8
eBook (PDF) ISBN 978-953-51-4322-2

Meet the editor

Medical Doctor (MD), Universidad Central de Venezuela, Caracas, Venezuela, 2003. Master of Sciences in Protozoology (MSc), Universidad de Los Andes, Trujillo, Venezuela, scholar 2004-2005. Diploma in Tropical Medicine & Hygiene (DTM&H), Universidad Peruana Cayetano Heredia, Lima, Peru and University of Alabama at Birmingham, Birmingham, Alabama, United States of America, 2009. Fellow of the Royal Society for Tropical Medicine & Hygiene (FRSTMH), London, United Kingdom, 1998. Fellow of the Faculty of Travel Medicine (FFTM) of the Royal College of Physicians and Surgeons of Glasgow (RCPSG), Glasgow, United Kingdom, 2010. Candidate to Doctor in Parasitologgy (PhD), Universidad Central de Venezuela, scholar 2005-2008. Editor, Journal of Infection in Developing Countries (JIDC). Chair, Comission of Scientific Publications and Teaching of the Latin American Society for Travel Medicine (SLAMVI). Editorial Board, Travel Medicine and Infectious Diseases (TMAID). Lead Guest Editor, Special Issue "Migration and Chagas' Disease", Epidemiology Research International (ERI). Guest Editor, Special Issue "Immunology of Zoonotic Infections", Clinical and Developmental Immunology (CDI).

Contents

Preface

Tropical medicine research holds a special place as an important activity that as a consequence of multiple factors, such as globalization and migration has extended and reaffirms its importance not only in tropical developing countries but also in non-endemic areas in the developed world. The update on different aspects related to the practice of tropical medicine and their multiple components needs to be frequently visited. Three of the most important infectious terminal diseases in the world that belong or significantly affect tropical areas are AIDS, Tuberculosis and Malaria. These pathologies, together with other important ones, represent relevant public health problems, particularly in Africa, Asia and Latin America (Franco-Paredes et al. 2007a, Franco-Paredes et al. 2007b, Rodríguez Morales AJ et al. 2006, Rodríguez Morales AJ et al. 2008), secondarily affecting, due to travel, Europe, North America and other areas of the world (Franco-Paredes et al. 2007c).

Diseases and conditions as object of the study of tropical medicine are diverse in organ compromise as well as in etiology, including infectious and non-infectious agents. With these concepts in mind, this book includes different topics of tropical medicine of current international interest, trying to update the most significant research in many of them as well as offer a multinational perspective on different relevant conditions. This book has been organized in three major sections: I. Tropical Diseases due to Bacteria and Viruses; II. Tropical Diseases due to Protozoa and Helminths; and III. Other Tropical Infectious and Non-Infectious Conditions. Section I includes topics covering bacterial diseases such as rickettsiosis, ehrlichiosis, leptospirosis, bartonellosis and tuberculosis; as well on viral diseases such as dengue, Lassa fever and Chikungunya. Section II includes topics covering protozoan diseases such as malaria, toxoplasmosis, amebiasis, leishmaniasis and Chagas disease; as well on helminthic diseases such as echinococcosis/hidatidosis, taeniosis/cysticercosis, schistosomiasis, filariasis, strongyloidiasis and soil-transmitted helminths. Section III includes topics on multiple-etiology conditions such as sexually transmitted diseases, new diagnostic tools for tropical diseases and vector-borne diseases; also includes non-infectious conditions particularly related to childhood health in the tropics.

This books does not intend to be an exhaustive compilation and this first edition has included not just multiple different topics but also a wide geographical participation from many countries where tropical medicine is of interest. Its online availability allows it to reach a worldwide audience.

I would like to give my thanks to InTech, and particularly to Mr. Vedran Greblo, for the opportunity to edit this interesting and important book. I want to dedicate this book to my family and particularly to my lovely wife, Diana, who actually represents my engine for every activity I made in my professional career up until now, also to my friends and my students around Latin America.

We hope our readers enjoy this publication as much as I did reading the chapters of Current Topics in Tropical Medicine.

References

Franco-Paredes C, Jones D, Rodriguez-Morales AJ, Santos-Preciado JI. 2007a. Commentary: improving the health of neglected populations in Latin America. *BMC Public Health* 7:11.

Franco-Paredes C, Von A, Hidron A, Rodriguez-Morales AJ, Tellez I et al. 2007b. Chagas disease: an impediment in achieving the Millennium Development Goals in Latin America. *BMC Int. Health Hum. Rights* 7:7.

Franco-Paredes C, Dismukes R, Nicolls D, Workowski K, Rodriguez-Morales A, Wilson M, Jones D, Manyang P, Kozarsky P. 2007c. Persistent and Untreated Tropical Infectious Diseases among Sudanese Refugees in the U.S. *Am J Trop Med & Hyg* 77: 633-635.

Rodríguez-Morales AJ, Barbella RA, Case C, Arria M, Ravelo M, Perez H, Urdaneta O, Gervasio G, Rubio N, Maldonado A, Aguilera Y, Viloria A, Blanco JJ, Colina M, Hernández E, Araujo E, Cabaniel G, Benitez J, Rifakis P. 2006. Intestinal Parasitic Infections among Pregnant Women in Venezuela. *Infect Dis Obstet Gynecol 14:23125*.

Rodríguez Morales AJ, Lorizio W, Vargas J, Fernández L, Durán B, Husband G, Rondón A, Vargas K, Barbella RA, Dickson SM. 2008. Malaria, Tuberculosis, VIH/SIDA e Influenza Aviar: ¿Asesinos de la Humanidad? *Rev Soc Med Quir Hosp Emerg Perez de Leon* 39:52-76.

Prof. Alfonso J. Rodriguez-Morales
MD, MSc, DTM&H, FRSTMH(Lon), FFTM RCPS(Glasg), PhD(c).
Editor, Current Topics in Tropical Medicine
Infection and Immunity Research Group, Universidad Tecnológica de Pereira, Pereira, Colombia
Immunoparasitology Section, Tropical Medicine Institute,
Universidad Central de Venezuela, Caracas,
Venezuela
Instituto José Witremundo Torrealba, Universidad de Los Andes, Trujillo,
Venezuela

Part 1

Tropical Diseases Due to Bacteria and Viruses

1

Rickettsiosis as Threat for the Traveller

Aránzazu Portillo and José A. Oteo
Hospital San Pedro-Centre of Biomedical Research (CIBIR)
Spain

1. Introduction

Over the past six decades, tourism has experienced continued expansion and diversification becoming one of the largest and fastest growing economic sectors in the world. Many new destinations have emerged alongside the traditional ones of Europe and North America. In the next years an increase of travelling is expected, and the number of related infections will also be higher (http://www.unwto.org/facts/menu.html). Rickettsioses are an important chapter in the field of travel medicine.

Rickettsiae are small gram-negative intracellular bacteria (belonging to the alpha-1 proteobacteria) mainly transmitted by arthropods (lice, fleas, ticks and other acari) with two genera: *Orientia* with a unique specie (*Orientia tsutsugamushi*) and *Rickettsia* with several species. The clinical pictures that they cause are named rickettsioses (Raoult, 2010a).

Rickettsioses have been a threat all along the History and nowadays they are an important cause of morbi-mortality in some areas of the world. To know the distribution of the different diseases caused by these bacteria and how the clinical pictures are recognized may be essential for a quick diagnoses and starting the correct treatment. Some of these infections can be also easily prevented with basic rules. Main rickettsioses with their distribution area are showed in the table 1.

In the 21st Century in most parts of the world hygienic conditions have improved and epidemic typhus is absent. To acquire this condition it is necessary to be in contact with body lice. Furthermore, if people have personal hygiene and change their clothing, body lice are removed. Nevertheless it is possible that if we travel for cooperation to catastrophic areas or other places with poverty, we may take body lice (refugees' camps) and may develop exanthematic typhus.

There are a lot of references of rickettsioses acquired by travellers and considered imported diseases (McDonald et al., 1988; Bottieau et al. 2006; Freedman et al., 2006; Askling et al. 2009; Chen & Wilson, 2009; Jensenius et al., 2009; Stokes & Walters, 2009).

Nowadays ticks cause most travel-associated rickettsioses. Ticks are considered to be one of the most important vectors of infectious diseases in the world, preceded only by mosquitoes. Therefore, tick-borne rickettsioses are endemic all over the world (Hechemy et al., 2006). The majority of travel-associated rickettsioses refer to Sub-Saharan Africa tourists who develop African tick-bite fever (ATBF), mainly transmitted by *Amblyomma hebraeum* (Figure 1). In addition to malaria, ATBF is an important cause of fever in people returning from the tropic (Field et al., 2010). Other reports describe Mediterranean spotted fever (MSF) acquired by tourists bitten by *Rhipicephalus* spp. ticks (Figure 2) when visiting Europe, being

more scarce references about other rickettsioses. Flea-borne rickettsioses and chigger-transmitted rickettsioses are less frequent in travellers and tourists, and some of them as murine typhus are associated with poor hygienic conditions. Most travel-acquired rickettsioses are related to outdoors leisure activities, like camping, trekking, hunting, safaris, etc.

It will be impossible to describe all rickettsioses in few pages. Since rickettsioses have very similar clinical pictures and they can be grouped in different syndromes, we will describe these syndromes emphasizing the typical features (i.e.: Presence of eschar or type of rash). Afterwards, distribution can be observed in the table 1. We will also write a specific paragraph for some infections (i.e.*:* Diseases caused by *Rickettsia akari* and *Orientia tsutsugamushi*).

Fig. 1. *Amblyomma hebraeum*, the principal vector of African-tick bite fever (ATBF) in southern Africa.

Fig. 2. *Rhipicephalus* spp., the main vector of Mediterranean spotted fever (MSF).

DISEASE	CAUSATIVE AGENT	VECTOR	DISTRIBUTION
Epidemic typhus	*R. prowazekii*	Body lice (*Pediculus humanus corporis*)	Peru, northern Africa, Senegal, Burundi, Rwanda, Russia, sporadic cases in USA associated with flying squirrels. Potentially, all over the world associated to poverty and dirt.
Murine typhus	*R. typhi*	Fleas (*Xenopsylla cheopis* and *Ctenocephalides felis*)	All over the world (more prevalent in tropical and subtropical areas)
Flea-borne spotted fever	*R. felis*	Cat fleas (*Ctenocephalides felis*)	All over the world

DISEASE	CAUSATIVE AGENT	VECTOR	DISTRIBUTION
Scrub typhus	*Orientia tsutsugamushi*	Trombiculid mite larvae (chiggers)	Thailand, Laos, India, Pakistan, Kashmir, Sri-Lanka, Afghanistan, Nepal, China, Japan, Korea, Indonesia. Philippines, Papua-New Guinea, Australia
Rickettsialpox	*R. akari*	Mouse mites (*Liponyssoides sanguineus*)	Eastern Europe, Korea, South Africa, USA
RMSF[1]	*R. rickettsii*	*Dermacentor variabilis* and other American ticks	USA, Mexico, Colombia, Brazil, Argentina, Panama, Costa Rica
RMSF-like	*R. parkeri*	*Amblyomma* spp. ticks	USA, Uruguay, Brazil, Argentina
MSF[2]	*R. conorii conorii*, *R. conorii israelensis*, *R. conorii caspia*, *R. conorii indica*	*Rhipicephalus* spp. ticks	Mediterranean area, Central Europe, Russia, India and Africa
MSF-like	*R. monacensis*	*Ixodes ricinus* ticks	Europe
MSF-like	*R. massiliae*	*Rhipicephalus sanguineus* ticks	Mediterranean area, Argentina, USA?
MSF-like	*R. aeschlimannii*	*Hyalomma marginatum* ticks	Africa, Europe?
DEBONEL / TIBOLA[2]	*R. slovaca* *R. rioja* *R. raoultii*	*Dermacentor marginatus* ticks	Europe
LAR[4]	*R. sibirica mongolitimonae*	*Hyalomma* spp. and *Rhipicephalus pusillus* ticks	Europe, Africa.
ATBF[5]	*R. africae*	*Amblyomma* spp. ticks	Sub-Saharan Africa and West Indies
Siberian tick typhus	*R. sibirica sibirica*	*Dermacentor* spp. ticks	Russia, Pakistan, China
R. helvetica infection	*R. helvetica*	*Ixodes ricinus* ticks	Central and northern Europe, Asia
Japanese spotted fever	*R. japonica*	Ticks	Japan
Queensland tick typhus	*R. australis*	*Ixodes* spp. ticks	Eastern Australia
Flinder's Island spotted fever	*R. honei*	Ticks	Australia, Southeast Asia
Far Eastern spotted fever	*R. heilonjgiangensis*	*Dermacentor* spp. ticks	Eastern Asia

[1]RMSF: Rocky Mountain spotted fever; [2]MSF: Mediterranean spotted fever; [3]DEBONEL/TIBOLA: *Dermacentor*-borne, necrosis, erythema, lymphadenopathy/Tick-borne lymphadenopathy; [4]LAR: Lymphangitis-associated rickettsiosis; [5]ATBF: African tick-bite fever.

Table 1. *Rickettsia* spp. causing medical diseases, vectors and distribution

2. Typhus syndrome

Typhus syndrome refers to a febrile syndrome with mental status impairment and rash. It is caused by *Rickettsia prowazekii* (epidemic typhus) and *Rickettsia typhi* (formerly, *R. mooseri*). *Rickettsia felis* may also produce a typhus syndrome named flea-borne spotted fever, which is similar to *R. typhi* infection (perhaps less severe) (Walker & Raoult, 2010; Dumler & Walker 2010; Oteo et al., 2006).

Nowadays epidemic typhus is only present in some regions of Africa, Russia and in Peru. It is associated with bad hygienic conditions that are necessary for body lice parasitization. Sporadic cases associated with contact with flying squirrels and their parasite arthropods, which have been involved as new reservoirs of the infection, have also been reported in USA. A possible source of *R. prowazekii* infection may be a recrudescent case (Brill-Zinsser disease) of *R. prowazekii* infection. If hygienic conditions are altered and an epidemic of body lice appears may be an epidemic of typhus, as occurred in Burundi with hundreds of affected people (Raoult et al., 1998). Some cases of louse-borne typhus in travellers have been published (Zanetti et al., 1998; Kelly et al., 2002).

Endemic typhus or murine typhus is associated with the presence of fleas. The main vector is the rat flea (*Xenopsylla cheopis*) associated with dirt and poor hygienic conditions. Flea-borne spotted fever is associated with the cat flea, and in this case bad hygienic conditions are not necessary. Murine typhus and flea-borne spotted fever are distributed all over the world. Although they are more frequent in tropical and subtropical areas, cases have also been reported in the Mediterranean area (Greece, Italy, Spain, France and Portugal) (Bernabeu-Wittel et al., 1999; Angel-Moreno et al., 2006; Gikas et al,. 2009; Pérez-Arellano et al., 2005; Oteo et al., 2006).

The clinical pictures of murine typhus and flea-borne spotted fever are less severe than the one of epidemic typhus. Thus, 1-2 weeks after the flea exposure, patients begin with fever, headache, myalgia, nausea and vomiting. Rash can be difficult to see in some cases, but is present until 80%. For *R. typhi* infection, rash is macular or maculo-papular and typically affects trunk and less frequently extremities. In epidemic typhus, petechial rash is more frequent than in endemic typhus, and cough, nausea and vomiting are frequent features. On the contrary of tick-borne rickettsioses or scrub typhus, an inoculation eschar (*tache noire*) is not observed. In most cases, fever and rash disappear in a few weeks but complications can be developed (central nervous, kidney involvement with renal insufficiency, respiratory failure, etc.). These complications are more frequent for epidemic typhus and in older people or patients suffering chronic diseases (Walker & Raoult, 2010; Dumler & Walker 2010). In all these conditions a raise in hepatic enzymes, C reactive protein as well as in leucocytes and platelets counts can be observed. We can also observe hepatosplenomegaly. In severe cases mainly associated with epidemic typhus, evolution to a multiple-organ dysfunction syndrome and coagulation disorders may appear.

Some references related to travellers are: Zanetti et al., 1998; Niang et al., 1999; Kelly et al., 2002; Jensenius et al., 2004; Azuma et al., 2006; Angelakis et al., 2010; Walter et al., 2011.

3. Tick-borne spotted fever

Tick-borne spotted fever are worldwide distributed and the clinical picture is very similar, although the severity is different related with the *Rickettsia* species involved.

ATBF and MSF are the most frequent tick-borne spotted fever rickettsioses in travellers (Smoak et al., 1996; Fournier et al., 1998; Oteo et al., 2004a; Raoult et al., 2001; Caruso et al., 2002; Jensenius et al., 2003; Roch et al., 2008; Tsai et al., 2008; Consigny et al., 2009; Stephany et al., 2009; Althaus et al., 2010; Jensenius et al., 2004; Boillat et al., 2008; Laurent et al., 2009). For this reason, we will refer to these conditions taking into account that few differences in the incubation period and severity may exist. For Rocky Mountain spotted fever (RMSF) and MSF caused by *R. conorii israelensis*, higher mortality than with the rest of spotted fever rickettsioses has been communicated (de Sousa et al., 2003). Some features of the main spotted fever rickettsioses are shown in table 2.

From 4 to 21 days after the tick bite, fever suddenly starts in 100% cases (less severe in ATBF). A characteristic inoculation lesion (eschar) (figure 3) is typically found until 72% of MSF cases and until 95% for ATBF. Multiple eschars are observed in some cases. This is more frequent in ATBF. Fever is accompanied of chills, headache, etc. (table 2). From 3 to 5 days after the onset of fever, the rash appears. This is a maculo-papular rash with purpuric elements in some cases (figure 4). It is more frequent in extremities and typically affects palms and soles. In ATBF the rash can be vesicular (figure 5), as occurs in *R. akari* and *R. australis* infections. For *R. sibirica mongolitimonae* infection, known as lymphangitis-associated rickettsiosis (LAR), lymphangitis from the eschar may appear in approximately

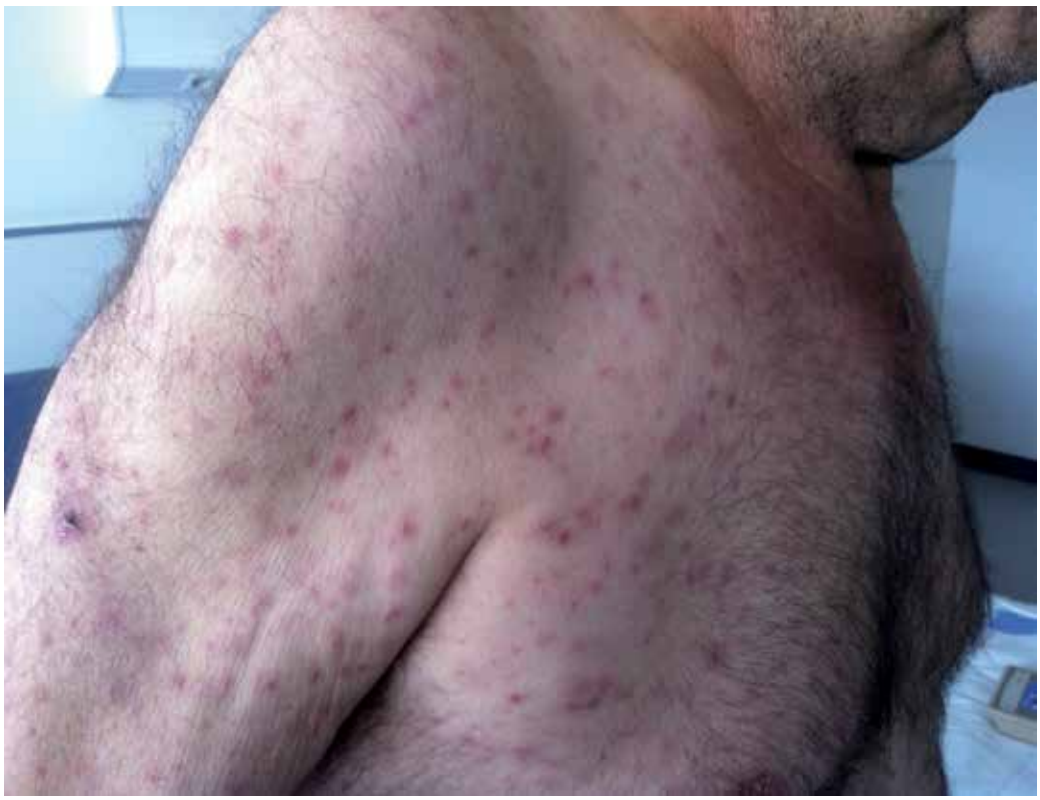


Fig. 3. Eschar (*tache noire*) and maculo-papular rash in a patient with Mediterranean spotted fever.

50% cases. It also can be observed in ATBF (Figure 6). There are few reported cases of tick-borne spotted fever caused by other of *Rickettsia* species (*R. monacensis, R. aeschlimannii, R. massiliae, R. helvetica, R. sibirica mongolitimonae, R. parkeri, R. japonica* and *R. honei*, among others) but it seems that the clinical pictures are very similar to the one of MSF cases. Data about the incidence of these infections among travellers to endemic areas are very scarce (Jensenius et al., 2004; Socolovschi et al., 2010).

For *R. helvetica* infections rash can be absent and fever is often the unique clinical manifestation. All these diseases are more frequent in spring and summer, when the vectors are more active. In all these conditions a raise in hepatic enzymes, C reactive protein and in leucocytes and platelets counts can be observed. We can also observe hepatosplenomegaly. In severe cases mainly associated to RMSF or MSF, evolution to a multiple-organ dysfunction syndrome and coagulation disorders may appear.

Distribution of human cases of tick-borne rickettsioses in Europe, Africa and Americas are showed in figures 7-10. Human cases of tick-borne rickettsioses and scrub typhus in Asia and Oceania are showed in figure 11.

DISEASE	**RASH**	**SPECIFITIES**	**ESCHAR**	FEVER
MSF[1]	>95%	10% purpuric rash	72%. Multiple in 32% (children)	100%
RMSF[2]	90%	45% purpuric rash	<1%	100%
ATBF[3]	30%	Vesicular rash	100%. Frequently multiple	100%
DEBONEL/TIBOLA[4]	Possible	Lymph nodes	100%. Larger than in other rickettsioses	30%
LAR[5]	>90%	50% lymphangitis	Frequent	100%
R. aeschlimannii infection	Possible	-	Possible	100%
R. helvetica infection	Possible	-	Absent	Not always
R. massilliae infection	Possible	Rash can be purpuric	Possible	100%
R. monacensis infection	Possible	-	?	100%
Queensland tick typhus	100%	Vesicular rash	65%	100%
Flinder's Island spotted fever	85%	8% purpuric rash	28%	100%
Siberian tick typhus	100%	-	77%	100%
Japanese spotted fever	100%	-	90%	100%
Far eastern spotted fever	Possible	-	Possible	100%

[1]MSF: Mediterranean spotted fever; [2]RMSF: Rocky Mountain spotted fever; [3]ATBF: African tick-bite fever; [4]DEBONEL/TIBOLA: *Dermacentor*-borne, necrosis, erythema, lymphadenopathy/Tick-borne lymphadenopathy; [5]LAR: Lymphangitis-associated rickettsiosis.

Table 2. Main clinical characteristics of tick-borne rickettsioses

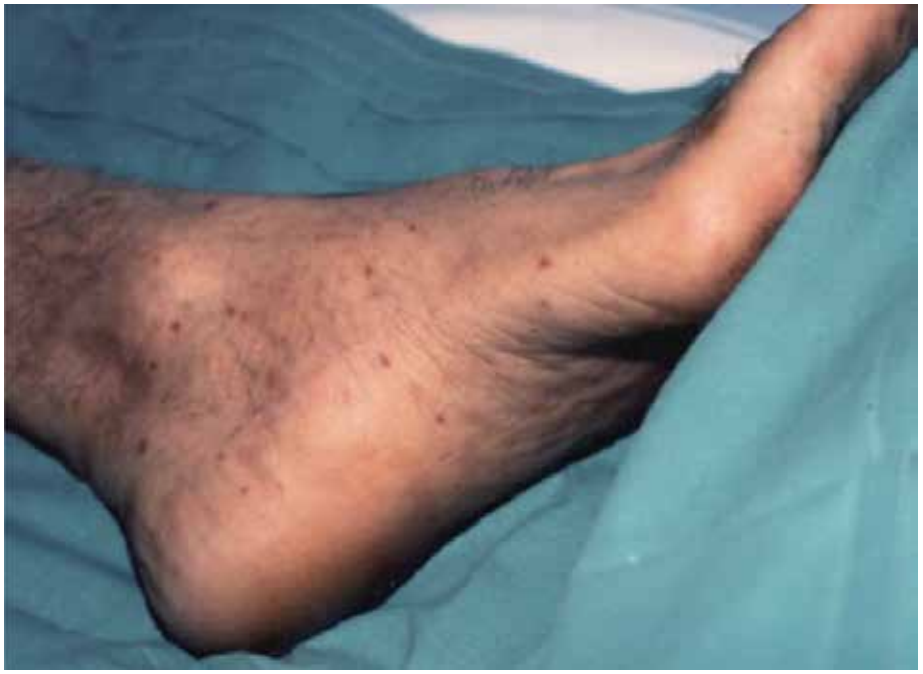


Fig. 4. Vasculitic rash affecting soles in a patient with Mediterranean spotted fever.

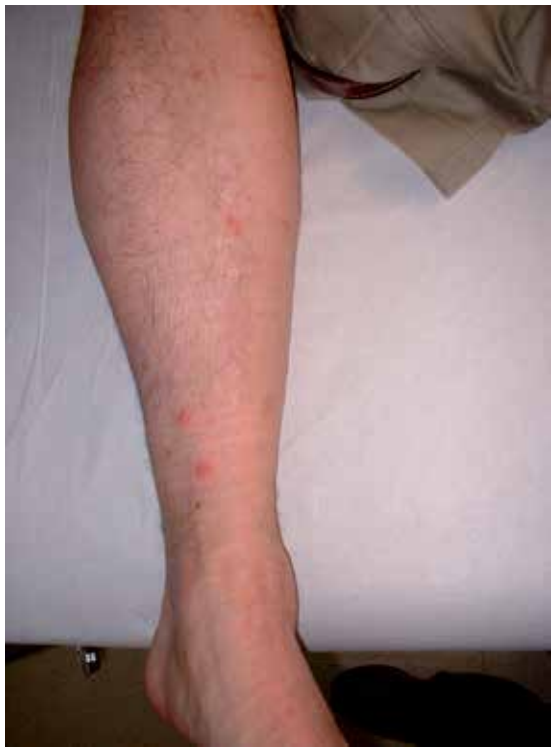


Fig. 5. Vesicular rash in a patient with African-tick bite fever.

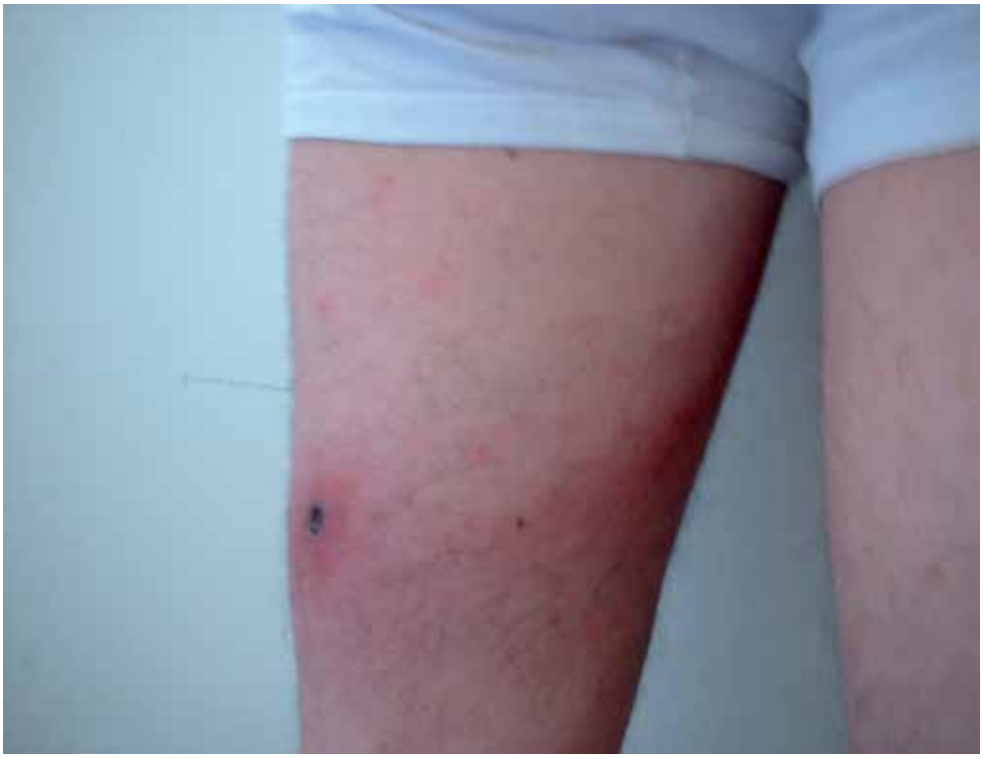


Fig. 6. Eschar and lymphangitis in a patient with African tick-bite fever.

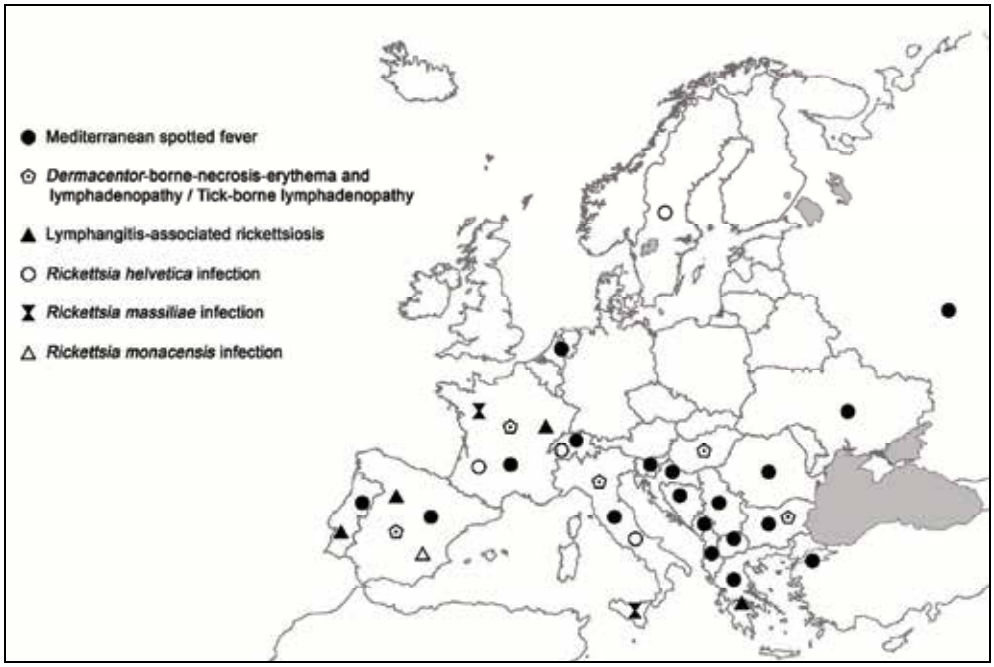


Fig. 7. Map showing distribution of human cases of tick-borne rickettsioses in Europe.

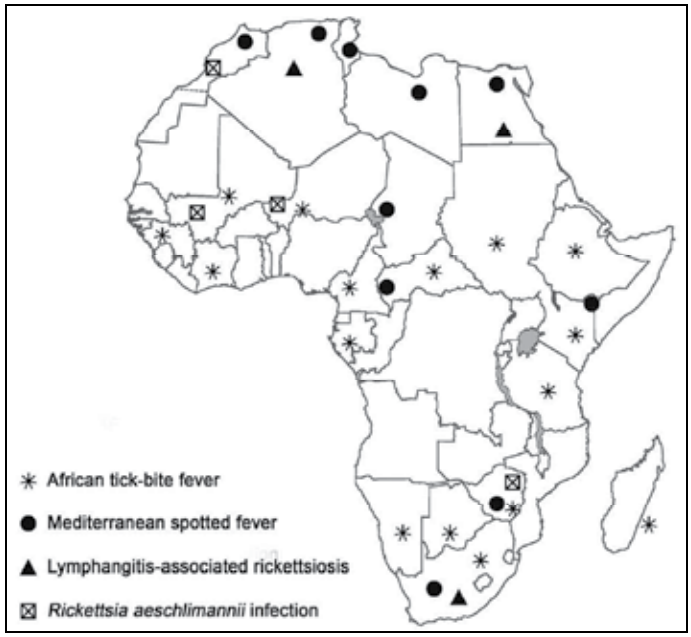


Fig. 8. Map showing distribution of human cases of tick-borne rickettsioses in Africa.

Fig. 9. Map showing distribution of human cases of tick-borne rickettsioses in Latin America.

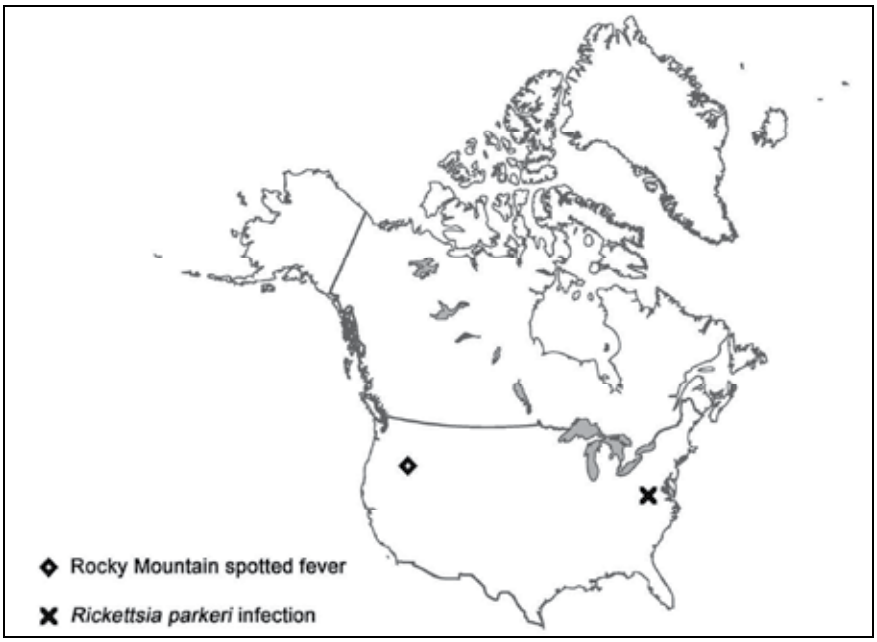


Fig. 10. Map showing distribution of human cases of tick-borne rickettsioses in North America.

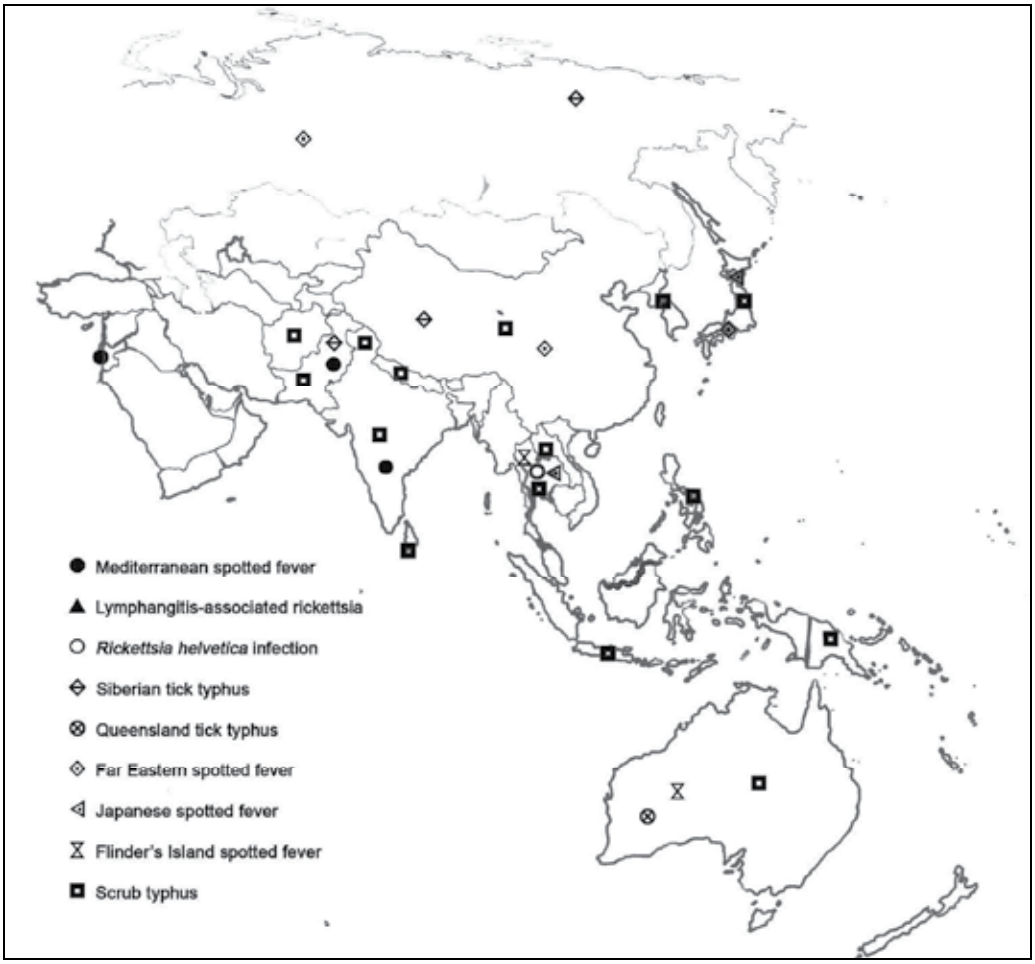


Fig. 11. Map showing distribution of human cases of tick-borne rickettsioses and srub typhus in Asia and Oceania.

4. Eschar and lymphadenopathy

This clinical syndrome has been recently reported in Europe, where it is named TIBOLA (TIck-BOrne LimphAdenopaty) or DEBONEL (*DErmacentor*-BOrne Necrosis-Erythema-Lymphadenopathy). *R. slovaca, R. rioja* and *R. raoultii* are the etiological agents, and *Dermacentor marginatus* is the main vector. This tick species is distributed all over Europe as well as in the North of Africa. Since this rickettsiosis appears in the coldest months of the year, the risk of acquisition for the travellers is lower than for the rickettsioses that are prevalent in spring and summer. In most cases (>90%) the tick-bite is located on the scalp (head) and always in the upper site of the body. After 1-15 days (mean: 4.8 days) of incubation period, the characteristic skin lesion starts as a crusted lesion at the site of the tick-bite (frequently on the scalp). A honey-like discharge from the lesion is observed in some cases. Few days later, a necrotic eschar appears (figure 12). This eschar is usually bigger than the one observed in MSF cases, and it is surrounded by an erythema. When the

tick-bite is out of the head, the skin lesion resembles the erythema migrans of Lyme disease. Other typical manifestation, which is always present when the bite is on the head, is the presence of regional and very painful lymphadenopathies.
On the contrary of other rickettsioses, in DEBONEL/TIBOLA there are not systemic clinical signs (or they are rare), such as fever or maculo-papular rash (Oteo et al., 2004b). The clinical course is sub-acute and no severe complications have been described.

Fig. 12. DEBONEL/TIBOLA patient with the typical crusted lesion on the scalp.

5. Scrub typhus

The etiological agent of scrub typhus is *Orientia tsutsugamushi,* which is transmitted by chigger bites (trombiculid mite larvae). It is mainly distributed in Afghanistan, India, Pakistan, Sri-Lanka, Kashmir, China, Nepal, Japan, Korea, Vietnam, Indonesia, Laos, Philippines, Papua New Guinea and Australia (Figure 11). Cases are mainly observed in autumn and spring, in temperate zones where the bite of this arthropod, which is on vegetation, is frequent. The incubation period is about 10 or more days and the clinical signs and symptoms are similar to typhus syndrome, including the rash which is transient and easily missed. A difference with typhus syndrome is the presence of eschar that is frequently multiple. The presence of regional lymphadenopathy is also more frequent. The mortality can be high despite the correct antimicrobial treatment. Outbreaks related to military operations have been reported (Pages et al., 2010). Most travel acquired cases of scrub typhus occur in patients returning from Southeast Asia (Jensenius et al., 2004, 2006).

6. Rickettsialpox

Rickettsialpox is a worldwide (North America, Eastern Europe, Korea and southern Africa) rickettsiosis caused by *Rickettsia akari* and transmitted by the bite of the mouse mite *Lyponyssoides sanguineus.* We can consider it a remerging infection since several cases have been detected in New York City after September 11 attacks (Paddock et al., 2003). Patients have fever, a prominent eschar -which is the best sign of the disease- and rash that, as occurs in ATBF and Queensland tick typhus, may be vesicular. Palms and soles are not involved. The presence of regional lymphadenopathy is common. Patients recover without treatment in most cases (Raoult, 2010b).

7. Laboratory diagnostic tools

As occurs for all infectious diseases, the most definitive diagnostic method is the rickettsial isolation in culture. The main problem is that *Rickettsia* spp. are strictly intracellular bacteria, conventional growth media cannot be used, and a laboratory with P3 safety level (not generally available in clinical microbiology labs) is necessary. Furthermore, culture is not very sensitive and the yield decreases when clinical samples are taken after antibiotic treatment or when samples are not processed within 24 hours. It is a slow technique that is used for research purposes but not for the routine clinical practice. Centrifugation shell-vial technique is a commercially available adaptation of cell cultures that is easier to handle, faster and less hazardous. Isolation attempts on cell cultures may be performed using buffy coat or tissue samples (eschar biopsies when possible). If not processed within 24 h, samples must be frozen at -70ºC or in liquid nitrogen.

Detection of rickettsiae by Giménez or Giemsa staining from blood and tissue samples would allow the confirmation of the diagnosis, but these techniques are non-specific and their sensitivity is very low.

In some laboratories molecular biology tools, such as polymerase chain reaction (PCR) and sequencing, are also available. PCR-based assays from anticoagulated blood, biopsies and arthropod tissue samples targeting *Rickettsia* spp. genes are quite sensitive and useful for a quick diagnosis of these infections. The evaluation of several primer sets for the molecular diagnosis of rickettsioses demonstrated that the performance of three sequential PCRs (nested or semi-nested ones) allowed the detection and identification of *Rickettsia* species in a high percentage of the samples with previous clinical diagnosis or microbiological confirmation (serological analysis) of rickettsiosis (Santibáñez et al., 2011). Blood and tissue samples should be stored at -20ºC or lower if PCR-based diagnosis is delayed for more than 24 hours. The European guidelines for the diagnosis of tick-borne bacterial diseases contain useful information for clinicians and microbiologists (Brouqui et al., 2004).

Indirect diagnostic tests and specifically, immunofluorescence assays (IFA) are considered the standard tests. Besides, since most traveller patients are investigated after returning, IFA are the most available tools for diagnosis. Acute and convalescent sera (collected 4-6 weeks after the onset of the illness) should be taken. In many cases we cannot observe seroconvertion but a high titre of antibodies. Cross-reactions among *Rickettsia* spp. make very difficult to definitively identify the causative agent by means of IFA. This can only be achieved in reference centres in which different antigens and other serological assays, such as western-blot, are available. Serum samples can be preserved at -20ºC or lower for several months without significant degradation of antibodies.

Ticks removed from patients can be used as tools for the diagnosis of tick-borne rickettsioses. The strategy includes the identification of the tick to the species level, and the detection or isolation of rickettsias (Table 3).

1. Identification of the ticks to the species level
2. Detection of bacteria in ticks with the use of staining tests (haemolymph for viable ticks; salivary glands if ticks were frozen), or PCR-based methods (using one-half of the tick, the other half being kept frozen). PCR may also be done using only ticks that stain positive.
3. Sequencing of the amplified PCR fragment and comparison with available sequences in sequence databases.
4. If there is 100% similarity between the tested sequence and the corresponding sequence of a known organism, the presumptive identification is confirmed
5. If the tested sequence appears to be different from all corresponding sequences available, the organism is probably a new strain and should be isolated and characterized from the stored frozen part of the tick

Table 3. Strategy for detecting and/or isolating rickettsias from ticks

Diagnostic scores with epidemiological, clinical and laboratory tests for some tick-borne rickettsioses (ATBF and MSF) have been proposed (Tables 4 and 5).

a. Direct evidence of *R. africae* infection by culture and/or PCR *or*
b. Clinical and epidemiological features highly suggestive of ATBF, such as multiple inoculation eschars and/or regional lymphadenitis and/or a vesicular rash and/or similar symptoms among other members of the same group of travellers coming back from an endemic area (sub-Saharan Africa or French West Indies) *and* Positive serology against spotted fever group rickettsiae *or*
c. Clinical and epidemiological features consistent with a spotted fever group rickettsiosis such as fever and/or any cutaneous rash and/or single inoculation eschar after travel to sub-Saharan Africa or French West Indies *and* Serology specific for a recent *R. africae* infection (seroconversion or presence of IgM ‡ 1:32), with antibodies to *R. africae* greater than those to *R. conorii* by at least two dilutions, and/or a Western blot or cross-absorption showing antibodies specific for *R. africae*

Table 4. Diagnostic criteria for African-tick bite fever (ATBF). A patient is considered to have ATBF when criteria A, B or C are met

CRITERIA	SCORE[a]
Epidemiological criteria	
Stay in endemic area	2
Occurrence in May–October	2
Contact (certain or possible) with dog ticks	2
Clinical criteria	
Fever > 39ºC	5
Eschar	5
Maculopapular or purpuric rash	5
Two of the above criteria	3
All three of the above criteria	5
Non-specific laboratory findings	
Platelets < 150 G/L	1
SGOT or SGPT > 50 U/L	1
Bacteriological criteria	
Blood culture positive for *Rickettsia conorii*	25
Detection of *Rickettsia conorii* in a skin biopsy	25
Serological criteria	
Single serum and IgG > 1/128	5
Single serum and IgG > 1/128 and IgM > 1/64	10
Four-fold increase in two sera obtained within a 2-week interval	10

SGOT, serum glutamate–oxaloacetate transaminase; SGPT, serum glutamate–pyruvate transaminase.
[a]A positive diagnosis is made when the overall score is ≥ 25.

Table 5. Diagnostic criteria for Mediterranean spotted fever caused by *Rickettsia conorii*

8. Prophylaxis

An important chapter in the field of rickettsioses is related to prophylaxis. Since the majority of rickettsioses associated to travels are transmitted by ticks, the main preventive measure is to avoid tick-bites. Measures to avoid chiggers' attacks are the same as the ones used against ticks. Only fleas can be more difficult to avoid when cats and other pets are abundant. If there is risk of getting lice, hygiene measures such as changing clothing (they live in the seams of clothing) may be sufficient.

How can we avoid tick-bites? There are some rules that can be useful to avoid arthropod-bites:

1. You must not wear dark clothes to see the ticks and remove them before attaching. Curiously, dark clothes attract less arthropods than clear ones. But, in our opinion, to look for the arthropods and remove them as soon as possible is more effective.
2. For outdoor activities (grass areas or mountains) you do not have to exposure your body to ticks. Thus, it is very useful to wear clothing that covers the majority of your body. The trousers must be tucked in your shocks with boots. Long sleeves shirt must be tucked into trousers. You must also wear a cap (especially children).
3. Permethrin-based repellents can be used on clothing, although their effect is short in time and the application should be repeated every few hours.
4. A careful inspection of clothing and body looking for ticks after returning from outdoors activities in endemic areas as well as removing them correctly has been

effective for the prevention of Lyme disease. The tick needs at least 24-48 hours for the transmission of *Borrelia burgdorferi*. This measure can be less efficient for *Rickettsia* spp. because these microorganisms can be transmitted since the first hours. But, anyway, the removal of the tick has to be done.

5. The contact with parasitized pets and wild animals must be avoided.

There are two questions that physicians have linked up with tick-bites: How must I remove the tick? Must I take prophylactic drugs after a tick-bite?

The first question is easy to answer. The most useful method to remove an attached tick is using forceps. Smooth forceps (without teeth) must be introduced between the tick's head and the skin in a 90º angle and then pull (Oteo et al., 1996). Other traditional methods as using oil, burning or freezing must be forgotten.

The other question is the use of prophylactic drugs after arthropod bites. There are no studies to answer this question. The transmission of rickettsias may be very quick, so we cannot extrapolate the recommendations for Lyme disease. Anyway, when people have been bitten by several ticks in an endemic area for a determinate disease (i.e.: Kruger National Park in South Africa and ATBF) and if the patient is anxious, we can offer doxycycline. It has been demonstrated that 3 doses of 100 mg. every 12 hours is safety and sufficient as treatment for the majority of rickettsioses. We must be cautious with the sun to avoid photo-sensibility. Children can take doxycycline for a short period of time. It is only contraindicated for pregnant women and in this case we can use macrolides (i.e. azythromycin).

Vaccine approaches for prevention of rickettsial diseases have been developed since the past century, but currently no vaccine is available. Major surface protein antigens (OmpA and OmpB) of *R. rickettsii* and *R. conorii* are candidate vaccine antigens. Molecular biology techniques such as selection, cloning and expression of genes encoding *R. prowazekii* virulence-associated proteins, offer the opportunity to develop new rickettsial vaccines against typhus group rickettsiae. Further research is needed to develop effective vaccines without undesirable toxic reactions (Azad & Radulovic, 2003; Walker, 2009).

9. Treatment

The treatment of rickettsiosis should be initiated as soon as possible. Antibiotics are very effective and may avoid severe complications and death. In all cases if rickettsiosis is suspected, samples should be sent for laboratory confirmation. In DEBONEL/TIBOLA, in which the clinical signs and symptoms are less severe, recovery without antimicrobials occurs but the use of antibiotics shortens the clinical course and improves the clinical picture (Ibarra et al., 2005).

Doxycycline is the most useful drug in children and adults. Doxycycline can be administered in short course (100 mg. every 12 hours for one day) for the treatment of typhus and scrub typhus. In the case of MSF, 2 doses of 200 mg./12 hous are also very effective (in children, 5 mg./kg./12hours); although most physicians use 100 mg. every 12 hours for 3-7 days after fever disappears. The same can be recommended for ATBF. This antibiotic regimen could probably be followed in other tick-borne rickettsioses but there are not good evidences (clinical assays) to support a recommendation. In RMSF the administration of doxycycline for 7 days is recommended. Other drugs that can be prescribed when not using doxycycline (allergy or pregnancy) are chloramphenicol (50-75 mg./kg./day given in 4 doses for 7-10 days) and azythromycin (500 mg./day for 5 days). Doxycycline for 7 days is the treatment of choice for rickettsialpox. Although there is *in vitro*

susceptibility to quinolones, the use of these drugs has been associated with worse clinical course (Botelho-Nevers et al., 2011).

10. Conclusion

In conclusion, rickettsioses are a worldwide threat that must be suspected in travellers returning from endemic areas. Most cases are caused by tick-bites, although in some areas of the world old diseases as typhus are present, and the risk exists. Rickettsiosis must be suspected in all patients with fever, exanthema with or without rash. Starting treatment with doxycycline when possible may be essential to rapidly recover and avoid complications. ATBF along with malaria is the leading cause of fever after returning from Sub-Saharan Africa.

11. Acknowledgment

We are grateful to all members from the Centre of Rickettsiosis and Arthropod-Borne Diseases, Hospital San Pedro-Centre of Biomedical Research (CIBIR), Logroño (La Rioja), Spain.
Financial support was provided in part by a grant from 'Instituto de Salud Carlos III' (EMER 07/033), Ministerio de Ciencia e Innovación (Spain).

12. References

Althaus, F., Greub, G., Raoult, D. & Genton, B. (2010). African tick-bite fever: a new entity in the differential diagnosis of multiple eschars in travelers. Description of five cases imported from South Africa to Switzerland. *International Journal of Infectious Diseases*, Vol.14, Suppl.3 (September 2010), pp. e274-276, ISSN 1201-9712

Angelakis, E., Botelho, E., Socolovschi, C., Sobas, C.R., Piketty, C., Parola, P. & Raoult, D. (2010). Murine typhus as a cause of fever in travelers from Tunisia and Mediterranean areas. *Journal of Travel Medicine*, Vol.17, No.5, (September 2010), pp. 310-315, ISSN 1195-1982

Angel-Moreno, A., Bolaños, M., Santana, E., Pérez-Arellano, J.L. (2006). Murine typhus imported from Senegal in a travelling immigrant. *Enfermedades Infecciosas y Microbiología Clínica*, Vol.24, No.6, (June-July 2006), pp. 406-407, ISSN 0213-005X

Askling, H.H., Lesko, B., Vene, S., Berndtson, A., Björkman, P., Bläckberg, J., Bronner, U., Follin, P., Hellgren, U., Palmerus, M., Ekdahl, K., Tegnell, A. & Struwe J. (2009). Serologic analysis of returned travelers with fever, Sweden. *Emerging Infectious Diseases*, Vol.15, No.11, (November 2009), pp. 1805-1808, ISSN 1080-6059

Azad, A.F. & Radulovic, S. (2003). Pathogenic rickettsiae as bioterrorism agents. *Annals of the New York Academy of Sciences*, Vol.990 (June 2003), pp. 734-8, ISSN 0077-8923

Azuma, M., Nishioka, Y., Ogawa, M., Takasaki, T., Sone, S. & Uchiyama, T. (2006). Murine typhus from Vietnam, imported into Japan. *Emerging Infectious Diseases*, Vol.12, No.9, (September 2006), pp. 1466-1468, ISSN 1080-6059

Bernabeu-Wittel, M., Pachón, J., Alarcón, A., López-Cortés, L.F., Viciana, P., Jiménez-Mejías, M.E., Villanueva, J.L., Torronteras, R. & Caballero-Granado, F.J. (1999). Murine typhus as a common cause of fever of intermediate duration: a 17-year study in the south of Spain. *Archives of Internal Medicine*, Vol.159, No.8, (April 1999), pp. 872-876, ISSN 0003-9926

Boillat, N., Genton, B., D'Acremont, V., Raoult, D. & Greub, G. (2008). Fatal case of Israeli spotted fever after Mediterranean cruise. *Emerging Infectious Diseases,* Vol.14, No.12, (December 2008), pp. 1944-1946, ISSN 1080-6059

Botelho-Nevers, E., Rovery, C., Richet, H. & Raoult, D. (2011). Analysis of risk factors for malignant Mediterranean spotted fever indicates that fluoroquinolone treatment has a deleterious effect. *Journal of Antimicrobial Chemotherapy,* Vol.66, (June 2011), pp. 1821-1830, ISSN 0305-7453

Bottieau, E., Clerinx, J., Schrooten, W., Van den Enden, E., Wouters, R., Van Esbroeck, M., Vervoort, T., Demey, H., Colebunders, R., Van Gompel, A. & Van Den Ende, J. (2006). Etiology and outcome of fever after a stay in the tropics. *Archives of Internal Medicine*, Vol.166, No.15, (August 2006), pp. 1642-1648, ISSN 0003-9926

Brouqui, P., Bacellar, F., Baranton, G., Birtles, R.J., Bjoërsdorff, A., Blanco, J.R., Caruso, G., Cinco, M., Fournier, P.E., Francavilla, E., Jensenius, M., Kazar, J., Laferl, H., Lakos, A., Lotric-Furlan, S., Maurin, M., Oteo, J.A., Parola, P., Perez-Eid, C., Peter, O., Postic, D., Raoult, D., Tellez, A., Tselentis, Y. & Wilske, B.; ESCMID Study Group on *Coxiella, Anaplasma, Rickettsia* and *Bartonella*; European Network for Surveillance of Tick-Borne Diseases. (2004). Guidelines for the diagnosis of tick-borne bacterial diseases in Europe. *Clinical Microbiology and Infection,* Vol.10, (December 2004), pp. 1108-1132, ISSN 1198-743X

Caruso, G., Zasio, C., Guzzo, F., Granata, C., Mondardini, V., Guerra, E., Macrì, E. & Benedetti, P. (2002). Outbreak of African tick-bite fever in six Italian tourists returning from South Africa. *European Journal of Clinical Microbiology & Infectious Diseases*, Vol.21, No.2, (February 2002), pp. 133-136, ISSN 0934-9723

Chen LH, Wilson ME. (2009). Tick-borne rickettsiosis in traveler returning from Honduras. *Emerging Infectious Diseases*, Vol.15, No.8, (August 2009), pp. 1321-3, ISSN 1080-6059

Consigny, P.H., Schuett, I., Fraitag, S., Rolain, J.M., Buffet, P. (2009). Unusual location of an inoculation lesion in a traveler with African tick-bite fever returning from South Africa. *Journal of Travel Medicine*, Vol.16, No.6, (November-December 2009), pp. 439-440, ISSN 1195-1982

de Sousa, R., Nóbrega, S.D., Bacellar, F., Torgal, J. (2003). Mediterranean spotted fever in Portugal: risk factors for fatal outcome in 105 hospitalized patients. *Annals of the New York Academy of Sciences*, Vol.990 (June 2003), pp. 285-294, ISSN 1749-6632

Dumler J.S. & Walker, D.H. (2010). *Rickettsia tyhi* (Murine typhus), In: *Mandell, Douglas, and Bennett´s Principles and Practice of Infectious Diseases*, Mandell G.L., Bennett J.E., Dolin R. (Eds.), pp. 2525-2528, Churchill Livingstone Elsevier, ISBN 978-0-4430-6839-3, Philadelphia-USA.

Field, V., Gautret, P., Schlagenhauf, P., Burchard, G.D., Caumes, E., Jensenius, M., Castelli, F., Gkrania-Klotsas, E., Weld, L., Lopez-Velez, R., de Vries, P., von Sonnenburg, F., Loutan, L. & Parola, P.; EuroTravNet network. (2010). Travel and migration associated infectious diseases morbidity in Europe, 2008. *BMC Infectious Diseases*, Vol.10, (November 2010), 330, ISSN 1471-2334

Fournier, P.E., Roux, V., Caumes, E., Donzel, M. & Raoult, D. (1998). Outbreak of *Rickettsia africae* infections in participants of an adventure race in South Africa. *Clinical Infectious Diseases*, Vol.27, No.2, (August 1998), pp. 316-323, ISSN 1058-4838

Freedman, D.O., Weld, L.H., Kozarsky, P.E., Fisk, T., Robins, R., von Sonnenburg, F., Keystone, J.S., Pandey, P. & Cetron, M.S.; GeoSentinel Surveillance Network.

(2006). Spectrum of disease and relation to place of exposure among ill returned travelers. *The New England Journal of Medicine*, Vol.354, No.2, (January 2006), pp. 119-130, ISSN 0028-4793

Gikas, A., Kokkini, S., Tsioutis, C., Athenessopoulos, D., Balomenaki, E., Blasak, S., Matheou, C., Tselentis, Y. & Psaroulaki, A. (2009). Murine typhus in children: clinical and laboratory features from 41 cases in Crete, Greece. *Clinical Microbiology and Infection*, Vol.15, Suppl. 2 (December 2009), pp. 211-212, ISSN 1198-743X

Hechemy, K.E., Oteo, J.A., Raoult, D., Silverman, D.J. & Blanco, J.R. (2006). A century of rickettsiology: emerging, reemerging rickettsioses, clinical, epidemiologic, and molecular diagnostic aspects and emerging veterinary rickettsioses: an overview. *Annals of the New York Academy of Sciences*, Vol.1078 (October 2006), pp. 1-14, ISSN 0077-8923

Ibarra, V., Blanco, J.R., Portillo, A., Santibáñez, S., Metola, L., Oteo, J.A. (2005). Effect of antibiotic treatment in patients with DEBONEL/TIBOLA. *Annals of the New York Academy of Sciences*, Vol.1063 (December 2005), pp. 257-258, ISSN 0077-8923

Jensenius, M., Davis, X., von Sonnenburg, F., Schwartz, E., Keystone, J.S., Leder, K., Lopéz-Véléz, R., Caumes, E., Cramer, J.P., Chen, L. & Parola, P.; GeoSentinel Surveillance Network. (2009). Multicenter GeoSentinel analysis of rickettsial diseases in international travelers, 1996-2008. *Emerging Infectious Diseases*, Vol.15, No.11, (November 2009), pp. 1791-1798, ISSN 1080-6059

Jensenius, M., Fournier, P.E. & Raoult, D. (2004). Rickettsioses and the international traveler. *Clinical Infectious Diseases*, Vol.39, No.10, (November 2004), pp. 1493-1499, ISSN 1058-4838

Jensenius, M., Fournier, P.E., Vene, S., Hoel, T., Hasle, G., Henriksen, A.Z., Hellum, K.B., Raoult, D. & Myrvang, B.; Norwegian African Tick Bite Fever Study Group. (2003). African tick bite fever in travelers to rural sub-Equatorial Africa. *Clinical Infectious Diseases*, Vol.36, No.11, (June 2003), pp. 1411-1417, ISSN 1058-4838

Jensenius, M., Montelius, R., Berild, D. & Vene, S. (2006). Scrub typhus imported to Scandinavia. *Scandinavian Journal of Infectious Diseases*, Vol.38, No.3, pp. 200-202, ISSN 0036-5548

Kelly, D.J., Richards, A.L., Temenak, J., Strickman, D., Dasch, G.A. (2002). The past and present threat of rickettsial diseases to military medicine and international public health. *Clinical Infectious Diseases*, Vol.34, Suppl.4, (June 2002), pp. S145-169, ISSN 1058-4838

Laurent, M., Voet, A., Libeer, C., Lambrechts, M., Van Wijngaerden, E. (2009). Mediterranean spotted fever, a diagnostic challenge in travellers. *Acta Clinica Belgica*, Vol.64, No.6, (November-December 2009), pp. 513-516, ISSN 0001-5512

McDonald, J.C., MacLean, J.D. & McDade, J.E. (1988). Imported rickettsial disease: clinical and epidemiologic features. *American Journal of Medicine*, Vol.85, No.6, (December 1988), pp. 799-805, ISSN 0002-9343

Niang, M., Brouqui, P., Raoult, D. (1999). Epidemic typhus imported from Algeria. *Emerging Infectious Diseases*, Vol.5, No.5, (September-October 1999), pp. 716-718, ISSN 1080-6059

Oteo, J.A., Ibarra, V., Blanco, J.R., Martínez de Artola, V., Márquez, F.J., Portillo, A., Raoult, D., Anda, P (2004b). *Dermacentor*-borne Necrosis Erythema and Lymphadenopathy:

Clinical and epidemiological features of a new tick-borne disease. *Clinical Microbiology and Infection,* Vol.10, pp. 327-331, ISSN 1198-743X

Oteo, J.A., Martínez de Artola, V., Gómez-Cadiñanos, R., Casas, J.M., Blanco, J.R. & Rosel, L. (1996). Evaluation of methods of tick removal in human ixodidiasis. *Revista Clínica Española* Vol.196, No.9, (September 1996), pp. 584-587, ISSN 0014-2565

Oteo, J.A., Portillo, A., Blanco, J.R., Ibarra, V. & Santibáñez, S. (2004a). Medicina Clínica, Vol.122, No.20, (May 2004), pp. 786-788, ISSN 0025-7753

Oteo, J.A., Portillo, A., Santibáñez, S., Blanco, J.R., Pérez-Martínez, L. & Ibarra, V. (2006). Cluster of cases of human *Rickettsia felis* infection from southern Europe (Spain) diagnosed by PCR. *Journal of Clinical Microbiology*, Vol.44, No.7, (July 2006), pp. 2669-2671, ISSN 0095- 1137

Paddock, C.D., Zaki, S.R., Koss, T., Singleton, J. Jr., Sumner, J.W., Comer, J.A., Eremeeva, M.E., Dasch, G.A., Cherry, B. & Childs, J.E. Rickettsialpox in New York City: a persistent urban zoonosis. *Annals of the New York Academy of Sciences*, Vol.990, (June 2003), pp. 36-44, ISSN 1749-6632

Pages F., Faulde M., Orlandi-Pradines E. & Parola P. (2010). The past and present threat of vector-borne diseases in deployed troops. *Clinical Microbiology and Infection*, Vol.16, No.3, (March 2010), pp. 209-224, ISSN 1198-743X

Pérez-Arellano, J.L., Fenollar, F., Angel-Moreno, A., Bolaños, M., Hernández, M., Santana, E., Hemmersbach-Miller, M., Martín, A.M. & Raoult, D. (2005). Human *Rickettsia felis* infection, Canary Islands, Spain. *Emerging Infectious Diseases*, Vol.11, No.12, (December 2005), pp. 1961-1964, ISSN 1080-6059

Raoult D. (2010b). *Rickettsia akari* (Rickettsialpox), In: *Mandell, Douglas, and Bennett´s Principles and Practice of Infectious Diseases*, Mandell G.L., Bennett J.E., Dolin R. (Eds.), pp. 2509-2510, Churchill Livingstone Elsevier, ISBN 978-0-4430-6839-3, Philadelphia-USA.

Raoult, D. (2010a). Introduction to Rickettsioses, Ehrlichioses, and Anaplasmosis, In: *Mandell, Douglas, and Bennett´s Principles and Practice of Infectious Diseases*, Mandell G.L., Bennett J.E., Dolin R. (Eds.), pp. 2495-2498, Churchill Livingstone Elsevier, ISBN 978-0-4430-6839-3, Philadelphia-USA.

Raoult, D., Fournier, P.E., Fenollar, F., Jensenius, M., Prioe, T., de Pina, J.J., Caruso, G., Jones, N., Laferl, H., Rosenblatt, J.E. & Marrie, T.J. (2001). *Rickettsia africae*, a tick-borne pathogen in travelers to sub-Saharan Africa. *The New England Journal of Medicine*, Vol.344, No.20, (May 2001), pp. 1504-1510, ISSN 0028-4793

Raoult, D., Ndihokubwayo, J.B., Tissot-Dupont, H., Roux, V., Faugere, B., Abegbinni, R. & Birtles, R.J. (1998). Outbreak of epidemic typhus associated with trench fever in Burundi. *Lancet*, Vol.352, No.9125, (August 1998), pp. 353-358, ISSN 0140-6736

Roch, N., Epaulard, O., Pelloux, I., Pavese, P., Brion, J.P., Raoult, D. & Maurin M. (2008). African tick bite fever in elderly patients: 8 cases in French tourists returning from South Africa. *Clinical Infectious Diseases*, Vol.47, No.3, (August 2008), pp. e28-35, ISSN 1058-4838

Santibáñez, S., Portillo, A., Santibáñez, P., Ibarra, V., Palomar, A., Oteo, J.A. (2011). Utility of five PCR targets for molecular diagnosis of human rickettsioses, *Proceedings of the 6th International Meeting on Rickettsiae and Rickettsial Diseases*, Heraklion, Crete, Greece, June 2011.

Smoak, B.L., McClain, J.B., Brundage, J.F., Broadhurst, L., Kelly, D.J., Dasch, G.A. & Miller, R.N. (1996). An outbreak of spotted fever rickettsiosis in U.S. Army troops deployed to Botswana. *Emerging Infectious Diseases*, Vol.2, No.3, (July-September 1996), pp. 217-221, ISSN 1080-6059

Socolovschi, C., Barbarot, S., Lefebvre, M., Parola, P. & Raoult, D. (2010). *Rickettsia sibirica mongolitimonae* in traveler from Egypt. *Emerging Infectious Diseases*, Vol.16, No.9, (September 2010), pp.1495-1496, ISSN 1080-6059

Stephany, D., Buffet, P., Rolain, J.M., Raoult, D. & Consigny, P.H. (2009). *Rickettsia africae* infection in man after travel to Ethiopia. *Emerging Infectious Diseases*, Vol.15, No.11, (November 2009), pp. 1867-1870, ISSN 1080-6059

Stokes, P.H. & Walters, B.J. (2009). Spotted fever rickettsiosis infection in a traveler from Sri Lanka. *Journal of Travel Medicine*, Vol.16, No.6, (November-December 2009), pp. 436-438, ISSN 1195-1982

Tsai, Y.S., Wu, Y.H., Kao, P.T., Lin, Y.C. (2008). African tick bite fever. *Journal Formosan Medical Association*, Vol.107, No.1, (January 2008), pp. 73-76, ISSN 0929-6646

Walker, D.H. & Raoult, D. (2010). *Rickettsia prowazekii* (Epidemic or louse-borne typhus), In: *Mandell, Douglas, and Bennett´s Principles and Practice of Infectious Diseases*, Mandell G.L., Bennett J.E., Dolin R. (Eds.), pp. 2521-2524, Churchill Livingstone Elsevier, ISBN 978-0-4430-6839-3, Philadelphia-USA.

Walker, D.H. (2009). The realities of biodefense vaccines against Rickettsia. *Vaccine*, Vol. 27, Suppl. 4 (November 2009), pp. D52-5, ISSN 0264-410X

Walter, G., Botelho-Nevers, E., Socolovschi, C., Raoult, D. & Parola, P. (2011). Murine typhus in returned travelers: a report of thirty-two cases, *Proceedings of the 6th International Meeting on Rickettsiae and Rickettsial Diseases*, Heraklion, Crete, Greece, June 2011.

Zanetti, G., Francioli, P., Tagan, D., Paddock, C.D. & Zaki, S.R. (1998). Imported epidemic typhus. *Lancet*, Vol.352, No.9141, (November 1998), pp. 1709, ISSN 0140-6736

2

Human Ehrlichioses and Rickettsioses in Cameroon

Lucy Ndip[1], Roland Ndip[1], David Walker[2] and Jere McBride[2]
[1]*University of Buea*
[2]*University of Texas Medical Branch, Galveston*
[1]*Cameroon*
[2]*USA*

1. Introduction

Human ehrlichioses and rickettsioses are important arthropod borne infectious diseases which are transmitted by ticks, mites, lice and fleas. Infections result in mild to fatal outcomes, with clinical presentations that resemble other tropical infectious diseases such as malaria making clinical diagnosis difficult. Despite recognition as important causes of life-threatening diseases in the United States, the geographic distribution of these diseases worldwide remains undefined due to their recent emergence, challenges in diagnosis and lack of comprehensive epidemiological studies needed to determine incidence in developing countries. Recently, the transfer of technological developments to other parts of the world especially developing countries has encouraged basic epidemiological inquiry and generated scientific interest in understanding the epidemiology of these tick borne diseases and their role as causes of undifferentiated febrile illnesses. In this chapter, we review the current knowledge of human monocytotropic ehrlichiosis (HME) and spotted fever rickettsiosis (African tick bite fever) in Cameroon.

2. Ehrlichiosis

2.1 Etiologic agents

Ehrlichioses are diseases caused by small (approximately 0.4–1.5 μm diameter) Gram negative, obligately intracellular bacteria belonging to the genus *Ehrlichia* of the family Anaplasmataceae, Order Rickettsiales and the alpha sub-division Proteobacteria (Dumler et al., 2001). Although they have a characteristic Gram negative cell wall structure, they lack the necessary enzymes to synthesize cell membrane components such as lipopolysaccharide and peptidoglycan (Lin & Rikihisa, 2003). As intracellular pathogens, *Ehrlichia* reside in cytoplasmic membrane-bound vacuoles inside host cells (granulocytes or monocytes) forming microcolonies called morulae, derived from the Latin word "morus" for mulberry (Popov et al., 1995; Paddock et al., 1997; Ismail et al., 2010). These morulae (ranging in size from 1.0 to 6.0 μm in diameter) may contain 1 to >40 organisms of uniform or mixed cell types (Popov et al., 1995; Rikihisa, 1999).

Organisms in the family *Anaplasmataceae* were first described in 1910 when Theiler described *Anaplasma marginale*, the etiologic agent of an economically important and severe disease of

cattle (Mahan, 1995). This discovery was followed shortly thereafter by the description of *E. ruminantium* (formerly *Cowdria ruminantium)* by Cowdry in 1925; *E. canis* by Donatien and Lestoquard in 1935; and *A. phagocytophilum* (formerly *E. phagocytophila*) by Gordon in 1940. Hence, the genus *Ehrlichia* was established in 1945 in honour of the German microbiologist Paul Ehrlich (Uilenberg, 1983).

Ehrlichia species cause significant diseases in their natural hosts (livestock and companion animals) and emerging zoonoses in humans (McBride & Walker, 2010). The first human ehrlichial infection (sennetsu fever) was reported in 1953 (Rapmund, 1984; Dumler et al., 2007). Sennetsu fever, caused by *Neorickettsia sennetsu,* was identified in Japan and Malaysia (Dumler et al., 2001; Dumler et al., 2007). However, recent phylogenetic reclassifications based on molecular analysis revealed that *E. sennetsu* is not a member of the *Ehrlichia* genus (Dumler et al., 2001). Presently, the genus *Ehrlichia* consists of five recognized species including *E. canis, E. chaffeensis*, *E. ewingii, E. muris,* and *E. ruminantium,* all of which are at least 97.7% similar in 16S rRNA gene sequence (Perez et al., 1996; Paddock et al., 1997; Dumler et al., 2001; Perez et al., 2006).

Ehrlichiae have relatively small genomes (0.8–1.5 Mb) with low G+C content and a high proportion of non-coding sequences but can synthesize all nucleotides, vitamins and cofactors (Dunning et al., 2006). They also have small subsets of genes associated with host-pathogen interactions (Ismail et al., 2010). *E. chaffeensis* have immunodominant outer membrane proteins (OMP-1/MSP2/P28) (Ohashi et al., 1998; Yu et al., 2000; Huang et al., 2008), and in infected macrophages ehrlichiae express the p28-Omp 19 and 20 genes as dominant protein products (Ganta et al., 2009; Peddireddi et al., 2009). Ehrlichiae also express several targets of the humoral immune response including tandem repeat and ankyrin repeat containing proteins (Yu et al., 1997; Sumner et al., 1999; McBride et al., 2003; McBride et al., 2007). *E. chaffeensis*, a human pathogen that was first recognised in the United States in 1986 and isolated in 1991 (Maeda et al., 1987; Dawson et al., 1991) is the cause of human monocytotropic ehrlichiosis (HME) (Anderson et al., 1992), a moderate to severe disease with a case fatality rate of 3% (Fishbein et al., 1994; McBride & Walker, 2010). *E. chaffeensis* is an obligately intracellular bacterium that primarily infects mononuclear leukocytes and replicates by binary fission. *E. chaffeensis* morulae can be detected in peripheral blood smears obtained from infected patients when observed with a light microscope (Rikihisa, 1991). When tissues (including clinical samples), mononuclear leucocytes or cell lines of mammalian origin infected with *E. chaffeensis* are viewed by electron microscopy, two distinct morphologic cell types are identified: a predominantly coccoid form which has a centrally condensed nucleoid DNA and ribosomes (dense-cored cells) measuring between 0.4 and 0.6 μm in diameter and reticulate or the coccobacillary form, which measures about 0.4 to 0.6 μm by 0.7 to 1.9 μm (Paddock et al., 1995; Popov et al., 1997).

2.2 Vectors and reservoirs

Investigative studies following the discovery of *E. chaffeensis* in the late 1980s revealed that the agent is transmitted to humans by the tick *Amblyomma americanum*, commonly referred to as the lone star tick which has a limited geographic distribution to the United States (Anderson et al., 1993). Molecular analysis (PCR) has demonstrated *E. chaffeensis* DNA in adult *A. americanum* ticks collected from different states. The increased recognition of *E. chaffeensis* as an emerging problem has evoked renewed interest in this and other tick borne diseases, and this has stimulated epidemiologic investigations of this pathogen and its vector in other regions where the tick *A. americanum* is not found. Results not only indicate

that *E. chaffeensis* has a wider distribution than the United States (Ndip et al., 2010), but also indicates that the pathogen exists outside of the known range of *A. americanum* and is harbored by other tick species. These tick species include *Ixodes pacificus* in California (Kramer et al., 1999), *Dermacentor variabilis* in Missouri (Roland et al., 1998), *Ixodes ricinus* in Russia (Alekseev et al., 2001), *Amblyomma testudinarium* in China, (Cao et al., 2000), *Haemaphysalis longicornis* (Lee et al., 2003), and *Ixodes persulcatus* (Kim et al., 2003) in Korea.

(a) (b)

Fig. 1. a) *Rhipicephalus sanguineus* (brown dog tick) and b) Male *Amblyomma variegatum* tick (courtesy Laboratory for Emerging Infectious Diseases, University of Buea)

Studies carried out by Ndip and colleagues in Cameroon identified *Ehrlichia chaffeensis* in *Rhipicephalus sanguineus* ticks. *R. sanguineus*, commonly known as the brown dog tick (Figure 1a) is a species that infests canids worldwide. In one study in Limbe, Cameroon, a very high prevalence of *E. chaffeensis* was detected in *R. sanguineus* ticks infesting dogs inhabiting one kennel (Ndip et al., 2010). *E. chaffeensis* DNA was detected in 33 (56%) of 63 *R. sanguineus* ticks collected from five dogs as opposed to 4 (6%) ticks infected with *E. canis*. Furthermore, co-infection with more than one pathogen was not uncommon. The *E. chaffeensis* strain circulating in Cameroon is similar to the North American strain AF403710 based on the analysis of the 378 bp fragment of the disulphide bond formation (Dsb) protein gene (Ndip et al., 2010). Earlier reports revealed *E. canis*, *E. chaffeensis*, and *E. ewingii* in *R. sanguineus* ticks collected from 51 dogs from different localities in Cameroon (Figure 2), suggesting that dogs could be a reservoir for *E. chaffeensis* and that *R. sanguineus* is the probable vector (Ndip et al., 2007).

In the United States, the white-tailed deer (*Odocoileus virginianus*) has been recognised as the primary natural reservoir of *E. chaffeensis* (Dugan et al., 2000). However, animals such as goats, dogs, and coyotes have also been identified as reservoirs which could play a limited role in the transmission of the pathogen to humans (Breitschwerdt et al., 1998; Dugan et al., 2000; Kocan et al., 2000). Unlike rickettsial species, ehrlichial species are not transmitted trans-ovarially (ie., larvae are uninfected) suggesting that the pathogen is maintained trans-stadially after the infection is acquired (Ismail et al., 2010). Although the reservoirs for *E. chaffeensis* in Cameroon have not yet been conclusively identified, preliminary studies detected antibodies reactive to *E. chaffeensis* in 56% of goats analysed suggesting a probable role of goats in maintaining the pathogen in nature. Moreover, *E. chaffeensis* DNA was detected in 17% of ticks collected from these animals (Ndip, unpubished data).

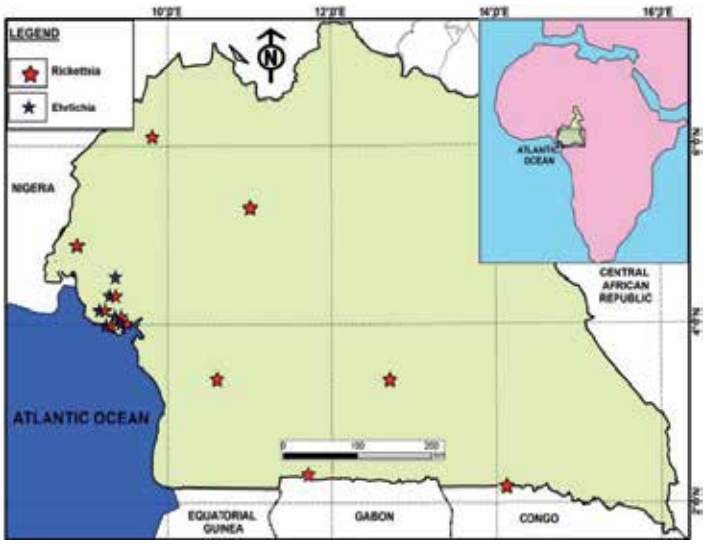


Fig. 2. Known distribution of ehrlichiae and rickettsiae in Cameroon

2.3 Clinical manifestations

The comprehensive data available in literature today on symptoms observed in HME infection is based on cases reported to the United States' Centers for Disease Control and Prevention in addition to a series of patients studied since the disease was described. After exposure to an infecting tick, an incubation period of 1 to 2 weeks (median, 9 days) ensues after which patients develop a febrile illness (often >39°C) characterized by general malaise, low-back pain, or gastrointestinal symptoms (Paddock & Childs, 2003). These signs and symptoms most often resemble manifestations caused by other infectious and non-infectious causes. After 3 to 4 days, symptoms progress and patients may seek medical attention presenting with fever (>95%), headache (60 to 75%), myalgias (40 to 60%), nausea (40 to 50%), arthralgias (30 to 35%), and malaise (30 to 80%) (Fishbein et al., 1994).

Some patients (10-40%) may present with cough, pharyngitis, diarrhea, or abdominal pain and may even progress to changes in mental status (Fishbein et al., 1994; Olano et al., 2003). Some populations especially HIV-infected patients (Paddock et al., 2001) and children (Jacobs & Schutze, 1997) may develop a rash on the extremities, trunk and face (Edwards, 1991). Hematological changes include leukopenia in approximately 60 to 70% of patients and thrombocytopenia (Fishbein et al., 1994; Olano & Walker, 2002). Liver enzymes (hepatic transaminases) may become slightly elevated (Nutt & Raufman, 1999). About 60 to 70% of patients require hospitalization and untreated cases last for 2-3 weeks or progress to a fatal outcome during the second week (Fishbein et al., 1994; Standaert et al., 2000). About 20% of patients develop neurologic signs, cough or other respiratory symptoms (Fishbein et al., 1994; Olano et al., 2003). Case-fatality ratio is approximately 3% (McQuiston et al., 2003) with risk factors for severe or fatal disease including older age (Paddock et al., 2001), underlying debilitating diseases such as HIV infection, immunosuppressive therapies (Olano & Walker, 2002) and sickle cell disease (Paddock & Childs, 2003).

These reported symptoms are quite similar to those manifested by Cameroonian HME patients. In one series of 206 acutely ill patients studied, 30 (14.6%) demonstrated anti-

ehrlichial IgM antibodies, and these probable HME patients presented with headache (83%), fatigue (37%), abdominal pain (47%), joint pain (60%), anorexia (37%) and diarrhoea (13%) in addition to fever (>38°C). Their mean hematocrit, AST and ALT values were 48±21%, 46±23% and 36± 21%, respectively. Five (17%) of the patients were anaemic while 10 (33%) and 5 (17%) had abnormal AST and ALT values, respectively (Ndip, unpublished data). In another series of 118 acutely ill febrile patients studied with HME diagnosed by detection of *E. chaffeensis* DNA in patient's blood (n=12), these patients presented with fever (100%), headache (seen in 72% of the patients), arthralgia (58%), myalgia (42%), cough (17%) and a diffuse maculopapular rash (17%). The rash was present on the trunk of one patient and the arms of another. One patient of the 12 with detectable *E. chaffeensis* DNA required hospitalization (see Table 1) (Ndip et al., 2009).

2.4 Epidemiology

The epidemiology and ecology of HME worldwide is not well documented. Since its description in 1986 more than 1000 cases of HME from at least 30 U.S. states have been reported to the Centers for Disease Control and Prevention in Atlanta, Georgia with nearly all occurring in the southeastern and south-central United States where the vector, *A. americanum* is common (Paddock & Childs, 2003; Dumler et al., 2007). However, the evidence of the disease and/or the pathogen is increasingly being reported in other parts of the world. This includes Africa (Uhaa et al., 1992; Brouqui et al., 1994; Ndip et al., 2009; Ndip et al., 2010), Israel (Dawson et al., 1991; Keysary et al., 1999; Brouqui & Dumler, 2000), Latin America (Gongora-Biachi et al., 1999; Calic et al., 2004;) and Asia (Heppner et al., 1997; Cao et al., 2000; Heo et al., 2002; Kim et al., 2003; Park et al., 2003, Lee & Chae, 2010).

In Cameroon, HME has been identified in patients along the coast of Cameroon, in Buea (4°10′0′′N9°14′0′′E), Limbe (4°01′N 9°13′13′′E), Muyuka (4°43′18′′N 9°38′27′′E), Tiko (4°4′0′′N 9°22′60′′E), and Kumba (4°38′38′′ N9°26′19′′E) and the agent, *E. chaffeensis,* in ticks collected from Limbe (Figure 2). HME was observed in both males and females as well as in children and adults although results suggested that older age was a risk factor for the disease (Ndip et al., 2009). The majority of the patients were adults which suggests that exposure to infected ticks may have occurred during outdoor activities such as farming. Another risk factor is that of owning a companion or domestic animal since most Cameroonian HME patients indicated they had tick-infested pets and domestic animals.

2.5 Microbiological diagnosis

The diagnosis of HME requires specialized microscopy equipment and skills which are not readily available in many diagnostic laboratories. Several methods have been proposed for the diagnosis of HME (Paddock & Childs, 2003; Ismail et al., 2010), including serologic tests such as immunofluorescent assay (IFA), western immunoblot employing specific proteins or ehrlichial whole cell antigens or the recently developed *Ehrlichia* recombinant protein or peptide ELISA for detection of the antibody (Cardenas et al., 2007; Luo et al., 2010; O'Connor et al., 2010). Though these tests can be used to confirm diagnosis retrospectively, some patients may not sero-convert during the early days of the disease and cannot be diagnosed with serologic tests. However, collecting paired sera (at acute and convalescent phases of illness) is confirmatory as a four-fold rise in titer indicates current infection. However, this

always presents a problem because patients who recover may not return to the hospital for follow up. Moreover, another issue with the interpretation of serological tests such as IFA is cross-reactive antibodies against other organisms, including *Anaplasma* species. PCR has also been employed to identify ehrlichial DNA in acutely ill patients when antibodies have not reached detectable levels. Several genes have been proposed and used including the VLPT gene (TRP32), TRP36, 16S rRNA, the TRP120, the Dsb, 28-kDa outer membrane protein gene have been used as genus or species specific targets (Yu et al., 1999; Doyle et al., 2005). IFA, western blot and PCR have been used to study the prevalence of ehrlichiae in blood of acutely ill patients, reservoirs, or in suspected tick vectors and anti-ehrlichial antibody in sera (Ndip et al., 2005; Ndip et al., 2007; Ndip et al., 2009; Ndip et al., 2010). Figure 3 shows IFA photomicrographs of whole cell of *E. chaffeensis* reacting with antibodies in an HME patient serum. A rapid method to detect *E. chaffeensis* is the observation of morulae in smears of peripheral blood buffy coat using the Diff Quik or Giemsa stain. However, this technique is very insensitive, and morulae are detected in leukocytes in only 10% of HME patients.

Patients	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Gender	F	F	M	F	M	M	F	M	F	F	M	M
Age (yr)	63	40	5	23	22	20	21	1	16	26	35	25
Location	A	B	C	D	C	D	A	C	A	A	B	A
Clinical Manifestations												
Fever >38^{o}C	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
*Day(s)	6	8	4	7	4	3	1	1	2	7	2	2
Headache	Yes	Yes	Yes	No	No	Yes	Yes	No	Yes	Yes	Yes	No
Myalgia	Yes	Yes	No	No	No	Yes	No	No	Yes	No	Yes	No
Arthralgia	Yes	Yes	No	No	No	Yes	Yes	No	Yes	Yes	Yes	No
Rash	No	No	No	Yes	No	No	No	Yes	No	No	No	No

*Days after onset (i.e., before collection of sample).
Locations: A - Buea , B – Limbe, C – Tiko, D - Muyuka

Table 1. Epidemiologic and clinical characteristics of twelve Cameroonian patients with HME

2.6 Treatment

The drug of choice for the treatment of *E. chaffeensis* infection is the tetracyclines (particularly doxycycline) and their derivatives. Generally, between 1 and 3 days after a patient with HME commences treatment with doxycycline, the patient becomes afebrile (Olano & Walker, 2002). However, treatment may continue for up to 10 days or at least 3 days after the patient becomes afebrile (Chapman et al., 2006). Clinical experience and *in-vitro* susceptibility testing of *E. chaffeensis* to some classes of antibiotics have revealed that fluoroquinolones, penicillins, aminoglycosides, macrolides and cotrimoxazole are not effective therapeutics (Dumler et al., 1993; Brouqui et al., 1994; Brouqui & Raoult, 1994; McBride & Walker, 2010).

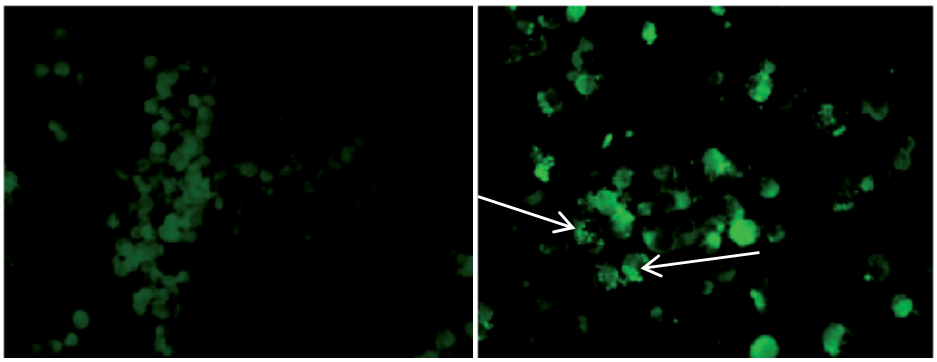


Fig. 3. Reactions of patient serum with *Ehrlichia chaffeensis* antigen and staining with Fluorescein isothiocynate (FITC)-labelled goat, IgG anti-human antibody (X40 magnification). **A**: Negative IFA slide, no inclusion bodies in cells. **B**: Positive IFA slide with morulae in monocytes (arrows) (Courtesy Laboratory for Emerging Infectious Diseases, University of Buea).

3. Rickettsiosis

3.1 The Genus *Rickettsia*

Rickettsial organisms are Gram-negative bacteria belonging to the order Rickettsiales, Family Rickettsiaceae and the Genus *Rickettsia*. They are strict intracellular parasites that are transmitted by arthropods including fleas, lice, mites and ticks (Kelly et al., 1992). These organisms are typically short rods (coccobacilli) measuring about 0.8-2.0µm in length and 0.3-0.5µm in diameter. They exhibit most of the biochemical and morphological characteristics of the Gram-negative cell (Gimenez, 1964; La Scola & Raoult, 1997). Based on antigenic characteristics, species of the genus *Rickettsia* have been divided into three groups; namely the typhus group (TG), the spotted fever group (SFG) and the transitional group (TRG). The TG has two members (*R. prowazekii* and *R. typhi*), which are mainly transmitted by lice and fleas, respectively (Raoult & Roux, 1997). The largest of the antigenic groups is the SFG that is made up of the tick-transmitted pathogens (except *R. bellii* and *R. canadensis*) (Parola et al., 2005). It has been grouped into several genogroups based on the *16S rRNA*, the *gltA*, *ompA*, *ompB* and *sca2* sequences. These groups include the *R. rickettsii* group, the *R. massiliae* group and *R. helvetica* group. The transitional group includes *R. akari, R. australis, and R. felis.* There are many ancestral organisms including *R. canadensis* and *R. bellii* (Parola et al., 2005), as well as numerous rickettsiae in herbivorous insects, and other hosts (leaches and amoeba). Also in the family Rickettsiaceae is *Orientia tsutsugamushi*, which is transmitted by *Leptotrombidium deliense (*Tamura et al., 1995).

Rickettsial organisms are of worldwide occurrence, although species/vector differences may exist along various geographical lines. In Africa, several species have been reported. These include *R. conorii* Malish strain, the cause of Mediterranean spotted fever or "boutonneuse fever". It was first documented in Tunis (Conor & Bruch, 1910), and today the disease continues to be reported in Tunisia (Romdhane et al., 2009; Sfar et al., 2009) and South Africa. The infection has the characteristic of a papular rash, in addition to an eschar at the site of the tick bite (Anton et al., 2003). The pathogen is transmitted by *R. sanguineus* ticks,

and is considered an urban disease (Font & Segura, 1983). Human infections with another strain of *R. conorii* (Israeli spotted fever strain) have been recently documented in Tunisia (Znazen et al., 2011). *R. conorii* Astrakhan strain is the cause of Astrakhan fever first detected in Astrakhan, Russia in the 1970s and transmitted by *R. sanguineus* and *R. pumilio* ticks (Parola et al., 2005). The Astrakhan strain has also been isolated from a patient in Chad (Fournier et al., 2003). *R. sibirica* mongolitimonae strain was identified in *Hyalomma truncatum* ticks in Niger in 2001 (Parola et al., 2001) and the first human case in Africa was documented in South Africa (Pretorius & Birtles, 2004). Other cases have been reported to have been acquired in Algeria (Fournier et al., 2005) and Egypt (Socolovschi et al., 2010). Another species, *R. aeschlimannii,* which was first isolated in *H. marginatum* ticks in Morocco (Beati et al., 1997) and later detected in *H. marginatum rufipes* in Mali and Niger, have been known to cause infections in tourists returning from Morocco and South Africa (Parola et al., 2001). *R. massiliae*, first isolated from *R. sanguineus* ticks in Marseille, France (Parola et al., 2005) was detected in *R. muhsame*, *R. lunalatus* and *R. sulcatus* from Central African Republic (Dupont et al., 1994) and in *R. muhsame* ticks collected from cattle in Mali 2001) and Ivory Coast (Berrelha et al., 2009). *Rickettsia felis* is a recently identified pathogen which was first detected in *Ctenocephalides felis* fleas (Bouyer et al., 2001). In Africa, the agent has been reported in Ivory Coast (Berrelha et al., 2009) and Senegal (Socolovsch et al., 2010), and human infections have been reported in Kenya (Richards et al., 2010). *Rickettsia africae,* the etiologic agent of African tick bite fever appears to be the most prevalent rickettsiosis in Africa. The disease was first reported in Mozambique and South Africa (McQuiston et al., 2004). The first isolate (*R. africae* strain ESF-5), was recovered from *A. variegatum* ticks in Ethiopia although it was only characterized as *R. africae* later (Roux et al., 1996). The agent was later isolated in *A. hebraeum* ticks in Zimbabwe in 1990, and in 1992, the first isolate from a patient was obtained (Kelly et al., 1991; Kelly et al., 1994). The pathogen has been detected in many other African countries including Senegal (Mediannikov et al., 2010), Ethiopia (Stephany et al., 2009) and Cameroon (Ndip et al., 2004a; Ndip et al., 2004b). In the following, we give a synopsis of our current knowledge of African tick bite fever in Cameroon.

3.2 Causative organism

R. africae, a member of the SFG is the only species that has been detected in Cameroon. The organism which measures about 0.4 μm by 1.0 μm, has an outer slime layer and a trilaminar cell wall which contains immunogenic lipopolysaccharide antigens responsible for cross-reactivity with the other SFG rickettsiae (Hechemy et al., 1989; Kelly et al., 1996). Like other Gram-negative organisms, rickettsiae have outer membrane proteins (dubbed OmpA and OmpB) present as species-specific antigens (Fournier et al., 1998; Roux & Raoult, 2000). The organism lives freely in the cytoplasm and usually infects endothelial cells. According to phylogenetic studies, this rickettsial species, which belongs to the *R. rickettsii* group is closely related to *R. parkeri* in North America and *R. sibirica* in northeast Asia (Parola et al., 2005).

3.3 Tick vectors and reservoirs

R. africae is a tick-borne pathogen, and ticks serve both as vectors and reservoirs. The pathogen is maintained in the tick through trans-stadial and trans-ovarial transmission, and this situation indicates the potential for transmission to humans by all stages (larvae, nymphs, and adults) of the feeding ticks. Ixodid ticks (hard ticks) of the genus *Amblyomma* have been recognized as the vectors (Kelly, 2001). In Cameroon, *A. variegatum* (Figure 1b)

has been identified as the potential vector with about 75% of ticks (male and female) collected from cattle found to be infected with *R. africae* (Ndip et al., 2004b). Reports from other studies have indicated that *R. africae* infection in *Amblyomma* ticks frequently has a high prevalence (up to 100%) reported in ticks collected in some disease-endemic countries (Dupont et al., 1994; Parola et al., 2001). Like any other tick borne disease, the ecological characteristics of the vector influence the epidemiology of the disease. The ticks are usually found all year round, but they peak during and after the rainy season when humidity is very high (Walker et al., 2003). *Ambylomma* are predominantly cattle ticks, and infestation of cattle can be very high (Kelly & Mason, 1991). *A. variegatum,* commonly found in central and west Africa typically enjoys a wide variety of different habitats although they have a preference for semi-arid and humid areas with tall grass, trees, and/or bush cover. These ticks usually quest on vegetation and would usually attack legs although they may crawl to other areas such as groin and perineum where they attach (Jensenius et al., 2003).

3.4 Clinical presentation

Since the description of ATBF in 1992, most of the knowledge available regarding the disease has been documented in travelers who become infected with *R. africae* during travel in Africa. After inoculation from a tick bite, the bacteria invade the vascular endothelial system causing a focal or disseminated vasculitis. Endothelial cells of small blood vessels become infected leading to the destruction of the endothelial cells (Toutous-Trellu et al., 2003) of the host where they have multiplied and eventually injured the host cells, leading to the disease symptoms. Multiple eschars typical of ATBF develop at the sites of tick bite, and following an incubation period between 5 and 7 days (up to two weeks in some cases), after the tick bite a febrile illness develops (Raoult et al., 2001). In most cases, symptoms of ATBF are usually mild and include headaches, nausea, chills, myalgia, lymphadenopathy and prominent neck ache (Jensenius et al., 2003; Raoult et al., 2001). Although there have been some controversies over the differences in the clinical presentations of African tick bite fever, our study of acutely ill patients in Cameroon revealed that the some individuals may manifest severe symptoms while in others the symptoms are mild. However, symptoms reported include fever >38°C (100%), headache (71%), myalgia (71%), arthralgia (57%), rash (15%) and pulmonary signs (28%).

3.5 Epidemiology

ATBF has been recognized as an emerging problem in sub-Saharan Africa, especially for international travelers to rural areas (Jensenius et al., 2003). Most of the victims reported are tourists who visit game reserves or participate in outdoor activities such as running, trekking and hiking in forested areas, usually inhabited by *Amblyomma* ticks. The patients acquire the disease in rural Africa, but most often symptoms manifest only after they have returned to their various countries in Europe and America. The first report suggesting that rickettsiosis could be prevalent in Cameroon was published in 1968 (Maurice et al., 1968). The report based on a serologic survey that used an unreliable technique demonstrated rickettsial antibodies in cattle and humans in the northern region of Cameroon and in other animals in the south of the country (Maurice et al., 1968; Le et al., 1977). Efforts to determine the epidemiology and ecology later re-emerged in 2004 when anti-rickettsial IgM antibodies were detected in some Cameroonian patients along the coastal region of Cameroon (Ndip et

al., 2004a). These results were further confirmed by detection of *R. africae* DNA in about 6% of acutely ill febrile patients (Ndip et al., 2004b). Human infections or the agent has been detected in all regions of southern Cameroon where epidemiologic investigations have been made (Figure 3). According to these studies, age appeared to be a risk factor of acquiring the disease, and it is suggested that activities such as game hunting usually constitutes a risk factor (Ndip et al., 2011). Other activities which could predispose to infection include cattle rearing and exposure to tick habitats.

Cameroon is a sub-saharan tropical country with a vast equatorial forest providing a good habitat for ticks (especially *A. variegatum* ticks). Individuals residing in lowland rainforest habitats have a higher risk of acquiring ATBF probably because these habitats are ideal for *A. variegatum* ticks because of their moderate canopy cover, providing microclimates favoring tick survival (Ndip et al., 2011). Although ATBF has been shown to be prevalent in the southern part of Cameroon (Figure 2), the actual epidemiology of the disease through wider disease surveillance needs to be documented.

3.6 Diagnosis

Diagnosis of ATBF can be achieved by either serological analysis of acute and convalescent serum samples or molecular detection of the DNA of the bacterium by real-time or conventional PCR. Target genes that have been utilized include the rickettsial *gltA* and *ompA* genes. For serological diagnosis, the indirect immunofluorescent test has been used in conjunction with western blot assay to detect antibodies reactive with whole cells or specific proteins of cell lysates of *R. africae*. However, these tests are not very reliable in distinguishing species because cross-reactivity may be observed among the SFG rickettsiae. However, some authors have proposed that a fourfold or greater titer for *R. africae* compared to other species is confirmatory (Raoult et al., 2001; Ndip et al., 2004a). The western immunoblot assay can also be used to detect antibodies against species-specific OmpA and OmpB proteins.

3.7 Treatment

The drug of choice for the treatment of ATBF is doxycycline (100 mg twice daily) for 3-7 days. *In-vitro* studies also indicate that *R. africae* is susceptible to tetracyclines, fluoroquinolones, some macrolides and chloramphenicol (Rolain et al., 1998). Mild cases of ATBF have also been shown to recover naturally (Jensenius et al., 1999).

4. Prevention of ehrlichiosis and rickettsiosis

Studies in Cameroon indicate that one risk factor for contracting *E. chaffeensis* infection and ATBF appears to be exposure to potential tick vectors. Many reports involving acquisition of rickettsial diseases have also indicated that exposure to ticks during safari tours and visit to parks constitute an important risk factor. Therefore, an important method of preventing ehrlichiosis and rickettsiosis is by reducing contact with infected ticks. Personal protective measures are quite important, including wearing light colored clothes when walking in tick infested areas, using insect repellents and examination of clothing after a visit to a tick infested area, and prompt removal of attached tick can all reduce the risk of infection. Companion animals and other domesticated animals should be taken care of and tick infestation controlled.

4.1 Conclusions

These data emphasize the importance of ehrlichiosis and ATBF as prevalent diseases in an indigenous Cameroonian population. Although these diseases present as febrile illnesses, they are rarely considered when evaluating patients with acute, undifferentiated febrile illnesses. This situation can be attributed in part to lack of adequate knowledge of the epidemiology and ecology of the disease to prompt diagnosis; unavailability of specific laboratory tests, equipment, and expertise and also the limited economic resources. Sharing new knowledge on these diseases and techniques to facilitate diagnosis are important factors that can change the types and frequencies of diseases diagnosed in febrile patients and necessitate surveillance for these diseases. Future efforts will attempt to address other issues requiring investigations such as the full description of the clinical spectrum of these diseases in African patients and risk factors for severe illness.

5. References

Alekseev, A.N., Dubinina, H.V., Semenov, A.V. & Bolshakov, C.V. (2001). Evidence of ehrlichiosis agents found in ticks (Acari: Ixodidae) collected from migratory birds. *Journal of Medical Entomolology.* 38 : 471-474.

Anderson, B.E., Sims, K.G., Olson, J.G., Childs, J.E., Piesman, J.F., Happ, C.M., Maupin, G.O. & Johnson, B.J. (1993). *Amblyomma americanum*: a potential vector of human ehrlichiosis. *American Journal of Tropical Medicine & Hygiene.* 49:239-244.

Anderson, B.E., Sumner, J.W., Dawson, J.E., Tzianabos, T., Greene, C.R., Olson, J.G., Fishbein, D.B., Olsen-Rasmussen, M., Holloway, B.P., George, E.H., et al. (1992). Detection of the etiologic agent of human ehrlichiosis by polymerase chain reaction. *Journal of Clinical Microbiology.* 30: 775-780.

Anton, E., Font, B., Munoz, T., Sanfeliu, I. & Segura, F. (2003). Clinical and laboratory characteristics of 144 patients with Mediterranean spotted fever. *European Journal of Clinical Microbiology & Infectious Diseases.* 22:126-128.

Beati, L., Meskini, M., Thiers, B. & Raoult, D. (1997). *Rickettsia aeschlimannii* sp. nov., a new spotted fever group rickettsia associated with *Hyalomma marginatum* ticks. *International Journal of Systematic Bacteriology.* 47:548-554.

Berrelha, J., Briolant, S., Muller, F., Rolain, J.M., Marie, J.L., Pages, F., Raoult, D. & Parola, P. (2009). *Rickettsia felis* and *Rickettsia massiliae* in Ivory Coast, Africa. *Clinical Microbiology & Infection.* 15: 251-252.

Bouyer, D.H., Stenos, J., Crocquet-Valdes, P., Moron, C.G., Popov, V.L., Zavala-Velazquez, J.E., Foil, L.D., Stothard, D.R., Azad, A.F. & Walker, D.H. (2001). *Rickettsia felis*: molecular characterization of a new member of the spotted fever group. *International Journal of Systematic Bacteriology.* 51: 339-347.

Breitschwerdt, E.B., Hegarty, B.C. & Hancock, S.I. (1998). Sequential evaluation of dogs naturally infected with *Ehrlichia canis, Ehrlichia chaffeensis, Ehrlichia equi, Ehrlichia ewingii,* or *Bartonella vinsonii. Journal of Clinical Microbiology.* 36:2645-2651.

Brouqui, P. & Dumler, J.S. (2000). Serologic evidence of human monocytic and granulocytic ehrlichiosis in Israel. *Emerging Infectious Diseases*. 6:314-315.

Brouqui, P., Le, C.C., Kelly, P.J., Laurens, R., Tounkara, A., Sawadogo, S., Velo, M., Gondao, L., Faugere, B., Delmont, J., et al. (1994). Serologic evidence for human ehrlichiosis in Africa. *European Journal of Epidemiology.* 10:695-698.

Brouqui, P. & Raoult, D. (1994). Human ehrlichiosis. *New England Journal of Medicine.* 330:1760-1761.

Calic, S.B., Galvao, M.A., Bacellar, F., Rocha, C.M., Mafra, C.L., Leite, R.C. & Walker, D.H. (2004). Human ehrlichioses in Brazil: first suspect cases. *Brazilian Journal of Infectious Diseases.* 8: 259-262.

Cao, W.C., Gao, Y.M., Zhang, P.H., Zhang, X.T., Dai, Q.H., Dumler, J.S., Fang, L.Q. & Yang, H. (2000). Identification of *Ehrlichia chaffeensis* by nested PCR in ticks from Southern China. *Journal of Clinical Microbiology.* 38: 2778-2780.

Cardenas, A.M., Doyle, C.K., Zhang, X., Nethery, K., Corstvet, R.E., Walker, D.H. & McBride, J.W. (2007). Enzyme-linked immunosorbent assay with conserved immunoreactive glycoproteins gp36 and gp19 has enhanced sensitivity and provides species-specific immunodiagnosis of *Ehrlichia canis* infection. *Clinical &Vaccine Immunology.* 14:123-128.

Chapman, A.S., Bakken, J.S., Folk, S.M., Paddock, C.D., Bloch, K.C., Krusell, A., Sexton, D.J., Buckingham, S.C., Marshall, G.S., Storch, G.A., Dasch, G.A., McQuiston, J.H., Swerdlow, D.L., Dumler, S.J., Nicholson, W.L., Walker, D.H., Eremeeva, M.E. & Ohl, C.A. (2006). Diagnosis and management of tickborne rickettsial diseases: Rocky Mountain spotted fever, ehrlichioses, and anaplasmosis--United States: a practical guide for physicians and other health-care and public health professionals. *MMWR Recommended Report.* 55: (RR-4) 1-27.

Dawson, J.E., Anderson, B.E., Fishbein, D.B., Sanchez, J.L., Goldsmith, C.S., Wilson, K.H. & Duntley, C.W. (1991). Isolation and characterization of an *Ehrlichia* sp. from a patient diagnosed with human ehrlichiosis. *Journal of Clinical Microbiology.* 29:2741-2745.

Doyle, C.K., Labruna, M.B., Breitschwerdt, E.B., Tang, Y.W., Corstvet, R.E., Hegarty, B.C., Bloch, K.C., Li, P., Walker, D.H. & McBride, J.W. (2005). Detection of medically important *Ehrlichia* by quantitative multicolor TaqMan real-time polymerase chain reaction of the *dsb* gene. *Journal of Molecular Diagnostics.* 7: 504-510.

Dugan, V.G., Little, S.E., Stallknecht, D.E., & Beall, A.D. (2000). Natural infection of domestic goats with *Ehrlichia chaffeensis*. *Journal of Clinical Microbiology.* 38: 448-449.

Dumler, J.S., Barbet, A.F., Bekker, C.P., Dasch, G.A., Palmer, G.H., Ray, S.C., Rikihisa, Y. & Rurangirwa, F.R. (2001). Reorganization of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with Neorickettsia, descriptions of six new species combinations and designation of *Ehrlichia equi* and 'HGE agent' as subjective synonyms of *Ehrlichia phagocytophila*. *International Journal of Systematic Bacteriology.* 51:2145-2165.

Dumler, J.S., Madigan, J.E., Pusterla, N. & Bakken, J.S. (2007). Ehrlichioses in humans: epidemiology, clinical presentation, diagnosis, and treatment. *Clinical InfectiousDiseases.* 45:S45-S51.

Dumler, J.S., Sutker, W.L. & Walker, D.H. (1993). Persistent infection with *Ehrlichia chaffeensis*. *Clinical Infectious Diseases.* 17:903-905.

Dunning Hotopp, J.C., Lin, M., Madupu, R., Crabtree, J., Angiuoli, S.V., Eisen, J.A., Seshadri, R., Ren, Q., Wu, M., Utterback, T.R., Smith, S., Lewis, M., Khouri, H., Zhang, C., Niu, H., Lin, Q., Ohashi, N., Zhi, N., Nelson, W., Brinkac, L.M., Dodson, R.J., Rosovitz, M.J., Sundaram, J., Daugherty, S.C., Davidsen, T., Durkin, A.S., Gwinn,

M., Haft, D.H., Selengut, J.D., Sullivan, S.A., Zafar, N., Zhou, L., Benahmed, F., Forberger, H., Halpin, R., Mulligan, S., Robinson, J., White, O., Rikihisa, Y. & Tettelin, H. (2006). Comparative genomics of emerging human ehrlichiosis agents. *PLoS.Genetics.* 2 (2): e21.

Dupont, H.T., Cornet, J.P. & Raoult, D. (1994). Identification of rickettsiae from ticks collected in the Central African Republic using the polymerase chain reaction. *American Journal of Tropical Medicine &Hygiene.* 50:373-380.

Edwards, M.S. (1991). Human ehrlichiosis. *Advances in Pediatric Infectious Diseases.* 6:163-178.

Fishbein, D.B., Dawson, J.E. & Robinson, L.E. (1994). Human ehrlichiosis in the United States, 1985 to 1990. *Annals of Internal Medicine.* 120:736-743.

Font, C.B. & Segura, P.F. (1983). Mediterranean boutonneuse fever. *Medicina Clinica (Barcelona).* 80:182-186.

Fournier, P.E., Gouriet, F., Brouqui, P., Lucht, F. & Raoult, D. (2005). Lymphangitis-associated rickettsiosis, a new rickettsiosis caused by *Rickettsia sibirica mongolotimonae*: seven new cases and review of the literature. *Clinical Infectious Diseases.* 40:1435-1444.

Fournier, P.E., Roux, V. & Raoult, D. (1998). Phylogenetic analysis of spotted fever group rickettsiae by study of the outer surface protein rOmpA. *International Journal of Systematic Bacteriology.* 48:839-849.

Fournier, P.E., Xeridat, B. & Raoult, D. (2003). Isolation of a rickettsia related to Astrakhan fever rickettsia from a patient in Chad. *Annals of the New York Academy of Sciences.* 990: 152-157.

Ganta, R.R., Peddireddi, L., Seo, G.M., Dedonder, S.E., Cheng, C. & Chapes, S.K. (2009). Molecular characterization of *Ehrlichia* interactions with tick cells and macrophages. *Frontiers in Bioscience.* 14: 3259-3273.

Gongora-Biachi, R.A., Zavala-Velazquez, J., Castro-Sansores, C.J. & Gonzalez-Martinez, P. (1999). First case of human ehrlichiosis in Mexico. *Emerging Infectious Diseases.* 5:481.

Hechemy, K.E., Raoult, D., Fox, J., Han, Y., Elliott, L.B. & Rawlings, J. (1989). Cross-reaction of immune sera from patients with rickettsial diseases. *Journal of Medical Microbiology.* 29:199-202.

Heo, E.J., Park, J.H., Koo, J.R., Park, M.S., Park, M.Y., Dumler, J.S. & Chae, J.S. (2002). Serologic and molecular detection of *Ehrlichia chaffeensis* and *Anaplasma phagocytophila* (human granulocytic ehrlichiosis agent) in Korean patients. *Journal of Clinical Microbiology.* 40:3082-3085.

Heppner, D.G., Wongsrichanalai, C., Walsh, D.S., McDaniel, P., Eamsila, C., Hanson, B. & Paxton, H. (1997). Human ehrlichiosis in Thailand. *The Lancet.* 350:785-786.

Huang, H., Lin, M., Wang, X., Kikuchi, T., Mottaz, H., Norbeck, A. & Rikihisa, Y. (2008). Proteomic analysis of and immune responses to *Ehrlichia chaffeensis* lipoproteins. *Infection & Immunity.* 76:3405-3414.

Ismail, N., Bloch, K.C. & McBride, J.W. (2010). Human ehrlichiosis and anaplasmosis. *Clinical Laboratory Medicine.* 30:261-292.

Jacobs, R.F. & Schutze, G.E. (1997). Ehrlichiosis in children. *Journal of Pediatrics.* 131:184-192.

Jensenius, M., Fournier, P.E., Vene, S., Hoel, T., Hasle, G., Henriksen, A.Z., Hellum, K.B., Raoult, D. & Myrvang, B. (2003). African tick bite fever in travelers to rural sub-Equatorial Africa. *Clinical Infectious Diseases.* 36:1411-1417.

Jensenius, M., Hasle, G., Henriksen, A.Z., Vene, S., Raoult, D., Bruu, A.L. & Myrvang, B. (1999). African tick-bite fever imported into Norway: presentation of 8 cases. *Scandinavian Journal of Infectious Diseases.* 31:131-133.

Kelly, P., Matthewman, L., Beati, L., Raoult, D., Mason, P., Dreary, M. & Makombe, R. (1992). African tick-bite fever: a new spotted fever group rickettsiosis under an old name. *The Lancet*. 340: 982-983.

Kelly, P.J. (2001). *Amblyomma hebraeum* is a vector of *Rickettsia africae* and not *R. conorii*. *Journal of South African Veterinary Association.* 72:182.

Kelly, P.J., Beati, L., Mason, P.R., Matthewman, L.A., Roux, V. & Raoult, D. (1996). *Rickettsia africae* sp. *nov*., the etiological agent of African tick bite fever. *International Journal Systematic Bacteriology.* 46:611-614.

Kelly, P.J., Beati, L., Matthewman, L.A., Mason, P.R., Dasch, G.A. & Raoult, D. (1994). A new pathogenic spotted fever group rickettsia from Africa. *Journal of Tropical Medicine & Hygiene. 97*:129-137.

Kelly, P.J. & Mason, P.R. (1991). Transmission of a spotted fever group rickettsia by *Amblyomma hebraeum* (Acari: Ixodidae). *Journal of Medical Entomology.* 28:598-600.

Kelly, P.J., Raoult, D. & Mason, P.R. (1991). Isolation of spotted fever group rickettsias from triturated ticks using a modification of the centrifugation-shell vial technique. *Transactions of the Royal Society of Tropical Medicine & Hygiene.* 85:397-398.

Keysary, A., Amram, L., Keren, G., Sthoeger, Z., Potasman, I., Jacob, A., Strenger, C., Dawson, J.E. & Waner, T. (1999). Serologic evidence of human monocytic and granulocytic ehrlichiosis in Israel. *Emerging Infectious Diseases.* 5:775-778.

Kim, C.M., Kim, M.S., Park, M.S., Park, J.H. & Chae, J.S. (2003). Identification of *Ehrlichia chaffeensis*, *Anaplasma phagocytophilum*, and *A. bovis* in *Haemaphysalis longicornis* and *Ixodes persulcatus* ticks from Korea. *Vector Borne & Zoonotic Diseases.* 3:17-26.

Kocan, A.A., Levesque, G.C., Whitworth, L.C., Murphy, G.L., Ewing, S.A. & Barker, R.W. (2000). Naturally occurring *Ehrlichia chaffeensis* infection in coyotes from Oklahoma. *Emerging Infectious Diseases.* 6: 477-480.

Kramer, V.L., Randolph, M.P., Hui, L.T., Irwin, W.E., Gutierrez, A.G., & Vugia, D.J. (1999). Detection of the agents of human ehrlichioses in ixodid ticks from California. *American Journal of Tropical Medicine & Hygiene.* 60:62-65.

Le, N.P., Rickenbach, A., Ravisse, P. & Le, N.D. (1977). Serological survey of animal rickettsioses in Cameroon. II. Results of the survey. *Bulletin de la Societe de Pathologie Exotique et de ses Filiales.* 70: 410-421.

Lee, J.H., Park, H.S., Jung, K.D., Jang, W.J., Koh, S.E., Kang, S.S., Lee, I.Y., Lee, W.J., Kim, B.J., Kook, Y.H., Park, K.H. & Lee, S.H. (2003). Identification of the spotted fever group rickettsiae detected from *Haemaphysalis longicornis* in Korea. *Microbiology & Immunology.* 47:301-304.

Lee, M.J. & Chae, J.S. (2010). Molecular detection of *Ehrlichia chaffeensis* and *Anaplasma bovis* in the salivary glands from *Haemaphysalis longicornis* ticks. *Vector Borne Zoonotic Diseases.* 10:411-413.

Lin, M. & Rikihisa, Y. (2003). *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* lack genes for lipid A biosynthesis and incorporate cholesterol for their survival. *Infection & Immunity.* 71: 5324-5331.

Luo, T., Zhang, X., Nicholson, W.L., Zhu, B. & McBride, J.W. (2010). Molecular characterization of antibody epitopes of *Ehrlichia chaffeensis* ankyrin protein 200 and

tandem repeat protein 47 and evaluation of synthetic immunodeterminants for serodiagnosis of human monocytotropic ehrlichiosis. *Clinical & Vaccine Immunology.* 17: 87-97 .

Maeda, K., Markowitz, N., Hawley, R.C., Ristic, M., Cox, D., & McDade, J.E. (1987). Human infection with *Ehrlichia canis*, a leukocytic rickettsia. *New England Journal of Medicine.* 316:853-856.

Mahan, S.M. (1995). Review of the molecular biology of *Cowdria ruminantium*. *Veterinary Parasitology.* 57:51-56.

Maurice, Y., Fernagut, R., & Gerome, R. (1968). Rickettsial diseases of North Cameroon; epidemiological study. *Revue d Elevage et de Medecine Veterinaire des Pays Tropicaux.* 21:341-349.

McBride, J.W., Comer, J.E. & Walker, D.H. (2003). Novel immunoreactive glycoprotein orthologs of *Ehrlichia spp*. *Annals of the New York Academy of Sciences.* 990:678-684.

McBride, J.W., Doyle, C.K., Zhang, X., Cardenas, A.M., Popov, V.L., Nethery, K.A. & Woods, M.E. (2007). Identification of a glycosylated *Ehrlichia canis* 19-kilodalton major immunoreactive protein with a species-specific serine-rich glycopeptide epitope. *Infection & Immunity.* 75:74-82.

McBride, J.W. & Walker, D.H. (2010). Progress and obstacles in vaccine development for the ehrlichioses. *Expert Review onVaccines.* 9:1071-1082 .

McQuiston, J.H., McCall, C.L. & Nicholson, W.L. (2003). Ehrlichiosis and related infections. *Journal of the American Veterinary Medical Association.* 223:1750-1756.

McQuiston, J.H., Paddock, C.D., Singleton, J., Jr., Wheeling, J.T., Zaki, S.R. & Childs, J.E. (2004). Imported spotted fever rickettsioses in United States travelers returning from Africa: a summary of cases confirmed by laboratory testing at the Centers for Disease Control and Prevention, 1999-2002. *American Journal of Tropical Medicine & Hygiene.* 70:98-101.

Mediannikov, O., Trape, J.F., Diatta, G., Parola, P., Fournier, P.E. & Raoult, D. (2010). *Rickettsia africae,* Western Africa. *Emerging Infectious Diseases.* 16:571-573.

Ndip, L.M., Bouyer, D.H., Travassos Da Rosa, A.P., Titanji, V.P., Tesh, R.B. & Walker, D.H. (2004a). Acute spotted fever rickettsiosis among febrile patients, Cameroon. *Emerging Infectious Diseases.* 10:432-437.

Ndip, L.M., Fokam, E.B., Bouyer, D.H., Ndip, R.N., Titanji, V.P., Walker, D.H. & McBride, J.W. (2004b). Detection of *Rickettsia africae* in patients and ticks along the coastal region of Cameroon. *American Journal of Tropical Medicine & Hygiene.* 71:363-366.

Ndip, L.M., Ndip, R.N., Esemu, S.N., Dickmu, V.L., Fokam, E.B., Walker, D.H. & McBride, J.W. (2005). Ehrlichial infection in Cameroonian canines by *Ehrlichia canis* and *Ehrlichia ewingii*. *Veterinary Microbiology.* 111:59-66.

Ndip, L.M., Ndip, R.N., Ndive, V.E., Awuh, J.A., Walker, D.H. & McBride, J.W. (2007). *Ehrlichia* species in *Rhipicephalus sanguineus* ticks in Cameroon. *Vector Borne & Zoonotic Diseases.* 7:221-227.

Ndip, L.M., Labruna, M., Ndip, R.N., Walker, D.H. & McBride, J.W. (2009). Molecular and clinical evidence of *Ehrlichia chaffeensis* infection in Cameroonian patients with undifferentiated febrile illness. *Annals of Tropical Medicine & Parasitology.* 103:19-725.

Ndip, L.M., Ndip, R.N., Esemu, S.N., Walker, D.H. & McBride, J.W. (2010). Predominance of *Ehrlichia chaffeensis* in *Rhipicephalus sanguineus* ticks from kennel-confined dogs in Limbe, Cameroon. *Experimental & Applied Acarology.* 50:163-168.

Ndip, L.M., Biswas, H.H., Nfonsam, L.E., LeBreton, M., Ndip, R.N., Bissong, M.A., Mpoudi-Ngole, E., Djoko, C., Tamoufe, U., Prosser, A.T., Burke, D.S. & Wolfe, N.D. (2011). Risk factors for African tick-bite fever in rural central Africa. *American Journal of Tropical Medicine &Hygiene.* 84:608-613.

Nutt, A.K. & Raufman, J. (1999). Gastrointestinal and hepatic manifestations of human ehrlichiosis: 8 cases and a review of the literature. *Digestive Diseases.* 17:37-43.

O'Connor, T.P., Saucier, J.M., Daniluk, D., Stillman, B.A., Krah, R., Rikihisa, Y., Xiong, Q., Yabsley, M.J., Adams, D.S., Diniz, P.P., Breitschwerdt, E.B., Gaunt, S.D. & Chandrashekar, R. (2010). Evaluation of peptide- and recombinant protein-based assays for detection of anti-*Ehrlichia ewingii* antibodies in experimentally and naturally infected dogs. *American Journal Veterinary Research.* 71: 1195-1200.

Ohashi, N., Zhi, N., Zhang, Y. & Rikihisa, Y. (1998). Immunodominant major outer membrane proteins of *Ehrlichia chaffeensis* are encoded by a polymorphic multigene family. *Infection & Immunity.* 66:132-139.

Olano, J.P. & Walker, D.H. (2002). Human ehrlichioses. *Medical Clinics of North America.* 86: 375-392.

Olano, J.P., Hogrefe, W., Seaton, B. & Walker, D.H. (2003). Clinical manifestations, epidemiology, and laboratory diagnosis of human monocytotropic ehrlichiosis in a commercial laboratory setting. *Clinical Diagnostic & Laboratory Immunology.* 10: 891-896.

Paddock, C.D., Sumner, J.W., Shore, G.M., Bartley, D.C., Elie, R.C., McQuade, J.G., Martin, C.R., Goldsmith, C.S. & Childs, J.E. (1997). Isolation and characterization of *Ehrlichia chaffeensis* strains from patients with fatal ehrlichiosis. *Journal of Clinical Microbiology.* 35:2496-2502.

Paddock, C.D., Folk, S.M., Shore, G.M., Machado, L.J., Huycke, M.M., Slater, L.N., Liddell, A.M., Buller, R.S., Storch, G.A., Monson, T.P., Rimland, D., Sumner, J.W., Singleton, J., Bloch, K.C., Tang, Y.W., Standaert, S.M. & Childs, J.E. (2001). Infections with *Ehrlichia chaffeensis* and *Ehrlichia ewingii* in persons coinfected with human immunodeficiency virus. *Clinical Infectious Diseases.* 33:1586-1594.

Paddock, C.D. & Childs, J.E. (2003). *Ehrlichia chaffeensis*: a prototypical emerging pathogen. *Clinical Microbiology Reviews.* 16: 37-64.

Park, J.H., Heo, E.J., Choi, K.S., Dumler, J.S. & Chae, J.S. (2003). Detection of antibodies to *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis* antigens in sera of Korean patients by western immunoblotting and indirect immunofluorescence assays. *Clinical & Diagnostic Laboratory Immunology.* 10:1059-1064.

Parola, P., Inokuma, H., Camicas, J.L., Brouqui, P. & Raoult, D. (2001). Detection and identification of spotted fever group Rickettsiae and Ehrlichiae in African ticks. *Emerging Infectious Diseases.* 7:1014-1017.

Parola, P., Paddock, C.D. & Raoult, D. (2005). Tick-borne rickettsioses around the world: emerging diseases challenging old concepts. *Clinical Microbiology Reviews.* 18:719-756.

Peddireddi, L., Cheng, C. & Ganta, R.R. (2009). Promoter analysis of macrophage- and tick cell-specific differentially expressed *Ehrlichia chaffeensis p28-Omp* genes. *BMC Microbiology.* 9:99.

Perez, M., Bodor, M., Zhang, C., Xiong, Q. & Rikihisa, Y. (2006). Human infection with *Ehrlichia canis* accompanied by clinical signs in Venezuela. *Annals of the New York Academy of Sciences.* 1078:110-117.

Perez, M., Rikihisa, Y. & Wen, B. (1996). *Ehrlichia canis*-like agent isolated from a man in Venezuela: antigenic and genetic characterization. *Journal of Clinical Microbiology.* 34:2133-2139.

Popov, V.L., Chen, S.M., Feng, H.M. & Walker, D.H. (1995). Ultrastructural variation of cultured *Ehrlichia chaffeensis*. *Journal of Medical Microbiology.* 43:411-421.

Pretorius, A.M. & Birtles, R.J. (2004). *Rickettsia mongolotimonae* infection in South Africa. *Emerging Infectious Diseases.* 10:125-126.

Raoult, D., Fournier, P.E., Fenollar, F., Jensenius, M., Prioe, T., de Pina, J.J., Caruso, G., Jones, N., Laferl, H., Rosenblatt, J.E. & Marrie, T.J. (2001). *Rickettsia africae*, a tick-borne pathogen in travelers to sub-Saharan Africa. *New England Journal of Medicine.* 344: 504-1510.

Raoult, D. & Roux, V. (1997). Rickettsioses as paradigms of new or emerging infectious diseases. *Clinical Microbiology Reviews.* 10:694-719.

Rapmund, G. (1984). Rickettsial diseases of the Far East: new perspectives. *Journal of Infectious Diseases.* 149:330-338.

Richards, A.L., Jiang, J., Omulo, S., Dare, R., Abdirahman, K., Ali, A., Sharif, S.K., Feikin, D.R., Breiman, R.F. & Njenga, M.K. (2010). Human infection with *Rickettsia felis*, Kenya. *Emerging Infectious Diseases.* 16:1081-1086.

Rikihisa, Y. (1991). The tribe Ehrlichieae and ehrlichial diseases. *Clinical Microbiology Reviews.* 4:286-308.

Rikihisa, Y. (1999). Clinical and biological aspects of infection caused by *Ehrlichia chaffeensis*. *Microbes & Infection.* 1:367-376.

Rolain, J.M., Maurin, M., Vestris, G. & Raoult, D. (1998). *In vitro* susceptibilities of 27 rickettsiae to 13 antimicrobials. *Antimicrobial Agents & Chemotherapy.* 42:1537-1541.

Roland, W.E., Everett, E.D., Cyr, T.L., Hasan, S.Z., Dommaraju, C.B. & McDonald, G.A. (1998). *Ehrlichia chaffeensis* in Missouri ticks. *American Journal of Tropical Medicine & Hygiene.* 59:641-643.

Romdhane, F.B., Loussaief, C., Toumi, A., Yahia, S.B., Khaiyrallah, M., Bouzouaia, N. & Chakroun, M. (2009). Mediterranean spotted fever: a report of 200 cases in Tunisia. *Clinical Microbiology & Infection.* 15(S2):209-210.

Roux, V., Fournier, P.E. & Raoult, D. (1996). Differentiation of spotted fever group rickettsiae by sequencing and analysis of restriction fragment length polymorphism of PCR-amplified DNA of the gene encoding the protein rOmpA. *Journal of Clinical Microbiology.* 34:2058-2065.

Roux, V. & Raoult, D. (2000). Phylogenetic analysis of members of the genus Rickettsia using the gene encoding the outer-membrane protein rOmpB (*ompB*). *International Journal of Systematic and Evolutionary Microbiology.* 50:1449-1455.

Sfar, N., Kaabia, N., Letaief, A., Rolain, J.M., Parola, P., Bouattour, A. & Raoult, D. (2009). First molecular detection of *R. conorii* subsp. *conorii* 99 years after the Conor

description of Mediterranean spotted fever, in Tunisia. *Clinical Microbiology & Infection.* 15(S2):309-310 .

Socolovschi, C., Barbarot, S., Lefebvre, M., Parola, P. & Raoult, D. (2010). *Rickettsia sibirica mongolitimonae* in traveler from Egypt. *Emerging Infectious Diseases.* 16:1495-1496.

Socolovschi, C., Mediannikov, O., Sokhna, C., Tall, A., Diatta, G., Bassene, H., Trape, J.F. & Raoult, D. (2010). *Rickettsia felis*-associated uneruptive fever, Senegal. *Emerging Infectious Diseases.* 16:1140-1142 .

Standaert, S.M., Yu, T., Scott, M.A., Childs, J.E., Paddock, C.D., Nicholson, W.L., Singleton, J., Jr. & Blaser, M.J. (2000). Primary isolation of *Ehrlichia chaffeensis* from patients with febrile illnesses: clinical and molecular characteristics. *Journal of Infectious Diseases.* 181:1082-1088.

Stephany, D., Buffet, P., Rolain, J.M., Raoult, D. & Consigny, P.H. (2009). R*ickettsia africae* infection in man after travel to Ethiopia. *Emerging Infectious Diseases.* 15:1867-1870.

Sumner, J.W., Childs, J.E. & Paddock, C.D. (1999). Molecular cloning and characterization of the *Ehrlichia chaffeensis* variable-length PCR target: an antigen-expressing gene that exhibits interstrain variation. *Journal of Clinical Microbiology.* 37:1447-1453.

Toutous-Trellu, L., Peter, O., Chavaz, P. & Saurat, J.H. (2003). African tick bite fever: not a spotless rickettsiosis! *Journal of the American Academy of Dermatology.* 48(2S):S18-S19.

Uhaa, I.J., MacLean, J.D., Greene, C.R. & Fishbein, D.B. (1992). A case of human ehrlichiosis acquired in Mali: clinical and laboratory findings. *American Journal of Tropical Medicine & Hygiene.* 46:161-164.

Uilenberg, G. (1983). Heartwater (*Cowdria ruminantium* infection): current status. *Advances in Veterinary Science & Comparative Medicine.* 27:427-480.

Yu, X.J., Crocquet-Valdes, P. & Walker, D.H. (1997). Cloning and sequencing of the gene for a 120-kDa immunodominant protein of *Ehrlichia chaffeensis*. *Gene*. 184:149-154.

Yu, X.J., McBride, J.W. & Walker, D.H. (1999). Genetic diversity of the 28-kilodalton outer membrane protein gene in human isolates of *Ehrlichia chaffeensis*. *Journal of Clinical Microbiology*. 37:1137-1143.

Yu, X., McBride, J.W., Zhang, X. & Walker, D.H. (2000). Characterization of the complete transcriptionally active *Ehrlichia chaffeensis* 28 kDa outer membrane protein multigene family. *Gene*. 2481-2):59-68.

Znazen, A., Hammami, B., Lahiani, D., Ben, J.M. & Hammami, A. (2011). Israeli spotted Fever, Tunisia. *Emerging Infectious Diseases.* 17:1328-1330.

3

Leptospirosis: Epidemiologic Factors, Pathophysiological and Immunopathogenic

Marcia Marinho
Laboratory of Microbiology, Department of Support, Animal Production and Health Course for Veterinary Medicine of UNESP, Campus Araçatuba, São Paulo Brazil

1. Introduction

Leptospirosis is a disease of worldwide distribution present on all continents except Antarctica (Adler & Montezuma, 2010) affecting wildlife, domestic and man. Leading consequently serious socio-economic and public health. It is currently the highest incidence of zoonosis in the world, also considers as an occupational disease, and reemerging infectious disease, occurring endemic and epidemic in developing countries with tropical and subtropical (Levett, 2001; Bharti et al., 2003, Ko et al , 2009). more frequently in tropical and developing countries (Bharti et al, 2003), acarrretando with this serious social and economic problems. The disease is an acute infection caused by a spirochete *Leptospiraceae* family, consisting of two genera, *Leptospira* and *Leptonema*. Recently, the genus *Leptospira* was divided into 17 species based on molecular classification (DNA), saprophytic and pathogenic species (Brazil 2002; Bharti et al. 2003). The pathogenic species are: *L. interrogans, L. alexanderi, L. fanei, L. inadai, L. kirschineri, L. meyeri, L. borgetersenii, L. weil, L. noguchi, L. santarosai,* Genomospecie 1, Genomospecie 4, 5 Genomospecie. The serotypes of *Leptospira* are interrogans Australis, Bratislava, Bataviae, Canicola, Hebdomadis, Icterohaemorrhagiae Copenhageni, Lai, Pomonoa, Pyrogenes, Hardjo and divided into serogroups (Ribeiro, 2006). The reservoir animals, mainly rats, are the most frequent disseminators, by eliminating spirochetes in the urine. *Leptospira* spp. can enter the body through intact skin or not, the oral mucosa, nasal and conjunctival (Kobayashi, 2001). The clinical manifestations of leptospirosis vary according to species, individual susceptibility, the pathogenicity and virulence of the serovar involved (Venugopal, 1990, Macedo 1991). After penetration of the bacteria likely, the organism spreads to the bloodstream to all organs (Hüttner et al, 2002). The incubation period is usually around 5-14 days, but have been described as short or long periods in some cases, such as 72 hours a month or more (Jezior, 2005). Leptospirosis is characterized by a vasculitis. The damage to capillary endothelial cells to the underlying cause of clinical manifestations such as renal tubular dysfunction, liver disease, myocarditis and pulmonary hemorrhage (Hill, 1997).

The clinical features are: a) kidneys: interstitial nephritis, tubular necrosis, decreased capillary permeability, and the combination of hypovolemia resulting in renal failure, b) in the liver: necrosis with central lobular proliferation of Kupffer cells and hepatocellular dysfunction c) in the lung, the lesions were secondary to vascular damage resulting in interstitial hemorrhage d) in the skin, the lesions occur as a result of vascular epithelial

injury, and) in skeletal muscle: the lesions were secondary to edema, vacuolation of the myofibril and damage of blood vessels, lesions of the vascular system in general, would result in capillary rupture, hypovolemia and shock (Jezior, 2005). In humans and dogs the most frequent clinical symptoms are severe hepatitis and nephritis (Mosier, 1957; Hagiwara et al., 1975). In dogs the most obvious symptom is jaundice (Greene et al. 1998; Sonrie et al., 2001), fever, myalgia, prostration and the evolution of the process, can present anuria, oliguria or polyuria, indicating different degrees of commitment renal (Masuzawa et al. 1991; Mcdonough, 2003). In cattle, the symptoms are related to the reproductive sphere as abortion and agalactia (Bercovich, 1989) and may have episodes of mastitis caused by serovar hardjo when determining the drop syndrome milk or "milk drop syndrome" (Higgins et al. , 1980, Pearson et al., 1980). In pigs, sheep and goats are seen sporadic reproductive disorders and, possibly, nervous and respiratory systems framework (Andre-Fontaine, 1985; Giles, 1993). Horses can be no abortion (Shapiro & Prescott, 1999) and ocular lesions (Jungherr 1944; Bohl and Ferguson 1952; Kemenes et al., 1985), such as recurrent uveitis, which have been observed after infection, particularly, by L. interrogans serovar pomona (Nick et al., 2000). The cats have to be refractory (Find and Szyfres, 1989). However, seroepidemiologic study in this species, conducted by different authors report seroconversion to multiple *Leptospira* spp (Langoni et al. 1998; Alves et al., 2003). From the epidemiological point of view, it is important to know the species of animals that act as reservoirs, and what the serovars prevalent in a given area. Some serovars have right choice for some species, so called primary hosts, in which cause mild disease with little damage. These can still host the leptospira in their renal tubules, where they remain free from the action of antibodies, and eliminate them through urine intermittently for long periods (Lamb et al., 1981), thus acting as a source of infection for man and other animals. The impact of leptospirosis in terms of public health is reflected in the high cost of treatment of humans afflicted with a fatality rate of about 5% to 20%. However, with regard to animal health, the consequences of infection are particularly the economic sphere, in view of the involvement of cattle, horses, pigs, goats and sheep, food producing animal species noble as meat, milk, and still products of industrial interest, such as wool and leather (Badke, 2001).

The disease course can vary from common symptomatic infection in endemic regions (Ashford et al., 2000), undifferentiated febrile illness, or syndrome to the presence of aseptic meningitis with low morbidity (Berman et al., 1973) or fulminant disease similar to toxic shock syndrome (Vernel-Pauillac and Merien, 2006) with jaundice, myocarditis, renal failure and cardiac hemorrhage, meningitis and death (Levett, 2001) have been described as epidemic in regions of severe leptospirosis in urban areas of Brazil (Ko et. al., 1999) The Jarisch-Herxheimer reaction is not an uncommon complication, when investigated (McBriede et al., 2005). The lung is a target organ that during leptospira infection, presents a hemorrhagic pneumonitis with varying degrees of severity. Under electron microscopy it is observed that the primary lesion is found in endothelial cells of capillaries (Huttner et al., 2002). Seijo et al (2002) classified the respiratory impairment present in leptospirosis in three groups: a) mild to moderate (20 to 70% of patients), pulmonary infiltrates frequently associated with jaundice and a slight alteration of renal function, b) with jaundice severe kidney disease and bleeding (Weill syndrome) occasionally death from kidney failure and myocarditis or cardiovascular collapse with extensive hemorrhage, c) pulmonary hemorrhage, often fatal, without the occurrence of jaundice, kidney disease or other bleeding.

Over the past year has been a frequent higher prevalence of leptospirosis with the observation of episodes of hemoptysis associated with pulmonary respiratory distress syndrome and death (Gill et al., 1992). The same authors, after review, mentioning that the death in Brazil is primarily linked with renal failure, 76.2% of cases, while 3.5% are related to pulmonary hemorrhage. In an outbreak of leptospirosis occurred in Nicaragua in 1995, 40% of fatal cases were associated with pulmonary hemorrhage (Trevejo et al. 1998).

The lung injury during inflammatory processes has been linked to excess stimulated cells in the lung, including alveolar macrophages, polymorphonuclear cells and production of reactive intermediates of oxygen and nitrogen, or other inflammatory mediators. The etiology of respiratory bleeding is unknown, however Nally et al. (2004) verified by immunofluorescence, the presence of immunoglobulins IgM, IgG, IgA and complement factor C3 deposited along the alveolar basement membrane, thus suggesting the existence of autoimmune process associated with the immunopathogenesis of pulmonary hemorrhage observed in fatal cases of leptospirosis.

The involvement of toxins or toxic factors in the pathogenesis of leptospirosis has long been contemplated, since the absence of the microorganism at the site of tissue injury is a factor that strengthens this hypothesis (Knight et al., 1973). Vinh et al. (1986) extracted a glycoprotein (GLP) present in cell walls of a strain of serovar L.interrogans copenhageni that had cytotoxic effect against the fibroblasts of mice (L929). Later it was demonstrated that GLP induced the production of cytokines, TNF-α and IL-10 by peripheral blood monocytes of healthy volunteers (Diament, et al. 2002). The mechanism by which leptospira activate the immune system has been the main focus of many studies, especially regarding the involvement of cytokines (Yang et al., 2000, Maragoni et al., 2004). High levels of TNF-α in serum of patients with leptospirosis were observed by Estavoyer et al. (1991) and Tajiki and Solomon (1996), and in the culture supernatant of macrophages from mice genetically selected Marinho et al. (2005, 2006) who also associated the severity of infection. Vernel-Pauillac and Merien (2006), tested using the technique of quantitative real-time PCR, found elevated levels of inflammatory cytokines, IL-4 and IL-10 in the late stage of infection with Leptospira interrogans icterohaemorrhagiae establishing a profile of involvement of cytokines in type 1 cellular immunity. It is believed that the naturally acquired immunity may result from humoral-mediated response (Adler and Faine, 1977, Adler et al., 1980), which in turn serovar-specific (Adler and Faine, 1977). The development of the humoral response is related to activation-dependent mechanism Recetor Tool-like type 2 (TLR-2), via the innate immune system that would be activated by LPS leptospiral (Werts et al., 2001). Klimpel et al. (2003), demonstrated that Leptospira can activate T cell proliferation and γ-δ α-β, suggesting therefore the involvement of these cell populations in host defense or in the pathology of leptospirosis.

The humoral immune response, compared to the exposure to leptospires, is demonstrated by serological tests, where there is an increased activity of immunoglobulins IgG and IgM after natural infection or immunization. In men there was a greater prevalence of immunoglobulin class IgM (Adler et al, 1980; Petchclai et al. 1991; and Ribeiro et al, 1992), in all the patients, but not all produce agglutinins IgG, after infection. The cause of this individual variation is unknown, however it is observed more frequently in patients afflicted with Weill syndrome (Adler et al., 1980).

Other factors such as hemolysins (Lee et al., 2002), hyaluronidases, phospholipases and glycoproteins (Yang et al. 2001; Sitprija et al., 1980) are implicated in the pathogenesis of leptospirosis. The spiral movement itself would facilitate adherence to renal tubular

epithelial cells by lipoproteins wall as Lip41, Lip 36 and LPS (Dobrin et al. 1995). Pathogenic Leptospira present several surface proteins that mediate the interactions between the bacteria with the extracellular matrix and host cells, proteins that facilitate adhesion and invasion of host cell proteins that allow motility in connective tissue, secreted proteins such as enzymes degradation (collagenase, hemolysins, phospholipids and sphingomyelin) and pore-forming proteins. No leptospires in protein secretion of type III and IV, as used by Gram-negative bacteria for introducing proteins into host cells (Ko et al, 2009). The cell apoptosis, or programmed cell death plays an important role in modulating the pathogenesis of many infectious processes. The occurrence of apoptosis in the mechanism of tissue injury is a well known event in renal disease processes (Wong et al., 2001). Cell death by an apoptotic process would regulate the number of cells during induction and resolution of renal injury (Savill, 1994, Ortiz et al., 2002). Leptospira interrogans has been considered as an agent inductor of apoptosis of macrophages (Merian et al. 1997) and guinea pig hepatocytes (Merien et al., 1998) However, the mechanism responsible for cell death remains desconheciso. Jin (2009) showed that L. interrogans induces apoptosis in cell line J774A.1 via dependent on caspase 3 and 8. Caspases (*cysteine-dependent aspartate-specific proteases*) signal for apoptosis and cleave substrates leading to condensation and nuclear fragmentation, externalization of membrane phospholipids that will signal to these cells were engulfed by macrophages (Nicholson et al. 1997, Boatright et al ., 2003).

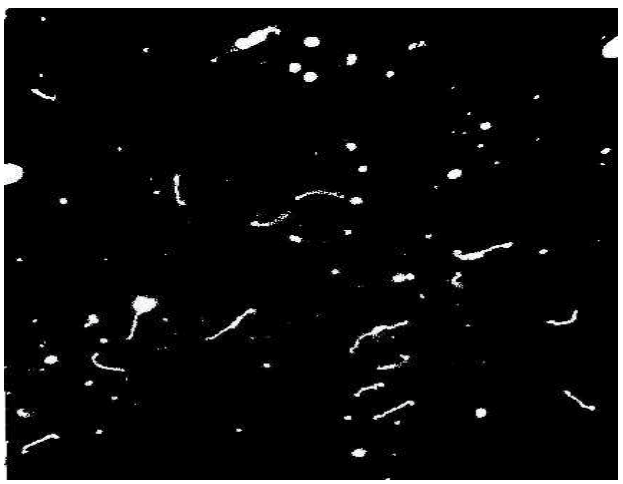


Fig. 1. *Leptospira* spp in dark field microscopy100 increased Microbiology Laboratory, Unesp Brazil Dr. Márcia Marinho /2011

The actual mechanisms that involve the immune response to leptospiral remain controversial and complex. The importance of understanding better the complexity of the mechanisms involved in leptospirosis, such as the virulence of the serovar, the immunocompetence of the host to the agent, the form of clinical manifestations presented, represents a major paradigm in the understanding of infectious diseases and factors related to imunofisiologia leptospirosis, foster the development of preventive and therapeutic strategies aimed at curbing the infection, contributing directly to reducing the prevalence of the disease. New studies are needed to determine the role of apotpose cell in the immunopathogenesis of leptospirosis and the mechanisms that underlie and induce

infection. Understanding these mechanisms and kinetics of their occurrence in the future will develop treatment strategies

2. References

Acha, P.N., Syfres, B. Leptospiroses. Publ. Cient. Org. Panam. De La Salud, 503, p.112-120, 1989.

Adler, B., De La Peña Moctezuma, A., 2010. Leptospira and leptospirosis. Vet. Microbiol. 140, 2010 287-296.

Adler, B., Faine,S. Host immunological mechanisms in the resistence of mice to leptospiral infections. Infect Immun. v.17, p.67-72, 1977.

Adler, B., Murphy, K.H., Locarnini,S.A., Faine,S. Detection of specific anti-leptospiral immunoglobulin M and immunoglobulin G in human serum by solid phase enzyme-linked immunosorbent assay. J Clin Microbiol v.11, p.452-457, 1980.

Alves, C.J., Vasconcellos, S.A., Morais Z.M., Andrade J.S.L., Clementino, I. J.,Azevedo, S.S., Santos, F.A. Avaliação dos níveis de aglutininas antileptospiras em gatos no município de Patos - PB Clínica Veterinária, v.46, p.48-54, 2003.

André – Fontaine,G., Caniere, J.P. Donnés actuelles sur la leptospirose des animaux d`élevage. NOTE I. Ëtiologie, Symptones et epidemiologie. Rev. Med. Vet., v. 136, p.627-637, 1985.

Ashford, D. A., Kaiser, R.M., Spiegel, R.A., Perkins, B.A., Weyant R.S., Bragg, S.L., Plikavtis, B., Jarquin, C., Reys, De J.H.L., Amador, I.J. Asymptomatic infection and risk factors for leptospirosis in Nicaragua. Am. J. Trop. Med. Hyg v.63, p.249, 2000.

Badke M. R. T Leptospirose 2001 Disponível em [www.cnpsa.embrapa.br/abraves-sc/pdf/Memorias2001/1_manoelrenato.pdf] Acesso em 13 /10 /2007.

Bercovich, Z., Taaijk E., Kokhout, B.A. Evaluation of an ELISA for the diagnosis of experimentally induced and naturally occurring *Leptospira hardjo* infections cattle. Vet. Microbiol v. 21,p. 255-262, 1989.

Berman, S.J.; Tsai, C.C.; Holmes, K.; Fresh, J.W.; Watten, R.H. Sporadic anicteric leptospirosis in South Vietnam. A study in 150 patients. Am. Intern. Med v. 79, p.167, 1973.

Bharti, A.R., Nally Je, Ricaldi Jn, Matthias Ma, Diaz Mm, Lovett M, Lovett Pn, Gilman Rh, Willig Mr, Gotuzzo E, Vinetz Jm. Leptospirosis: a zoonotic disease of global importance Lancet Infect Dis v.3, p.757-771, 2003.

Boatright KM, Salvesen GS. Mechanisms of caspase activation. Curr Opin Cell Biol. 2003;15:725-31.

Bohl, E.H., Ferguson, L.C. Leptospirosis in domestic animals. J. Am. Vet. Med. Assoc v.121, p.421-423, 1952.

Brasil. Ministério Da Saúde. Secretaria Nacional de Ações Básicas de Saúde Manual de controle da Leptospirose. Ministério da Saúde. Divisão Nacional de Zoonoses – Brasília: Centro de Documentação do Ministério da Saúde, 2002. 726p. (Série A: Normas e manuais técnicos).

Cordeiro, F., Sulzer, C.R., Ramos, A.A. *Leptospira interrogans* in several wildlife species in Southeast,Brazil. Pesquisa Agropecuária Brasileira, Série Veterinária, v. 1, p.19-29, 1981.

Diament, D., Karina M., Brunialti, C., Romero E.C., Kallas E.G., Salomao R. Peripheral blood mononuclear cell activation induced by *Leptospira interrogans* glycoliprotein. Infection and Immun v.70, p.1677-1687, 2002.

Dobrina A., Nardon E., Vecicle E., Cinco M. & Patriarca P. 1995. *Leptospira* Icterohaemorrhagiae and *Leptospira peptideoglicans* induce endothelial cell adhesiveness for polymorphonuclear leukocytes. Infect. Immun. 63:2995-2999.

Estavouyer, J.M.; Rcadot, E.; Couetdic, G.; Lerroy, J.; Grosperrin, L. Tumor necrosis factor in patiens with leptospirosis [letter]. Rev. Infect. Dis v.13 p.1245-1246, 1991.

Faber, N.C., Crawford, M., .Lefebvre, R.B.,Buyukmihci, N.C., Madigan, J.E., Willits, N.H. Detection of *Leptospira* spp. In the aqueous humor of horses with naturally acquired recurrent uveitis J. Clin. Microbiol v.38, p.2731-2733, 2000.

Giles,N., Hataway,S.C., StevenS, A.E. Isolation of Leptospira interrognas serovar hardjo from a viable premature calf. Vet. Rec v. 113, p.153-154, 1993.

Gonçalves, A.J, De Carvalho, M., Guedes, J.B.S, Rozembaum, R., Riera, A.R.M. Hemoptise e síndrome de angustia respiratória do adulto como causas da morte na leptospirose: mudanças de padrões clínicos e anatomopatologicos. Rev Bras Med Trop v.25, p.261. 1992.

Greene, C. E.; Miller, M. A.; Brown, C. A. Leptospirosis. In: Infectious Diseases of the Dog and Cat. 2 ed. W. B. Saunders, Philadelphia, 1998. p. 273-281.

Hagiwara, M. K.; Santa Rosa, C. A. Leptospirose canina em São Paulo. Arq Inst Biol São Paulo. v. 42, p. 111-118, 1975.

Higgins, R.J.; Harbourne, J.F.; Little, T.W.A.; Stevens, A.E. Mastitis and abortion in dairy cattle associated with *Leptospira* serotype *hardjo*. Vet. Rec v.107, p. 307-10, 1980.

Hill, M.K, Sanders, C.V. Leptospiral pneumonia. Semin Respir Infect v.12, p.44-49. 1997.

Huttner, M.D., Pereira, H.C.P., Tanaka, R.M. Pneumonia por Leptospirose J Pneumol v.28, p.229-232, 2002

Jezior, M. R. Leptospirosis. 2005 Disponível em [URL: http://www.emedicine.com/med/topic1283.htm] Acesso em 28 de abril de 2006

Jin D. (2009)Leptospira interrogans induces apoptosis in macrophages via caspase-8- and caspase-3-dependent pathways. Inf Immun 77:799

Jungherr, W. Bovine Leptospirosis. J. Am. Vet. Med. Assoc v. 105, p.276-281, 1944.

Kemenes, F., Surjan, J., Kasza, L. Studies on equine leptospirosis with emphasis on eye-lesions (equine periodic ophthalmia) Ann. Immunol. Hung. v.24, p.345-355, 1985.

Klimpel G.R., Mathias M.A., Vinetz, J. *Leptospira interrogans* activation of human peripheral blood mononuclaer cells: preferential expacion of TCR gamma delta + cells vs TCR alpha beta + T cells J. Immunol. v .171 p.1447-1455, 2003.

Knight, L.L., Miller, N.G., White, R.J. Cytotoxic factor in the blood and plasma of animals during Leptospirosis Infect. Immunity v.8 p.401-405, 1973.

Ko, 2009 Leptospirosis: a zoonotic disease of global importance. Lancet Infect. Dis. 3, 757-

Ko, A. I. M., Reis, G., Dourado, C.M.R., Johnson, W.D., Rielly,L. W. Urban Epidemic of severe leptospirosis in Brazil. Lancet v .354, p.820, 1999.

Kobayashi ,Y. Clinical observation and treatment of leptospirosis. J Infect Chemother. v. 7 p. 59-68, 2001.

Langoni, H., Cabral, K.G., Kronfly, C.S. Pesquisa de aglutininas anti-leptospíricas em gatos Clínica Veterinária n.17, p.24-6, 1998.

Lee, S.H.; Kim, S.; Park, S.C.; Kim,M. J., Cytotoxic activies of Leptospira interrogans hemolysin SphH as a pore: foirming protein on mammalian cells. Infect. Immun., v. 70, nn1, p., 315-322,2002.

Levett, P.N. Leptospirosis Clin. Microbiol v. 14, p.296, 2001.

Macedo, N.A. Influência da via de inoculação sobre o estabelecimento e a evolução da leptospirose em hamster (*Mesocricetus auratus*) experimentalmente infectados com L. *interrogans* sorovar Pomona. São Paulo, 1991, 45 p. Dissertação (Mestrado) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo.

Marangoni, A. R. Aldini, V., Sambri, L., Giacani, K., Di Leo, Cevenini R. Production of tumor necrosis factor alpha by *Treponema pallidum, Borrelia burgdorferi,* s.l., and *Leptospira interrogans* in isolated rat Kupffer cells. FEMS Immunol. Med. Microbiol v.40, p.187-191, 2004.

Marinho, M., Langoni, H., Oliveira S.L., Lima V.M.F.., Peiró J.R., Perri, S.H.V., Carreira, R. Role of cytokines, NO, and H_2O_2 on the immunopathology of Leptospirosis in genetically selected mice. J. Venom. Anim. Toxins incl. Trop. Dis. v. 11, p.198-212, 2005.

Marinho, M., Silva, C., Lima, V.M.F, Peiró, J.R., Perri, S.H.V. Citokine and antibody production during murine leptospirosis. J. Venom. Anim. Toxins incl. Trop. Dis.v 12 , p.595-603, 2006.

Masuzawa, T.; Suzuki, R.; Yanagihara, Y.Comparison of protective effects with tetravalentglicolipid antigens and whole cell inactivated vaccine inexperimental infection of *Leptospira*. Microbiol Immunol v. 35, p. 199-208, 1991.

Mcbried, A.J.A, ATHANAZIO, D.A, REIS, M. G., KO A.I. Leptospirosis Curr Opin Infect Dis v.18 p. 595-603, 2005

Mcdonough, P. L. Leptospirosis in dogs – Currentstatus. In: CARMICHAEL, L. Recent Advances in Canine Infectious Diseases. International Veterinary Information Service (www.ivis.org). Ithaca. 2001. Último acesso em julho 2003.

Merien, F., Baranton, G., Perolat, P. (1997). Invasion of Vero cells and induction of apoptosis in macrophages by pathogenic Leptospira interrogans are correlated with virulence. Infection and Immunity 65, 729–738

Merien, F., Truccolo, J, Rougier Y (1998). In vivo apoptosis of hepatocytes in guinea pigs infected with Leptopira interrogans serovar Icterohaemorrhagiae. FEMS Microb Letters, 169:95-102.

Mosier,J.E. Leptospirosis of pet animals. Vet. Med v. 52, p. 537-539, 1957.

Nally, J.E., Chantranuwat, C., Wu, X-Yang, Fishbein, M.C., Pereira, M.M., Silva, J. J. Pereira, Blanco D.R., Lovett, M.A. Alveolar septal deposition of immunoglobulin and complement parallels pulmonary hemorrhage in guinea pig model of severe pulmonary leptospirosis. Am J Pathol. v.164 p.1115-1127. 2004.

Nicholson Dw, Thornberry NA. Caspases: killer proteases. Trends Biochem Sci.1997;22:299-306.

Ortiz A, Justo P, Catalán Mp (2002). Apoptotic cell death in renal injury: the rationale for intervention. Curr Drug Targ- Imm Endoc Metabo Disords 2: 181-192.

Pearson, J.K.L., Mackie, D.P., Ellis, W.A. Milk drop syndrome resulting from *Leptospira* Hardjo. Vet. Rec v.107, p. 135-37, 1980.

Petchclai, B., Hiranra, S. S., Potha,U. Gold immunoblot analysis of IgM-specific antibody in the diagnosis of human leptospirosis. Am J Trop Med Hyg v. 45, p.672-675, 1991.

Ribeiro, A.F. Leptospirose, avaliação de fatores prognósticos da doença, município de São Paulo Boletim Epidemiológico Paulista BEPA, v.3, n.28, 2006. Disponível em:[hhtp://www.cve.saude.sp.gov.br/gencia/bepa28_lepto.htm]. Acesso em: 7 jun.2007.

Ribeiro, M.A., Sakata, E.E., Silva, M.V., Camargo, E.D., Vaz,A.J. De Brito,T. Antigens involvrd in the human antibody response to natural infections with *Leptospira interrogans* serovar Copenhageni J Trop Med Hyg v.95 p.239-245, 1992.

Savill J (1994) Apoptosis and kidney. J Am Soc Nephrol 5:12-21.

Seijo, A, Coto H., San Juan J., Videla J., Deodato, B., Cernigoi B et al. Lethal leptospirosis pulmonary hemorrhage: Na emerging disease in Buenos Aires, Argentina Emerg Infect Dis v.8 p.491-499, 2002.

Shapiro, J.L., Prescott, J.F., Equine abortions in eastern Ontario due to leptospirosis. Can Vet J. v.40 p. 350-351, 1999.

Sitprija V, Pipatanagul V, Mertowidjojo K (1980) Pathogenesis of renal disease in leptospirosis:clinical and experimental studies. Kidney Intern,17:827-6.

Sonrier, C.; Branger, C.; Michel, V.; Ruvoënclouet,N.; Ganière, J. P.; André-Fontaine, G.Evidence of cross-protection within *Leptospira interrogans* in an experimental model. Vaccine. v. 19, p. 86-94, 2001.

Tajiki, M.H., Salomao, R. Association of plasma levels of tumor necrosis factor α with severity of diseases and mortality among patients with leptospirosis. CID v.23 p.1177-1178, 1996.

Trevejo, R.T., Rigau-Perz, J.G., Ashford D.A., Zaki, S.R., Shieh, W.J., Peters, C.U.J. et al. Epidemic Leptospirosis associated with pulmonary hemorrhage-Nicaragua, 1995 J infect Dis v.178, p.1457-1463, 1998.

Venugopal, K. Ratnam, S. Lesions and immune responses produced in hamsters and guinea pigs inoculated with some strains of *Leptospira* Indian J. Exp. Biol., v.28, p.1075-1077, 1990.

Vernel-Pauillac, F; Merien F. Proinflammatory and immunomodulatory cytokine mRNA Time Course Profiles in Hamsters Infected with a virulent Variant of *Leptospira interrogns* Infect. Immun v.6 p.4172-4179, 2006.

Vinh,T., Adler,B., Faine, S. Glycolipoprotein Cytotoxin from *Leptospira interrogans serovar copenhageni J. Gen. Micob.* v. 132 p.111-123, 1986.

Werts, C.,R.,I., Tapping, J.C., Mathinson, T. H., Chuang, V., Kravchenko, I. Saint Giron, D.A., Haake, P.J., Godowski, F., Hayashi,A.,Ozinsky, D.M., Underhill,C.J., Kirsching, H,. Wagner, A. Aderem, P. S., Tobias A.N.D., Ulevitch, R.J. Leptospiral lipopolysaccharid activates cells through a TLR-2 dependent mechanism. Nat. Immunol. v. 2 p.346-452, 2001.

Wong Vy,Keller Pm, Nuttall Me(2001). Role of caspases in human renal proximal tubular epithelial cell apoptosis. Eur J Pharmacol, 433:135-140.

Yang Cw,Wu Ms, Pan Mj. (2001) Leptospirosis renal disease, Nephrol Dial Transplant, 16 (Suppl 5):73-77.

Yang, C.W., Wu, M.S., Pan, M.J., Hong, J.J., Yu, C.C., Vandewalle, A., Huang, C.C. Leptospira outer membrane protein actives NF-kappa B and downstream genes expressed in medullary thick ascending limb cells. J.Am. Soc. Nephrol. v.11, p.2017-2026, 2000.

4

Bartonella Infections in Rodents and Bats in Tropics

Ying Bai and Michael Kosoy
Division of Vector-Borne Diseases, National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention USA

1. Introduction

Bacteria of genus *Bartonella* are mainly hemotropic, intracellular gram-negative bacteria associated with erythrocytes and endothelial cells of mammals and other vertebrates (Anderson & Neuman, 1997; Schülein et al., 2001). Members within the genus have been expanded during last three decades with over 30 species or subspecies having been described. In addition to the well-known human pathogens *B. bacilliformis* (agent of Carrión's disease), *B. quintana* (agent of trench fever), and *B. henselae* (agent of cat-scratch disease), a growing number of *Bartonella* species, such as *B. alsatica*, *B. elizabethae*, *B. grahamii*, *B. koehlerae*, *B. clarridgeiae*, *B. washoensis, B. vinsonii* subsp. *berkhoffii*, *B. vinsonii* subsp. *arupensis*, *B. tamiae*, and *B. rochalimae*, have been identified as human pathogens (Kordick et al., 1997; Margileth & Baehren, 1998; Kerkhoff et al., 1999; Welch et al., 1999; Roux et al., 2000; Sander et al., 2000; Kosoy et al., 2003 & 2008; Raoult et al., 2006; Eremeeva et al., 2007). Infections caused by these microorganisms have been encountered in vertebrates of virtually all species surveyed, which to date have extended to members of at least eight different orders of mammals, including Artiodactyla, Cetacea, Carnivora, Chiroptera, Insectivora, Lagomorpha, Primates, and Rodentia (Boulouis et al., 2005; Concannon et al., 2005; Maggi et al., 2005). Results have demonstrated that the prevalence of bacteremia can range from 0 to almost 100% in vertebrate populations. Persistent infections in domestic and wild animals result in a substantial reservoir of bartonellae in nature. Several mammalian species, such as rodents, cats, and dogs are reservoir hosts of some of these pathogenic *Bartonella* species. However, animal reservoirs remain unknown for some newly identified human *Bartonella* species, such as *B. tamiae* and *B. rochalimae*. Knowledge of the transmission of *Bartonella* bacteria between mammalian hosts is incomplete. However, hematophagous arthropods, such as fleas, flies, lice, mites, and ticks, have been found naturally infected and are frequently implicated in transmitting *Bartonella* species (Baker, 1946; Garcia-Caceres & Garcia, 1991; Chomel et al., 1995& 1996; Higgins et al., 1996; Pappalardo et al., 1997; Roux & Raoult, 1999; Welch et al., 1999).

Bartonella infections can cause a wide spectrum of emerging and reemerging diseases, ranging from a short-term fever that resolves quickly on its own to potentially fatal diseases with cardiovascular, nervous system, or hepatosplenic involvement (Anderson & Neuman, 1997; Koehler, 1996). These findings have shown the emerging medical importance of bartonellae. In fact, bartonella infections have become a big world-wide issue. This review

presents the current findings of bartonella infections in rodents and bats from tropics. We are proposing the urgent need to expand studies of bartonella infections in tropics for better understanding the ecology, reservoir potential, vector transmission, pathogenesis of bartonellosis, and their roles in tropical medicine.

2. Bartonella infections in rodents in tropics

The order Rodentia contains over 2,000 species and makes up the largest group of mammals. Rodents can carry many different zoonotic pathogens, such as *Leptospira*, *Yersinia pestis, Toxoplasma gondii*, *Campylobacter*, and *Bartonella* species. With their broad distribution and close contact with humans, rodents play an important role in serving as natural reservoir hosts of these zoonotic pathogens. The first *Bartonella* species found in rodents was isolated from the blood of the vole *Microtus pennsylvanicus*. Originally described as a rickettsial agent (Baker, 1946), this bacterium was later reclassified as *Bartonella vinsonii* (Weiss & Dasch, 1982). During the last three decades, numerous surveys have been conducted in a variety of rodent communities at many locations. These surveys demonstrated that bartonellae are widely distributed in rodents of numerous species in all continents (Birtles et al.,1994; Kosoy et al., 1997; Heller et al., 1998; Hofmeister et al., 1998; Laakkonen et al., 1998; Bermond et al., 2000; Fichet-Calvet et al., 2000; Bajer et al., 2001; Bown et al., 2002; Holmberg et al., 2003; Engbaek & Lawson, 2004; Gundi et al., 2004; Pawelczyk et al., 2004; Pretorius et al., 2004; Tea et al., 2004; Jardine et al., 2005; Kim et al., 2005; Telfer et al., 2005; Markov et al., 2006; Knap et al., 2007).

The very first investigation of bartonella infection in rodents from tropic areas was conducted in Yunnan, a province located in southwestern China (Ying et al., 2002). This study revealed the important finding that *Rattus* rats are the reservoir hosts of *B. elizabethae*, a bartonella strain associating with human diseases. With this discovery, more investigations of bartonella infections in rodents were later carried out in several other tropical countries, including Bangladesh, Thailand, Vietnam, Indonesia, Kenya, and others (Castle et al., 2004; Winoto et al., 2005; Bai et al., 2007b & 2009b; Kosoy et al., 2009 & unpublished data). In this section, we compare the composition of rodent community, bartonella prevalence, and genetic diversity of the *Bartonella* strains, mainly based on three most complete studies that were conducted in southwestern China, Bangladesh, and Thailand (Ying et al., 2002; Bai et al., 2007b & 2009b). We discuss the epidemiological significance of these findings.

2.1 Rodent community

As an environment with a moderate climate, ample food, and plenty of water, the tropics harbor highly diverse rodent communities. The rodents tested for bartonella infections from different regions of tropics represented over 20 species of 10 genera, including *Apodemus chevrieri*, *A. draco*, *A. peninsulae*, *Bandicota bengalensis*, *B. indica*, *B. savilei*, *Berylmys berdmorei*, *Eothenomys miletus*, *Lemniscomys striatus*, *Mastomys natalensis*, *Mus caroli*, *M. cervicolor*, *M. minutoides*, *M. musculus*, *Rattus argentiventer*, *R. exulans*, *R. losea*, *R. nitidus*, *R. norvegicus*, *R. rattus*, *R. remotes*, *R. surifer*, and *R. tanezumi* subsp. *flavipectus*. Among these, rats of the genus *Rattus* were the most widely distributed and prevalent, being found in all study areas. For example, in the survey of bartonella infections in rodents from 17 provinces of Thailand, the total number of *Rattus* rats accounted for more than 80% of the tested rodents (Bai et al., 2009b); in studies in southwestern China and Bangladesh, more than 50% rodents also were

Rattus rats (Ying et al., 2002; Bai et al., 2007b). Nevertheless, the most common *Rattus* species varied among the study sites. In Thailand, the *R. rattus*, *R. norvegicus*, and *R. exulans* were the most common species; in southwestern China, *R. norvegicus* and *R. tanezumi* subsp. *flavipectus* were the most common species; and *R. rattus* were the most common species in Bangladesh (Table 1). In addition to *Rattus* rats, rats of the genus *Bandicot*a also were commonly distributed in Bangladesh and Thailand. *Bandicota bengalensis*, for example, accounted for 41% in the local rodent community in Dhaka, Bangladesh, and were actually the most common species; *Bandicota indica* and *Bandicota savilei* accounted for 16% of all rodents in Thailand (Table 1). In fact, *Bandicota indica* alone accounted for 78% of tested rodents in another study conducted in Chiang Rai, a northern province of Thailand (Castle et al., 2004), indicating that *Bandicota* rats could be more common than *Rattus* rats in some areas in Thailand. Mice of the genus *Apodemus* were found more popular in rural areas in southwestern China, and accounted for 35% of local rodents. Rodents of some other genera, including *Mus*, *Berylmys*, and *Eothenomys* were also found in different areas but in smaller numbers.

2.2 Bartonella prevalence in rodents

Ecologic and bacteriologic observations of rodents in different regions of the world have shown the wide spread of bartonella infection in rats and mice of various species. Nevertheless, large variations in prevalence of infection have been observed among different studies and rodents of different genera, or even species, ranging from 0 to >80% (Birtles et al., 1994; Kosoy et al., 1997; Bai et al., 2009a & 2011). A possible explanation for such variation is the different composition of rodent communities in which the biodiversity can affect the prevalence in a local community (Bai et al., 2009a).

Similar observations were reported from studies of bartonella infection in rodents conducted in tropical areas. A relatively low prevalence of bartonella infection in rodents was reported from Kenya (15%) (Kosoy et al., 2009), while high prevalence was demonstrated in studies conducted in several countries of Southeast Asia. More interestingly, the overall prevalence of bartonella infection in rodents reported from these countries was very similar, with 42.8%, 44.5%, and 41.5% in Bangladesh, southwestern China, and Thailand, respectively (Ying et al., 2002; Bai et al., 2007b & 2009b), although composition of the rodent communities differed among the study sites.

Nevertheless, the bartonella prevalence varied by rodent species. Generally, rats of the genus *Rattus* are highly infected with *Bartonella* species. In Thailand, bartonella prevalence in *Rattus* rats was 43% with a range of 0-86% among eight investigated species. *R. norvegicus* and *R. rattus*, as the most common species present, exhibited very high prevalence of bartonella infection with 86% and 65% in each, respectively, while only 3% of another common tropical species, *R. exulans*, were infected with *Bartonella* species. In one southwestern China study, *Rattus tanezumi* subsp. *flavipectus* was the predominant species among the local rodents and also highly infected by *Bartonella* species with 41% prevalence. In addition to the variation in prevalence between rodent species, the same rat species can exhibit different degrees of susceptibility to infection with *Bartonella* species at different locations. For example, the infection rate in *R. rattus* was 32% in Bangladesh, but 65% in Thailand; the infection rate in *R. norvegicus* was 43% in southwestern China, but 86% in Thailand (Table 1).

Rats of the genus *Bandicota* were also frequently infected with *Bartonella* species. In Bangladesh, 63% of *B. bengalensis* were infected; in Thailand, 33% and 57% of *B. indica* and *B. savilei* were infected with *Bartonella* species, respectively.

Mice of genus *Apodemus* were also highly susceptible to bartonella infection, with 33-71% prevalence in different species in southwestern China; in Kenya, 63% of *Mastomys natalensis* had bartonella infection; rodents of the genus *Mus* and several other genera seem to exhibit lower susceptibility to bartonella infection. In Bangladesh, none of the 12 tested *Mus musculus* had bartonella infection; but in Thailand, three of seven *Mus cervicolor* were infected with *Bartonella* species.

2.3 Diversity of *Bartonella* species in rodents

Studies from different regions of the world have demonstrated that rodents harbor extremely diverse *Bartonella* strains. Although many strains remain uncharacterized or were only partially characterized, quite a few novel rodent-associated *Bartonella* species and subspecies have been described, including *B. birtlesii*, *B. coopersplainensis*, *B. elizabethae*, *B. doshiae*, *B. grahamii*, *B. phoceensis*, *B. queenslandensis*, *B. rattimassiliensis*, *B. taylorii*, *B. tribocorum*, *B. vinsonii* subsp. *arupensis*, and *B. washoensis* (Daly et al., 1993; Birtles et al., 1995; Heller et al., 1998; Kosoy et al., 2003; Gundi et al., 2004 & 2009; Bai et al., 2011). Among these, *B. coopersplainensis*, *B. elizabethae*, *B. phoceensis*, *B. queenslandensis*, *B. rattimassiliensis*, and *B. tribocorum* were all associated with rats of the genus *Rattus*, and they are genetically closer to each other than to other *Bartonella* species that are associated with *Apodemus* spp., *Peromyscus* spp., *Spermophilus* spp., *Myodes* spp., and other rodent genera.

Comparative analyses of bartonella cultures obtained from the rodents in the tropics also revealed diverse assemblages of *Bartonella* strains, many of which appear to represent a variety of distinct species. These bartonella isolates clustered into different lineages that mostly had a close association with their host genus or species. In southwestern China, *Bartonella* isolates obtained from *Rattus norvegicus* and *R. tanazumi.* subsp. *flavipectus* were closely related to *B. elizabethae* or to the closely related *B. tribocorum*. In fact, isolates obtained from the *R. norvegicus* in Vietnam were identical to the type strain of *B. elizabethae* (Kosoy et al., unpublished). Subsequent studies from Bangladesh, Thailand, and Kenya showed that *B. elizabethae*-like bacteria are highly prevalent in a large portion of the local populations of *Rattus* rats. In addition to *B. elizabethae*, several more *Bartonella* species were identified, including *B. tribocorum*, *B. coopersplainensis*, *B. phoceensis*, *B. queenslandensis*, and *B. rattimassiliensis*, all of which were previously described from *Rattus* rats captured in France and Australia (Heller et al., 1998; Gundi et al., 2004 and 2009), as well as the tropics (Castle et al., 2004; Bai et al., 2007b & 2009b; Kosoy et al., 2009). These results suggested that these *Bartonella* species probably co-speciated with rats of the genus *Rattus*.

The spectrum of *Bartonella* species found in rats of the genus *Bandicota* from Bangladesh and Thailand were very similar to those of the genus *Rattus* (Bai et al., 2007b; Castle et al., 2004), demonstrating sharing of *Bartonella* strains among rodents of these species. In fact, *Bandicota* rats share the same habitat with *Rattus* rats and these rats are phylogenetically related as well. *Rattus* rats and *Bandicota* rats may play equally important roles in serving as reservoir hosts of these *Bartonella* strains.

Interestingly, in Kenya, all bartonella isolates obtained from rodents of *Mastomys natalensis* and *Lemniscomys striatus* were relatively closely related but not identical to *Bartonella tribocorum* and *B. elizabethae*. It is questionable whether these mice can also serve as reservoirs of these highly rat-associated *Bartonella* species.

Bartonella isolates obtained from rodents of other species, such as *Apodemus* mice, *Eothenomys* voles, and others, were distant from strains obtained from *Rattus* rats and *Bandicota* rats, and were classified into different phylogenetic groups of *Bartonella*. Further characterization is needed for fully description of these strains.

Rodent species	Bangladesh		China		Thailand	
	No. tested	Prevalence (%)	No. tested	Prevalence (%)	No. tested	Prevalence (%)
Apodemus chevrieri			32	62.5		
Apodemus draco			6	33.3		
Apodemus peninsulae			7	71.4		
Bandicota bengalensis	76	63.2				
Bandicota indica					46	32.6
Bandicota savilei					7	57.1
Berylmys berdmorei					1	100
Eothenomys miletus			16	18.8		
Mus caroli					3	0
Mus cervicolor					7	42.9
Mus musculus	*12*	0	1	0		
Rattus argentiventer					3	66.7
Rattus exulans					95	3.2
Rattus losea					4	0
Rattus nitidus					3	33.3
Rattus norvegicus			*7*	42.9	22	86.4
Rattus rattus	*99*	*32.3*			135	65.2
Rattus remotus					2	50
Rattus surifer					2	0
Rattus tanezumi subsp. *flavipectus*			58	41.4		
Total	187	42.8	127	44.2	330	41.5

Table 1. Bartonella in rodents from Bangladesh, China, and Thailand

2.4 Host-specificity relationships between *Bartonella* spp. and rodents

Studies from different regions of the world have shown controversial relationships between *Bartonella* species and their natural rodent hosts. A study of bartonella infection in rodents from the United Kingdoms by Birtles and his colleagues (1994), questioned host-specificity of *Bartonella* species by finding that three *Bartonella* species (*B. grahamii, B taylorii,* and *B. doshiae*) were circulating among woodland mammals of all dominant rodent species (*Apodemus sylvaticus, A. flavicollis, Myomys glareolus, Microtus agrestis* and *Neomys fodiens*). Subsequent investigations of bartonella infections in rodent communities in central Sweden reported similar results, demonstrating that *Bartonella grahamii* frequently infected *Microtus* voles (*M. glareolus*), *Apodemus* mice (*A. flavicollis*, *A. sylvaticus*) and house mice (*Mus musculus*) (Holmberg et al., 2003). By contrast, investigations from North America suggested a completely different picture of *Bartonella* species - rodent relationships from those found in Europe. In these North American studies, *Bartonella* species specific to a particular rodent species have been reported, such as those found in mice of the genus *Peromyscus*, rats of the genus *Neotoma*, chipmunks of the genus *Tamias*, ground squirrels of the genus *Spermopnilus*, prairie dogs of the genus *Cynomys*, and other rodents (Kosoy et al., 1997 & 2003; Stevenson et al., 2003; Jardine et al., 2006; Bai et al., 2008), indicating definite host-specific relationships exist between these *Bartonella* strains and their rodent hosts.

Observations of the studies of rodent-borne bartonella infections in the tropics showed some different views in regards to a relationship between *Bartonella* species and rodents. In southwestern China and Vietnam, *Barotnella* isolates obtained from *Rattus* rats were all classified as *B. elizabethae* and/or genetically very closely related to *B. tribocorum*, showing a very specific relationship (Ying et al., 2002; Kosoy et al., unpublished data); in Bangladesh and Thailand, all isolates obtained from the *Rattus* rats also fell within the cluster of *Rattus* rats-associated *Bartonella* species, including *B. elizabethae*, *B. tribocorum*, *B. coopersplainensis*, *B. phoceensis*, *B. queenslandensis*, and *B. rattimassiliensis*. However, all of these strains were also frequently harbored by *Bandicota* rats in these same regions. Sharing of the same *Bartonella* strains by rats of two genera might suggest a lower level of host-specificity in these areas or reflect a phylogenetic relatedness between rats belonging to both genera. In Kenya, *B. elizabethae*-like bartonellae were even more widely spread, being found not only in *Rattus* rats, but also in *Mastomys natalensis* and *Lemniscomys striatus*, both which are taxonomically much further from *Rattus* rats than *Bandicota* rats are from *Rattus* rats. Such results implied that *B. elizabethae* and related *Bartonella* species, as the dominant species, may have extended the range of their animal reservoir hosts because long periods of coexistence have provided numerous opportunities to infect local rodent.

2.5 *Rattus* rats as reservoir hosts of zoonotic bartonellae

Bartonella species usually do not cause diseases or pathologic changes to their natural animal hosts. However, some *Bartonella* species can become opportunistic pathogens following a host switch, such as could occur when a strain of rodent bartonella infects humans. During recent years, more and more evidence has accumulated showing that bartonella infections are indeed associated with human illnesses and can be considered as emerging infections. This has raised public health concern and drawn the attention of scientists studying zoonotic diseases. Some rodents often live with or near humans. Close contact between rodents and humans throughout the world makes the study of rodent-borne *Bartonella* essential in order to determine the extent to which rodents may serve as sources of human infections. The epidemiological importance of rodent-borne bartonellae as causes of disease in animals and humans is emerging. Rodents of some species have been found to be reservoir hosts of some *Bartonella* species that are human pathogens, such as *B. elizabethae*, *B. grahamii*, *B. vinsonii* subsp. *arupensis*, and *B. washoensis* (Daly et al., 1993; Birtles et al., 1995; Ellis et al., 1999; Kerkhoff et al., 1999; Welch et al., 1999; Kosoy et al., 2003; Iralu et al., 2006). It is likely that new rodent-borne bartonellae will be identified in the near future, and some of these possibly can be proven to be as zoonotic pathogens.

The most intriguing result of studying bartonella infection in rodents in tropics was the finding of a large number of *Bartonella* strains that are genetically related to the recognized human pathogen *B. elizabethae.* These strains widely infect rats of genera *Rattus* and *Bandicota* in Bangladesh, southwestern China, Thailand, Kenya, and other areas (Ying et al., 2002; Castle et al., 2004; Bai et al., 2007b & 2009b; Kosoy et al., 2009). *B. elizabethae* was originally isolated from the blood of a patient with endocarditis in Massachusetts, USA (Daly et al., 1993). Subsequent studies have implicated *B. elizabethae* as a cause of additional cases of endocarditis, as well as a case of Leber's neuroretinitis, and some have shown the presence of *B. elizabethae*-reactive antibodies in a high proportion of intravenous drug users (O'Halloran et al., 1998; Comer et al., 1996).

In Thailand, researchers have reported that febrile illnesses in human patients were associated with infections of several *Bartonella* species, including *B. elizabethae*, *B.*

rattimassiliensis, *B. tribocorum*, *B. vinsonii* subsp. *arupensis*, *B. tamiae*, and others (Kosoy et al., 2010). Homologous sequences comparison indicated that the *Bartonella* genotypes identified as *B. elizabethae*, *B. rattimassiliensis*, and *B. tribocorum* in the patients were completely identical or very close to *Bartonella* strains that were derived from black rats, bandicoot rats, Norway rats, and other rodents from Bangladesh, China, Thailand, and other Asian countries. These results suggested that the rodents are the potential source of the infection. Very recently, a serological survey studying source of undiagnosed febrile illness conducted in Nepal also reported antibodies specific to *B. elizabethae*, *B. tamiae*, *B. vinsonii* supsp. *arupensis*, and other *Bartonella* species (Myint et al., 2.11).

A natural reservoir for *B. elizabethae* was not implicated until 1996 when Birtles & Raoult identified a strain of *Bartonella* obtained in Peru from a *R. norvegicus* that had *gltA* and 16SrRNA gene sequences that matched the sequences for the respective genes of *B. elizabethae* (Birtles & Raoult, 1996). Numerous isolates were later obtained from *R. norvegicus* in United States and from *R. rattus* in Portugal. Genetic analyses demonstrated that these isolates formed a phylogenetic group along with the genotypes of *B. elizabethae* and the *Bartonella* strains found in rats from Peru (Ellis et al., 1999). *Rattus* rats occupy many different ecologic niches in the sites in Southeast Asia where these animals initially evolved (Eisenberg, 1981). These rats were introduced into other continents through the aid of humans and have become common and widespread in urban and rural environments in Europe, North America, and South America. The findings of *B. elizabethae* in *Rattus* rats from Peru, United States, Portugal, France, and other areas have led to the hypothesis of an Old World origin of *B. elizabethae* and related *Bartonella* bacteria (Childs, et al., 1999; Ellis et al., 1999). These bacteria could have spread from the Old World to other parts of the world through infected rats traveling by ship. Investigations conducted in southwestern China, Bangladesh, Thailand, and Kenya provided evidence in support of the Old World origin hypothesis. The finding of *B. elizabethae*-like agents in a high proportion of the rats raised potential public health concerns of humans acquiring the bartonella infection and the need to study whether these agents are responsible for cases of non-culturable bacterial endocarditis and febrile illnesses of unknown etiology in tropics.

B. vinsonii supsp. *arupensis* was first isolated from a bacteremic cattle rancher in USA (Welch et al., 1999). This bacterium is highly prevalent among deer mice (*Peromyscus maniculatus*), a strict North American rodent species, and has never been detected in any rodents from elsewhere, including tropics. However, the strain was found in stray dogs in Thailand (Bai et al., 2010). It is logical to suggest that this bacterium was acquired by dogs from wild rodents in North America and then, relocated to other continents through the translocation of infected dogs. Regardless, further investigations are needed to define the role of domestic animals as potential sources for human bartonellosis in Thailand and other tropical areas.

3. Bartonella infections in bats in tropics

Like rodents, bats (Order: Chiroptera) are another group of very abundant, diverse, and geographically dispersed vertebrates on the earth (Simmons, 2005; Calisher et al., 2006). Multiple studies have highlighted that bats may play an important role in serving as natural reservoirs to a variety of pathogens (Schneider et al., 2009). Transmission of pathogenic bat-borne viruses capable of causing disease with high human mortality has been demonstrated for a number of viruses, including rabies virus and related lyssaviruses, Nipah and Hendra

viruses, Marburg virus, Ebola viruses, and the very recently emerged inferred for SARS-CoV-like virus and other coronaviruses and others (Halpin et al., 2000; Li et al., 2005; Williams, 2005; Tang et al., 2006). The high mobility, broad distribution, social behavior (communal roosting, fission-fusion social structure) and longevity of bats make them ideal reservoir hosts and sources of infection for various etiologic agents.

There is very limited information regarding *Bartonella* infections in bats. In England, detection of *Bartonella* DNA in bats was reported recently (Concannon et al., 2005). A few studies from Egypt and United States reported presence of *Bartonella* species in ectoparasites collected from bats (Loftis et al., 2005; Reeves et al., 2005, 2006, & 2007).

From tropic areas, two studies of bartonella infections in bats were conducted in Kenya and Guatemala very recently (Kosoy et al., 2010; Bai et al., 2011). These studies brought large information regarding distribution of bartonellae in bats. Here we present the findings from these two studies. We compare the composition of bat communities, prevalence of bartonella infections in bat populations, genetic diversity of *Bartonella* strains circulating among the bat populations. We also discuss the epidemiological significance of these findings.

3.1 Bat community

Belonging to 28 species, bats collected from these two studies showed large diversity. Species composition was completely different in the two studies. Bats collected from Kenya represented 13 species of 9 genera (Table 2), including *Chaerephon* sp., *Coleura afra*, *Eidolon helvum*, *Epomophorus* spp., *Hipposideros commersoni*, *Miniopterus* spp., *Rhinolophus* spp., *Rousettus aegyptiacus*, and *Triaenops persicus*. Accounted for 32% of all bats, *Rousettus aegyptiacus* was the most prevalent species in Kenya. The other common species included *Eidolon helvum* (27%) and *Miniopterus* spp. (26). Other species only accounted for a very small portion (Table 2).

Bat species	No. tested	No. pos	Prevalence (%)
Chaerephon sp.	1	0	0
Coleura afra	9	4	44.4
Eidolon helvum	88	23	26.1
Epomophorus spp.	23	0	0
Hipposideros commersoni	4	1	25
Miniopterus spp.	87	49	56.3
Rhinolophus spp.	6	0	0
Rousettus aegyptiacus	105	22	20.9
Triaenops persicus	8	7	87.5
Total	331	106	32

Table 2. Bartonella in bats, Kenya

Bats collected from Guatemala represented 15 species of 10 genera (Table 3), including *Artibeus jamaicensis, Artibeus lituratus, Artibeus toltecus, Carollia castanea, Carollia perspicillata, Desmodus rotundus, Glossophaga soricina, Micronycteris microtis, Myotis elegans, Myotis nigricans, Phyllostomus discolor, Platyrrhinus helleri, Pteronotus davyi, Sturnira lilium*, and *Sturnira ludovici. Desmodus rotundus* comprised 26% of all bats and was the most prevalent species. *Glossophaga soricina, Carollia perspicillata, Artibeus jamaicensis*, and *Sturnira lilium* comprised 13%, 12%, 11%, and 10%, respectively, also were frequently found. The other six species comprised a smaller portion (Table 3).

3.2 Bartonella prevalence in bats

Although composition of bat species was completely different in the Kenya and Guatemala, interestingly, the overall prevalence of bartonella infection in bats was quite similar: 32% in Kenya and 33% in Guatemala. Such high prevalence may suggest persistent infection of long-lived bats with *Bartonella* species, similar to their infection with some viruses (Sulkin & Allen, 1974). Nevertheless, large variations of bartonella prevalence were observed among the bat specie. *Bartonella* species exhibit high, low, or no infectivity depending on the bat species. In Kenya, the bartonella prevalence was 88%, 56%, 44%, 26%, 25%, and 21% for *Triaenops persicus* bats, *Miniopterus* spp. bats, *Coleura afra* bats, *Eidolon helvum* bats, *Hipposideros commersoni* bats, and *Rousettus aegyptiacus* bats, respectively. In Guatemala, *Phyllostomus discolor* bats, *Pteronotus davyi* bats, and *Desmodus rotundus* bats were highly infected with *Bartonella* species, with prevalence of 89%, 70%, and 48% in each, respectively. Bartonella prevalence was relatively low in *Sturnira lilium* bats (8%) and *Glossophaga soricina* bats (13%), and no bartonellae were discovered in some bat species, such as *Epomophorus* spp., *Rhinolophus* spp., and *Artibeus jamaicensis* (Table 2 & Table 3).

3.3 Bartonella genetic heterogeneity and relationships with bat species

Genetic analyses of a portion of citrate synthase gene (*glt*A) demonstrated that the *Bartonella* strains obtained from bats in both Kenya and Guatemala represent a variety of distinct phylogroups, including 11 from Kenya and 13 from Guatemala. Further characterization is necessary to verify whether the *Bartonella* strains represent novel *Bartonella* species.

In Kenya, a definite host-specificity was observed for *Bartonella* strains in bat species. All *Bartonella* isolates obtained from *Rousettus aegyptiacus* bats are similar to each other (>96%) and clustered in a monophyletic genogroup that is distant from all other *Bartonella* species; similarly, *Bartonella* cultures obtained from *Coleura afra* bats, *Triaenops persicus* bats also clearly belonged to the specific *Bartonella* species group found exclusively in the particular bat species. By contrast, *Bartonella* cultures obtained from *Eidolon helvum* bats and *Miniopterus* bats showed great variation, clustering into three or four clades, each representing a distinct *Bartonella* phylogroup. Nevertheless, all strains of *Bartonella* species recovered from *Eidolon helvum* bats were typical for this species of bats only. Similarly, the *gltA* sequences from all strains obtained from *Miniopterus* spp. bats have not been found in bats of other bat genera.

Unlike the discovery in bats in Kenya, host specificity of *Bartonella* species was not found in bats in Guatemala. In some instances, bats of two or more species may share the same *Bartonella* strains. For example, one *Bartonella* strain recovered in *Desmodus rotundus* bats was also found in *Carollia perspicillata* bats. Similarly, same *Bartonella* strain was found in both *Glossophaga soricina* bats and *Pteronotus davyi* bats, or both *Carollia perspicillata* bats and *Phyllostomus discolor* bats. On the other hand, co-infection with multiple *Bartonella* strains in the same bat species was observed. For example, *Desmodus rotundus* bats and *Carollia perspicillata* bats each were infected with two *Bartonella* strains; while *Pteronotus davyi* bats and *Phyllostomus discolor* bats were infected with four *Bartonella* strains, respectively. The tendency of some bat species to share roosts, reach large population densities, and roost crowded together creates the potential for dynamic intraspecies and interspecies transmission of infections (Streicker et al., 2010). The observations in the Guatemala study suggested active interspecies transmission of *Bartonella* species likely occurs among bats in Guatemala, which may have contributed to the lack of host-specificity. Arthropod vectors that parasitize bats may also be partly associated with none host-specificity.

Bat species	No. cultured	No. positive	Prevalence (%)
Artibeus jamaicensis	13	0	0
Artibeus lituratus	3	0	0
Artibeus toltecus	1	1	100
Carollia castanea	1	0	0
Carollia perspicillata	14	4	28.6
Desmodus rotundus	31	15	48.4
Glossophaga soricina	15	2	13.3
Micronycteris microtis	3	1	33.3
Myotis elegans	2	0	0
Myotis nigricans	1	0	0
Phyllostomus discolor	9	8	88.9
Platyrrhinus helleri	1	0	0
Pteronotus davyi	10	7	70
Sturnira lilium	12	1	8.3
Sturnira ludovici	2	0	0
Total	118	39	33.1

Table 3. Bartonella in bats, Guatemala

3.4 Epidemiology significance

Bartonellae were virtually unrecognized as pathogens of humans until 1990s. Identifications of bartonellae as agents of cat-scratch disease, bacillary angiomatosis, urban trench fever, and recent outbreaks of Carrión's disease have left no doubt about the emerging medical importance of these bacteria. Within the last two decades, new bacteria of the genus of *Bartonella* were isolated from large number of several mammalian reservoirs, including rodents, cats, dogs, and rabbits, and recognized as emerging zoonotic agents. At least 13 *Bartonella* species or subspecies have been recognized as emerging human pathogens or zoonotic agents, causing a wide range of syndromes, from a self-limiting to life-threaten endocarditis, myocarditis, and meningoencephalitis. All of these emphasize the concept that inadvertent transmission of known or currently uncharacterized *Bartonella* spp. from both wild animals and domestic animals occurs in nature.

Although evidence of overt disease in bats caused by *Bartonella* species has not been demonstrated to date, high incidence of bartonella infection in bats from the studies carried out in Guatemala, Kenya, and other regions suggested that bats may be natural reservoirs in maintaining circulation of *Bartonella* species in nature. Bats have very long life spans compared to other mammals of similar body size, such as rodents. This may make them serve as reservoirs contributing to the maintenance and transmission of *Bartonella* to other animals and/or humans. Some bat species have been known to directly transmit infections to humans. For example, the common vampire bat (*Desmodus rotundus*) has been long recognized to transmit rabies virus to humans by biting throughout Latin America (Schneider et al., 2009). These bats typically feed on the blood of mammals, including domestic animals, such as cattle, horses, pigs, dogs, but also feed on the blood of humans (Turner & Bateson, 1975). Predation of vampire bats on humans is a major problem in Latin America (Schneider et al., 2009). If *Bartonella* species can be transmitted to humans through the bite of bats, the need for further studies with vampire bats is imperative. Findings of bartonella in bats highlight the need to study whether the bat-originated *Bartonella* species

are responsible for the etiology of local undiagnosed illnesses in humans and domestic animals in tropics.

In addition to the large number of documented reservoir hosts, an increasing number of arthropos vectors, includingbiting flies, fleas, keds, lice, sandflies, and ticks have been confirmed or suspected to be associated with the transmission of *Barotellla* spp. among animal populations (Billeter et al., 2008). *Bartonella* species–specific DNA has been detected in ectoparasites collected from bats (Loftis et al., 2005; Reeves et al., 2005 & 2007). Presumably, if *Bartonella* species are transmitted through a bat ectoparasite vector, some, if not all, bat-associated *Bartonella* species could be transmitted to humans because bats are frequent hosts to a wide variety of ectoparasites, including bat flies, fleas, soft ticks, and mites.

Very recently, two novel *Bartonella* species, *B. tamiae*, isolated from febrile Thai patients (Kosoy et al., in press), and *B. rochalimae*, isolated from an American patient who traveled in Peru and developed fever and splenomegaly after return (Eremeeva et al., 2007). However, the reservoir remains unknown as do the mode of transmission, pathogenesis, and many other characteristics of these organisms. There is the need to identify the animal reservoirs of these novel *Bartonella* species and to understand their disease ecology. These studies of *Bartonella* species in bats have enlarged the scope of this zoonotic potential as we search for the reservoirs that harbor novel and known *Bartonella* species.

4. References

Anderson, B. & Neuman, M. (1997). *Bartonella* spp. as emerging human pathogens. Clin. Microbiol. Rev. 10:203-219.

Bai, Y., Kosoy M. Y., Cully, J. F., Bala, T., Ray, C., & Collinge, S. K. (2007a). Acquisition of nonspecific Bartonella strains by the northern grasshopper mouse (*Onychomys leucogaster*). FEMS Microbiol. Ecol. 61:438-448.

Bai, Y., Montgomery, S. P., Sheff, K. W., Chowdhury, M. A., Breiman, R. F., Kabeya, H., et al. (2007b.) *Bartonella* strains in small mammals from Dhaka, Bangladesh, related to *Bartonella* in America and Europe. Am. J. Trop. Med. Hyg. 77:567-570.

Bai, Y., Kosoy, M. Y., Martin, A., Ray, C., Sheff, K., Chalcraft, L., et al. (2008). Characterization of *Bartonella* strains isolated from black-tailed prairie dogs (*Cynomys ludovicianus*). Vector Borne Zoonotic Dis. 8:1-5.

Bai, Y., Kosoy, M. Y., Calisher, C. H., Cully, J. F. Jr, & Collinge, S. K. (2009a). Effects of rodent community diversity and composition on prevalence of an endemic bacterial pathogen – Bartonella. Biodiversity. 10:3-11.

Bai, Y., Kosoy, M. Y., Lerdthusnee, K., Peruski, L. F., & Richardson, J. H. (2009b). Prevalence and Genetic Heterogeneity of *Bartonella* Strains Cultured from Rodents from 17 Provinces in Thailand. Am. J. Trop. Med. Hyg. 81:811-816.

Bai, Y., Calisher, C., Kosoy, M., Root, J., & Doty, J. (2011). Persistent Infection or Successive Reinfection of Deer Mice with *Bartonella vinsonii* subsp. *arupensis*. Appl. Environ. Microbiol. 77:1728-1731. doi:10.1128/AEM.02203-10.

Bai, Y., Kosoy, M. Y., Boonmar, S., Sawatwong, P., Sangmaneedet, S., & Peruski, L. F. (2010). Enrichment culture and molecular identification of diverse *Bartonella* species in stray dogs. Vet. Microbiol. 146:314-319.

Bai, Y., Kosoy, M., Recuenco, S., Alvarez, D., Moran, D., Turmelle, A., et al. (2011). *Bartonella* spp. in Bats, Guatemala. Emerg. Infects. Dis. 17:1269-1271.

Bajer, A., Pawelczyk, A., Behnke, J. M., Gilbert, F. S., & Sinski, F. (2001). Factors affecting the component community structure of haemoparasites in bank voles (*Clethrionomys glareolus*) from the Mazury Lake District region of Poland. Parasitology 122:43-54.

Baker, J. A. 1946. A rickettsial infection in Canadian voles. J. Exp. Med. 84:37- 51.

Bermond, D., Heller, R., Barrat, F., Delacour, G., Dehio, C., Alliot, A., et al. (2000). *Bartonella birtlesii* sp. nov., isolated from small mammals (*Apodemus* spp.). Int. J. Syst. Evol. Microbiol. 50:1973-1979.

Billeter, S. A., Levy, M. G., Chomel, B. B., & Breitschwerdt, E. B. (2008). Vector transmission of *Bartonella* species with emphasis on the potential for tick transmission. Med. Vet. Entomol. 22:1-15.

Birtles, R. J., Harrison, T. G., & Molyneux, D. H. (1994). *Grahamella* in small woodland mammals in the UK - isolation, prevalence and host-specificity. Ann. Trop. Med. Parasitol. 88:317-327.

Birtles, R. J., Harrison, T. G., Saunders, N. A., & Molyneux, D. H. (1995). Proposals to unify the genera *Grahamella* and *Bartonella,* with descriptions of *Bartonella talpae* comb. nov., *Bartonella peromysci* comb. nov., and three new species, *Bartonella grahamii* sp. nov., *Bartonella taylorii* sp. nov., and *Bartonella doshiae* sp. nov. Int. J. Syst. Bacteriol. 45:1-8.

Birtles, R. J., & D. Raoult. (1996). Comparison of partial citrate synthase gene *(gltA)* sequences for phylogenetic analysis of *Bartonella* species. Int. J. Syst. Bacteriol. 46: 891-897.

Boulouis, H. J., C. C. Chang, J. B. Henn, R. W. Kasten, et al. 2005. Factors associated with the rapid emergence of zoonotic Bartonella infections. Vet. Res. 36:383-410.

Bown, K. J., Ellis, B. A., Birtles, R. J., Durden, L. A., Lelllo, J., Begon, M., et al. (2002). New world origins for haemoparasites infecting United Kingdom grey squirrels (*Sciurus carolinensis*), as revealed by phylogenetic analysis of bartonella infecting squirrel populations in England and the United States. Epidemiol. Infect. 129:647-653.

Calisher, C. H., Childs, J. E., Field, H. E., Holmes, K. V., & Schountz, T. (2006).Bats: important reservoir hosts of emerging viruses. Clin. Microbiol. Rev.19:531–545. doi:10.1128/CMR.00017-06.

Castle, K., Kosoy, M., Lerdthusnee, K., Phelan, L., Bai, Y., Gage, K. L., et al. (2004). Prevalence and diversity of *Bartonella* in rodents of Northern Thailand: A comparison with *Bartonella* in rodents from southern China. Am. J. Trop. Med. Hyg. 70:429-433.

Childs, J. E., Ellis, B. A., Nicholson, W. L., Kosoy, M. Y., & Sumner, J. W. (1999). Shared vector-borne zoonoses of the Old World and New World: home grown or translocated? Schweiz Med. Wochenschr.129:1099-1105.

Chomel, B. B., Abbott, R. C., Kasten, R. W., Floyd-Hawkins, K. A., Kass, P. H., Glaser, C. A., et al. (1995). *Bartonella henselae* prevalence in domestic cats in California: Risk factors and association between bacteremia and antibody titers. J. Clin. Microbiol. 33:2445-2450.

Chomel, B. B., Kasten, R. W., Floyd-Hawkins, K. A., Chi, B., Yamamoto, K., Roberts-Wilson, J., et al. (1996) Experimental transmission of *Bartonella henselae* by the cat flea. J. Clin. Microbiol. 34:1952-1956.

Comer, J. A., Flynn, C., Regnery, R. L., Vlahov, D., & Childs, J. E. (1996). Antibodies to *Bartonella* species in inner-city intravenous drug users in Baltimore, MD. Arch. Intern. Med. 156:2491-2495.

Concannon, R., Wynn-Owen, K., Simpson, V. R., & Birtles, R. J. (2005). Molecular characterisation of haemoparasites infecting bats (Microchiroptera) in Cornwall, United Kingdom. Parasitology. 131:489-496.

Daly, J. S., Worthington, M. G., Brenner, D. J., Moss, C. W., Hollis, D. G., Weyant, R. S., et al. (1993). *Rochalimaea elizabethae* sp. nov. isolated from a patient with endocarditis. J. Clin. Microbiol. 31: 872–881.

Eisenberg, J. F., (1981). The Mammalian Radiations: An Analysis of Trends in Evolution, Adaptation, and Behavior. Chicago: The University of Chicago Press.

Ellis, B. A., Regnery, R. L., Beati, L., Bacellar, F., Rood, M., Glass, G. G., et al. (1999). Rats of the genus *Rattus* are reservoir hosts for pathogenic *Bartonella* species: an Old World origin for a New World disease? J. Infect. Dis.180: 220-224.

Engbaek, K., & Lawson, P. A. (2004). Identification of *Bartonella* species in rodents, shrews and cats in Denmark: detection of two *B. henselae* variants, one in cats and the other in the long-tailed field mouse. APMIS. 112:336-341.

Eremeeva, M. E., Gerns, H. L., Lydy, S. L., Goo, J. S., Ryan, E. T., Mathew, S. S., et al. (2007). Bacteremia, fever, and splenomegaly caused by a newly recognized *Bartonella* species. N. Engl. J. Med. 356:2381-2387.

Fichet-Calvet, E., Jomaa, I., Ben Ismail, R., & Ashford, R. W. (2000). Pattern of infection of haemoparasites in the fat sand rat, *Psammomys obesus,* in Tunisia, and effect on the host. Ann. Trop. Med. Parasitol. 94:55-68.

Garcia-Caceres, U., & Garcia, F. U. (1991). Bartonellosis: an immunodepressive disease and the life of Daniel Alcides Carrion. J. Clin. Pathol .95 (Suppl): 58-66.

Gundi, V. A., Davoust, B., Khamis, A., Boni, M., Raoult, D., & La Scola, B. (2004). Isolation of *Bartonella rattimassiliensis* sp. nov. and *Bartonella phoceensis* sp. nov. from European Rattus norvegicus. J. Clin. Microbiol.42:3816-3818.

Gundi, V. A., Taylor, C., Raoult, D., & La Scola, B. (2009). *Bartonella rattaustraliani* sp. nov., *Bartonella queenslandensis* sp. nov. and *Bartonella coopersplainsensis* sp. nov., identified in Australian rats. Int. J. Syst. Evol. Microbiol. 59:2956-2961.

Halpin, K., Young, P. L., Field, H. E., & Mackenzie, J. S. (2000). Isolation of Hendra virus from pteropid bats: a natural reservoir of Hendra virus. J. Gen. Virol. 81:1927-1932.

Heller, R., Riegler, P., Hansmann, Y., Delacour, G., Bermound, D., Dehio, C., et al. (1998). *Bartonella tribocorum* sp. nov., a new *Bartonella* species isolated from the blood of wild rats. Int. J. Syst. Bacteriol. 48: 1333-1339.

Higgins, J. A., Radulovic, S., Jaworski, D. C., & Azad, A. F. (1996). Acquisition of the cat scratch disease agent *Bartonella henselae* by cat fleas (Siphonaptera: Pulicidae). J. Med. Entomol. 33:490-495.

Hofmeister, E. K., Kolbert, C. P., Abdulkarim, A. S., Magera, J. M., Hopkins, M. K., Uhl, J. R., et al. (1998). Cosegregation of a novel *Bartonella* species with *Borrelia burgdorferi* and *Babesia microti* in *Peromyscus leucopus*. J. Infect. Dis. 177:409-416.

Holmberg, M., Mills, J. N., McGill, S., Benjamin, G., & Ellis, B. A. (2003). *Bartonella* infection in sylvatic small mammals of central Sweden. Epidemiol Infect 130:149-157.

Iralu, J., Bai, Y., Crook, L., Tempest, B., Simpson, G., Mckenzie, T., et al. (2006). Rodent-associated bartonella febrile illness, southwestern United States. Emerg. Infect. Dis. 12:1081-1086.

Jardine, C., Appleyard, G., Kosoy, M. Y., McColl, D., Chirino-Trejo, M., Wobeser, G., et al. (2005). Rodent-associated *Bartonella* in Saskatchewan, Canada. Vector Borne Zoonotic Dis. 5:402-409.

Jardine, C., McColl, D., Wobeser, G., & Leighton, F. A. (2006). Diversity of *Bartonella* genotypes in Richardson's ground squirrel populations. Vector Borne Zoonotic Dis. 6:395-403.

Kerkhoff, F.T., Bergmans, A. M., van Der Zee, A., & Rothova, A. (1999). Demonstration of *Bartonella grahamii* DNA in ocular fluids of a patient with neuroretinitis. J. Clin. Microbiol. 37: 4034-4038.

Kim, C. M., Kim, J. Y., Yi, Y. H., Lee, M. J., Cho, M. R., Shah, D. H., et al. (2005). Detection of *Bartonella* species from ticks, mites and small mammals in Korea. J. Vet. Sci. 6:327-334.

Knap, N., Duh, D., Birtles, R., Trilar, T., Petrovec, M., & Avsic-Zupanc, T. (2007). Molecular detection of *Bartonella* species infecting rodents in Slovenia. FEMS Immunol. Med. Microbiol. 50:45-50.

Koehler, J. E. (1996). Bartonella infections. Adv. Pediatr. Infect. Dis. 11:1-27.

Kordick, D. L., Hilyard, E. J., Hadfield, T. L., Wilson, K. H., Steigerwalt, A. G., Brenner, D. J., et al. (1997). *Bartonella clarridgeiae*, a newly recognized zoonotic pathogen causing inoculation papules, fever, and lymphadenopathy (cat scratch disease). J. Clin. Microbiol. 35:1813-1818.

Kosoy, M. Y., Regnery, R. L., Tzianabos, T., Marston, E. L., Jones, D. C., Green, D., et al. (1997). Distribution, diversity, and host specificity of *Bartonella* in rodents from the Southeastern United States. Am. J. Trop. Med. Hyg. 57:578-588.

Kosoy, M., Murray, Gilmore, M., R., Bai, Y., & Gage, K. (2003). *Bartonella* strains obtained from ground squirrels in Nevada are identical by sequencing of three genes to *Bartonella washoensis* isolated from a patient with cardiac disease. J. Clin. Microbiol. 41: 645-650.

Kosoy, M., Morway, C., Sheff, K. W., Bai, Y., Colborn, J., Chalcraft, L., et al., (2008). *Bartonella tamiae* sp. nov., a Newly recognized pathogen isolated from three human patients from Thailand. J. Clin. Microbiol. 46:772-775.

Kosoy, M., Iverson. J., Bai. Y., Knobel. D., Halliday J., Agwanda B., et al. (2009). Identification of *Bartonella* species in small mammals from urban and sylvatic foci in Kenya. The International Conference on Emerging Infectious Diseases.

Kosoy M., Bai, Y., Sheff, K., Morway, C., Baggett, H., Maloney S. A., et al. (2010). Identification of *Bartonella* Infections in Febrile Human Patients from Thailand and Their Potential Animal Reservoirs. Am. J. Trop. Med. Hyg. 82:1140-1145.

Kosoy, M., Bai, Y., Lunch, T., Kuzmin, I., Niezgoda, M., Franka, R., et al. (2010). *Bartonella* spp. in bats, Kenya. Emerg. Infects. Dis. 16:1875-1881.

Laakkonen, J., Haukisalmi ,V., & Merritt. J. F. (1998). Blood parasites of shrews from Pennsylvania. J. Parasitol. 84:1300-1303.

Li, W., Shi, Z., Yu, M., Ren, W., Smith, C., Epstein, J. H., et al. (2005). Bats are natural reservoirs of SARS-like coronaviruses. Science. 310:676-679.

Loftis, A. D., Gill, J. S., Schriefer, M. E., Levin, M. L., Eremeeva, M. E., Gilchrist, M. J., et al. (2005). Detection of *Rickettsia*, *Borrelia*, and *Bartonella* in *Carios kelleyi* (Acari: Aragasidae). J. Med. Entomol. 42:473-480.

Maggi, R. G., Harms, C. A., Hohn, A. A., Pabst, D. A., McLellan, W. A,, Walton, W.J., et al. (2005). *Bartonella henselae* in porpoise blood. Emerg. Infect. Dis. 11:1894-1898.

Margileth, A. M., & Baehren, D. F. (1998). Chest-wall abscess due to cat-scratch disease (CSD) in an adult with antibodies to *Bartonella clarridgeiae*: case report and review of the thoracopulmonary manifestations of CSD. Clin. Infect. Dis. 27:353-357.

Markov, A. P., Lopyrev, I. V., Irkhin, A. I., Khliap, L. A., Levitskii, S. A., Kinillov, M., et al. (2006). Wild small mammals are the reservoir hosts of the *Bartonella* genus bacteria in the south of Moscow region. Mol. Gen. Mikrobiol. Virusol. 4:8-13.

Myint, K. S. A., Gibbons, R. V., Iverson, J., Shrestha, S. K., Pavlin, J. A., Mongkolsirichaikul, D., et al. Serological response to *Bartonella* species in febrile patients from Nepal. Trans. R. Soc. Trop. Med. Hyg. *In press.*

O'Halloran, H. S., Draud, K., Minix, M., Rivard, A. K., & Pearson, P. A. (1998). Leber's neuroretinitis in a atient with serologic evidence of *Bartonella elizabethae.* Retina 18: 276–278.

Pappalardo, B. L., Correa, M. T., York, C. C., Peat, C. Y., & Breitschwerdt, E. B. (1997). Epidemiologic evaluation of the risk factors associated with exposure and seroreactivity to *Bartonella vinsonii* in dogs. Am. J. Vet. Res. *58*:467-471.

Pawelczyk, A., Bajer, A., Behnke, J. M., Gilbert, F. S., & Sinski, E. (2004). Factors affecting the component community structure of haemoparasites in common voles (*Microtus arvalis*) from the Mazury Lake District region of Poland. Parasitol. Res. *92*:270-284.

Pretorius, A. M., Beati, L., & Birtles, R. J. (2004). Diversity of bartonellae associated with small mammals inhabiting Free State Province, South Africa. Int. J. Syst. Evol. Microbiol .54:1959-1967.

Reeves, W. K., Loftis, A. D., Gore, J. A., & Dasch, G. A. (2005). Molecular evidence for novel *Bartonella* species in *Trichobius major* (Diptera: Streblidae) and *Cimex adjunctus* (Hemiptera: Cimicidae) from two southeastern bat caves, U.S.A. J. Vector. Ecol. 30:339-341.

Reeves, W. K., Streicker, D. G., Loftis, A. D., &Dasch, G. A. (2006). Serologic survey of *Eptesicus fuscus* from Georgia, U.S.A. for *Rickettsia* and *Borrelia* and laboratory transmission of a *Rickettsia* by bat ticks. J. Vector. Ecol. 31:386-389.

Reeves, W. K., Rogers, T. E., Durden, L. A., & Dasch, G. A. (2007). Association of *Bartonella* with the fleas (Siphonaptera) of rodents and bats using molecular techniques. J. Vector. Ecol. 32:118-122.

Roux, V., & D. Raoult. (1999). Body lice as tools for diagnosis and surveillance of reemerging diseases. J. Clin. Microbiol. 37:596-599.

Roux, V., Eykyn, S. J., Wyllie, S., & Raoult, D. (2000). *Bartonella vinsonii* subsp. *berkhoffii* as an agent of afebrile blood culture-negative endocarditis in a human. J. Clin. Microbiol. 38:1698-1700.

Raoult, D., Roblot, F., Rolain, J. M., Besnier, J. M., Loulergue, J., Bastides, F., et al. (2006). First isolation of *Bartonella alsatica* from a valve of a patient with endocarditis. J. Clin. Microbiol. 44:278-279.

Sander, A., Zagrosek, A., Bredt, W., Schiltz, E., Piemont, Y., Lanz, C., et al. (2000). Characterization of *Bartonella clarridgeiae* flagellin (FlaA) and detection of

antiflagellin antibodies in patients with lymphadenopathy. J. Clin. Microbiol. 38:2943-2948.

Schneider, M. C., Romijn, P. C., Uieda, W., Tamayo, H., da Silva, D. F., Belotto, A., et al. (2009). Rabies transmitted by vampire bats to humans: an emerging zoonotic disease in Latin America? Rev. Panam. Salud. Publica. 25:260–269. doi:10.1590/S1020-49892009000300010

Schülein, R., Seubert, A., Gille, C., Lanz, C., Hansmann, Y., Piemont, Y., et al. (2001). Invasion and persistent intracellular colonization of erythrocytes: a unique parasitic strategy of the emerging pathogen bartonella. J. Exp. Med. 193:1077-1086.

Simmons, N. B. (2005). Order Chiroptera, p. 312-529. *In* D. E. Wilson and D. M. Reeder (ed.), Mammal species of the world: a taxonomic and geographic reference, 3rd ed. Johns Hopkins University Press, Baltimore, Md.

Stevenson, H., Bai, Y., Kosoy, M., Montenieri, J., Lowell, J., Chu, M., et al. (2003). Detection of novel *Bartonella* strains and *Yersinia pestis* in prairie dogs and their fleas (*Siphonaptera:* Ceratophyllidae and *Pulicidae*) using multiplex PCR. J. Med. Entomol. 40:329-337.

Streicker, D. G., Turmelle, A. S., Vonhof, M. J., Kuzmin, I. V., McCracken, G. F., & Rupprecht, C. E. (2010). Host phylogeny constrains cross-species emergence and establishment of rabies virus in bats. Science. 329:676–379. doi:10.1126/science.1188836

Sulkin, S. E., & Allen, R. (1974). Virus infections in bats. Monogr. Virol.8:1–103.

Tang, X. C., Zhang, J. X., Zhang, S. Y., Wang, P., Fan, X. H., Li, L. et al. (2006). Prevalence and genetic diversity of coronaviruses in bats from China. J. Virol. 80:7481-7490.

Tea, A., Alexiou-Daniel, S., Papoutsi, A., Papa, A., & Antoniadis, A. (2004). *Bartonella* species isolated from rodents, Greece. Emerg. Infect. Dis. 10: 963-964.

Telfer, S., Bown, K. J., Sekules, R., Begon, M., Hayden, T., & Birtles, R. (2005). Disruption of a host-parasite system following the introduction of an exotic host species. Parasitology. 130:661-668.

Turner, D. C., & Bateson, P. (1975).The vampire bat: a fi eld study in behavior and ecology. Baltimore: Johns Hopkins University Press; p. 1–7.

Welch, D. F., Carroll, K. C., Hofmeister, E. K., Persing, D. H., Robison, D. A., Steigerwalt, A. G., et al. (1999). Isolation of a new subspecies, *Bartonella vinsonii* subsp. *arupensis* , from a cattle rancher: identity with isolates found in conjunction with *Borrelia burgdorferi* and *Babesia microti* among naturally infected mice. J. Clin. Microbiol. 37: 2598-2601.

Weiss, E., & Dasch, G. (1982). Differential characteristics of strains of *Rochalimae: Rochalimae vinsonii* sp. nov., the Canadian vole agent. Int. J. Syst. Bacteriol. 32: 305-314.

Williams, C. J. 2005. Bats as the reservoir for outbreaks of emerging infectious diseases. Erup. Serveill. 10:E051110.4.

Winoto, I. L., Goethert, H., Ibrahim, I. N., Yuniherlina, I., Stoops, C., Susanti, I., et al. (2005). *Bartonella* species in rodents and shrews in the greater Jakarta area. Southeast Asian J. Trop. Med. Public Health 36:1523-1529.

Ying, B., Kosoy, M., Maupin, G., Tsuchiya, K., & Gage, K. (2002). Genetic and ecological characteristics of *Bartonella* communities in rodents in southern China. Am. J. Trop. Med. Hyg. 66:622-627.

5

Social Networking in Tuberculosis: Experience in Colombia

Diana M. Castañeda-Hernández and Alfonso J. Rodriguez-Morales
[1]*Tuberculosis Control Program, Health and Social Security Secretary, Pereira and Fundación Universitaria del Área Andina, Pereira*
[2]*Instituto José Witremundo Torrealba, Universidad de Los Andes, Trujillo; Faculty of Health Sciences, Universidad Tecnológica de Pereira, Pereira and Office of Scientific Research, Cooperativa de Entidades de Salud de Risaralda (COODESURIS), Pereira*
[1,2]*Colombia*
[2]*Venezuela*

1. Introduction

Tuberculosis (TB) is an infectious disease caused by different species of *Mycobacteria*. Human disease is usually caused by *Mycobacterium tuberculosis*, also know as the Koch's bacilli, which can affect any organ or tissue in the body. Although this, pulmonary disease, with their particular hallmarks such as occurrence of cough with expectoration lasting more than 15 days, is the main corporal area affected by this mainly tropical pathogen (Rodríguez-Morales et al. 2008). In such cases, previous to a microbiological diagnosis, individuals in such state are so-called respiratory symptomatic.

Besides those symptoms/signs, disease can be manifested with hemoptisis, fever, night sweating, general malaise, thoracic pain, anorexia and weight lost. This disease is still a significant public heal problem due to is highly transmissibility, but is highly potentially preventable and treatable condition (Curto et al. 2010, Dim et al. 2011, Orcau et al. 2011, Marais & Schaaf 2010, Glaziou et al. 2009). Even more, in the context of HIV and newer immunosuppressive conditions mycobacterial diseases emerge as public health threat in the World (Vargas et al. 2005).

According to the World Health Organization (WHO), in 2010, there were 8.8 million (range, 8.5–9.2 million) incident cases of TB, 1.1 million (range, 0.9–1.2 million) deaths from TB among HIV-negative people and an additional 0.35 million (range, 0.32–0.39 million) deaths from HIV-associated TB. Important new findings at the global level are: a) the absolute number of TB cases has been falling since 2006 (rather than rising slowly as indicated in previous global reports); b) TB incidence rates have been falling since 2002 (two years earlier than previously suggested); c) Estimates of the number of deaths from TB each year have been revised downwards; d) In 2009 there were almost 10 million children who were orphans as a result of parental deaths caused by TB (World Health Organization 2011).

Beyond its epidemiology, particularly mostly due to pulmonary disease, other important forms of disease represent also a significant burden in thee World. When the infection affects organ other than the lung is called extrapulmonary TB. The most common form of this disease is at the pleura, followed by the lymphatic nodes. Extrapulmonary TB includes

various manifestations according to the affected organ. Prognosis and time to develop disease also can vary according to the affected organ.

Disease can ranges a spectrum that can begin from a latent infection or reactivation slowly evolving into a focal or whole spread and involvement of multiple organs, which makes it difficult to diagnosis by clinicians and health care workers, who many times could not identify it timely (Castañeda-Hernández et al. 2012a). One of the most severe forms of extrapulmonary TB is the meningitis (TB meningitis), which occurs as a result of hematogenous spread of bacilli into the subarachnoid space. This is known as a complication of primary TB and may occur years later as an endogenous reactivation of a latent tuberculosis or as a result of exogenous reinfection (Glaziou et al. 2009, Hoek et al. 2011, Galimi 2011, Garcia-Rodriguez et al. 2011).

Tuberculosis is a complex disease in terms of the multiple factors that are involved in its occurrence and persistence in the human societies. In first place there are factors associated with the bacillus (viability, transmissibility and virulence), with the host as a biological individual (immune status, genetic susceptibility, duration and intensity of exposure) as well, at the bacillus-host interaction (place of affection, severity of illness).

At a second, clinical level, the occurrence of pulmonary tuberculosis undiagnosed or untreated, overcrowding, malnutrition, immunosuppresion from any cause (HIV infection, use of immunosuppressive drugs, diabetes, cancer, chronic renal failure, silicosis, alcoholism and drug addiction), are also important factors.

At community public health interventional level, protective factors include the BCG (Bacille Calmette Guerin) vaccine, applied in developing countries, which provides protection before exposure and prevent severe infection forms, especially in infants and young children, reaching up to 80% of protection against the development of forms of the disease such as meningeal and miliary TB (Garcia-Rodriguez et al. 2011, Garg 2010, Black et al. 2003, Francis et al. 2002, Arbelaez et al. 2000, Ginsberg 2000).

Additional to those clinical implications, changes in the susceptibility of the etiological agent to the therapy used drugs has imposed more challenges in the management of TB. The magnitude of problem with TB now lies in the fact that one third of the world population is infected by *Mycobacterium tuberculosis*. Even in the 21st century, TB kills more people than any other infective agent. This, then, occurs in part as a result of a progressive decrease in its susceptibility to anti-TB drugs or resistance emergence. Cases of resistant TB, defined by the recommendations of the World Health Organization (WHO) as primary, initial, acquired multidrug resistant (MDR-TB) or extensively drug resistant TB (XDR-TB) are emerging in different areas of the World.

The development of resistance TB may result from the administration of mono-therapy or inadequate combinations of anti-TB drugs. A possible role of health care workers in the development of multi drug-resistant TB is very important. Actually, multi drug-resistant TB is a direct consequence of mistakes in prescribing chemotherapy, provision of anti-tuberculosis drugs, surveillance of the patient and decision-making regarding further treatment as well as in a wrong way of administration of anti-TB drugs. The problem of XDR-TB in the world has become very alarming. Only adequate treatment according to directly supervised short regiment for correctly categorized cases of TB can stop the escalation of MDR-TB or XDR-TB, which is actually, in large magnitude, a global threat in the 21st century (Torres et al. 2011, Solari et al. 2011, Chadha et al. 2011, Arenas-Suarez et al. 2010, Ferro et al. 2011, Martins 2011).

Another important issue in TB is the social component, related to a complex background and multiple interacting factors that internally and externally affect individuals affected by

the disease, which still represents a significant stigma in many communities in the World. Given this setting, TB approach is complex and requires not only medical but also psychological and especially sociological approaches in order to improve its management from a collective medicine perspective as well better acceptability by non-affected people surrounding infected individuals at their communities or neighborhoods. In this way, programs approaching taking all these considerations in count will benefit with better strategies that allow good interactions between social actors involve in the complex social matrix in which sometimes TB can be present at societies. Taking advantage from this, regular activities, such as proper diagnosis and treatment would be achieve in a more efficient way (Murray et al. 2011, Santin & Navas 2011, Juniarti & Evans 2011).
This chapter will cover how using social networks in the context of tuberculosis control program would achieve a better management of cases at individual and at a collective level in a western area of Colombia, where TB is a highly prevalent condition and where available resources for disease management and program are still limited in multiple aspects.

2. Social networking

Human societies can be regarded as large numbers of locally interacting agents, connected by a broad range of social and economic relationships. These relational ties are highly diverse in nature and can represent, e.g., the feeling a person has for another (friendship, enmity, love), communication, exchange of goods (trade), or behavioral interactions (cooperation or punishment). Each type of relation spans a social network of its own. A systemic understanding of a whole society can only be achieved by understanding these individual networks and how they influence and co-construct each other. The shape of one network influences the topologies of the others, as networks of one type may act as a constraint, an inhibitor, or a catalyst on networks of another type of relation.
For instance, the network of communications poses constraints on the network of friendships, trading networks are usually constrained to positively connoted interactions such as trust, and networks representing hostile actions may serve as a catalyst for the network of punishments. A society is therefore characterized by the superposition of its constitutive socioeconomic networks, all defined on the same set of nodes. This superposition is usually called multiplex, multirelational, multimodal, or multivariate network (Szell et al. 2010). Summarizing, a social network is a social structure made up of individuals (or organizations) called "nodes", which are tied (connected) by one or more specific types of interdependency, such as friendship, kinship, common interest, financial exchange, dislike, sexual relationships, or relationships of beliefs, knowledge or prestige (Palinkas et al. 2011, Szell et al. 2010).
Understanding and modeling network structures have been a focus of attention in a number of diverse fields, including physics, biology, computer science, statistics, and social sciences. Applications of network analysis include friendship and social networks, marketing and recommender systems, the World Wide Web, disease models, and food webs, among others (Zhao et al. 2011). Social network analysis (SNA) is the study of structure. It involves relational datasets. That is, structure is derived from the regularities in the patterning of relationships among social entities, which might be people, groups, or organizations. Social network analysis is quantitative, but qualitative interpretation also its necessary. It has a long history in sociology and mathematics and it is creeping into health research as its analytical methods become more accessible with user friendly software (Hawe et al. 2004). SNA views social relationships in terms of network theory consisting of nodes and ties (also called edges, links, or connections).

Nodes are the individual actors within the networks, and ties are the relationships between the actors. The resulting graph-based structures are often very complex. There can be many kinds of ties between the nodes. Research in a number of academic fields has shown that social networks operate on many levels, from families up to the level of nations, and play a critical role in determining the way problems are solved, organizations are run, and the degree to which individuals succeed in achieving their goals (McGrath 1988, Palinkas et al. 2011, Szell et al. 2010, Zhao et al. 2011, Hawe et al. 2004).

In its simplest form, a social network is a map of specified ties, such as friendship, between the nodes being studied. The nodes to which an individual is thus connected are the social contacts of that individual. The network can also be used to measure social capital – the value that an individual gets from the social network. These concepts are often displayed in a social network diagram, where nodes are the points and ties are the lines.

Its use in health (Bhardwaj et al. 2010, Lawrence & Fudge 2009), and more on in infectious diseases (Klovdahl et al. 2002), has been recently highlighted, including sexually transmitted infections (Perisse & Costa Nery 2007), as well in TB (Boffa et al. 2011, Waisbord 2007, Curto et al. 2010, Burlandy & Labra 2007, Santos Filho & Santos Gomes 2007, Freudenberg 1995, Murray et al. 2011).

3. Tuberculosis as a social issue

Multiple studies have evidenced links between social, economic and biologic determinants to TB, recently using modeling approaches that have been used to understand their contribution to the epidemic dynamics of TB (Murray et al. 2011). Specifically, different authors have evidence for associations between smoking, indoor air pollution, diabetes mellitus, alcohol, nutritional status, crowding, migration, aging and economic trends, and the occurrence of TB infection and/or disease. We outline some methodological problems inherent to the study of these associations; these include study design issues, reverse causality and misclassification of both exposure and outcomes. From a social perspective, multiple analyses can be useful and approaches to modeling the impact of determinants and the effect of interventions as the follow will help: the population attributable fraction model, which estimates the proportion of the TB burden that would be averted if exposure to a risk factor were eliminated from the population, and deterministic epidemic models that capture transmission dynamics and the indirect effects of interventions. Can be stated that by defining research priorities in both the study of specific determinants and the development of appropriate models to assess the impact of addressing these determinants (Murray et al. 2011, Santin & Navas 2011, Juniarti & Evans 2011).

Although not considered neglected, TB disproportionally affect resource-constrained areas of the World, including Latin America. In tropical and subtropical areas of this region, the vicious cycle of poverty, disease and underdevelopment is widespread, including TB as one of the significant pathologies involved. The burden of disease associated to TB in this region is highly significant in some countries (eg. Bolivia, Haiti, Brazil, among others). TB has burdened Latin America throughout centuries and has directly influenced their ability to develop and become competitive societies in the current climate of globalization.

Therefore, the need for a new paradigm that integrates various public health policies, programs, and a strategy with the collaboration of all responsible sectors is long overdue. In this regard, innovative approaches are required to ensure the availability of low-cost, simple, sustainable, and locally acceptable strategies to improve the health of neglected

populations to prevent, control, and potentially eliminate poverty diseases, such as TB. Improving the health of these forgotten populations will place them in an environment more conducive to development and will likely contribute significantly to the achievement of the Millennium Development Goals in this area of the globe (Franco-Paredes et al. 2007). For example in Colombia, TB is still a significant public health problem. Figure 1 shows the WHO profile for TB in Colombia for 2010.

4. Social networks in tuberculosis

Multiple studies have evidenced links between social, economic and biologic determinants to TB, recently using modeling approaches (Guzzetta et al. 2011, Drewe et al. 2011, Wilson et al. 2011, Bohm et al. 2008, Cook et al. 2007, Cohen et al. 2007, Ayala & Kroeger 2002). Tuberculosis is the archetypal disease of poverty, and social inequalities undermine TB control (Rocha et al. 2011, Lonnroth et al. 2010). Poverty predisposes individuals to TB through multiple mechanisms, such as malnutrition (Rocha et al. 2011, Lonnroth et al. 2010, Cegielski & McMurray 2004), and TB worsens poverty as it increases expenses and reduces income (Rocha et al. 2011, Pantoja et al. 2009, Pantoja et al. 2009, Kemp et al. 2007, Lonnroth et al. 2007, Rajeswari et al. 1999).

Furthermore, poor TB-affected households often experience stigmatization; adding barriers to TB control (Rocha et al. 2011, Atre et al. 2011, Dhingra & Khan 2010, Pungrassami et al. 2010, Jittimanee et al. 2009). Poor people at the greatest risk of TB are therefore, in many settings, also the least able to access TB care (Rocha et al. 2011). Then, socio-economic interventions adapted to the needs of TB-affected households living in impoverished peri-urban shantytowns and other demographical settings.

The socio-economic interventions can successfully engaged most TB-affected households in an active civil society that was associated with marked improvements in uptake of TB prevention, diagnosis and treatment, resulting in strengthened TB control (Rocha et al. 2011). The development of social networks and SNA, however, has been mostly approached only for investigation of TB outbreaks (Fitzpatrick et al. 2001, Sterling et al. 2000) and fewly in the support with the strategies of the WHO for TB Control (World Health Organization 2011).

The WHO Stop TB Strategy, recently revised (World Health Organization 2011), stated a vision for a TB-free world, with a goal of to dramatically reduce the global burden of TB by 2015 in line with the Stop TB Partnership targets and the Millennium Development Goals (MDGs) which pursue the significant reduction in endemic diseases, such as TB and others, even regional diseases (e.g. Chagas disease), that can represent an impediment in achieving the MDGs (Franco-Paredes et al. 2007). In their components, it is included Empower people with TB, and communities through partnership through: a. Pursue advocacy, communication and social mobilization; b. Foster community participation in TB care, prevention and health promotion; and c. Promote use of the Patients' Charter for Tuberculosis Care (World Health Organization 2011).

In Brazil, one of the countries in Latin America where TB is a major public health problem, recent experiences suggest the importance of networking and civil society participation for TB control (Santos Filho & Santos Gomes 2007). In that country, until 2003, the presence of civil society in the fight against TB took place by means of several initiatives from researchers, healthcare professionals and medicine students, especially from the Sociedade Brasileira de Pneumologia e Tisiologia (Brazilian Thoracic and Tuberculosis Society), Rede

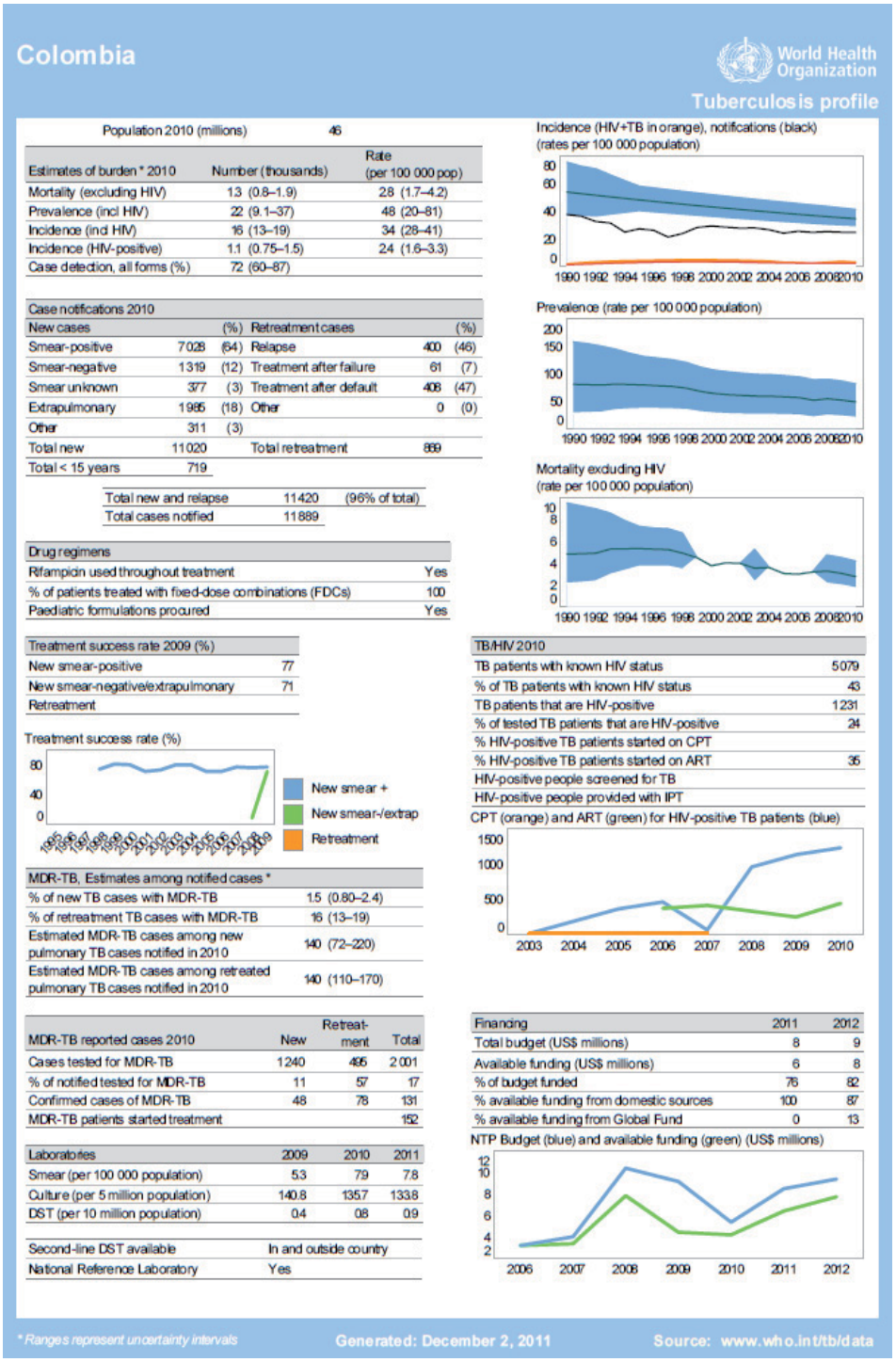


Fig. 1. Tuberculosis epidemiological profile for Colombia according to the World Health Organization, 2010.

TB (TB Network) and Liga Científica contra a Tuberculose (Scientific League against Tuberculosis). Since their creation, these entities have been constituted by people who are committed to TB control, though lacking the "community" component represented by people who are affected by and live with the disease (Santos Filho & Santos Gomes 2007). After that in recent years more organizations were involved in the fight against TB in the country.

The actions by the community entities in the fight against tuberculosis have been particularly concentrated on the networking among diverse social and govern mental actors; plus, on making the problem noticeable to their target populations or the general population, aiming their sensitization (Santos Filho & Santos Gomes 2007). For some relevant social actors, such as the Rede TB (TB Network) and the Liga Científi ca (Scientific League), the participation of the community sector in their activities aims at contributing to greater efficacy of their actions and responses to certain problems that are presented. Without the user's voice and perspective, there is the risk of repeating mistakes of not evaluating correctly the efficacy of actions such as applied methods and methodologies in health services (Santos Filho & Santos Gomes 2007). Then, multiple strategies are important in this context of development of new alternatives in the control of TB. The practice of participation, networking, advocacy and multi-sector cooperation will provide the necessary conditions for an effective control of tuberculosis in Brazil, as well in other countries where they would be applied (Santos Filho & Santos Gomes 2007).

5. Social networks in tuberculosis in Pereira, Colombia

Taking in account general epidemiology of TB in Colombia and particularly at a municipality where this strategy of social networking was developed, social conditions were analyzed (Collazos et al. 2010, Jalil-Paier & Donado 2010, Ascuntar et al. 2010, Mateus-Solarte & Carvajal-Barona 2008, Jaramillo 1999). Also, in the scenarios were considered the recent impacts of the health sector reform (Carvajal et al. 2004, Ayala & Kroeger 2002), that also have influenced the TB control programs from a national to a local perspective. Pereira is the capital municipality of the Department of Risaralda (Figure 2). It stands in the center of the western region of the country, located in a small valley that descends from a part of the western Andes mountain chain. Its strategic location in the coffee producing area makes the city an urban center in Colombia, as does its proximity to Bogotá, Cali and Medellín.

For 2011, Pereira municipality has an estimated population of 459,690. Official reported records for TB in Risaralda registered a disease incidence for 2011 of 66 cases per 100,000 pop (as 15 December) (which is above the national average rate of 24 cases per 100,000 pop). Pereira is divided into 19 urban submunicipalities: Ferrocarril, Olímpica, San Joaquín, Cuba, Del Café, El oso, Perla del Otún, Consota, El Rocío, El poblado, El jardín, San Nicolás, Centro, Río Otún, Boston, Universidad, Villavicencio, Oriente y Villasantana. Additionally also has rural townships which include Altagracia, Arabia, Caimalito, Cerritos, La Florida, Puerto Caldas, Combia Alta, Combia Baja, La Bella, Estrella- La Palmilla, Morelia, Tribunas. The municipality of Pereira has a diversified economy: the primary sector accounts for 5.7% of domestic product, the secondary sector shows a relative weight of 26.2%, while the tertiary sector is the most representative with a 68.1%. The GDP of Pereira grew by 3.7% in 2004. For 2010, Pereira reported 301 cases of TB (incidence rate of 65.85 cases per 100,000pop). In Pereira, previously reported interventions have been developed and working intersectorially with the academia in order to increase the impact of activities in TB control (Castañeda-Hernández et al. 2012b).

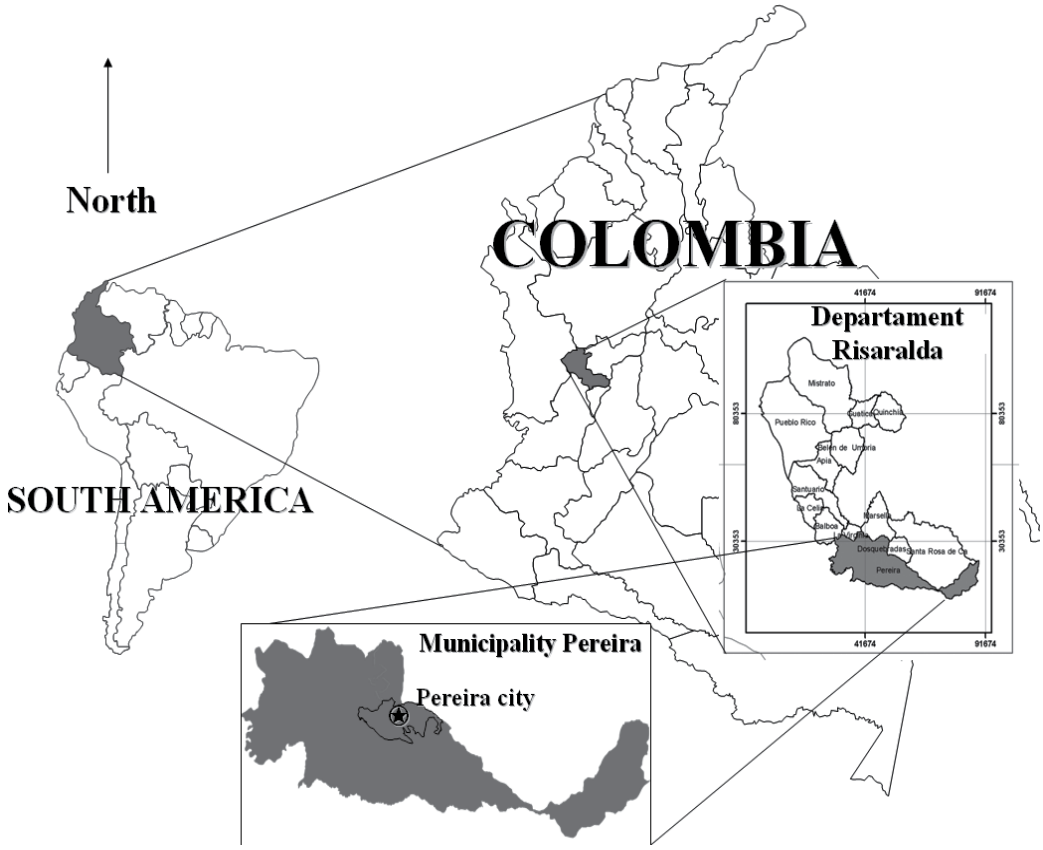


Fig. 2. Relative location of Pereira, Risaralda, Colombia, South America.

In the country, the strategic plan "*Colombia Libre de Tuberculosis para la Expansión y Fortalecimiento de la Estrategia Alto a la TB, 2010-2015*" (Colombia Free of TB for the Expansion and Enhancement of the Strategy Stop TB, 2010-2015), define as goal the achievement of notifications of new positive baciloscopy cases in more than 70% and a curation rate of at least 85%. In this context the routine surveillance allow to follow management and measurement of the impact of the realized actions by the control programs at municipal, departmental and national level, in order to generate interventions that contribute to achievement of the established goals to stop the advance of TB in the country.

Those considered strategies in the referred plan include the previously mentioned pursue advocacy, communication and social mobilization (ACMS), from the WHO Global Plan to STOP TB (World Health Organization 2011). In the context of wide-ranging partnerships for TB control, advocacy, communication and social mobilization (ACSM) embrace: advocacy to influence policy changes and sustain political and financial commitment; two-way communication between the care providers and people with TB as well as communities to improve knowledge of TB control policies, programmes and services; and social mobilization to engage society, especially the poor, and all allies and partners in the campaign to Stop TB. Each of these activities can help build greater commitment to fighting TB.

Advocacy is intended to secure the support of key constituencies in relevant local, national and international policy discussions and is expected to prompt greater accountability from governmental and international actors. Communication is concerned with informing, and enhancing knowledge among, the general public and people with TB and empowering them to express their needs and take action. Equally, encouraging providers to be more receptive to the expressed wants and views of people with TB and community members will make TB services more responsive to community needs. Social mobilization is the process of bringing together all feasible and practical intersectoral allies to raise people's knowledge of and demand for good-quality TB care and health care in general, assist in the delivery of resources and services and strengthen community participation for sustainability. Thus, ACSM is essential for achieving a world free of TB and is relevant to all aspects of the Stop TB Strategy. ACSM efforts in TB control should be linked with overarching efforts to promote public health and social development (World Health Organization 2011).

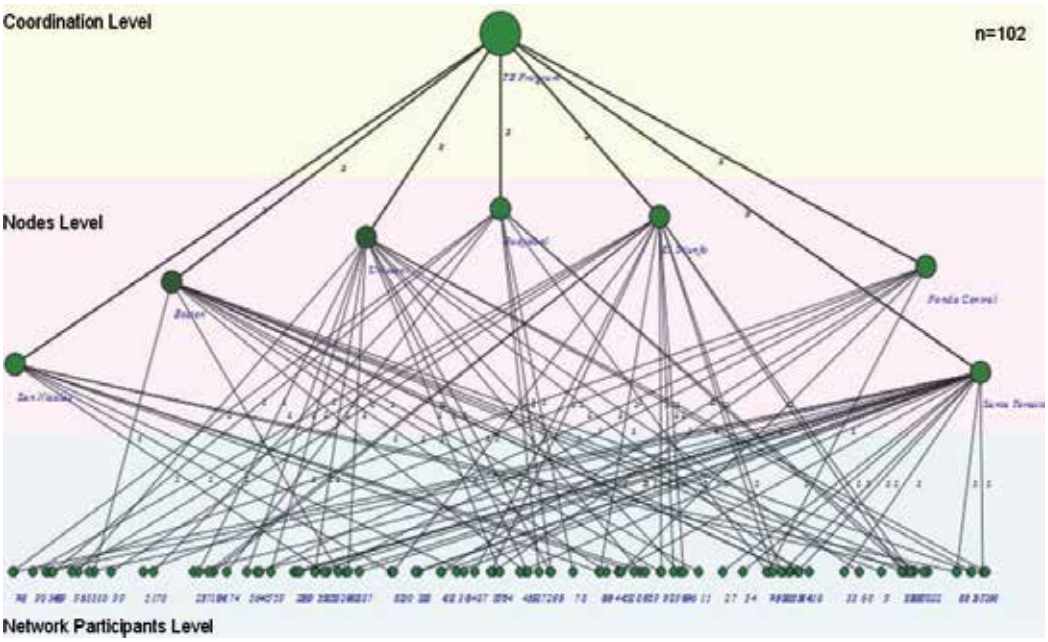


Fig. 3. SNA for Tuberculosis Network in Pereira, Colombia (developed with the Software SocNETV 0.81).

With these considerations in mind, in Pereira a social network for TB was developed. This network include the participation of ex-patients with TB, also healthy general population participated. All of them through the creation of nodes of the network, which were considered for this purposes as communitarian organizations constituted for a common objective and which are present regularly at neighborhoods in the municipality. Nodes were located at the 7 most highly prevalent areas of the municipality, previous to epidemiological analyses of those areas. Then, these locations were oriented to focalize the actions through the impact that, once established, this network would have on the control activities for TB in the areas of the municipality where most cases are concentrated.

In Pereira, with coordination of the TB control program on the top of the organization, a social network was developed with 7 strategically located and voluntary participation nodes

(Figure 3). This social network included more than 100 people supporting the program of TB in the municipality, then strengthening the control and surveillance activities necessary to reduce and to impact more the actions against the disease.
The nodes were constituted as communitarian organization, recognized by the communities and by the different related institutions, seen as long-lasting entities, with clear objectives and work plans for continued activity at the community. In all cases the participation was completely voluntary and non-profit.
As one of the key aspects of this network, multiple programmed activities were developed, including regularly meetings for discussions and for education on TB, giving multiple and different type of incentives in order to increase the interest on participation, helding workshops and different age-oriented designed activities that include games and handy-craft works, but in every case taking in consideration an structured and varied programation to include activities for TB education.
With this social TB network the municipal TB control program pursue to improve case detection and treatment adherence, combat stigma and discrimination, empower people affected by TB and mobilize political commitment and resources for TB.
Further implications of this social network, however, should be analyzed in the long term in order to measure its impact of epidemiological indicators of TB in the municipality.

6. Conclusions

Tuberculosis control in the XXI century requires new approaches and interventions, particularly those based in education and prevention with a community-based orientation. Programs such as the social network developed in Pereira TB control program, should performed in other highly endemic places. As the WHO recommends to pursue the ACMS (advocacy, communication and social mobilization), strategies as the social network allow to enhance particularly the communication and social mobilization components. Unfortunately at many national plans of TB control, how translate the ACMS in specific actions is not well defined in most occasions.
As has been previously stated, in the establishment of a social network for TB, previous diagnosis, including geo-referenced characterization, it is necessary to select the areas where the nodes will be established, taking also in consideration the suitability as the willingness of the potential participants of the network in each area and node. Finally, with the mining of the activities described, but also beginning with the idea of raise the awareness about the disease, taking in consideration a high level of diversity on the activities, as has been stated in order to warrant the continuous interest and participation of the network members on it.
In the future, in order to enhance the function and structure of the whole social network, further meetings between the nodes are expected. As now, only nodes interact internally, but the idea for the future activities in this setting is increase the links internally, but also between the main nodes in order to potentially increase the participation in the whole network.
Activities such as the development of social network of TB in Pereira will enhance the prevention, education and surveillance in the community, allowing a better integrated approach to the TB control in these scenarios and increasing the health profile in the community decreasing the lost opportunities for diagnosis and treatment of TB cases, finally leading to an improvement of the TB prevention and control.

7. References

Arbelaez MP, Nelson KE, Munoz A. 2000. BCG vaccine effectiveness in preventing tuberculosis and its interaction with human immunodeficiency virus infection. *Int. J Epidemiol.* 29(6):1085-91

Ascuntar JM, Gaviria MB, Uribe L, Ochoa J. 2010. Fear, infection and compassion: social representations of tuberculosis in Medellin, Colombia, 2007. *Int. J Tuberc. Lung Dis.* 14(10):1323-9

Atre S, Kudale A, Morankar S, Gosoniu D, Weiss MG. 2011. Gender and community views of stigma and tuberculosis in rural Maharashtra, India. *Glob. Public Health* 6(1):56-71

Ayala CC, Kroeger A. 2002. Health sector reform in Colombia and its effects on tuberculosis control and immunization programs. *Cad. Saude Publica* 18(6):1771-81

Bhardwaj N, Yan KK, Gerstein MB. 2010. Analysis of diverse regulatory networks in a hierarchical context shows consistent tendencies for collaboration in the middle levels. *Proceedings of the National Academy of Sciences of the United States of America* 107(15):6841-6

Black GF, Weir RE, Chaguluka SD, Warndorff D, Crampin AC et al. 2003. Gamma interferon responses induced by a panel of recombinant and purified mycobacterial antigens in healthy, non-mycobacterium bovis BCG-vaccinated Malawian young adults. *Clin. Diagn. Lab Immunol.* 10(4):602-11

Bohm M, Palphramand KL, Newton-Cross G, Hutchings MR, White PC. 2008. Dynamic interactions among badgers: implications for sociality and disease transmission. *J Anim Ecol.* 77(4):735-45

Carvajal R, Cabrera GA, Mateus JC. 2004. Effects of the health sector reform upon tuberculosis control interventions in Valle del Cauca, Colombia. *Biomedica.* 24 Supp 1:138-48

Castañeda-Hernández DM, Rodríguez-Morales AJ, Sepulveda-Arias JC. 2012a Importancia del uso de pruebas de medicion de la liberacion de interferon-gamma en la vigilancia epidemiologica de la tuberculosis. *Rev Med Chile* 140:(1)in press

Castañeda-Hernández DM, Mondragón-Cardona A, Campo-Betancourth CF, Tobón-García D, Alzate-Carvajal V, Jiménez-Canizales CE, Rodríguez Morales AJ. 2012b. Impacto de una Actividad Formativa en los Conocimientos, Actitudes y Percepciones (CAP) sobre Tuberculosis (TB) de Estudiantes de Medicina de una Universidad de Risaralda, Colombia. *Gaceta Médica de Caracas* 120(1):40-47

Cegielski JP, McMurray DN. 2004. The relationship between malnutrition and tuberculosis: evidence from studies in humans and experimental animals. *Int. J Tuberc. Lung Dis.* 8(3):286-98

Cohen T, Colijn C, Finklea B, Murray M. 2007. Exogenous re-infection and the dynamics of tuberculosis epidemics: local effects in a network model of transmission. *J R. Soc Interface* 4(14):523-31

Collazos C, Carrasquilla G, Ibanez M, Lopez LE. 2010. Prevalence of respiratory symptomatic in health institutions of Bogota, Colombia. *Biomedica.* 30(4):519-29

Cook VJ, Sun SJ, Tapia J, Muth SQ, Arguello DF et al. 2007. Transmission network analysis in tuberculosis contact investigations. *J Infect Dis.* 196(10):1517-27

Curto M, Scatena LM, de Paula Andrade RL, Palha PF, de Assis EG et al. 2010. Tuberculosis control: patient perception regarding orientation for the community and community participation. *Rev. Lat. Am. Enfermagem.* 18(5):983-9

Dhingra VK, Khan S. 2010. A sociological study on stigma among TB patients in Delhi. *Indian J Tuberc.* 57(1):12-8

Dim CC, Dim NR, Morkve O. 2011. Tuberculosis: a review of current concepts and control programme in Nigeria. *Niger. J Med* 20(2):200-6

Drewe JA, Eames KT, Madden JR, Pearce GP. 2011. Integrating contact network structure into tuberculosis epidemiology in meerkats in South Africa: Implications for control. *Prev. Vet. Med* 101(1-2):113-20

Fitzpatrick LK, Hardacker JA, Heirendt W, Agerton T, Streicher A et al. 2001. A preventable outbreak of tuberculosis investigated through an intricate social network. *Clin. Infect Dis.* 33(11):1801-6

Francis J, Reed A, Yohannes F, Dodard M, Fournier AM. 2002. Screening for tuberculosis among orphans in a developing country. *Am. J Prev. Med* 22(2):117-9

Franco-Paredes C, Jones D, Rodriguez-Morales AJ, Santos-Preciado JI. 2007. Commentary: improving the health of neglected populations in Latin America. *BMC Public Health* 7:11

Franco-Paredes C, Von A, Hidron A, Rodriguez-Morales AJ, Tellez I et al. 2007. Chagas disease: an impediment in achieving the Millennium Development Goals in Latin America. *BMC Int. Health Hum. Rights* 7:7

Galimi R. 2011. Extrapulmonary tuberculosis: tuberculous meningitis new developments. *Eur. Rev Med Pharmacol. Sci* 15(4):365-86

Garcia-Rodriguez JF, Alvarez-Diaz H, Lorenzo-Garcia MV, Marino-Callejo A, Fernandez-Rial A, Sesma-Sanchez P. 2011. Extrapulmonary tuberculosis: epidemiology and risk factors. *Enferm. Infecc. Microbiol. Clin.* 29(7):502-9

Garg RK. 2010. Tuberculous meningitis. *Acta Neurol. Scand.* 122(2):75-90

Ginsberg AM. 2000. A proposed national strategy for tuberculosis vaccine development. *Clin. Infect Dis.* 30 Suppl 3:S233-S242

Glaziou P, Floyd K, Raviglione M. 2009. Global burden and epidemiology of tuberculosis. *Clin. Chest Med* 30(4):621-36, vii

Guzzetta G, Ajelli M, Yang Z, Merler S, Furlanello C, Kirschner D. 2011. Modeling socio-demography to capture tuberculosis transmission dynamics in a low burden setting. *J Theor. Biol.* 289:197-205

Hawe P, Webster C, Shiell A. 2004. A glossary of terms for navigating the field of social network analysis. *J Epidemiol. Community Health* 58(12):971-5

Hoek KG, Van RA, van Helden PD, Warren RM, Victor TC. 2011. Detecting drug-resistant tuberculosis: the importance of rapid testing. *Mol. Diagn. Ther.* 15(4):189-94

Jalil-Paier H, Donado G. 2010. Socio-political implications of the fight against alcoholism and tuberculosis in Colombia, 1910-1925. *Rev Salud Publica (Bogota.)* 12(3):486-96

Jaramillo E. 1999. Tuberculosis and stigma: predictors of prejudice against people with tuberculosis. *J Health Psychol.* 4(1):71-9

Jittimanee SX, Nateniyom S, Kittikraisak W, Burapat C, Akksilp S et al. 2009. Social stigma and knowledge of tuberculosis and HIV among patients with both diseases in Thailand. *PLoS. One.* 4(7):e6360

Kemp JR, Mann G, Simwaka BN, Salaniponi FM, Squire SB. 2007. Can Malawi's poor afford free tuberculosis services? Patient and household costs associated with a tuberculosis diagnosis in Lilongwe. *Bull. World Health Organ* 85(8):580-5

Klovdahl AS, Graviss EA, Musser JM. Infectious disease control: combining molecular biological and network methods. 8, 73-99. 2002.

Lawrence RJ, Fudge C. 2009. Healthy Cities in a global and regional context. *Health promotion international* 24 Suppl 1:i11-i18

Lonnroth K, Aung T, Maung W, Kluge H, Uplekar M. 2007. Social franchising of TB care through private GPs in Myanmar: an assessment of treatment results, access, equity and financial protection. *Health Policy Plan.* 22(3):156-66

Lonnroth K, Castro KG, Chakaya JM, Chauhan LS, Floyd K et al. 2010. Tuberculosis control and elimination 2010-50: cure, care, and social development. *Lancet* 375(9728):1814-29

Lonnroth K, Williams BG, Cegielski P, Dye C. 2010. A consistent log-linear relationship between tuberculosis incidence and body mass index. *Int. J Epidemiol.* 39(1):149-55

Marais BJ, Schaaf HS. 2010. Childhood tuberculosis: an emerging and previously neglected problem. *Infect Dis. Clin. North Am.* 24(3):727-49

Mateus-Solarte JC, Carvajal-Barona R. 2008. Factors predictive of adherence to tuberculosis treatment, Valle del Cauca, Colombia. *Int. J Tuberc. Lung Dis.* 12(5):520-6

Murray M, Oxlade O, Lin HH. 2011. Modeling social, environmental and biological determinants of tuberculosis. *Int. J Tuberc. Lung Dis.* 15 Suppl 2:S64-S70

Orcau A, Cayla JA, Martinez JA. 2011. Present epidemiology of tuberculosis. Prevention and control programs. *Enferm. Infecc. Microbiol. Clin.* 29 Suppl 1:2-7

Palinkas LA, Holloway IW, Rice E, Fuentes D, Wu Q, Chamberlain P. 2011. Social networks and implementation of evidence-based practices in public youth-serving systems: a mixed-methods study. *Implement. Sci* 6:113

Pantoja A, Floyd K, Unnikrishnan KP, Jitendra R, Padma MR et al. 2009. Economic evaluation of public-private mix for tuberculosis care and control, India. Part I. Socio-economic profile and costs among tuberculosis patients. *Int. J Tuberc. Lung Dis.* 13(6):698-704

Pantoja A, Lonnroth K, Lal SS, Chauhan LS, Uplekar M et al. 2009. Economic evaluation of public-private mix for tuberculosis care and control, India. Part II. Cost and cost-effectiveness. *Int. J Tuberc. Lung Dis.* 13(6):705-12

Perisse AR, Costa Nery JA. 2007. The relevance of social network analysis on the epidemiology and prevention of sexually transmitted diseases. *Cad. Saude Publica* 23 Suppl 3:S361-S369

Pungrassami P, Kipp AM, Stewart PW, Chongsuvivatwong V, Strauss RP, Van RA. 2010. Tuberculosis and AIDS stigma among patients who delay seeking care for tuberculosis symptoms. *Int. J Tuberc. Lung Dis.* 14(2):181-7

Rajeswari R, Balasubramanian R, Muniyandi M, Geetharamani S, Thresa X, Venkatesan P. 1999. Socio-economic impact of tuberculosis on patients and family in India. *Int. J Tuberc. Lung Dis.* 3(10):869-77

Rocha C, Montoya R, Zevallos K, Curatola A, Ynga W et al. 2011. The Innovative Socio-economic Interventions Against Tuberculosis (ISIAT) project: an operational assessment. *Int. J Tuberc. Lung Dis.* 15 Suppl 2:S50-S57

Rodríguez Morales AJ, Lorizio W, Vargas J, Fernández L, Durán B, Husband G, Rondón A, Vargas K, Barbella RA, Dickson SM. 2008. Malaria, Tuberculosis, VIH/SIDA e Influenza Aviar: ¿Asesinos de la Humanidad? *Rev Soc Med Quir Hosp Emerg Perez de Leon* 39(1):52-76

Santos Filho ET, Santos Gomes ZM. 2007. Strategies for tuberculosis control in Brazil: networking and civil society participation. *Rev. Saude Publica* 41 Suppl 1:111-6

Sterling TR, Thompson D, Stanley RL, McElroy PD, Madison A et al. 2000. A multi-state outbreak of tuberculosis among members of a highly mobile social network: implications for tuberculosis elimination. *Int. J Tuberc. Lung Dis.* 4(11):1066-73

Szell M, Lambiotte R, Thurner S. 2010. Multirelational organization of large-scale social networks in an online world. *Proc. Natl. Acad. Sci U. S. A* 107(31):13636-41

Vargas J, Gamboa C, Negrin D, Correa M, Sandoval C et al. 2005. Disseminated Mycobacterium mucogenicum infection in a patient with idiopathic CD4+ T lymphocytopenia manifesting as fever of unknown origin. *Clin. Infect Dis.* 41(5):759-60

Wilson GJ, Carter SP, Delahay RJ. 2011. Advances and prospects for management of TB transmission between badgers and cattle. *Vet. Microbiol.* 151(1-2):43-50

World Health Organization. 2011. *2011 Global Tuberculosis Control,* Geneva: WHO.

Zhao Y, Levina E, Zhu J. 2011. Community extraction for social networks. *Proc. Natl. Acad. Sci U. S. A* 108(18):7321-6

6

Molecular Characterization of Dengue Virus Circulating in Manaus, the Capital City of the State of Amazonas, Brazil

Regina Maria Pinto de Figueiredo
Foundation for Tropical Medicine Dr. Hector Vieira Dourado (FMT/HVD)
Brazil

1. Introduction

The term dengue, of Spanish origin, was used to describe joint pain from an illness that attacked the British during the epidemic that affected the Spanish West Indies from 1927-1928. Dengue was brought to the American continent to the Old World during the colonization in the late eighteenth century. However, it is not possible to say according to the historical record if the outbreaks were caused by dengue virus, as its symptoms are similar to those of several other infections, especially yellow fever (Holmes et al., 1998). The etiology of dengue has been credited to the miasma theory, bacterial or protozoan infection and finally to an ultramicroscopic agent. Similarly, transmission has been considered by respiratory airway and finally, by mosquitoes.

The isolation of dengue virus (DENV) occurred in the 1940s during the epidemics of Nagasaki (1943) and Osaka (1944). The first known strain is coined DENV Mochizuki (Kimura & Hotta, 1944). In 1945, the Hawaii strain was isolated, and that same year other DENVs showing antigenic characteristics of different serotypes were isolated in New Guinea. The first two strains were designated serotype 1 and serotype 2. In 1956, other strains designated as serotype 3 and 4 were isolated. Thus, four dengue serotypes are known to date: DENV-1, DENV-2, DENV-3 and DENV-4 (Martinez Torres, 1990).

Genetic variation within each serotype was first demonstrated by serological techniques. Subsequently analysis of the viral genome showed that DENV-1and DENV-2 can be classified as having five genotypes or subtypes each while DENV-3 and DENV-4having four and two respectively (Rico-Hesse, 1990, Lanciotti et al., 1994). Recently, Rico-Hesse et al (2003) reviewed the classification of DENV genotypes by the analysis and comparison of the nucleotide sequence of the complete E gene of various strains. As a result it was defined thatDENV-2 and DENV-3 have four genotypes while DENV-1 and DENV-4 have five and three genotypes respectively (Cunha & Nogueira, 2005).

1.1 Etiology

Dengue Fever, a vector-borne disease, is the most important arboviral disease worldwide. Dengue viruses (DENVs) belong to the genus *Flavivirus*, family *Flaviviridae*. These are single-stranded positive-sense RNA viruses. DENV are grouped into four antigenically related but distinct serotypes named DENV-1, 2, 3 and 4.The four serotypes of DENV are diverse and

not phylogenetically related but strongly related to flavivirus transmitted by mosquitoes. It is reported that these viruses have emerged about 1000 years ago from a monkey virus and its transmission to humans occurred in the last 320 years. Some studies indicated its origin in Africa and others in Asia (Weaver et al., 2004).

1.2 Clinical characteristics

Dengue has two main clinical forms: classic dengue fever (DF) and dengue hemorrhagic fever (DHF) with or without shock (PAN AMERICAN HEALTH ORGANIZATION, 1994). The symptoms of DF are headache, retro-orbital pain, breaking bones sensation, muscles or joints pain, rash and leucopenia. Dengue hemorrhagic fever is characterized by high fever, hemorrhagic phenomena often with hepatomegaly and, in severe cases, signs of circulatory failure. Patients with DHF may develop hypovolemic shock resulting from plasma leakage. This clinical manifestation is called dengue shock syndrome (DSS) and can be fatal (WORLD HEALTH ORGANIZATION, 2001).

1.3 Transmission

The dengue virus is transmitted to humans through the bite of hematophagous Diptera, the mosquito *Aedes aegypti*. In the Americas, *A. aegypti* is one of the most efficient vectors of Arboviruses and is highly anthropophilic thriving in close proximity to humans and adapts very well indoors, generally in humid environment (WORLD HEALTH ORGANIZATION, 2001).

Once contracted the virus, the mosquito remains infected during its entire life and may transmit the virus to individuals during blood meals. The infected *A.aegypti* females may also transmit the virus to the next generation of mosquitoes by transovarial. Although very seldom, this means of transmission is of great epidemiological significance demonstrating that the vector play an important role in the persistence of the virus in the environment and act as reservoirs (WORLD HEALTH ORGANIZATION, 2001; Castro et al., 2004; Joshi et al., 2006).

Human beings are the main host and the virus replicates in the blood stream. Uninfected mosquitoes can contract the virus during blood meals from an infected individual. The virus multiplies in the cells of the mosquito during a period of 8 to 10 days. After this period, the vector is able to transmit the virus to humans again. In humans, the incubation period of dengue fever ranges from 2 to 7 days. Laboratory experiments showed that the mosquito *A. aegypti* may be infected simultaneously by different arboviruses and is also capable of transmitting them simultaneously (Araújo et al., 2006). According Wenming et al. (2005) it is possible that mosquitoes infected with DENV-2 and DENV-3 can transmit both in areas where two or more serotypes circulate. The poor environmental conditions of urban centers, the humidity and temperature as in Brazil associated with resistance of eggs of *A. aegypti* for long periods of desiccation favor the proliferation of mosquitoes and contribute to the spread of DENV. Dengue is currently considered the most important arbovirus and is a public health problem in tropical and subtropical countries (Guzman et al. 2006; WORLD HEALTH ORGANIZATION, 2001).

1.4 Dengue in Manaus

Infection by dengue virus in Brazil has increased significantly over the last decade, particularly after 1994, as a consequence of the spread of *A.aegypti*. The following serotypes,

DENV-1, DENV-2 and DENV-3 were common in most Brazilian cities. The DENV-4, co-circulating with DENV-1,was detected during the first epidemic reported in Brazil in Boa Vista - Roraima in 1981-1982 (Osanai, 1984).

In early 1998, the Laboratory of Arbovirology at the Foundation for Tropical Medicine Dr. Hector Vieira Gold (FMT/HVD) in Manaus in the state of Amazonas, started a program of monitoring and diagnosing of viral diseases transmitted by arthropods (arboviruses) to determine their etiological agents. The diagnosis was made by serologic studies using the MAC-ELISA test for detection of IgM antibodies. Sera samples from 8557 patients suspected of dengue were analyzed and 40% of the sera were ELISA positive for dengue virus. The DENV-1 was considered responsible for this epidemic (Figueiredo et al., 2004). In 2001, DENV-1 and DENV-2 were isolated in the State of Amazons and dengue hemorrhagic fever cases were registered (Figueiredo et al., 2002). In 2003, DENV-3 was isolated for the first time in the state of Amazonas from a patient coming from the state of Bahia (Figueiredo et al., 2003).

A variety of acute febrile diseases with and without hemorrhagic manifestations were diagnosed as dengue in Manaus in March 1998 by detection of specific IgM antibodies. Mayaro (MAYV) and *Oropouche* (OROV) viruses were also diagnosed. Infections with rubella and parvovirus were also observed (Figueiredo et al., 2004).

In 2008, DENV-4 was first identified in Manaus (Figueiredo et al., 2008). The Amazon is situated in the Northern Region of Brazil, bordered to the north with the State of Roraima, Venezuela and Colombia. Serotype DENV-4 was endemic to Venezuela and Colombia and this may have influenced the detection of this serotype in the Amazon due to its proximity to these countries (Figueiredo, 2008). The four serotypes of dengue and cases of DENV-3/DENV-4 co-infection are shown in Figure 1.

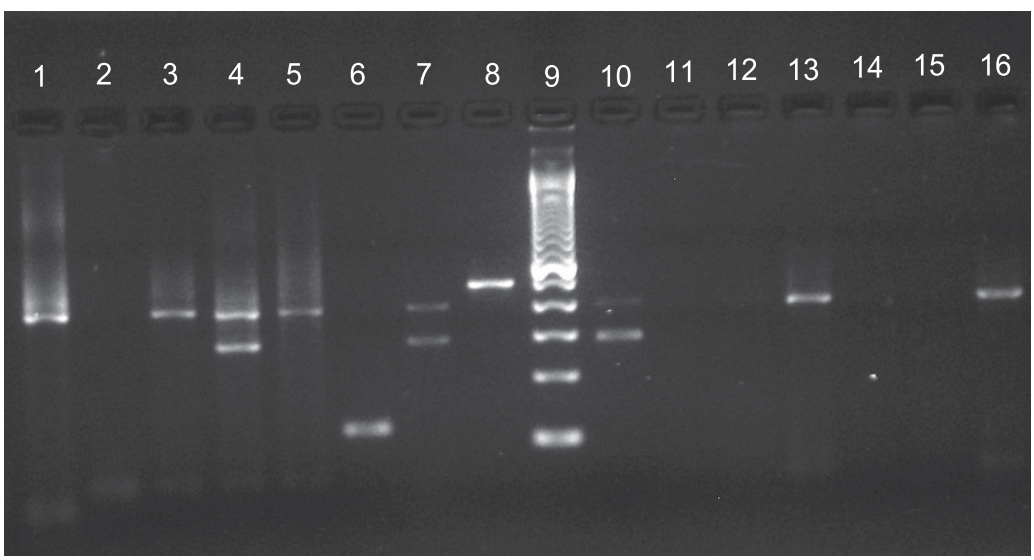


Fig. 1. Agarose gel electrophoresis. Lanes 1,3, 13, 16: DENV-4; Lane 4,7 and 10: DENV-3/DENV-4; Lane 6: DENV-2, Lane 8: DENV-1; Lane 9: DNA size standards 100Kb;

Nucleotide sequence analysis of DENV-4 present in the State of Amazonas showed to be of genotype I which is only present in the Asian continent and never described in the

Americas (De Melo et al., 2009). The introduction of this virus in Manaus is probably due to the fact that this is a city with eco-tourism development, and possess an Industrial Center (Free Zone of Manaus), with over 450 factories of large, medium and small size (PORTAL AMAZÔNIA) and many of them are of Asian origin (Figueiredo, 2008). It is possible that the virus has been introduced from an Asian-infected visitor or vector *A.aegypti*-infected.

1.5 Co-infection

Natural co-infection with dengue virus can occur in highly endemic areas where several serotypes have been transmitted for many years. Cases of simultaneous infection by more than one species of arboviruses in mosquito or human host were reported (Meyers& Carey, 1967; Gubler et al., 1985). In Brazil, one case of co-infection by DENV-1 and DENV-2 was reported in the patient with classic dengue fever (DF) from Southeastern region through immunofluorescence and RT-PCR (Santos et al., 2003). Another case of co-infection by DENV-2 and DENV-3, was observed in 2005 in the Northeastern region from a patient with DF (Araújo et al., 2006). Simultaneous infection by different strains of dengue virus in mosquitoes and humans underscores the potential for recombination (Santos et al., 2003). Although recombination is rarely recorded in positive-stranded RNA viruses (Lai, 1992), recombination occurrence in picornavirus, coronavirus, and alphavirus have been suggested. The latter viruses are also transmitted by mosquitoes (Hahn et al., 1998.Variations in dengue virus and the occurrence of co-infections with different DENV serotype may lead to genetic exchange between strains increasing the likelihood of recombination (Kuno, 1997).

During an outbreak of dengue in São José do Rio Preto, State of São Paulo, 365 samples were positives to DENV-3, 5 samples were to DENV-2, and 8 to Saint Louis encephalitis flavivirus (SLEV). Among the positive samples, one co-infection was detected for DENV-2 and DENV-3. Co-infection of each distint DENV serotype or other flavivirus during dengue outbreaks seems to be common (Terzian et al., 2010).

2. Laboratory diagnosis

2.1 Isolation of virus

The two basic methods for establishing a laboratory diagnosis of dengue fever are: the detection of viruses (egg, culture), or detection of IgM antibodies anti-dengue (serology). Blood samples for viral isolation should be collected within five days from the onset of symptoms. Serum is obtained by centrifugation and stored at -70°C.The inoculation of clinical specimens in adult mosquitoes or larvae in the culture technique is more sensitive for the detection of DENV. In laboratories where colonized mosquitoes are not available, samples can be inoculated in any mosquito cell lines available such as C6/36 (*Aedes albopictus* clone) that has a high sensitivity to DENV and other arboviruses.

2.2 Serological diagnosis

Sera should be collected from patients from the sixth day of illness and stored at -20°C. The MAC-ELISA is an antibody-capture assay of IgM from sera of patients suspected of DF. Briefy, the plate is sensitized with an anti-human IgM, and after various steps of the assay (blocking, dilution, washing, incubation overnight with the pool of antigen (DENV-1,

DENV-2 and DENV-3), the presence of specific IgM antibodies to dengue in the patient serum is shown by color change of the substrate that undergo enzymatic action of the conjugate. The color intensity is directly proportional to the amount of IgM antibodies contained in serum (Kuno et al., 1987).

2.3 Polymerase chain reaction (PCR) coupled with reverse transcription(RT-PCR)

The technique of PCR can be used to detect the presence of the DENV. Viral RNA is extracted using commercial kits. The RNA is reverse transcribed to cDNA in a first step. Then specific primers for DENV nucleotide sequence are used for the amplification of targeted sequence so as to detect a small amount of RNA molecules of DENV. This method is fast and rapid compared to cell culture. Reverse transcription and PCR technique was shown for the first time as a powerful technique for the detection of the DENV during convalescence, when the antibodies would limit its detection (WORLD HEALTH ORGANIZATION, 2001).

However, this method should still be considered an experimental approach. Is implementation on a large scale awaits further experiments. Moreover, a consensus on the adequate preparation of samples and for determining the sequences of bases in oligonucleotides capable of detecting all or most circulating genotypes of dengue need to be reached (WORLD HEALTH ORGANIZATION, 2001).

2.4 Virological diagnosis

The technique used to isolate DENV is continuous cell lines of mosquito cells C6/36 (*Aedes albopictus* clone) (Igarashi, 1978), grown in 25 cm flask with growth medium L-15 plus 5% of fetal bovine serum. To 1.5 mL of theL-15 plus 5% of fetal bovine serum containing the C6/36 cells in disposable falcon tubes (15 mL), 70 µl of serum from patients suspected of DF in acute phase was added and incubated at 28° C for two week. Culture media is changed two times per week and incubated. After 10 days of incubation, immunofluorescence (IF) technique is used for the identification of serotypes of dengue.

3. Discussion

In Brazil, first cases of DHF occurred after the introduction of DENV-2 in the State of Rio de Janeiro. The cases of DHF by DENV-2 occurred after an epidemic by DENV-1 in Rio de Janeiro four years ago (Dias et al. 1991; Zagne et al., 1994). The same pattern was seen in Cuba during the 1981 epidemic with sequential infection by two serotypes (DENV-1 and DENV-2), and an interval of six months to five years or so (Kouri et al., 1986).The dynamics of epidemic of dengue in the Amazon is similar to that of other regions of Brazil and America. In Manaus – Amazonas, the first cases of dengue were registered in March 1998 by serological studies during the first epidemic of dengue (Figueiredo et al., 2004). In 2001, it was possible to identify the DENV-1 as the causative agent of that epidemic (Figueiredo et al., 2002). That same year, cases of dengue hemorrhagic fever were registered in the State of Amazonas, with the viral isolation of DENV-2 (Figueiredo et al., 2002). In 2002, DENV3 was first isolated from a patient coming from Bahia. Since then, DENV-3 was diagnosed by viral isolation from several patients with DF (Figueiredo et al., 2003).The virus DENV-4 was detected during the first epidemic reported in Brazil in Boa Vista-Roraima in 1981-1982. At

that time the DENV-1 was also found (Osanai, 1984). Since then, no isolate of DENV-4 was recorded anywhere else in the country until later in the year 2008 DENV-4 was isolated in Manaus from patients with DF (Figueiredo et al., 2008).
The DENV-4 present in the State of Amazonas and analyzed by nucleotide sequence was shown to belong to genotype I, only present in the Asian continent and never described in the Americas (De Melo et al., 2009).
Dengue fever in uncomplicated cases of co-infection has also been observed by other authors, contradicting the hypothesis that simultaneous infection with dengue virus permits the emergence of a more severe disease (Santos et al., 2003; Araújo et al.,2006). In areas where more than one serotype are transmitted at the same time, clinical cases caused by more than one serotype of dengue fever can be common (Lorono et al., 1999). The high rate of cases occurring during epidemics can result in many infections with multiple serotypes in humans (both clinical and subclinical), and also provide opportunity for mosquitoes to become infected with two or more serotypes (Gubler et al., 1985; Burke et al., 1988). This suggests that co-infection by multiple dengue serotypes may influence the clinical expression of disease and it was initially considered as an explanation for the emergence of DHF (Hammon, 1973).The Amazon is situated in the Northern Region of Brazil, bordered to the north with the State of Roraima, Venezuela and Colombia to the east with the State of Pará, the southeast by the State of Mato Grosso, to the south with the State of Rondônia and southwest with the State of Acre and Peru (Viverde Tourism). The proximity to other countries where endemic DENV-4 is observed as in Venezuela and Colombia may have influenced the detection of this serotype first in the Amazon. Besides the geographical location, being today a city with eco-tourism development, and the Industrial Pole of Manaus, with over 450 factories of large, medium and small size (PORTAL AMAZON), which attracts investors from around the world. Manaus receives many people from these and other states and countries.
It is important to remember that the DENV-3 was first detected in Manaus from a patient coming from Salvador-Bahia (Figueiredo et al., 2003).The analysis of the region the C/prM of the DENV-3 in this study belonged to genotype III strains. All DENV-3 isolated in the Amazon were very close to the Indian strain GWL-60 (N°acessoAY770512) and the Brazilian strain BR-74 886 (N access AY679147). Subtype III is related to outbreaks of DHF in India (Dash et al., 2006). In Brazil, the prevalence of DENV-3 two years after its introduction in 2000 was associated with major epidemics in terms of more severe clinical manifestations, and the number of deaths (Nogueira et al., 2005). Twenty-two isolates were classified as DENV-3 subtype III (Miagostovich et al., 2002). The similarity of these strains to other represented by the same genotype III ranged from 96% to 98% and 98-99% for sequences nucleotides and amino acids, respectively. These data demonstrate that this virus is circulating around the world, again indicating high potential for distribution, adaptation in in various geographic areas of the world. This subtype has been implicated in outbreaks of DHF in Asia, Africa and the Americas, and has high potential to cause a pandemic of dengue (Messer et al., 2003).

4. Conclusions

The emergence of dengue virus in Manaus follows the same pattern as to what occurs in other Brazilian cities. Every two to three years there are new outbreaks of dengue and the

emergence of new serotypes followed by cases of dengue hemorrhagic. The emergence of DENV-4 in 2008 did not just caused a major epidemic with severe and fatal cases. Other serotypes were also observed. It is possible due to competition between with other serotypes, DENV-4 took some time to become established and the population might have been protected by antibodies obtained in heterologous infections by heterologous serotypes. The viral strain should be studied in detail since it is an Asian genotype that has been associated with DHF in the Asian continent.

- In relation to the DENV-2 which appears in 2011 with very high frequency, Manaus already has the DENV-2 genotype II for some time and often associated with cases of hemorrhagic dengue fever in other regions.
- Another important point is the co-infections with DENV-3 and DENV-4 in patients with DF. Most of them are associated with mild DF.
- Considering the large number of samples negative for dengue fever in our study, and to take into account the occurrence of other viruses, clinically similar to dengue fever, and the possibilities available to new viruses emerging or reemerging as Manaus is surrounded by the Amazon rainforest and also a large urban center that welcomes visitors from around the world, other viruses need to be investigated.

5. Recognition

Laboratory of Virology at the Foundation for Tropical Medicine Dr. Hector Vieira Gold (FMT/HVD); Dr. RajendranathRamasawmy for review of the manuscript; ConselhoNacional de DesenvolvimentoCientífico e Tecnológico (CNPq) for financial support.

6. References

Araújo et al. Concurrent infection with dengue virus type-2 and DENV-3 in apatient from Ceará, Brazil.*MemInstOswaldo Cruz,* Rio de Janeiro, 2006. 101(8): 925-928.

Burke DS, Nisalak A, Johnson De, Scott RM, 1988. A prospective study of dengue infections in Bangkok.Am J TropMed Hyg, 1988,38:172– 180.

Castro, M.G.; Nogueira, R.M.; Schatzmayr, H.G.; Miagostovich, M.P.; Lourenco-De-Oliveira, R. Dengue vírus detection by using reverse transcriptionpolymerase chain reaction in saliva and progeny of experimentally infected *Aedesalbopictus* from Brazil. *MemInstOswaldo Cruz*, 2004.99: 809-814.

Cunha, R.V. & Nogueira, R.M. Dengue E Dengue Hemorrágico. In: *Dinâmica das doençasInfecciosas e Parasitarias,* 2005. p.1767-1781.

Dash, P.K. et al. Reemergence of dengue vírus type-3 (subtype-III) in Índia.Implications for increased incidence of DHF & DSS.*Virology Journal*, 2006.

De Melo FL, Romano CM, De Andrade Zanotto PM. Introduction of Dengue virus 4 (DENV-4) genotype I in Brazil from Asia?PLoSNeglTrop Dis. 2009; 3(4):390.

Dias, M.; Zagne, S.M.O.; Pacheco, M.; Stavola, M.S.; Costa, A.J.L. Dengue hemorrágicoemNiterói.*Revista da SociedadeBrasileira de Medicina Tropical*, 1991. 24 (2): 122.

Figueiredo, R.M.P.; Bastos, M.S.; Lima, M.L.; Almeida, T.C.; Alecrim, W.D. Dinâmica da sorologia e isolamento viral na epidemia de dengue em Manaus (1998-2001). In:

Resumos do XXXVIII Congresso da Sociedade Brasileira de Medicina Tropical, 2002.

Figueiredo, R.M.P; Silva, M.N.R.; Almeida, T.C.; Lopes, H.C.; Bastos, M.S. Diagnóstico virológico de dengue em pacientes com quadro febril não diferenciado. In: Resumos do XXXIX Congresso da Sociedade Brasileira de Medicina Tropical, 2003.

Figueiredo, R.M.P.; Thatcher, B.D.; Lima, M.L.; Almeida, T.C.; Alecrim, W.D.; Guerra, M.V.F. Doençasexantemáticas e primeiraepidemia de dengue ocorridaem Manaus, Amazonas no período de 1998 -1999. *Revista da SociedadeBrasileira de Medicina Tropical,*2004.37: 476-479.

Figueiredo RMP. Caracterização molecular e epidemiológica dos vírus dengue no Estado do Amazonas, Brasil. Tese – Universidade Federal do Amazonas-UFAM, Manaus-Amazonas, 2008.

Figueiredo RM, Naveca FG, Bastos SM, Melo MN,Viana SS, Mourão MP, Costa Ca,Farias IP. Dengue virus type 4, Manaus, Brazil. Emerg Infect Dis. 2008; 14: 667–669.

Gubler, D.J.; Kuno, G.; Sather, G. E. & Waterman, S.H. A case of natural concurrent human infection with two dengue viruses. Amer. J. trop. Med. Hyg., 34: 170-173, 1985.

Guzmán, M.G.; Garcia, G.; Kouri, G. El dengue y el dengue hemorragico: prioridades de investigacion. *Rev PanamSaludPublica*. 2006. 19(3): 204-215.

Hahn, C.S.; Lustig, S.; Strauss, E.G. and Strauss, J.H. Western equine encephalitis virus is a recombination virus. *Proc. Natl. Acad. Sci*. USA, 1998. 85: 5997-6001.

Hammon WM. Dengue hemorrhagic fever - do we know its cause? Am J Trop Med Hyg, 1973, 22*:* 82–91.

Holmes, E.C.;Bartley, L.M.; Garnet, G.P. The emergence of dengue past, present and future. In: KRAUSE, R. M. Emerging infectors, London: Academic Press, 1998. p. 301-25.

Igarashi, A. Isolation of SinghsAedesalbopictus cell clone sensitive to dengue andchikungunya viruses.*Journal of General Virology*, 1978. 40: 531-544.

Joshi, V.; Sharma, R.C.; Sharma, Y.; Adha, S.; Sharma, K.; Singh, H.; Purohit, A.; Singhi, M. Importance of socioeconomic status and tree holes i distribution of *Aedes* mosquitoes (Diptera:Culicidae) in Jodhpur, Rajasthan, India. *J. Med Entomol, 2006*. 43: 330-336.

Kimura, R.; Hotta, S.On the inoculation of dengue virus into mice.Nippon Igakka, 1994.bp.629-633.

Kourí, G.P.; Guzman, M.G; Bravo, J. Dengue hemorrágicoem Cuba.Crônica de umaepidemia.*Boletin de la Oficina Sanitaria Panamericana*, 1986.100: 322- 329.

Kuno, G.; Gomez, I.; Gubler, D.J. Detecting artificial antidengueIgM immune complexes using an enzyme-linked immunosorbent assay.*American Journal of Tropical Medicine andHygiene,* 1987.36: 153-159.

Kuno, G. Factors Influencing The Transmission Of Dengue Viruses.Pp. 61-88 In D.J. Gubler And G. Kuno, Eds. Dengue And Dengue Hemorrhagic Fever. CAB International, Wallingford, U.K, 1997.

Lai, M.M.C. Rna recombination in animal and plant virases.*Microbiol. Rev.*,1992. *56*: 61-79.

Lanciotti, R.S.; Lewis, J.G.; Gubler, D.J.; Trent, D.W. Molecular evolution and epidemiology of dengue 3 viruses.*J Gen Virol*, 1994. 75: 65-75.

Lorono PM, Cropp CB, Farfan AJ, Vorndam AV, Rodriguez-Ângulo EM, Rosado-Paredes Ep, Flores-Flores Lf, Beaty Bj, And Gubler DJ. Common occurrence of concurrent infections by multiple dengue virus serotypes. American Journal Tropical Medicine and Hygiene, 61(5), 1999, pp. 725–730.

Martinez-Torres, M.E. Dengue hemorrágicoemcrianças: editorial. Havana: 1990. p.180.

Messer, WB, Gubler, D.J.; Harris, E.; Sivananthan K.; De Silva Am; Emergence and global spread of a dengue serotype 3, subtype III virus. *Emerg Infect Dis,* 2003. 9: 800-9.

Meyers, R.M. & Carey, D.E. Concurrent isolation from patient of two arboviruses,chikungunya and dengue type 2. *Science*, 1967. 157: 1307-1308.

Miagostovich et al. Genetic characterization of dengue vírus type 3 isolates in the State of Rio de Janeiro, 2001. *Brazillian Journal of Medical and Biological Research*, 2002. 35:869-872.

Nogueira, R.M.; Schatzma,Y.R.H.G.; Filippis, A.M.B.; Santos, F.V.; Cunha, R.V.; Coelho, J.O.; Souza, L.J.; Guimarães, F.R.; Araújo, E.S.M.; De Simone, T.S.; Baran, M.; Teixeira, G.; Miagostovich, M.P. Dengue virus type 3, Brazil, 2002. *Emerg Infect Dis*, 2005. 11: 1376-1381.

Osanai, C.H. Aepidemia de Dengue em Boa Vista, território Federal de Roraima, 1981-1982. Dissertação – Escola Nacional de SaúdePública, Rio de Janeiro, 1984.

Pan American Health Organization. Dengue and Dengue Haemorragic fever in the Americas: Guidelines for prevention and control. Washington: 1994.

Portal Amazon Zona Franca de Manaus Disponível no site www.manausonline.com.

Rico - Hesse, R. Molecular evolution and distribution of dengue viruses type 1 and 2 in nature. *Virology,* 1990. 174: 479-493.

Rico-Hesse, R. Microevolution and virulence of dengue viruses.*AdvVir Res*, 2003.59: 315-341.

Santos, C.L.S.; Bastos, M.A.A.; Sallum, M.A.M. & Rocco, I.M. Molecular characterization of dengue virases type 1 and 2 isolated from a concurrent human infection. *Rev. Inst. Med. Trop. S. Paulo,* 2003.*45 (*1): 11-16.

Terzian ACB, Mondini A, Bronzoni RVM, Drumond BP, Ferro BP ,Cabrera Sem, Figueiredo LTM, Chiaravalloti-Neto F, Nogueira ML . Detection of Saint Louis Encephalitis Virus in Dengue-Suspected Cases During a Dengue 3 Outbreak.Vector Borne and Zoonotic Diseases, 2010. Doi:10.1089/vbz.2009.0200.

Viverde Tourism -Informações cientificas da Amazônia. Disponível no site www.viverde.com.br

Zagne, S.M.O. et al. Dengue Haemorragic fever in the state of Rio de Janeiro, Brazil: a study 113 of 56 confirmed cases. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 1994.88: 677-679.

Weaver, S.C.; Barret, A.D.T. Transmission cycles, host range, evolution and emergence of arboviraldisease.*Nat Rev Microbiol*., 2004. 2: 789-801.

Wenming, P.; Man, Y.; Baochang, F.; Yongqiang, D.; Tao, J.; Hongyuan, D. Simultaneous infection with dengue 2 and 3 viruses in a Chinese patient return from Sri Lanka. *JClinVirol,* 2005. 32: 194-198.

World Health Organization.Dengue Haemorrhagic Fever: Diagnosis, Treatment, Prevention and Control, 2 nd ed., Geneva: WHO, 1997.

7

Genetic Diversity of Dengue Virus and Associated Clinical Severity During Periodic Epidemics in South East Asia

E. Khan, R. Hasan, J. Mehraj and S. Mahmood
Department of Pathology and Microbilogy
Aga Khan University, Stadium Road, Karachi
Pakistan

1. Introduction

The geographic distribution and genetic diversity of dengue virus is deeply rooted in Asia suggesting its origin from this region, with first reported out-break of DHF from Philippine in 1953 (Halstead, 1980). One of the characteristics notable in Asian regions, where the disease is endemic is that dengue hemorrhagic fever outbreaks occur in repetitive cycles of 3-5 years, (Ferguson et al, 1999). The incidence of disease and its severity varies across different dengue virus serotypes and also between primary and secondary infections of same serotypes(Vaughn DW et al).

Due to lack of in-vivo study models, there is little information about factors contributing to disease severity and its variation across dengue virus genotypes and the cyclical nature of dengue outbreaks. It is however critical to study these factors particularly in the South East Asian region where incidence of dengue cases is thought to be associated with variables such as water, sanitation, population density and rate of literacy as opposed to developed countries where ambient temperature, moisture and rainfall perhaps plays the major role. A better understanding of disease epidemiology and pathogenesis will help identify optimum control measures in the region. It will also develop systems for predicting the outcome of mass vaccination when the vaccine becomes available in this region.

The chapter has been divided in three parts: the first part will discuss the historical evolution of the dengue virus in the region its spatial and temporal distribution. It will also look at the effects of covariates such as poverty, water supply, sanitation and global warming on expansion of the dengue endemic regions. .

The second part of the chapter will focus on the genetic evolution of the viral isolates circulating in the region. Phylogenetic studies of dengue viruses have uncovered genetic variation within each serotypes, these variations have been organized in discrete clusters on dendograms. Analyses of such studies have broadened our horizon to relate the mutational changes with disease evolution and factors like seasonality and incidence variability. This part of chapter will focus on the common mutational variations that have been reported so far and how these relate with the disease dynamics in the endemic region.

In the third and final part of the chapter an attempt has been made to relate the mutational changes of dengue genotypes with disease severity. Vast array of literature has been published investigating relationship of genetic variation with disease severity. The structure

of virus E- protein that confers the viral infectivity and host immune response of the virus (E.Descloux,2009) remains the focus of such studies. Sequence variation at different loci such as CprM, E/NS1, preM/E, C/prM/M and untranslated regions etc. have been investigated for its association with disease severity. This part of chapter will throw some light on our current understanding of disease severity and it relation with genetic variation.

2. Historical background of dengue virus in South East Asian

Geographically South East Asia comprises of land south of China to east of India extending as far as to the north of Australia. Although geographically the region is well defined, the list of countries included in this region varies due to political reasons. For the purpose of this review W.H.O based definition has been used. In addition status of dengue virus in further south of the region; including countries like Pakistan and Bangladesh have also been included to encompass the broader spectrum of the region.

2.1 Dengue vector evolution

Evidences suggest that vectors *Ades aegypti* and *Ades albopictus* originated from darker sylvan forms found in African tropical forests. It is believed to have reached New World from West Africa via slave ships during the 17th century (Gubler, D.J.1998). *Adesaegypti* was introduced into the coastal cities of South East Asia from East Africa around nineteenth century via the shipping industry. With the eruption of World War II it became deeply entrenched in many cities (Gubler, D.J.1998). The mitochondrial genetic diversity studies have revealed circulation of two distinct clusters of *Ades aegypti* in South East Asia one with strains from French Polynesia, Guinea and Brazil while the other cluster is of strains that migrated from Europa Island in Mozambique and Amazonia (Mousson et al 2005). In contrast; *A. albopictus* is known to be native to South East Asia. It has spread within past few decades to various countries primarily due to introduction of trade of used tyres worldwide. Using ecological niche modeling Benedict and co-workers have predicted the risk of global invasion by *Ades albopitus* secondary to cargo trade and increasing air travel. Although temperate and humid climates are prerequisites for the optimum survival of both the vectors but *A. albopictus* is known to better acclimatize to the cold and dry weather due to its ability of efficient egg diapause during the extreme conditions, thus favoring its survival in the regions with exotic temperature ranges (Benedict, M.Q, *et al* 2007).

2.2 Factors leading to disease spread in SEA

The factors responsible for the insurmountable expansions of dengue in the region are complex and thought to be intricately linked with vector-host-virus triad, socioeconomic stresses and climatic variations. There are excellent reviews that discuss the impact of these factors in details (Aiken, S.R. 1978, Kendall, C. et al 1991, Halstead, S.B. 1966). Only salient factors in context of SEA will be discussed here. The distribution of DHF outbreaks in SEA correlates with emergence of mosquito A. aegypti in South East Asian countries perhaps due to displacement of indigenous A. albpictus in the region. This is considered to be associated with uncontrolled urbanization leading to shanty towns with inadequate pipe water supply and poor sanitation.

A. albopictus is semi domestic species that breeds on natural and man-made breeding sights; it feeds on variety of animals, birds and man. The A. aegypti on the other hand is more

acclimatized to urban set-up, once established the density of this mosquito is directly proportional to density of human population and artificial breeding sites (Merril S.A *et al* 2005), it feeds almost exclusively on humans. Moreover A. aegypti is considered to be more competent vector for dengue virus. Genetic traits that determines successful midgut infection by DEN virus have been mapped on several loci on A. aegypti chromosomes (Benedict, M.Q, *et al* 2007) indicating that vector competence is genetically determined.
The extent to which these mosquitoes compete with each other in the environment is not clear, nonetheless the balance of two species in the region is important, and the socioeconomic factors in SEA appear to be displacing *A.albopictus* in favour of *A.aegypti* leaving the population more susceptible. The poor socioeconomic conditions are major contributing factor to sustained vector activity with severe form of disease in the South East Asia. The breeding habitats of A.aegypti have been strongly associated with squatter settlements, inadequate piped water supply and sewage facilities (Halstead, S.B. 1966). In addition, there are impacts of higher environmental temperature in the region. High temperature is inversely related to the mosquito gonotropic cycle and viral extrinsic incubation period; this increases the egg laying episodes resulting in more blood meals and increased risk for viral transmission. In addition shorten extrinsic viral incubation period culminate to increase virus load at time of inoculation (Focks D.A. et al 1993). These effects have been proven for dengue vectors in simulation studies conducted by (Cox J et al 2001) and it has been projected that increase in global temperature would increase the length of transmission season in temperate regions.

2.3 Dengue fever and dengue hemorrhagic fever

The word dengue is believed to have originated from Swahili language *"ki denga pepo"*, which describes sudden cramp like seizure. The clinical symptoms suggestive of dengue virus infection can be traced back to Chinese Chin Dynasty (265-420 AD) where disease was considered as water poison and was known to be associated with water and insects (anonymous 2006).
Emergence of the disease in the new world can be traced back to the transmigration of the vector in the 17th century. There are reports that suggest possible epidemics of dengue like illness in three major continents (Asia, Africa and North America) as early as 1779 and 1780, within Asia Batavia (now known as Jakarta) was affected by this outbreak (Halstead,S.B. 1966). By early nineteenth century Dengue fever was known to be endemic in the rural areas of South East Asia probably due to the indigenous vector *A.albopictus*. It manifested as self limiting disease to which native population developed immunity at early age. With the advent of *A. aegypti* at Asian ports, the disease spread to the main inland cities and towns. It is assumed that unlike rural population, the urban populations of South East Asia remained susceptible to dengue virus and were then infected by newly imported vector. Dengue epidemics progressively became less frequent as urban population became immune to the disease, until 1953 when a new form of dengue fever was reported from Thailand and Manila, where children suffered from fever followed by bleeding diathesis; the disease was then called as *Philippine Fever* (Aiken, S.R. 1978). By 1960's the hemorrhagic form of disease had spread to Malaysia, Vietnam, Sri Lanka, Singapore and Indonesia (Halstead, S.B. 1966). The disease epidemiology extended and outbreaks of dengue hemorrhagic fever (DHF) were reported from India 1988) French Polynesia (1990), Pakistan (1992) and Bangladesh (2000).Until recently, DHF was considered to be disease of childhood, especially in South

East Asia where mean age of cases under fifteen, and the modal age of five or slightly higher was reported from countries such as Thailand, Philippines and Malaysia, however, recent reports are now documenting increasing number of DHF and DSS in adult population as well (Khan E et al 2007). The precise cause of DHF/DSS remains elusive despite enormous research in this area. Evidences suggest interplay of multiple factors such as host genetic make-up with unique immune response and viral virulence may play a role in determining the severity of the disease.

2.4 Pathogenesis of severe dengue disease

There are two form of Severe disease, namely dengue shock syndrome (DSS) and DHF without shock. It is proposed that devastating coagulation derangements due to host immune response leads to heamorrhage and shock in severe cases. The concept of original antibody sin leading to immune enhancement is considered to be the main reason whereby infection with one type of dengue virus sensitizes an individual and that subsequent infection with different virus type elicits a hypersensitivity reaction (secondary infection). Various studies have been conducted to show the association of elevated cytokines in patients presenting with DHF and DSS. Elevated serum levels of cytokine and chemokines such as IL-2, IL-8, IL-6, IL-10, IL-13, TNF and INF-γ have been found to be significantly associated with patients presenting with DHF and DSS in clinical setting (Azeredo et al., 2001; Hung, et al., 2004, Clyde. K. et al., 2006). It has been proposed that the pro-inflammatory cytokines released by the cross reactive memory T-cellls, induce plasma leakage by its effects on the endothelial cells (Eva.H. et al 2004; Aviruntanan et al., 1998). In fact in-vitro studies have rendered endothelial cell monolayers permeable by the application of chemokine such as IL-1β (Cardier et al.,2005). In vitro-and in-vivo models of studies also suggest role of decreased nitric oxide levels and its relation with IL-10 and raised viral load (Simmons et al., 2007). There is evidence that suggests relation of increased expression of certain cytokines such as IL-1β, TNF-γ, and IL-6 with elevated NO production (Guzik et al., 2003).

With the advances in genomic and bioinformatics tools the scope of genetic studies has greatly expanded particularly in depth data on genomic changes and its association with disease epidemiology, seasonality and severity has been made available. Growing availability of comparative genome sequence data has provided important insights into the molecular evolution of dengue virus. Evidence strongly suggests appearance of new strains correlating with DHF/DSS epidemics. Despite the wealth of genomic data now available the exact cause and effect of viral virulence and clade changes is yet to be proven, however it is quite evident that different serotypes and viral linage is continually changing with local extinction and emergence of new clade and that the introduction of new clade in the region translates in form of outbreaks of DHF and DSS.

3. Distribution of dengue virus serotypes in SEA

Dengue like other RNA viruses is prone to genetic mutations as it replicates using RNA-Polymerase; enzyme that lacks proof reading mechanism. The mutation rates in the order of 10^{-3}has been reported for dengue (ElodieDes et al 2009) in different host settings. Such mutations often result in variants that become targets of selection; an outcome of underlying genotype and its environment. Despite these facts dengue virus do not evolve as fast as other RNA viruses. The only **macro evolutionary** divergence is perhaps the radiations in its four serotypes in its primate host (sylvatic strains) around one thousand years ago (ElodieDes

et al 2009). There after genetic mutation in the envelope protein and receptor binding domains resulted in its emergence as infectious pathogen in human population. The divergent forms of these sylvatic strains are often found to be circulating in human habitat, suggesting that enzootic cycles with some spill over in the surrounding human population. This has been shown in Malaysian populations settled near forest and marshy habitats (Wang, E. et al., 2000). The phylogenetic studies conducted based on envelope gene sequences of basal portion of sylvatic linage, DENV 1,-2,-4 of Malaysian descent suggest that endemic /epidemic strains of these viruses diverged from sylvatic ancestors more than 1000 years ago (Wang, E. et al., 2000). Thereafter, only micro evolutionary change within dengue serotypes have taken place, these changes have nevertheless resulted in substantial genetic diversity with emergence of endemic and epidemic strains in different parts of the region.

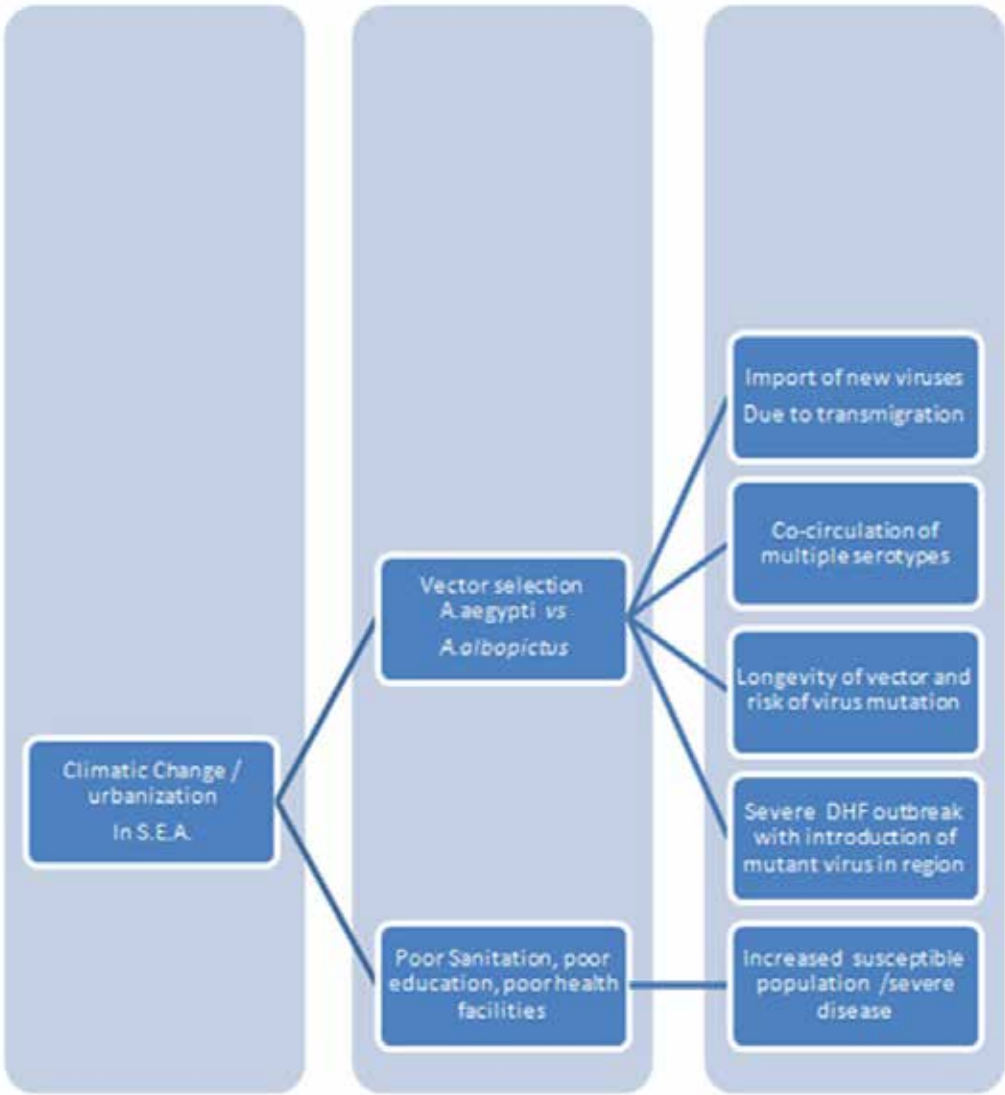


Fig. 1. The effects of climatic and social change on vector evolution and disease severity

DEN-3 viruses have undergone independent evolution which has resulted in emergence of four genetic subtypes of which subtype I-III circulate in the South East Asian Region. Subtype I comprises of viruses from Indonesia, Malaysia and the Philippines; subtype II of viruses from Thailand and subtype III includes viruses from Sri Lanka India and Pakistan. The genetic evolution in these subtypes is primarily reported mutations in the prM/M and E structural protein genes. In spite of these mutations, the genomic region has retained greater than 95% amino acid sequence similarity (Lanciotti, R.S et al.,1994), suggesting that these are highly conserved regions responsible for protein architecture and / or biological function.

Phylogenetic studies suggest that there are regional foci of virus extinction and selection, one such region is Thailand where the indigenous DEN-3 virus circulating up to 1992 disappeared and was replaced by two new lineages perhaps from a common ancestor (Wittke, V. et al. 2002). The sequence of all Thai DEN-3 isolates recovered after 1992 had T at position 2370 in contrast to the C at this site in the pre-1992 samples(Wittke, V. et al. 2002), and nucleotides difference was observed in at least 45 sites of total 96 sites studied. It appears that the post-1992 strains have replaced the pre-1992 strains). These studies point towards potential of regular extinctions of strains of DEN-3 virus and replacement by new variants in the region (Wittke, V. et al. 2002). Natural selection and / or genetic bottle neck could be the plausible causes for this variation. Since the extinction of pre 1992 strains and appearance of new epidemic strain in Thailand occurred during inter-epidemic period it is therefore hypothesized that the genetic bottleneck is perhaps the cause of regional replacement. This is further supported by studies from India reporting shift and dominance of the dengue virus serotype-3 (subtype III) replacing the earlier circulating serotype-2 (subtype IV) with emergence of increased incidence of DHF and DSS in subsequent outbreaks (Dash, P.K.et al. 2006). Strains from the 2005 outbreak in Karachi (Pakistan) were found to be similar to those from Indian strains of dengue serotype 3, and were responsible for deadly outbreak in 2005-06 (Jamil. B. et al. 2007). Thus over the period 1989 and 2000, a new clades of DENV-3 genotype III viruses have replaced older genotype and clades in this region and emergence of new clades coincided with severe epidemics. The epidemiologic data suggests that the DEN-3 virus responsible for recent epidemic outbreaks in Mozambiques, Gutamealla, Pakistan and SriLanka may have been introduced from India, and changing age structure of dengue patients from 1996–2005 may also be indicative of the selected virus moving into new areas(Kanakaratne, N. et al.2009).

4. Genetic evolution and disease severity in SEA

The micro evolutionary change within dengue serotypes has resulted in substantial genetic diversity with emergence of endemic and epidemic genotypes. With current advances in the field of genetic and molecular techniques scientists are now trying to decipher relation of changing clades with disease severity and epidemic potential. With the availability of complete genomic sequence of the Dengue virus different genetic loci have been investigated to find this relationship. Envelope –gene (E-gene) sequence is the most frequently investigated locus, (Wittke,V.et al., 2002;-Thu, H.M. et al.,2004;Islam, M.A.et al., 2006,27) followed by capsuler C-prM gene (Kukreti, H. 2008;,Dash, P.K. 2006;,Kanakaratne, N. 2009;Jamil B,2007). In addition non-structural (NS) viral proteins such as NS1 and untranslated genomic region 3'-UTR, 5' UTR along with complete genomic sequences have been investigated to relate the genetic changes with the disease severity (Mangada, M.N. et al., 1997;Zhou,Y.et al.,2006;Islam, M.A.et al.,2006. Despite the wealth of genomic data

available the exact cause and effect of viral virulence and clade changes is yet to be proven, however, viral linage is continually changing with local extinction and emergence of new clade. The introduction of new clade in the region translates in form of outbreaks of DHF and DSS. In order to analyze if there is a selection of specific clade in South East Asia that is circulating in the region and causing DHF outbreaks we conducted a meta-analysis. Studies conducted from 1950 to 2009 in South East Asian region that have investigated association of disease severity with specific sequence mutations in the dengue virus genome were retrieved. The objective was to analyze association of disease severity with the specific genomic mutation in the clade circulating and causing periodic epidemics in South East Asia. Since DENV-2 and DENV-3 are more common in this region our study was focused on these two genotypes only. Objectives of the metaanalysis were to identify association of specific genetic mutation in DENV-2 and DENV-3 with clinical severity seen during periodic epidemics in South East Asia. The specific review question was:Is clinical severity of dengue in the South East Asian region associated with emergence of specific mutations in genomes of DENV-2 and DENV-3 genotypes? We hypothesized that there is changing pattern of dengue virus genotypes in South East Asia and these mutations are associated with clinical severity of the disease.

4.1 Methods

4.1.1 Literature search

The literature search was performed from February 2010 to June 2010. Data sources include Medline via Pubmed (1950-February 2010), Cochrane data base of systematic reviews, Google scholar and experts in the field. Secondary references and review articles were scanned for thematic review. Hand search of the journal was also carried out. However, unpublished and ongoing studies could not be explored. Terminologies i.e. dengue type 1-4,

Author's name	**Why excluded?**
Araujo J.M.G. *et al,* (2009)	Conducted in Brazil, (also include dengue strains from regions other than South east Asia) but country of origin of these isolates was not clear, Sequences were selected from gene bank.
Lanciotti RS.*et al*, (1994)	Conducted in USA, origin of isolates not clear (also include dengue strains from other geographical regions other than South east Asia) The DEN-3 viruses used in this study were obtained from the collection at the Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, Colo., U.S.A.
Soundravally R, *et al* (2007)	Focused on host factors as cause of disease severity rather than on virus gene mutations
Soundravally R., *et al* (2008 a)	Focused on host factors as cause of disease severity rather than on virus gene mutations
Soundravally R., *et al,* (2008b)	Focused on host factors as cause of disease severity rather than on virus gene mutations
Gibbons RV. *et al,* (2007)	Does not give sequence analysis in detail

Table 1. Features of the excluded studies

dengue fever, dengue hemorrhagic fever, genetic variation, sequence analysis, south East Asia were used individually as well as in various combinations. Two independent reviewers; reviewed the titles, abstracts and full text articles and selected potentially relevant studies based on inclusion criteria established prior to the literature search. Discrepancy between the reviewers were sought to reach on consensus in consultation with third reviewer. Those potentially irrelevant studies that were ultimately excluded are listed together with the reason for exclusion in Table 1.

4.1.2 Inclusion criteria

Studies which reported dengue virus genotype (mutation / sequencing of viral genetic material) and clinical features of dengue fever patients were included.

4.1.3 Design of the studies

All type of observational studies i.e. case report, case series, surveys and descriptive cross-sectional studies which were focusing on genotype and clinical presentation of dengue patients were included in the review. *Population:* Population includes patients of dengue fever of all age groups. No age and sex restriction were applied. *Outcome of interest:* Difference in nucleotide and protein sequences were analyzed and compared according to geographical origin, the sampling period and the clinical presentation. Clinical severity of the disease is defined as presence of DF, DHF or DSS. *Language:* Only articles in English language were included in the review.

4.1.4 Exclusion criteria

All those studies focusing on dengue vector control, clinical trials on vaccines, clinical trials on drugs, pure prevalence or incidence, unusual case report or case series without genotype and studies conducted in countries other than south East Asian region were excluded.

4.1.5 Data extraction

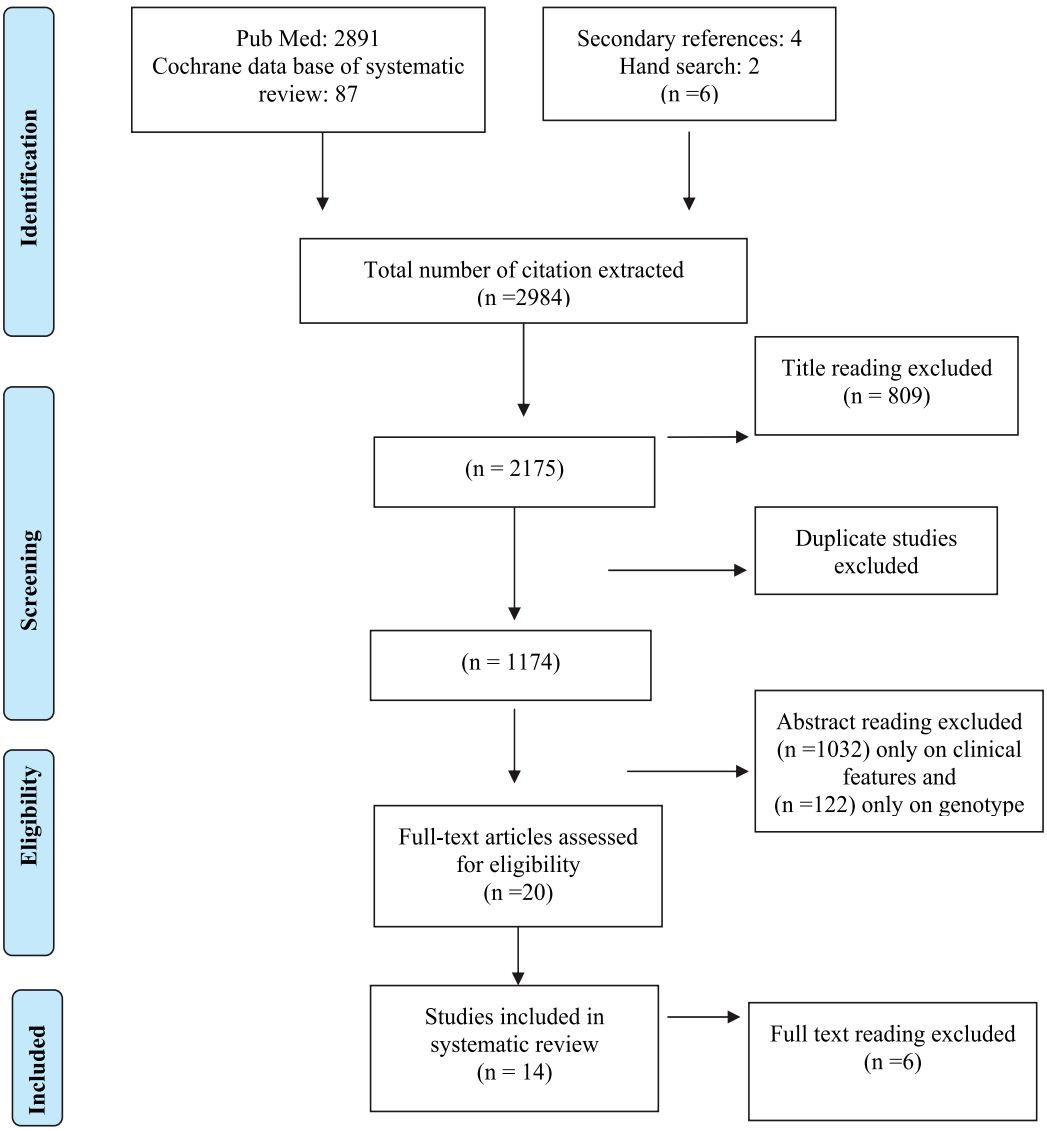
Data extraction of the included studies was done by using structured data extraction form specifically made for the review. Data was extracted for country of origin, year of publication, clarification of objectives, type of study, its duration and setting, results on both genotype and clinical severity etc.

4.1.6 Data synthesis

A narrative data synthesis was carried out to show result summary of all included studies which include description of clinical features and genotype of dengue virus. However, meta-analysis could not be performed due to non availability of required data i.e. measure of strength of association. Hence, pooled effect of genetic variation on clinical severities among dengue patients could not be provided.

4.1.7 Quality assessment

According to Cochrane Collaboration's recommendation, the quality of included studies have been assessed by using criterion which asses the quality of studies by focusing on study type, sample size calculation, clarity of objective, selection of cases, and internal validity of selected studies.



PRISMA Flow Diagram

Fourteen studies were finally selected based on inclusion criteria i.e., association of dengue genotype and clinical severity of the diseases in the patients and were conducted in different countries of South East Asia. Setting of these studies were; Thailand 6 (Zhang, C. et al 2006, Rico-Hesse R. et al. 1998, Wittke,V.et al.,2002), Myanmar (Thu H.M. et al.,2004), India (Kukreti, H. et al.,2008;Dash, P.K.et al.,2006), Bangladesh (Islam, M.A.et al.,2006), Sri Lanka (Kanakaratne, N. et al.,2009), Taiwan (King, C.C. et al.,2008) and Pakistan (Jamil B, et al 2007). These studies were published from 1997 to 2009. Since the focus of our study was on DEN-2 and DEN3 viruses 12 studies out of these 14 were finally included in this study

4.1.8 Study sample characteristics

Eight studies were conducted in the hospital settings (Zhang, C. et al 2006, Rico-Hesse R. et al. 1998, Wittke,V.et al.,2002, Kukreti, H. et al.,2008;Dash, P.K.et al.,2006, Islam, M.A.et al.,2006, al,Kanakaratne, N. et al.,2009), three in the community (Zhou Y, et al 2006, Dash PK, et al 2006, Kanakaratne, N. et al.,2009), where as data was extracted from laboratory records in two studies (Jamil B, et al, 2007, King, C.C. et al.,2008) and dengue virus strain were taken from frozen stock of clinical serum samples in two (18, 21).

Age ranges for dengue patients in these studies varied from 1 year to 70 years. The total numbers of dengue patients were 7663 in these studies. Characteristics of the studies included in this review have been summarized in table 2. A total of 285 virus isolates were subjected to genotyping/ sequence analysis in these studies. All four genotypes were studied in three studies (Zhou Y, et al 2006,Jarman RG, et al 2008, Rico-Hesse R. et al. 1998); only DEN 3 in five studies (Wittke,V.et al.,2002, Kukreti, H. et al.,2008; Islam, M.A.et al.,2006), only DEN 2 in three studies (Zhou Y, et al 2006,Mangada MNM et al 1997, Zhang, C. et al 2006), only DEN 4 in one studies (KlungthongC, et al, 2004), whereas DEN 1 and DEN 3 in one study (Kukerti H, et al 2008) and DEN 1, DEN 2 and DEN 3 studied in one study (Jarman RG et al 2008).

4.1.9 Clinical definition

Dengue case was defined on the bases of presence of IgM, IgG, or fourfold or greater rise in hemagglutination inhibiting (HI) antibody titer against dengue virus, and presence of dengue virus specific nucleic acids in RT-PCR. Clinical severity was defined as presence of hemorrhagic manifestation and DHF related symptoms such as thrombocytopenia, skin rash, gum bleeding, gastrointestinal bleeding, hemorrhagic sclera, epistaxis, edema and ascitis. Where as other studies simply defined as presence of DF, DHF grade I, II and III and DSS as per WHO criteria.

5. Nucleotide sequencing and phylogenetic analysis

Envelope –gene (E-gene) sequence was most frequently investigated loci, nine studies were focused on this region followed by C-prM gene, in three studies both genetic loci studied in one study (9) and one study included NS1 along with PrM and E loci. The 3′-UTR, 5′ and 3′ UTR and complete genomic sequences were studied in one each

Homology search and comparisons of most obtained sequences were performed using commercially available software systems such as DNASIS, DNAStar, 3′ –UTR secondary structures were estimated using MFOLD package, while nucleotide sequence alignments (Phylogenetic analysis) were performed using CLUSTAL X, MEGA version, and maximum likely hood methods available e.g. PAUP PROGRM

The quality of included studies was assessed by using criterion which asses the quality of studies by focusing on study type, sample size calculation, clarity of objective, selection of cases, and internal validity of selected studies. From total of 16 points scale, individual score on quality assessment criteria was as follows 8.5 (Rico-Hesse R. et al. 1998), 7.0 (Zhou Y, et al 2006,Jarman RG, et al 2008, Jamil B, et al, 2007), 10.5 (Dash PK, et al 2006, Kukreti, H. et al.,2008), 5.0 (Wittke,V.et al.,2002), 6.5 (Zhang, C. et al 2006,), 10 (Zhou Y, et al 2006, Mangada MNM et al 1997), 7.5 (Wittke,V.et al.,2002, Jarman RG, et al 2008, King, C.C. et al.,2008), 12(KlungthongC, et al, 2004). Since most of the severe DHF outbreaks in SEA have been associated with DEN-2 and DEN-3, mutational changes and its relation to disease severity of these two serotypes will be discussed here in detail.

5.1 Mutations observed

5.1.1 E-gene mutations

In case of DEN-2 virus, maximum numbers of viral isolates have been analyzed in studies from Thailand. The E-NS1 region of 77 different variants of DEN-2 studied using Maximum Parsimony analysis of 240 nucleotide sequence, showed 11 of 240 nucleotide variation; 4.6% divergence but did not reveal significant segregation of virus according to geographic location (Rico-Hesse R. et al. 1998). Similarly, Phylogenetic analysis of 120 E gene of DEN-2 by another group from Thailand has confirmed existence of six genotypes of this virus; however evolutionary relationships among the genotypes is difficult to determine (Zhang, C. et al 2006). In terms of dengue pathogenesis these studies failed to show segregation of DF versus DHF-associated viruses on the evolutionary tree. There are no clear-cut evolutionary divergence or branching of DF versus DHF isolates, suggesting that nucleotides from this region of the genome encode amino acids that are apparently not under immune selection (Rico-Hesse R. et al. 1998 and Zhang, C. et al 2006,).

DEN-3 has replaced DEN-2 as most frequently isolated virus in Thailand since late 1980's (Wittke,V.et al.,2002). The evolutionary history of Thai DEN-3 viruses, has been studied by comparative analysis of the nucleotide sequence of E protein genes of currently prevailing isolates with those from all previously published E gene sequences of DEN-3 virus available in Gen Bank (Wittke,V et al. 200218), this study has shown E-gene of DEN-3 to be relatively conserved at amino acid level, however, four amino acid changes have been identified within genotype II of Thai strains. The amino acid changes observed at positions (E172 I-V) and (E479 A-V) are the only difference found between pre and post-1992 viruses. Similarly, there is little evidence to support in-situ evolution among the virus samples that were studied over prolong period ranging from days to months in a selected community in Thailand(Jarman, R.G.et al.,2008) very few mutational changes were noted, and association of these mutations with disease severity could not be delineated either. Analysis by E-sequence of eight DEN-3 strains from Bangladesh (2002 out-break strains) were found to be very closely related to Thai isolates that caused out-break in 1998 in Thailand. The multiple alignment of amino acid (aa) sequence revealed that Bangladeshi isolates and Thai isolates shared common aa changes at position E127 (I-V), suggesting that 2002 outbreak in Bangladesh was due to introduction of Thai isolates (Islam, M.A.et al.,2006), however the statistical association of aa changes with disease severity could not be delineated. In case of Sri Lankan DEN-3, type III is the most frequent strain with two distinct clades IIIA and IIIB linked to mild and severe disease epidemics on the island respectively (Kanakaratne, N. et al.,2009). Phylogenetic studies of E-NS1 junction of DEN-2 isolates from Sri Lanka has categorized the isolates into 4 genotypes designated as Malaysian/Indian subcontinent, Southeast Asian, American, and West African (Sylvatic) and Sri Lankan isolates are closely related to Indian / Malaysian genotype.

5.2 C-prM mutations

The phylogenetic analysis of 433 base pair region (nucleotides 180—612) of the DEN-3 *CprM* gene junction showed that sequences of Delhi isolates (2006 outbreak) were closely related to sequences from Guatemala (1998) and presented a nucleotide identity of 95.9—98.2% (mean 97.05%). On comparison of Delhi 2006 sequences with other Indian sequences from years 2003, 2004, and 2005, mean sequence divergence of 2.85%, 2.15%, and 1.6%, respectively, were observed (Kukreti, H. et al.,2008. Common amino acid mutations observed in 2006 DENV-3 sequences are given in table 3. Similar study performed on DEN-3 isolates of 2003-04 outbreaks in New Delhi, found them to be closely related and belonged to subtype III from Sri Lanka (Dash, P.K.et al.,2006). Moreover, Phylogenetic analysis of C/PrM/M region of

DEN-3 isolates from Pakistan (2004-05 outbreak isolates) also showed sequence homology with 2003-04 New Delhi outbreak strains suggesting that circulation of common isolates of DEN-3 subtype III in the region . There was no clear statistical association of disease severity

DENV-Protein	Geographical Origin	year	aa change (positions)	Relation to Disease severity	Ref
Envelope (E) Den 3	Thailand	2002	E124 (P-S) E132 (H-T) E172 (I-V) E479 (V-A)	Could not be ascertained	(Rico-Hesse R *et al*)
Envelope (E) Den 3	Thailand	2008	Phylogenetic analysis= multiple genetic variants (mutational positions not mentioned)	Could not be ascertained	(Jarman RG)
DEN 3 E region	Taiwan	2008	E301 (L to T)		(King, C.C. et al.,2008)
Envelope (E) Den 3	Bangladesh	2005/6	E81 (I-T) E140 (I-T) E127 (I-V)	Distinct clade causing epidemic outbreak	(Islam, M.A.et al.,2006)
Den 3 C-preM/E	Srilanka	2003-06	Phylogenetic analysis= multiple genetic variants (mutational positions not mentioned)	2 distinct clades linked to mild (IIIA) and severe (IIIB) disease epidemics	(Kanaka-ratne et al)
CprM Den 3	India	2005/6	CprM88 (I-V) CprM 121(A-A) CprM127 (I-P) CprM122 (G-G) CprM55 (A-L) CprM 128(V-G)	No association of any particular variant with serious dengue disease	(Kukreti H)
C-prM	India	2006	C-prM108(M-I) C-prM112(T-A)	may be attributed to increased incidence of DHF & DSS in India	(Dash PK)
CprM Den 3	Pakistan	2005-2006	C-prM	Similar to New Delhi strain 2004	Jamil B, et al
DEN 3 prM region	Taiwan	2008	CprM 55 (L-H) PrM 57 (T-A)	No association with disease severity could be determined	(King CC)

Table 2. Genetic Characteristics and relation to disease severity in patients with DEN-3 infections reports from South East Asian Region

with specific serotype, as viruses isolated from DHF patients fell at different locations on the phylogenetic tree (Kukreti, H. et al.,2008;Dash, P.K.et al.,2006).

Using maximum likelihood and Bayesian approaches, phylogenetic analysis of Taiwan's indigenous DENV-3 isolated from 1994 and 1998 dengue/DHF epidemics were found to be of three different genotypes –I, II and III each associated with DEN-3 circulating in Indonesia, Thailand and Sri Lanka, respectively(King, C.C. et al.,2008). The authors of this study analyzed complete nucleotide sequence of DEN-3 for its mutation and its relation with regional evolution. The highest level of nucleotide sequence diversity, and the positive selection site was detected at position 178 of the NS1 gene.Although the authors have identified the NS 1 gene as the positive selection site and the envelope protein site for purifying selection pressure, however direct association of these changes with disease severity was not determined. Study from Bangkok Thailand performed sequence analysis on E/NS-1 region of Thai isolates to determine if viral strains from less severe DENV infections had distinct evolutionary nucleotide pattern then those with more severe form (Rico-Hesse R. et al.,1998). This study found that two distinct genotypes were identifiable from both DF and DHF cases, suggesting its evolution from common progenitor that perhaps shares the potential to cause severe disease.

DENV-Protein	Geographical Origin	year	aa change (positions)	Relation to Disease severity	Ref
E/NS1 (77 DEN-2 virus strains studied)	Thailand	1998 from 1980	11 nucleotides (4.6% divergence) between Strain PUO-218-280. 22 nt or 9.2% divergence PUO-218-D80141	No specific association with disease severity	(Klungthong C)
E/NS1 junction Den 2	Srilanka	2003-06	239-nt (from positions 2311–2550)	Could not be ascertained	(Kanaka-ratne et al)
3′ and 5′ UTR Den 2	Thailand	1996-97	5′ NCR homologus 3′ UTR trinucleotide change 297± 299 (two transversions and one transition)	Trinucleotide change may alter the functional characteristic of Secondary structure	(Mangada MNM)
Den 2 E /C/NS2A	Thailand	2006	approx 10^{-3} substitutions	no apparent association	(Zhang C)
3′-UTR	Thailand	1973 to 2003	Variable secondary structures were detected	No clear association	(Zhou Y)

Table 3. Genetic Characteristics and relation to disease severity in patients with DEN-2 infections reports from South East Asian Region

5.3 Untranslated Region (UTR) mutations

The 3′ UTR region is thought to play a pivotal role in the DENV biology; it contains several conserved regions as well as 3′ long Stable Hair Pin structure which is conserved among all the members of the family *Flaviviridae*. It has been proposed that this structure interacts with viral and host nucleic acid and protein factors to form a complex to regulate transcription and replication (Zhou, Y. et al.,2006. Therefore it appears to play a significant role in the efficiency of RNA- translation, and virus ability to cause infection, hence the role of 3-UTR in determining the severity of dengue disease seems plausible. The literature reviewed under this study did show considerable intra-serotype diversity at 3-UTR region with greatest variability seen in DEN-4 followed by DEN-1.

A comparative analysis of 3′ UTR conducted for DENV isolates from Bangkok, Thailand compared Thai sequences with 61 globally sampled isolates of DENV taken from patients with varying disease severity. Although some genetic variations were found both within and among the serotypes notably at 3′ Long Hairpin Stable structure, however these mutations did not show consistent association with the clinical outcome of the DENV infection (Zhou, Y. et al.,2006). Study focusing on terminal 3′ 5′UTR sequences of four DEN-2 from Thailand 1998 outbreak strains, showed complete homology for sequences at 5′ UTR (highly conserved region) when compared with the prototype virus New Guinea C strain

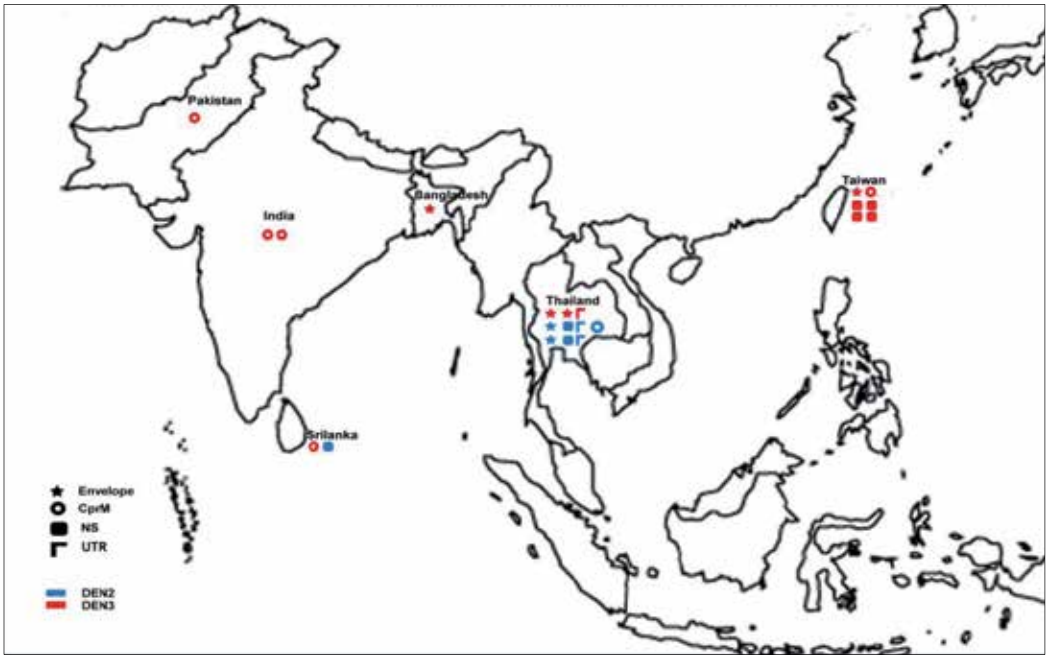


Fig. 2. The Geographical distribution of mutations in DEN-2 and DEN-3 viruses detected at different genomic loci of isolates from South East Asia

6. Conclusion

Ades aegypti was introduced into the coastal cities of South East Asia from East Africa around nineteenth century via the shipping industry. With the eruption of World War II it

deeply entrenched in many cities. The distribution of DHF outbreaks in SEA correlates with emergence of mosquito *A. egypti* in South East Asian countries due to uncontrolled urbanization leading to displacement of indigenous *A. albpictus* from the region.

Phylogenetic analysis suggests that there are foci of virus extinction and selection in South East Asian region, one such region is Thailand where the indigenous DEN-3 virus circulating up to 1992 has disappeared and replaced by two new lineages perhaps from a common ancestor. These studies point towards potential of regular extinctions of strains of dengue virus particularly DEN-3 virus and replacement by new variants in the region. Natural selection and / or genetic bottle neck are plausible causes for this variation. Since the extinction of pre 1992 strains and appearance of new epidemic strain in Thailand occurred during inter-epidemic period we therefore hypothesize that the genetic bottleneck is perhaps major cause of regional replacement. This is further supported by studies from India reporting shifting and dominance of the dengue virus serotype-3 (subtype III) replacing the earlier circulating serotype-2 (subtype IV) with emergence of increased incidence of DHF and DSS in subsequent outbreaks. Strains from the 2005 outbreak in Karachi (Pakistan) were found to be similar to those from Indian strains of dengue serotype 3, and were responsible for deadly outbreak in 2005-06.

Despite the growing genomic data base in the gene bank there are fundamental gaps in our understanding of epidemiological and evolutionary dynamics and its relation with disease severity. There are two possibilities that explain the association between clade replacement and increased viral virulence. The first is the possibility of these viruses to be better fit and therefore produce high viremia in infected humans, consequently with better transmission of virus by the vector. The other hypothesis to explain the possible virulence of emerging clades in the region is its improved ability to avoid neutralization by serotypes cross reactive antibodies (Kochel et al., 2005). Thus there is relative abundance of different serotypes and viral linage is continually changing in South East Asia. In face changing threshold of host immunity, periodic epidemics of DHF and DSS is due to local extinction and emergence of new clades. Over the period 1989 and 2000, a new genotype of DENV-1 and new clades of DENV-3 genotype III viruses have replaced older genotype and clades in this region and emergence of new clades coincided with severe epidemics.

Thus South East Asia displays greatest degree of genetic diversity, suggesting that it is the hub for the evolution of new epidemic strain. However, selection of specific clade and association of specific sequence variation with disease severity at various genomic levels reported in the literature reviewed in this study lacks strength of association i.e. reporting Relative Risk (RR)/ Odds Ratio (OR) limits our interpretation regarding causality or pin pointing specific clade with virus virulence, and therefore further studies are recommended.

7. Acknowledgment

This work was supported by University Research Council of the Aga Khan University, special thanks are extended to Mr. Faisal Malik for expert help in formatting of figures and tables

8. References

Aiken S R, Leigh C H, (1978),Dengue haemorrhagic fever in South East Asia.Transactions of the institute of British geaographers,New series ,Vol. 3, No. 4, (1978), pp.476-497

Annonymous.(2006) etymologia dengue. Emerg.Infec. Dis 2006; vol.12 (6) 893

Araújo JMG, Nogueira RMR, Schatzmayr HG, Zanotto PMA, Bello G. Phylogeography and evolutionary history of dengue virus type 3. Infection, Genetics and Evolution. 2009;9(4):716-25.

Benedict M Q, Levine R S, Hawley W A, Lounibos L P. (2007).Spread of the Tiger:Global risk of invasion by the mosquito Aedesalbopictus. Vector born zoonotic Dis. Vol .7, No. 1,(Jan 2008),pp.76-85.

Benedict, MQ., Levine, R.S., Hawley, W.A. and Lounibos, L.P. (2007). Spread of the Tiger: global risk of invasion by the Mosquito Aedesalbopictus. Vector Borne Zoonotic Dis. 2007 ; Vol.7(1): pp.76–85.

Cox J, Brown H E, Rico-Hesse R. (2011). Variation in vector competence for dengue viruses does not depend on mosquito midgut binding affinity. PLoS: Neglected Tropical Diseases, Vol. 5, No. 5, (May 2011)

Dash PK, Parida MM, Saxena P, Abhyankar A, Singh CP, Tewari KN, et al. Reemergence of dengue virus type-3 (subtype-III) in India: Implications for increased incidence of DHF & DSS. Virology Journal. 2006;3(1):55.

Desprès P, Frenkiel MP, Deubel V. Differences between cell membrane fusion activities of two dengue type-1 isolates reflect modifications of viral structure. Virology. 1993;196(1):209-19.

Downs SH, Black N. The feasibility of creating a checklist for the assessment of the methodological quality both of randomised and non-randomised studies of health care interventions.Journal of Epidemiology and Community Health. 1998;52(6):377.

ElodieDescloux, Van-Mai Cao-Lormeau, Claudine Roche, Lamballerie XD. Dengue 1 Diversity and Microevolution, French Polynesia 2001–2006: Connection with Epidemiology and Clinics. PLoSNegl Trop Dis. 2009;3(8).

Ferguson N, Anderson R, Gupta S.The effect of antibody-dependent enhancement on the transmission dynamics and persistence of multiple-strain pathogens.Proceedings of the National Academy of Sciences of the United States of America. 1999;96(2):790

Focks, D.A., Haile, D.C., Daniels, E., Moun, G.A., 1993. Dynamics life table model for aedes aegypti: Analysis of the literature and model development. J. Med. Entomol. 30, 1003–1018.

Gibbons RV, Kalanarooj S, Jarman RG, Nisalak A, Vaughn DW, Endy TP, et al. Analysis of repeat hospital admissions for dengue to estimate the frequency of third or fourth dengue infections resulting in admissions and dengue hemorrhagic fever, and serotype sequences. The American journal of tropical medicine and hygiene. 2007;77(5):910.

Gubler D J,Reiter P, Ebi K L, Yap W, Nasci R, Patz J A. (2001). Climate variability and change in the United States: Potential vector and rodent born diseases. Enviromental health perspectives, Vol.109, no.2, (May 2001), pp. 223- 233.

Gubler DJ. (1997). Dengue and dengue heamorrhagic fever fever: its history and resurgence as a global public health problem. In: Dengue and dengue heamorrhagic fever, D.J. Gubler, and G.Kuno, eds. (oxford, UK: CAB international) pp.1-22

Gubler DJ. (1998). Dengue and dengue heamorrhagic fever. ClinMicrobiol Rev 11:480-496

Halstead S B. (1966).Mosquito-borne haemorrhagic fevers of South and South-East Asia. Bulletin of world health organization , Vol. 35, No.1,(1966) ,pp.3-15

Halstead SB. Dengue haemorrhagic fever—a public health problem and a field for research. Bulletin of the World Health Organization. 1980;58(1):1.

Hawley WA, Pumpuni CB, Brady RH, Craig GB Jr. (1989).Overwintering survival of Aedesalbopictus(Diptera: Culicidae) eggs in Indiana. Journal of Medical Entomology, Vol. 26,No.2 (Mar 1989),pp122-129.

Henry A, Thongsripong P, Gonzalz I F, Ocampo N J, Dujardin J P. (2009). Wing shap of dengue vectors from around the world. Infection, Genetics and Evolution, Vol. 10, No. 2 (Mar 2010),pp. 207-214

Islam MA, Ahmed MU, Begum N, Chowdhury NA, Khan AH, Parquet MC, et al. Molecular characterization and clinical evaluation of dengue outbreak in 2002 in Bangladesh. Jpn J Infect Dis. 2006.

Jamil B, Hasan R, Zafar A, Bewley K, Chamberlain J, Mioulet V, et al. Dengue virus serotype 3, Karachi, Pakistan. Emerging Infectious Diseases. 2007.

Jarman RG, Holmes EC, Rodpradit P, Klungthong C, Gibbons RV, Nisalak A, et al. Microevolution of Dengue viruses circulating among primary school children in KamphaengPhet, Thailand. Journal of virology. 2008;82(11):5494.

Kanakaratne N, Wahala W, Messer WB, Tissera HA, Shahani A, Abeysinghe N, et al. Severe dengue epidemics in Sri Lanka, 2003–2006. Emerging infectious diseases. 2009;15(2):192.

Kendall C, Hudelson P, Leontsini E, Winch P, Lloyd L, Cruz F.(1991).Urbanization, Dengue, and the health transition: Anthropological contributions to international health. Medical anthropology quarterly , new series,contemprory issues of anthropology in international Health.Vol.5,No.3, (Sep.,1991),pp.257-268.

Khan E, Siddiqui J, Shakoor S, Mehraj V. et al. Dengue outbreak in Karachi, Pakistan 2006: experience at tertiary care center. Trans. R. Soc. Trop. Med. Hyg (2007) 101, 1114-1119

King CC, Chao DY, Chien LJ, Chang GJJ, Lin TH, Wu YC, et al. Comparative analysis of full genomic sequences among different genotypes of dengue virus type 3.Virology Journal. 2008;5(1):63.

Klungthong C, Zhang C, MammenJr MP, Ubol S, Holmes EC. The molecular epidemiology of dengue virus serotype 4 in Bangkok, Thailand. Virology. 2004;329(1):168-79.

Kukreti H, Chaudhary A, Rautela RS, Anand R, Mittal V, Chhabra M, et al. Emergence of an independent lineage of dengue virus type 1 (DENV-1) and its co-circulation with predominant DENV-3 during the 2006 dengue fever outbreak in Delhi. International journal of infectious diseases. 2008;12(5):542-9.

Lanciotti RS, Lewis JG, Gubler DJ, Trent DW.Molecular evolution and epidemiology of dengue-3 viruses.Journal of General Virology. 1994;75(1):65.

Mangada MNM, Igarashi A. Sequences of terminal non-coding regions from four dengue-2 viruses isolated from patients exhibiting different disease severities. Virus genes. 1997;14(1):5-12.

Merrill S A, Ramberg F B,Hagedorn H H.(2005). Phylogeography and population structure of Aedesaegypti in Arizona. The American journal of tropical medicine and hygeine, Vol. 72, No. 3, (2005), pp.304-310

Messer WB, Gubler DJ, Harris E, Sivananthan K, de Silva AM. Emergence and global spread of a dengue serotype 3, subtype III virus. Emerging infectious diseases. 2003;9(7):800-9.

Mousseon, L., Dauga, C., Garrigues, T., Schaffner, F., Vazeille, M. (2005).Phylogeography of Ades (Stegomyia) aegypti (L) and Ades (Stegomyia) albopictus (Skuse) (Diptera:Culicidae) based on mitochondrial DNA variations. Genet. Res. Camb. Vol. 86., pp. 1-11

Nagao Y, Svasti P, Tawatsin A, Thavara U. (2007). Geographical structure of dengue transmission and its determinants in thailand. Epidemiol.Infec, Vol.136 , (2008),pp.843- 851.

Rico-Hesse R, Harrison LM, Nisalak A, Vaughn DW, Kalayanarooj S, Green S, et al. Molecular evolution of dengue type 2 virus in Thailand.The American journal of tropical medicine and hygiene. 1998;58(1):96.

Rico-Hesse R, Harrison LM, Salas RA, Tovar D, Nisalak A, Ramos C, et al. Origins of Dengue Type 2 Viruses Associated with Increased Pathogenicity in the Americas* 1. Virology. 1997;230(2):244-51.

Riesenberg LA, Leitzsch J, Massucci JL, Jaeger J, Rosenfeld JC, Patow C, et al. Residents' and attending physicians' handoffs: a systematic review of the literature. Academic Medicine. 2009;84(12):1775.

Soundravally R, Hoti SL. Immunopathogenesis of dengue hemorrhagic fever and shock syndrome: role of TAP and HPA gene polymorphism. Human immunology. 2007;68(12):973-9.

Soundravally R, Hoti SL. Polymorphisms of the TAP 1 and 2 gene may influence clinical outcome of primary dengue viral infection. Scandinavian journal of immunology. 2008;67(6):618-25.

Soundravally R, Hoti SL. Significance of transporter associated with antigen processing 2 (TAP2) gene polymorphisms in susceptibility to dengue viral infection. Journal of Clinical Immunology. 2008;28(3):256-62.

Thu HM, Lowry K, Myint TT, Shwe TN, Han AM, Khin KK, et al. Myanmar dengue outbreak associated with displacement of serotypes 2, 3, and 4 by dengue 1. Emerg Infect Dis. 2004;10(4):593-7.

Twidddy, S., Holmes, E. and Rambaut, A. (2003).Inferring the rate and time scale of dengue virus evolution.Mol.Biol.Evol. Vol:20, pp.122-129

Vaughn DW, Green S, Kalayanarooj S, Innis BL, Nimmannitya S, Suntayakorn S, et al. Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. The Journal of infectious diseases. 2000;181:2-9.

Wang,E., Ni,H.,Xu,R., Barrett, A.,Watowich,S., GublerD., and Weaver,S. (2000)Evolutionary relationships of endemic/epidemic and sylvatic dengue viruses. J.Virol. Vol(74): pp.3227-3234

Wittke V, Robb TE, Thu HM, Nisalak A, Nimmannitya S, Kalayanrooj S, et al. Extinction and rapid emergence of strains of dengue 3 virus during an interepidemic period. Virology. 2002;301(1):148-56.

Zhang C, MammenJr MP, Chinnawirotpisan P, Klungthong C, Rodpradit P, Nisalak A, et al. Structure and age of genetic diversity of dengue virus type 2 in Thailand. Journal of General Virology. 2006;87(4):873.

Zhou Y, MammenJr MP, Klungthong C, Chinnawirotpisan P, Vaughn DW, Nimmannitya S, et al. Comparative analysis reveals no consistent association between the secondary structure of the 3'-untranslated region of dengue viruses and disease syndrome. Journal of General Virology. 2006;87(9):2595.

8

Lassa Fever in the Tropics

Ute Inegbenebor
Department of Physiology
College of Medicine
Ambrose Alli University, Ekpoma
Nigeria

1. Introduction

Lassa fever is a frequently underestimated but socially and economically devastating disease. Lassa fever first came into limelight in 1969, when two nuns died as a result of complications of a hemorrhagic fever in Lassa town in the Borno State of Nigeria. Since then it has become endemic in many parts of West Africa. Out breaks of Lassa fever are usually associated with high mortality rates as the cases usually present late to the hospitals. Besides, many doctors find it difficult to diagnose Lassa fever until complications have set in because of the similarity of presentation to other more common febrile illnesses such as malaria and typhoid. In a study carried out in Sierra Leone in 1987, Lassa fever was found to be responsible for 10-16% of admissions and 30% of deaths in a major referral center. In another study of adult medical admissions in a special center for the management of Lassa fever in Nigeria in 2008, Lassa fever was responsible for 7% of admissions and 13% of deaths with a case fatality rate of 28%. However, the enlightenment campaign for the prevention of Lassa fever and diagnostic facilities are either lacking or rudimentary in most countries where Lassa fever is endemic. Compared to HIV/AIDS, Lassa fever is more infectious to close associates and it rapidly kills in dozens. However, Lassa fever does not get the global attention it deserves.

2. Etiology/pathogenesis-

Lassa fever is caused by Lassa fever virus, a member of the family Arenaviridae. It is an enveloped single stranded bi-segmented rna virus Replication for Lassa virus is very rapid, while also demonstrating temporal control in replication. There are two genome segments. The first replication step is transcription of messenger RNA copies of the negative- or minus sense genome. This ensures an adequate supply of viral proteins for subsequent steps of replication, as proteins known as N and L are translated from the mRNA. The positive- or plus-sense genome then makes viral complementary (vcRNA) copies of itself, which are + sense. The vcRNA is a template for producing minus sense progeny but mRNA is also synthesized from it. The mRNA synthesized from vcRNA are translated to make the G (spike) proteins and Z proteins. Thus, with this temporal control, the spike proteins, which are on the outside of the virus particle, are produced last, making the infection more difficult for the host immune system to detect. Nucleotide studies of the genome have shown that Lassa has four lineages: three found in Nigeria and the fourth in Guinea, Liberia, and Sierra

Leone. The Nigerian strains seem likely to have been ancestral to the others but further research is required to confirm this.
The Lassa virus gains entry into the host cell by means of the cell-surface receptor the alpha-dystroglycan (alpha-DG), a versatile receptor for proteins of the extracellular matrix. It shares this receptor with the prototypic arenavirus lymphocytic choriomeningitis virus. Receptor recognition depends on a specific sugar modification of alpha-dystroglycan by a group of glycosyltransferases known as the LARGE proteins. Specific variants of the genes encoding these proteins appear to be under positive selection in West Africa where Lassa is endemic. Alpha-dystroglycan is also used as a receptor by viruses of the New World clade C arenaviruses (Oliveros and Latino viruses). In contrast, the New World areanviruses of clades A and B, which include the important viruses Machupo, Guanarito, Junin, and Sabia in addition to the non pathogenic Amapari virus, use the transferrin receptor 1. A small aliphatic amino acid at the GP1 glycoprotein amino acid position 260 is required for high-affinity binding to alpha-DG. In addition, GP1 amino acid position 259 also appears to be important, since all arenaviruses showing high-affinity alpha-DG binding possess a bulky aromatic amino acid (tyrosine or phenylalanine) at this position.
Unlike most enveloped viruses which use clathrin coated pits for cellular entry and bind to their receptors in a pH dependent fashion, Lassa and lymphocytic choriomeningitis virus instead use an endocytotic pathway independent of clathrin, caveolin, dynamin and actin. Once within the cell the viruses are rapidly delivered to endosomes via vesicular trafficking albeit one that is largely independent of the small GTPases Rab5 and Rab7. On contact with the endosome pH-dependent membrane fusion occurs mediated by the envelope glycoprotein.
Lassa virus will infect almost every tissue in the human body. It starts with the mucosa, intestine, lungs and urinary system, and then progresses to the vascular system.

2.1 Predisposing factors

- Use of rat meat as a source of protein by people in some communities; contamination of exposed food by rat feces and urine;
- Traditional autopsy, where the operator may injure himself with scalpel and contaminate the injury with the blood of the deceased, who may have died of Lassa fever
- Forceful ingestion of water used in bathing a dead husband, by a widow suspected to be involved in his death. In many communities, family members may be forced to drink water used in bathing dead relatives in order to prove their innocence.
- Corrupt practices by staple food producers, which involve drying garri (cassava flour) in the open air in the daytime and sometimes at night. This enables all types of rat including mastomys natalensis to contaminate the flour with their excreta. This constitutes a public health hazard when the infected garri is sold to consumers in the market. The common habit of eating garri soaked in water may favor Lassa fever infection.
- Many other types of staple foods are also processed in the open sun, which is the major natural drier. These include rice, plantain chips, yam chips and cassava chips, which are processed into rice flour, plantain flour, yam flour, and raw cassava flour. Though these are also processed into staple foods such as tuwo shinkafa, plantain based amala, yam based amala and lafun respectively, the amount of heat involved in processing them into edible pastes, may be enough to denature lassa fever virus, which is heat labile.
- Bush burning of savannahs may be carried out by meat-hungry youths, during the dry season, in order to be able to have access to rodents and other animals. This habit often

drives mastomys natalensis, the reservoir of lassa fever virus, into peoples homes and may be responsible for outbreaks of lassa fever in the dry season.

3. Epidemiology

3.1 Distribution

Lassa fever is endemic in West Africa. However the world is now a global village and the previous geographical gap between the tropics and the developed world has been bridged by international travel. The 6 – 21 days incubation period indicates that a person who contacts Lassa fever in an endemic area in West Africa may travel to a developed country within the incubation period and cause an epidemic.

3.2 Prevalence

The prevalence of Lassa fever can be assesed by determining the prevalence of antibodies to Lassa fever in communities. The prevalence of Lassa fever in Nigeria, Guinea and Sierra Leone can be up to 21%, 55% and 52% respectively.

3.3 Reservoir

The reservoir of infection is mastomys natalensis. It is a species of rodent in the Muridae family. It is also known as the Natal multimammate rat, the common African rat, or the African soft-furred rat. It is found in Angola, Benin, Botswana, Burkina Faso, Burundi, Cameroon, Central African Republic, Chad, Republic of the Congo, Democratic Republic of the Congo, Ivory Coast, Equatorial Guinea, Ethiopia, Gabon, Ghana, Guinea, Guinea-Bissau, Kenya, Lesotho, Malawi, Mali, Mauritania, Mozambique, Namibia, Niger, Nigeria, Rwanda, Senegal, Sierra Leone, Somalia, South Africa, Sudan, Swaziland, Tanzania, Togo, Uganda, Zambia, and Zimbabwe. Its natural habitats are subtropical or tropical dry forests, subtropical or tropical moist lowland forests, dry savanna, moist savanna, subtropical or tropical dry shrubland, subtropical or tropical moist shrubland, arable land, pastureland, rural gardens, urban areas, irrigated land, and seasonally flooded agricultural land. In 1972, the Natal multimammate Mouse was found to be the natural host of the deadly Lassa fever virus.

3.4 Transmission

Lassa fever is transmitted to humans when they ingest food contaminated by the feces and urine of mastomys natalensis. Once humans are infected, transmission also occurs from human to human through contact with fluid and aerosol secretions in the form of sneezing, sputum, seminal fluid, stool, urine and blood. Vertical transmission through breast milk has been observed.

3.5 Host factors

Men are more commonly affected than women. However the case fatality rate is nearly two times higher in women. Men are more likely to buy food from food vendors especially at lunch time while women are more likely to eat personally cooked food. Contamination of food from this source may be responsible for the higher incidence of Lassa fever in men. Although the high case-fatality of Lassa fever is due to delayed cellular immunity, development of partial immunity as a result of frequent exposure to contaminated food may be responsible for the milder forms of the disease and lower case-fatality rate in men. Research is needed to find out whether Lassa fever infection confers partial or full immunity on affected people.

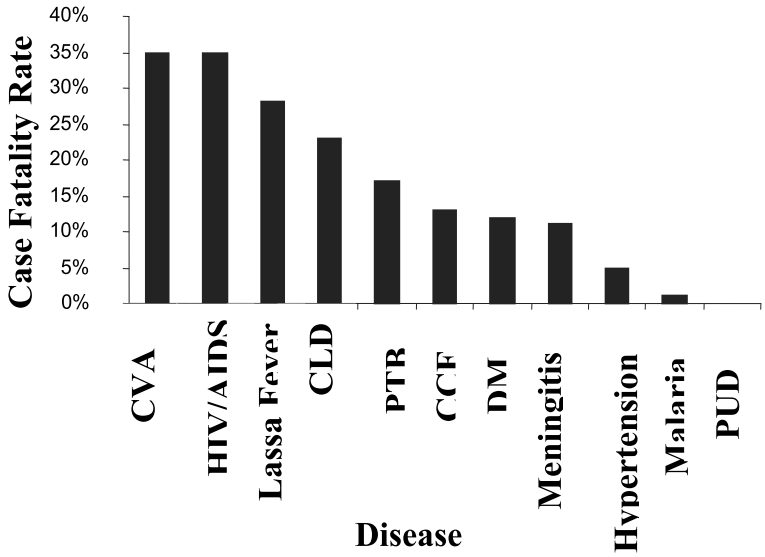


Fig. 1. Bar Chart showing Case Fatality Rates of Common Diseases of Medical Inpatients in Irrua Specialist Teaching Hospital (ISTH), Irrua, Nigeria in 2007

4. Clinical features

Signs and symptoms typically occur after an incubation period of 6 –21 days. The onset of illness is insidious, with fever and shivering accompanied by malaise, headache and generalized aching. Sore throat is a common early symptom. In some cases the tonsils and pharynx may be inflamed with patches of white or yellowish exudate and occasionally small vesicles or shallow ulcers. (Importantly, a similar appearance may be seen in cases of malignant tertian malaria). As the illness progresses the body temperature may rise to 41^{o}C with daily fluctuations of 2-3^{o}C. The duration and severity of fever is very variable. The average duration is 16 days but extremes of 6-30 days have been reported. A feature of severe attacks is lethargy or prostration disproportionate to the fever. During the second week of illness there may be edema of the head and neck, encephalopathy, pleural effusion and ascites. Vomiting and diarrhea may aggravate the effects of renal and circulatory failure. Severe cases develop significant hemorrhage and multi-organ failure with widespread edema and bleeding into the skin, mucosae and deeper tissues. In non-fatal cases, the fever subsides and the patient's condition improves rapidly although tiredness may persist for several weeks. There is usually a leucopenia early in the course, though a high polymorphonuclear leucocytosis may occur with severe tissue damage. Another common late complication is sensorineural deafness. Clinically, a Lassa fever infection is difficult to distinguish from other viral hemorrhagic fevers, such as Ebola and Marburg, and from more common febrile illnesses such as malaria and typhoid.

5. Complications of Lassa fever

Various complications may occur in the course of Lassa fever. These complications vary with duration of illness and sex of the victim. Thes complications include hypovolemic

shock, Electrolyte imbalance, Disseminated intravascular coagulation, Renal failure, Sensorineural deafness, pregnancy complications.

5.1 Hypovolemic shock

Lassa fever viremia causes endothelial and platelet dysfunction with consequent leaky capillary syndrome. Bleeding occurs in all organs and from all mucosae leading to hypovolemic shock.

5.2 Electrolyte imbalance

Most Lassa fever victims lose fluid through vomiting and diarrhea and therefore develop electrolyte imbalance.

5.3 Renal failure

Renal tubular damage may also occur on Lassa fever and in conjunction with the hypovolemic shock predispose to renal failure

5.4 Complications of lassa fever in pregnancy

Lassa fever is especially dangerous in pregnant women. Abortion is common in early pregnancy and intrauterine fetal death is common in later pregnancies. Abortion reduces the mortality rate in affected pregnant women. Prognosis is very poor in pregnant women as mortality rate may be up to 80%.

5.5 Sensorineural deafness

This is the commonest complication of Lassa fever. It is not related to the severity of disease as it may occur with the same frequency in both mild and severe forms of the disease.

6. Laboratory diagnosis

Lassa fever is most often diagnosed using ELISAs. The virus can also be detected by reverse transcription PCR (RT-PCR) in all patients by the third day of illness, but immunofluorescence identifies only 52% of the patients.

6.1 Treatment

Ribavirin, an antiviral drug, is the current treatment of Lassa fever. The drug is to be administered in a volume of 50-100 ml of normal saline to be infused over 30-40 minutes.

- Loading dose: 33 mg/kg (maximum dose 2.64 g)
- Followed by a dose of 16 mg/kg (max dose 1.28 g) every 6 hours for the first 4 days
- Followed by a dose of 8 mg/kg (maximum dose 0.64 g) every 8 hours for the subsequent 6 days

Supportive treatment is usually carried out with intravenous fluids, and treatment of complications such as renal failure and infections may be necessary.

Although Lassa fever can be treated with ribavirin, early diagnosis and treatment is essential in all cases of Lassa fever. Ribavirin is most effective when given within 6 days of illness. Self-diagnosis and treatment is common in the tropics because of ignorance and poverty. It is only when there is no remission of fever that the patient seeks treatment in a health-care facility. However, many health-care providers are unable to make early

Disease	Number of Admissions			Laboratory Diagnosis	Treatment	Number of Deaths			Case Fatality Rate		
	Male	Female	Total			Male	Female	Total	Male	Female	General
Cerebrovascular accident (CVA)	44	35	79	Clinical diagnosis/ Occasional CT Scans.	Supportive Treatment; Aspirin 300mg daily Treatment for underlying problems e.g Hypertension, Diabetes Mellitus	18	10	28	41%	29%	35%
HIV/AIDS	36	56	92	ELISA/ Confirmatorry Test	Supportive treatment; Treatment for opportunistic infections followed by Combined therapy with AZT and lamivudine	13	19	32	36%	34%	35%
Lassa fever	48	16	64	Clinical triad of Pharygitis, Retrosternal pain and Proteinuria.; Confirmation wirh RT-PCR test	Supportive treatment Intravenous Ribavirin for 8 days	11	7	18	23%	44%	28%
Chronic liver disease (CLD)	13	9	22	Liver Function Tests	Supportive treatment, Interferon, Lipid clearing agents e.g . Litrison, Essentiale Lamivudine for chronic Hepatitis B infection	2	3	5	15%	33%	23%
Pulmonary Tuberculosis (PTB)	45	27	72	Clinical Diagnosis/ Chest X-Ray/ 3 early morning samples of Sputum for Ziehl Neelsen Stain and Microscopy for Acid Fast Bacilli	Supportive treatment; Rifampicin, Isoniazid, Ethambutol, Pyrazinimide	7	5	12	16%	19%	17%
Congestive Cardiac Failure(CCF)	52	41	93	Clinical Diagnosis/ Chest X Ray	Cardiac position, Oxygen therapy, Diuretic e.g. Furosemide ± Digoxin	6	6	12	12%	15%	13%
Diabetes Mellitus (DM)	73	84	157	Clinical Diagnosis Fasting Blood Sugar ≥8mmol/l Random Blood Sugar ≥11mmol/l	Soluble Insulin/ Lente Insulin (70:30) treatment	6	12	18	8%	14%	12%
Meningitis	23	23	46	Clinical Diagnosis Lumbar Puncture Cerebrospinal spinal fluid for Microscopy, culture & Sensitivity	Supportive therapy; Initial high dose Cephalosporins followed by Antimicrobial therapy based on sensitivity results	3	2	5	13%	9%	11%
Hypertension (HTN)	41	42	83	Blood Pressure ≥ 160/100mmHg	Anti hypertensive drugs e.g Nifedipine	2	2	4	5%	5%	5%
Malaria	82	76	158	Thick and Thin Blood Film for microscopy.	Supportive treatment; Intravenous Quinnine or Artesunate/ amodiaquine	1	0	1	1%	0%	<1%
Peptic Ulcer (PUD)	24	18	42	Clinical Diagnosis/ Barium meal	Supportive treatment; Ranitidine/ Proton inhibitors	0	0	0	0%	0%	0%
Total	**481**	**427**	908			69	66	**135**			

Table 1. Case fatality rates of common Diseases among Medical inpatients in ISTH, Irrua, Nigeria.

diagnosis, and are very likely to make a diagnosis of resistant malaria or typhoid. Besides most health care providers have no access to diagnostic facilities, which are only available in tertiary health centers. This allows the patient to get to terminal stages before they are transferred to a tertiary center. Sometimes the life of the health-care provider is claimed along with that of the patient.

7. Prognosis

Prognosis depends on how early a patient presents at the clinic. Most patients recover completely if diagnosed early and when treatment with ribavirin is commenced within 6 days of illness. In studies carried out in special referral centers in Nigeria and Sierra Leone, Lassa fever was responsible for 13% and 30% of adult deaths respectively. The death rates were in adult medical wards where only 7% and 10-16 % respectively of the total number of admissions were for Lassa fever. Prognosis is probably better in males who may acquire partial immunity due to the habit of patronizing food vendors. In a study done in Nigeria, the case fatality rate in males was 23% compared to women with 44%, though males were four times more commonly affected than females.

8. Control

8.1 The individual

The affected person should be admitted to a special center for the treatment of Lassa fever. Where this is not possible, the patient should be barrier-nursed. Health care providers and close associates of the patient should wear protective clothing, masks and gloves. Excrements from affected persons should be properly disposed.

8.2 The community

Legislation is needed to prevent widowhood rites, traditional autopsies, bush burning and unhygienic preparation of garri and other staple foods. Animal husbandry and fisheries should be encouraged in order to provide alternative sources of first-class proteins for rat eaters. Regular and sustainable environmental sanitation is needed to prevent rat breeding. The public should be made aware of the mode of contact of Lassa fever and its high case-fatality rate using print and electronic media. Community involvement and participation is necessary to provide sustainable Lassa fever control. Food vendors should be educated on the need to prevent food contamination with Lassa fever virus. Grains, flours and left-over foods should be adequately covered to prevent contamination by rats. Rodenticides should be used for the destruction of rats in homes, and development of Lassa fever vaccine should be facilitated. Regular seminars should be held for health-care providers on early diagnosis and treatment of Lassa fever, while diagnostic kits should be made available in district hospitals. Affected people should be referred early to the special center in order to prevent or limit disability, while those with disabilities should be rehabilitated functionally, socially and psychologically so that they can be gainfully employed.

9. Prospects

Though vaccines are not currently available for Lassa fever, there is evidence that they will be produced in the near future. Research done with non-human primates have revealed that survivors exhibit fewer lesions and a **lower** viral load than non-survivors.

Although all animals develop strong humoral responses, antibodies appear more rapidly in survivors and are directed against GP(1), GP(2), and NP. Activated T lymphocytes circulate in survivors, whereas T-cell activation is **low** and delayed in fatalities

A single injection of ML29 reassortant vaccine for **Lassa fever** induces **low**, transient viremia, and **low** or moderate levels of ML29 replication in tissues of common marmosets depending on the dose of the vaccination. The vaccination elicits specific immune responses and completely protects marmosets against fatal disease by induction of sterilizing cell-mediated immunity. DNA array analysis of human peripheral blood mononuclear cells from healthy donors exposed to ML29 revealed that gene expression patterns in ML29-exposed PBMC and **control**, media-exposed PBMC, clustered together confirming safety profile of the ML29 in non-human primates. The ML29 reassortant is a promising vaccine candidate for **Lassa fever**.

10. References

Baize S, Marianneau P, Loth P, et al. Early and strong immune responses are associated with control of viral replication and recovery in lassa virus-infected cynomolgus monkeys. J Virol (United States), Jun 2009, 83(11) p5890-903

Demby AH, Chamberlain J, Brown DW, Clegg CS. Early diagnosis of Lassa fever by reverse transcription PCR. J Clin Microbiol 1994; 32:2898–903

Donaldson, Ross I. (2009). The Lassa Ward:One Man's Fight Against One of the World's Deadliest Diseases. St. Martin's Press. ISBN 0312377002. ISBN 978-0312377007.

Frame JD, Baldwin JM, Gocke DJ, Troup JM. Lassa fever, a new virus disease of man from West Africa. I. Clinical description and pathological findings. Am J Trop Med Hyg 1970; 19:670–6.

Granjon, L., Lavrenchenko, L., Corti, M., Coetzee, N. & Rahman, E.A. Mastomys natalensis. 2006 IUCN Red List of Threatened Species. Accessed 09-06-2011.

Inegbenebor, U, Okosun, J, Inegbenebor, J. Prevention of Lassa fever in Nigeria. Trans R. Soc Trop Med Hyg, 2010; 4(1):51-54 PMID: 19712954

Lukashevich IS, Carrion R, Salvato MS, et al. Safety, immunogenicity, and efficacy of the ML29 reassortant vaccine for Lassa fever in small non-human primates. Vaccine (Netherlands), Sep 26 2008, 26(41) p5246-54

McCormick JB, King IJ, Webb PA, Johnson KM, O'Sullivan R, Smith ES. A case-control study of the clinical diagnosis and course of Lassa fever. J Infect Dis 1987;155:445–55.

McCormick JB, King IJ, Webb PA, Scribner CL, Craven RB, Johnson KM, et al. Lassa fever: effective therapy with ribavirin. N Engl J Med. 1986;314:20–6.

Ogbu O, Ajuluchukwu E, Uneke CJ. Lassa fever in West African subregion: an overview. J Vector Borne Dis 2007; 44:1–11.

Richmond JK, Baglole DJ. Lassa fever: epidemiology, clinical features,and social consequences. BMJ 2003;327:1271–5.

WHO. Lassa fever fact sheet no. 179. Geneva: World Health Organization; 2005.

9

The Re-Emergence of an Old Disease: Chikungunya Fever

Bordi Licia et al.*
Laboratory of Virology, National Institute for Infectious Diseases "L. Spallanzani", Rome Italy

1. Introduction

Until recently, very few physicians in industrialized countries had heard the word "Chikungunya", and fewer knew how to spell it. Chikungunya, a viral infection transmitted by mosquitoes, derives its name from Makonde, a language spoken in south Tanzania, and means "that which bends up", referring to the posture of patients afflicted with severe joint paints characterizing this infection. Chikungunya virus (CHIKV) was first isolated in Tanzania in 1952 (Robinson, 1955) and has come to the world attention recently, when it caused a massive outbreak in the Indian Ocean region and India (Enserik, 2006). Since 1952, CHIKV has caused a number of epidemics, both in Africa and Southeast Asia, many of them having involved hundreds-of-thousands people. In 2005 the largest Chikungunya fever epidemic on record occurred. The most affected region was La Reunion Island, where CHIKV infected more than a third of the population and killed hundred of people. The 2005/2006 outbreak, started from Comoro Islands, rapidly spread to several countries in the Indian Ocean and India (Enserik, 2006; Mavalankar et al., 2007). Compared to earlier outbreaks, this episode was massive, occurred in highly medicalized areas such as La Reunion, and had very significant economic and social impact. More than 1000 imported CHIKV cases have been detected among European and American travellers returning from the affected areas since the beginning of the outbreak in the Indian Ocean region (Fusco et al., 2006; Taubiz et al., 2007), giving rise, in 2007, to the first autochthonous European outbreak in Italy (Charrel & de Lambellerie, 2008; Rezza et al., 2007). Since 2006, the Regional Office of the French Institute For Public Health Surveillance in the Indian Ocean has conducted epidemiological and biological surveillance for CHIKV infection. During the period December 2006-july 2009, no confirmed case was detected on Reunion Island and Mayotte, but new outbreak were reported in Madacascar. After few years of relative dormancy in Réunion Island, CHIKV transmission has restarted in 2009 and 2010, with one case imported in France (May 2010) (D'Ortenzio et al., 2010). This episode has refreshed the concerns about the possibility of renewed autochthonous transmission in Mediterranean countries.

* Meschi Silvia[1], Selleri Marina[1], Lalle Eleonora[1], Castilletti Concetta[1], Carletti Fabrizio[2], Di Caro Antonino[2] and Capobianchi Maria Rosaria[1]
[1]*Laboratory of Virology, National Institute for Infectious Diseases "L. Spallanzani", Rome, Italy*
[2]*Laboratory of Microbiology and Infectious Disease Biorepository, National Institute for Infectious Diseases "L. Spallanzani", Rome, Italy*

2. Microbiology

CHIKV is an alphavirus belonging to the *Togaviridae* family. Alphaviruses are small and spherical, with a 60-70 nm diameter capsid and a phospholipid envelope. The RNA single-strand of positive polarity encodes four non structural proteins (nsP1-4) and three structural proteins (C,E1,E2). Viral replication is initiated from the time of attachment of viral envelope to cellular host receptors (Strauss & Strauss, 1994). Endocitosis of the virus occurs, following which, delivery of the viral nucleocapsid into cytoplasm takes place. The replication cycle is considerably fast, taking around 4hours. Alphaviruses are sensitive to disseccation and to temperatures above 58°C (Khan et al., 2002; Strauss J.H. & StraussE.M., 1994). About 30 species of arthropod-borne viruses are included in the alphavirus genus, antigenically classified into 7 complexes. These viruses are widely distributed throughout the world, with the exception of Antarctica, and 7 of them cause a syndrome similar to Chikungunya fever, arthralgia and rash: Barmah Forest and Ross river viruses (Oceania), O'nyong-nyong and Semliki Forest viruses (Africa), Mayaro (South America), Sindbis and Sindbis-like (Africa, Asia, Scandinavia and Russia) (Taubiz et al., 2007).

3. Vector and reservoir

In Asia and the Indian Ocean region the main CHIKV vectors are *A. aegypti* and *A. albopictus.*(Jeandel et al. 2004 ; Zeller, 1998). A larger range of *Aedes* species *(A. furcifer, A. vittatus, A. fulgens, A. luteocephalus, A. dalzieli, A. vigilax, A. camptorhynchites)* transmit the virus in Africa, and *Culex annulirostris, Mansonia uniformis*, and anopheles mosquitoes have also occasionally been incriminated (Jupp P.G. et al., 1981; Jupp P.G. & McIntosh, 1990; Lam et al., 2001;). In India, the dominant carrier of Chikungunya virus is *A. aegypti*, which breeds mainly in stored fresh water in urban and semi-urban environments (Yergolkar, 2006). *A. albopictus* has a wide geographical distribution, is particularly resilient, and can survive in both rural and urban environments. The mosquito's eggs are highly resistant and can remain viable throughout the dry season, giving rise to larvae and adults the following rainy season. Originating from Asia, and initially sylvatic, *A. albopictus* has shown a remarkable capacity to adapt to human beings and to urbanisation, allowing it to supersede *A. aegypti* in many places, and to become a secondary but important vector of dengue and other arboviruses (Knudsen, 1995). *A. albopictus* is zoophilic and anthropophilic, is aggressive, silent, active all-day long, and has a lifespan longer than other mosquitoes (up to 8 weeks) and, in the last decades has expanded to several areas previously known to be *Aedes*-free (Charrel et al., 2007). It seems that most new introductions of *A. albopictus* have been caused by vegetative eggs contained in timber and tyres exported from Asia throughout the world. Other emerging events also contributed to the introduction of *A. albopictus* mosquitoes into previously unaffected areas, such as climate change and the increasing use of plastic containers in developing countries. Indeed, climate changes may have several effects on vector biology: increasing temperatures may improve survival at higher latitudes and altitudes, increase the growth rates of vector populations, and alter their seasonality; increased rainfall may have an effect on the larval habitat and population size, and finally an increase in humidity could favourably affect vector survival (Gubler et al., 2001). The use of plastic containers in developing countries, where they are usually not correctly disposed and remain in the environment for years, has also been linked with the spread of the mosquitoes: acting as rain-water receptacles, and being exposed to sunlight,

they can become perfect "incubators" for mosquito eggs, where the ideal conditions of temperature and humidity are achieved easily and naturally.
Human beings serve as the Chikungunya virus reservoir during epidemic periods. In Africa some animals (monkeys, rodents, and birds) constitute the virus reservoir during not-epidemic periods, sustaining virus circulation in the environment in the absence of human cases. Outbreaks might occur in monkeys when herd immunity is low; the animals develop viraemia but no pronounced physical manifestations (Inoue et al., 2003; Wolfe et al., 2001). An animal reservoir has not been identified in Asia, where humans appear to be the only host.

4. Clinical manifestation

4.1 General features

After infection with Chikungunya virus, there is a silent incubation period lasting 2–4 days on average (range 1–12 days) (Lam et al., 2001). Clinical onset is abrupt, with high fever, headache, back pain, myalgia, and arthralgia; the latter can be intense, affecting mainly the extremities (ankles, wrists, phalanges) but also the large joints (Hochedez et al., 2006; Lam et al., 2001; Quatresous, 2006; Robinson, 1955; Saxena et al., 2006). Skin involvement present in about 40–50% of cases, and consists of (1) a pruriginous maculopapular rash predominating on the thorax, (2) facial oedema, or (3) in children, a bullous rash with pronounced sloughing, and (4) localised petechiae and gingivorrhagia (mainly in children) (Brighton et al., 1983; Fourie & Morrison, 1979). Radiological findings are normal, and biological markers of inflammation (erythrocyte sedimentation rate and C-reactive protein) are normal or moderately elevated (Fourie & Morrison, 1979; Kennedy et al., 1980). Iridocyclitis and retinitis are the most common ocular manifestations associated with Chikungunya fever; less frequent ocular lesions include episcleritis. All ocular manifestations have a benign course with complete resolution and preservation of vision. Retinitis shows gradual resolution over a period of 6 to 8 weeks (Mahendradas et al., 2008). CHIKV infection seems to elicit long-lasting protective immunity, and experiments performed using animal models have shown a partial cross-protection among CHIKV and other alphaviruses (Edelman et al., 2000; Hearn Jr. & Rainey, 1963).

4.2 Arthralgia

Erratic, relapsing, and incapacitating arthralgia is the hallmark of Chikungunya, although it rarely affects children. These manifestations are normally migratory and involve small joints of hands, wrists, ankles, and feet with pain on movement. Symptoms generally resolve within 7–10 days, except for joint stiffness and pain: up to 12% of patients still have chronic arthralgia three years after onset of the illness. Arthralgia experienced by CHIKF patients closely resembles the symptoms induced by other viruses like Ross River Virus (RRV) and Barmah Forest virus (BFV) (Jacups et al, 2008; Mahalingam et al., 2002). Such alphavirus-induced arthralgia mirrors rheumatoid arthritis, a condition which is characterised by severe joint pains due to inflammation and tissue destruction caused by inflammatory cytokines such as IL-1b, IL-6 and TNF-a (Barksby et al., 2007). It is thus plausible that CHIKV infection induces similar pro-inflammatory cytokines that cause arthralgia, explaining why joint pains are constant ailments of many patients infected with CHIKV even years after recovery from the initial febrile phase (Lakshmi et al., 2008). More recently, global analyses on the specific involvement of cytokines and chemokines have showed that IL-1b, IL-6, and RANTES were associated with disease severity (Ng et al.,

2009). Moreover, since high concentrations of these pro-inflammatory factors were found in the joints of humans afflicted with RRV-induced polyarthritis, they probably have a causative role in chronic joint and muscle pains that plague patients (Lidbury et al., 2008). The finding that aberrant Type I interferon signalling in mice led to severe forms of CHIKF (Couderc et al., 2008) further highlighted the important role cytokines play in the pathology of CHIKV infection.

4.3 Other pathologies

Chikungunya is not generally considered to be a life-threatening disease. Usually the clinical course is fairly mild, but fatal cases directly or indirectly linked to infection with CHIKV have been observed during the Indian-Ocean outbreak (Josseran et al., 2006). The main evidence of a mortality linked to Chikungunya fever epidemics has been obtained in La Reunion, Mauritius, and India by comparing expected and observed mortality data. In all cases, during the months when the epidemics were raging, the observed mortality significantly exceeded the expected one. In particular, in La Reunion the monthly crude death rates in February and March 2006 were 34.4%and 25.2% higher, respectively, than expected. These corresponded to 260 excess deaths (an increase of 18.4%) with a rough estimate of the case-fatality rate for Chikungunya fever of ≈1/1,000 cases. The case-fatality rate calculated on increased crude death rates in Mauritius and Ahmedabad, India, is substantially higher than that calculated in La Reunion: approximately 4.5% (15,760 confirmed or suspected cases and 743 excess deaths) and 4,9% (60,777confirmed or suspected cases and 2,944 excess deaths), respectively (Beesoon et al., 2008; Mavalankar et al., 2008). These differences may be attributed to many factors (greater disease severity, preexisting patient conditions, different patient management, or coincident excess deaths from other causes) but may also be due to a different efficacy of the surveillance systems for Chikungunya fever, that probably worked poorly in Mauritius and India, leading to underestimating the total number of cases (Fusco et al., 2010). The possible link between CHIKV infection and multiorgan failure is still under investigation.

Neurological complications such as meningo-encephalitis were reported in a few patients during the first Indian outbreak in 1973, and during the 2006 Indian outbreak (Chatterjee et al., 1965; Ravi, 2006). The possible mechanisms underlying these processes remain unknown. Studies performed on animal models showed that CHIKV-infected young mice had weakness and walking difficulties which could be due to necrosis and inflammation of skeletal muscles (Ziegler et al., 2008). CHIKV antigens and viral replication have been detected in human myogenic precursors such as satellite cells but not in muscle fibers (Ozden et al., 2007), suggesting that muscle satellite cells could be potential virus reservoirs. The pathologic symptoms of encephalitis owing to CHIKV infection as well as central nervous system (CNS) infections (Chatterjee & Sarkar, 1965) were expected, since *in vitro* experiments showed that the virus could infect and replicate for extended periods in mouse brain cells (Precious et al., 1974). More recently, it was found that mouse CNS tissues such as the choroid plexi could also be targets of CHIKV, lending more credence to the fact that CHIKV infections do affect CNS cells and tissues (Couderc et al., 2008). Work is currently underway by several research groups around the world to decipher this mechanism in CHIKV infections. Moreover, during the 2006 Indian-Ocean outbreak, rare cases of Guillain-Barré syndrome (GBS) associated with CHIKV infection have been described (Lebrun et al., 2009; Wielanek et al., 2007).

Other rare complications described after CHIKV infection are mild hemorrhage, myocarditis, hepatitis (Lemant et al., 2008) .

5. Diagnosis

Diagnosis of infection with CHIKV is based on molecular biology (RT-PCR) and serology methods. The first one is useful during the initial viraemic phase, at the onset of symptoms and normally for the following 5-10 days, when CHIKV RNA reaches very high levels (viral loads of 3.3 x 10^9 copies/ml) and can be detected (Carletti et al., 2007; Parola et al., 2006). Afterwards, the diagnosis is based on serological methods (ELISA, immunofluorescence, hemoagglutination inhibition (HI) and infectivity neutralization (Nt)).
IgM specific against CHIKV are detectable 2-3 days after the onset of symptoms by ELISA immunofluorescent assay and persists for several weeks, up to 3 months (Litzba et al, 2008; Sam & AbuBakar, 2006); rarely, IgM can be detected for longer periods, up to 1 year. IgG specific against CHIKV appear soon after IgM antibodies (2-3 days) and persists for years. Testing of a couple of sera collected in the acute and the convalescent phases of the disease is mandatory for the identification of recent infection using serology methods that cannot distinguish IgG Ab from IgM Ab (i.e. HI and Nt). It is also very useful to confirm results obtained with other methods, especially taking into account the although rare persistence of IgM antibodies. Viral isolation can be performed from serum of infected patient on insect or mammalian cell lines (i.e. C6/36 or Vero E6) during the early phase of the disease, when the viral load is very high and the immune response is still not detectable; however it is useful only for epidemiology or pathogenesis studies or for thorough molecular characterization (Fusco et al., 2010). The sensitivity and specificity of rapid bedside tests commercially available are poorly established, and the possibility of false-positive reactions resulting from cross-reactivity with dengue or other arboviruses such as o'nyong-nyong virus has to be considered (Blackburn et al., 1995). Serologically, chikungunya virus is most closely related to o'nyong-nyong virus and is a member of the Semliki Forest antigenic complex. Individual serological testing is not particularly useful, except when faced with atypical or severe forms, or in travellers returning from an epidemic zone (Pile et al., 1999).

6. Treatment

Currently, there are no available specific therapeutics against CHIKV. Treatment is purely symptomatic and can include rest, fluids, and medicines to relieve symptoms of fever and aching, such as ibuprofen, naproxen, acetaminophen, or paracetamol. Non-steroidal anti-inflammatory drugs (NSAIDs) are primarily used to treat inflammation but high doses, administrated to control arthralgia, could cause thrombocytopenia, gastrointestinal bleeding, nausea, vomiting and gastritis (Jain et al, 2008; Pialoux et al., 2007). Steroids have been occasionally used but their efficacy was not significant (Taubitz et al., 2007). Some time ago chloroquine, a drug useful for prophylaxis and treatment of malaria, showed promising results for treating chronic Chikungunya arthritis (Brighton, 1984), while a recent trial conducted on French Reunion Island proved that there is currently no justification for the use of chloroquine to treat acute chikungunya diseases (De Lamballerie et al., 2008). However, the usefulness of chloroquine in the treatment of Chikungunya infection deserves further investigation that could take advantage on the availability of a non-human primate animal model (Labadie et al., 2010). Ribavirin (200 mg twice a day for seven days) given to

patients who continued to have crippling lower limb pains and arthritis for at least two weeks after a febrile episode, had a direct antiviral property against CHIKV, leading to faster resolution of joint and soft tissue manifestations (Ravichandran & Manian, 2008). Briolant and collegues screened various active antiviral compounds against viruses of the Alphavirus genus *in vitro* and demonstrated that 6-azauridinet was more effective against CHIKV, as compared to ribavirin. Moreover, the combination of IFN-alpha2b and ribavirin had synergistic antiviral effect on Chikungunya virus (Briolant et al., 2004).

It is widely recognized that passive vaccination is an appropriate preventive and therapeutic option for many viral infections in human, including those spread by viral vertical transmission, especially when no alternative therapy is available (Dessain et al., 2008). Human polyvalent immunoglobulins purified from plasma samples obtained from donors in the convalescent phase of CHIKV infection exhibited a high *in vitro* neutralizing activity and a powerful prophylactic and therapeutic efficacy against CHIKV infection *in vivo* in mouse models (Couderc et al., 2009). Due to the demonstrated efficacy of human anti-CHIKV antibodies in a mouse model, purified polyvalent CHIKIg (commercialized under the brand Tégéline) could be used in humans for prevention and treatment, especially in individuals at risk of severe CHIKV disease, such as neonates born to viraemic mothers and adults with underlying conditions. Polyclonal immune globulins present the advantage of a broad reactivity but the therapeutic intervention is limited, due to the short viremia in acute phase of CHIKV infection: thus the only benefit this treatment has to offer would be to help reducing viremia faster (Kam et al., 2009). As an alternative, more specific human monoclonal antibodies (MAbs) could be used. In a recent study two unique human mAbs, specific for the CHIKV envelope glicoproteins, strongly and specifically neutralized CHIKV infection *in vitro* (Warter et al., 2011).

7. Prevention

Although no licensed vaccines are currently available for CHIKV, potential vaccine candidates have been tested in humans and animals with varying success. Due to the easiness in preparation, the first developed vaccines were formulations of whole-virus grew on cells and inactivated either by formalin or tween-ether (Eckels et al., 1970; Harrison et al., 1967, 1971; White et al., 1972).

Further vaccines are focused on attenuated strains of CHIK obtained after serial passages in cells cultures (Edelman et al., 2000; Levitt et al., 1986). One of these promising candidates is TSI-GSD-218, a serially passaged and plaque-purified live CHIK vaccine, tested for safety and immunogenicity in human Phase II trials by the US Army Medical Research Institute (Edelman et al., 2000). Seroconversion was obtained in 98% of vaccinees volunteers by day 28 and neutralizing antibodies persisted in 85% of cases at one year after immunization. However transient arthralgia occurred in 8% of the volunteers. Some chimeric candidates vaccines were developed using either Venezuelan equine encephalitis (VEEV) attenuated vaccine strain TC-83, a naturally attenuated strain of eastern equine encephalitis virus (EEEV), or Sindbis virus (SV) as a backbone and the structural protein genes of CHIKV. Vaccinated mice were fully protected against disease and viraemia after CHIKV challenge (Wang et al., 2008). The maturity of reverse genetic technology has provided unprecedented opportunities for manipulation of the alphaviral genome to improve attenuation strategies. Thus, unlike traditional attenuation approaches that rely on cell culture passages, which typically result in attenuation that depends only on small numbers of attenuating point

mutations, alternative genetic strategies such as viral chimeras offer the promise of more stable attenuation (Kennedy et al., 2011). In addition to the risk of reactogenicity, attenuation based on small numbers of mutations can also result in residual alphavirus infectivity for mosquito vectors. This risk, which was underscored by the isolation of the TC-83 VEEV vaccine strain from mosquitoes in Louisiana during an equine vaccination campaign designed to control the 1971 epidemic (Pedersen et al., 1972), is especially high when a vaccine that relies on a small number of point mutations is used in a nonendemic location that could support a local transmission cycle. In a recent study chimeric alphaviruses, encoded CHIKV-specific structural genes (but no structural or nonstructural proteins capable of interfering with development of cellular antiviral response) induced protective immune response against subsequent CHIKV challenge (Wang et al., 2011). More in detail, recombinant chikungunya virus vaccine, comprising a non-replicating complex adenovirus vector encoding the structural polyprotein cassette of chikungunya virus, consistently induced in mice high titres of anti-chikungunya virus antibodies that neutralised both an old Asian isolate and a Réunion Island isolate from the recent epidemic (Wang et al., 2011).

A novel CHIK vaccine candidate, CHIKV/IRES, was generated by manipulation of the structural protein expression of a wt-CHIKV strain via the EMCV IRES (Plante et al., 2011). In particular, the internal ribosome entry site (IRES) from encephalomyocarditis virus replaced the subgenomic promoter in a cDNA CHIKV clone, thus altering the levels and host-specific mechanism of structural protein gene expression. This vaccine candidate exhibited a high degree of murine attenuation that was not dependent on an intact interferon type I response, highly attenuated and efficacious after a single dose.

Another approach was the selective expression of CHIK viral structural proteins recently obtained by Akata and collegues using virus-like particles (VLPs) *in vitro,* that resemble replication-competent alphaviruses (Akahata et al., 2010). Immunization of monkeys with these VLPs elicited neutralizing antibodies against envelope proteins from different CHIKV strains and obtained antibodies transferred into mice protective against subsequent lethal CHIKV challenge. The last frontier in the approach of CHIK vaccine design is the DNA vaccine strategy. An adaptive constant-current electroporation technique was used to immunize mice (Muthumani et al., 2008) and rhesus macaques (Mallilankaraman et al., 2011) with an intramuscular injection of plasmid coding for the CHIK-Capsid, E1 and E2. Vaccination induced robust antigen-specific cellular and humoral immune responses in either case.

To date a number of CHIKV vaccines have been developed, but none have been licensed. While a number of significant questions remain to be addressed related to vaccine validation, such as the most appropriate animal models (species, age, immune status), the dose and route of immunization, the potential interference from multiple vaccinations against different viruses, and last, the practical cost of the vaccine, since most of the epidemic geographical regions belong to the developing countries, there is real hope that a vaccine to prevent this disease will not be too long in arriving.

Since a vaccine is not available actually, protection against mosquito bites and vector control are the main preventive measures. Individual protection relies on the use of mosquito repellents and measures in order to limit skin exposure to mosquitoes. Bednets should be used during the night in hospitals and day-care facilities but *Aedes* mosquitoes are active all-day-long. Control of both adult and larval mosquito populations uses the same model as for dengue and has been relatively effective in many countries and settings. Breeding sites must be removed, destroyed, frequently emptied, and cleaned or treated with insecticides. Large-

scale prevention campaigns using DDT have been effective against *A. aegypti* but not *A. albopictus*. Control of *A. aegypti* has rarely been achieved and never sustained (Reiter et al., 2006). Recent data show the different degrees of insecticide resistance in *A. albopictus* and *A. aegypti* (Cui et al., 2006). However, vector control is an endless, costly, and labour-intensive measure and is not always well accepted by local populations, whose cooperation is crucial. Control of CHIKV infection, other than use of drugs for treatment of disease, development of vaccines, individual protection from mosquitoes and vector control programs, also involves surveillance that is fundamental for early identification of cases and quarantine measurement. A model used in investigation of the transmission potential of CHIKV in Italy has proven useful to provide insight into the possible impact of future outbreaks in temperate climate regions and the effectiveness of the interventions performed during the outbreak (Poletti et al., 2011).

8. Geographic distribution and map

Chikungunya fever has an epidemiological pattern with both sporadic cases and epidemics in west Africa, from Senegal to Cameroun, and in many other African countries (Democratic Republic of Congo, Nigeria, Angola, Uganda, Guinea, Malawi, Central African Republic, Burundi, and South Africa). Moreover, many epidemics occurred in Asia (Burma, Thailand, Cambodia, Vietnam, India, Sri Lanka, Timor, Indonesia, and the Philippines) in the 1960s and in the 1990s (Jain et al., 2008; Pialoux et al., 2007).

Major epidemics appear and disappear cyclically, usually with an inter-epidemic period ranging from 7 to 20 years. The huge outbreak that increased concern about CHIKV started in Kenya in 2004, where the seroprevalence rates reached 75% in Lamu island (Pialoux et al., 2007), before reaching the Comores, Seychelles, and Mauritius islands. The virus reached La Reunion island, a French overseas district, in March–April 2005, probably as a result of importation of cases among immigrants from the Comores. The outbreak had two phases: after some thousands of cases which occurred in March-April 2005, very few cases were reported during the austral winter, while the second epidemic peak arose in the initial months of 2006. For the first time, a substantial number of deaths (254) were attributed, directly or indirectly, to CHIKV. From late 2005 onwards, hospitals in some Indian states found themselves swamped with patients complaining of fever and joint pain, which turned out to be Chikungunya fever (Fusco et al., 2010). The World Health Organization Regional Office for South-East Asia has reported that 151 districts in nine states/provinces of India have been affected by Chikungunya fever between February and October 2006 (Pialoux et al., 2007).

Several imported cases were reported in industrialized countries among travellers returning from endemic areas, mainly tourists and immigrants (Depoortere & Coulombier, 2006). In particular, many cases were detected in early 2006, when the outbreak involved the Indian Ocean islands. The Indian Ocean islands, India, and Malaysia are popular tourist destinations. According to the World Tourism Organization, an estimated 1 474 218 people travelled from Madagascar, Mauritius, Mayotte, Reunion, and the Seychelles to European countries in 2004 (Depoortere & Coulombier, 2006; Parola et al., 2006).

The European country with the highest number of imported cases was France, especially the south-eastern region of Provence-Alpes-Côte d'Azur, and Marseille in particular, home to a large Comorian community (Cordel et al., 2006; Hochedez et al., 2007). Other European countries that reported imported cases include Belgium, Bosnia, Czech Republic, Croatia,

Germany, Greece, Italy, Serbia, Spain, Switzerland, Norway, and the United Kingdom (Beltrame, A. 2007; Deporteere & Coulombier, 2006; Fusco, F.M. 2006; Pialoux et al., 2007; Taubitz et al., 2007). In 2006, CHIK fever cases have also been reported in traveller returning from known outbreak areas to Canada, the Caribbean (Martinique), and South America (French Guyana). During 2005-2006, 12 cases of CHIK fever were diagnosed serologically and virologically at CDC in travellers who arrived in the United States from areas known to be epidemic or endemic for CHIK fever, and 26 additional imported cases with onset in 2006 underscores the importance of recognizing such cases among travellers (CDC, 2006; CDC 2007).

Moreover, CHIKV gave rise in 2007 to the first autochthonous European outbreak in Italy, in the northern region of Emilia-Romagna (Rezza et al., 2007; Charrel et al., 2008).

In June 2007, an Indian citizen returned to Italy after a visit to relatives in Kerala, India, developed 2 episodes of fever. During the second febrile episode, he visited his cousin in Castiglione di Cervia. The cousin had an onset of symptoms, with fever and arthralgia, on July 4. This sequence of events started the first Chikungunya fever outbreak in a temperate country, that lasted approximately 2 months with a total 247 cases of Chikungunya fever occurred in the region (217 laboratory-confirmed, 30 suspected) (Fusco et al., 2010). A unique sequence of events seems to have contributed to the establishment of local transmission in Emilia-Romagna: the high concentration of competent vectors *A. albopictus* in the area at the time of arrival of the index case, the presence of a sufficient human population density and the temporal overlapping of arthropod activity (seasonal syncronicity) (Charrel et al., 2008; Rezza et al., 2007).

During 2008, cases of Chikungunya fever have been reported from many countries in Asia other than India, as well as active epidemics from Singapore, Sri Lanka, and Malaysia (Leo et al., 2009).

Since 2006, the Regional Office of the French Institute For Public Health Surveillance in the Indian Ocean has conducted epidemiological and biological surveillance for CHIKV infection. During the period December 2006-July 2009, no confirmed case was detected on Reunion Island and Mayotte, but new outbreaks were reported in Madacascar. After few years of relative dormancy in Réunion Island, in August 2009, a cluster of cases was identified on the western coast of Réunion Island (D'Ortenzio et al., 2009) and, subsequently, an outbreak of CHIKV infection was described on Réunion Island in 2010 (D'Ortenzio et al., 2011). Moreover, recent publications described cases of Chikungunya fever in tourist returning from Maldives, confirming the circulation of the virus by the end of 2009 (Pfeffer et al., 2010; Receveur et al., 2010)

These episodes have refreshed the concerns about the possibility of renewed autochthonous transmission in Mediterranean countries and highlight the need for surveillance in countries where emerging infections may be introduced by returning travellers. Travellers can serve as sentinel population providing information regarding the emergence or re-emergence of an infectious pathogen in a source region. Travellers can thus act as carriers who inadvertently ferry pathogens that can be used to map the location, dynamics and movement of pathogenic strains (Pistone et al, 2009). Thus, with the increase in intercontinental travel, travellers can provide insights into the level of the risk of transmission of infections in other geographical regions.

The geographic range of CHIKV is mainly in Africa and Asia (Fig. 1)

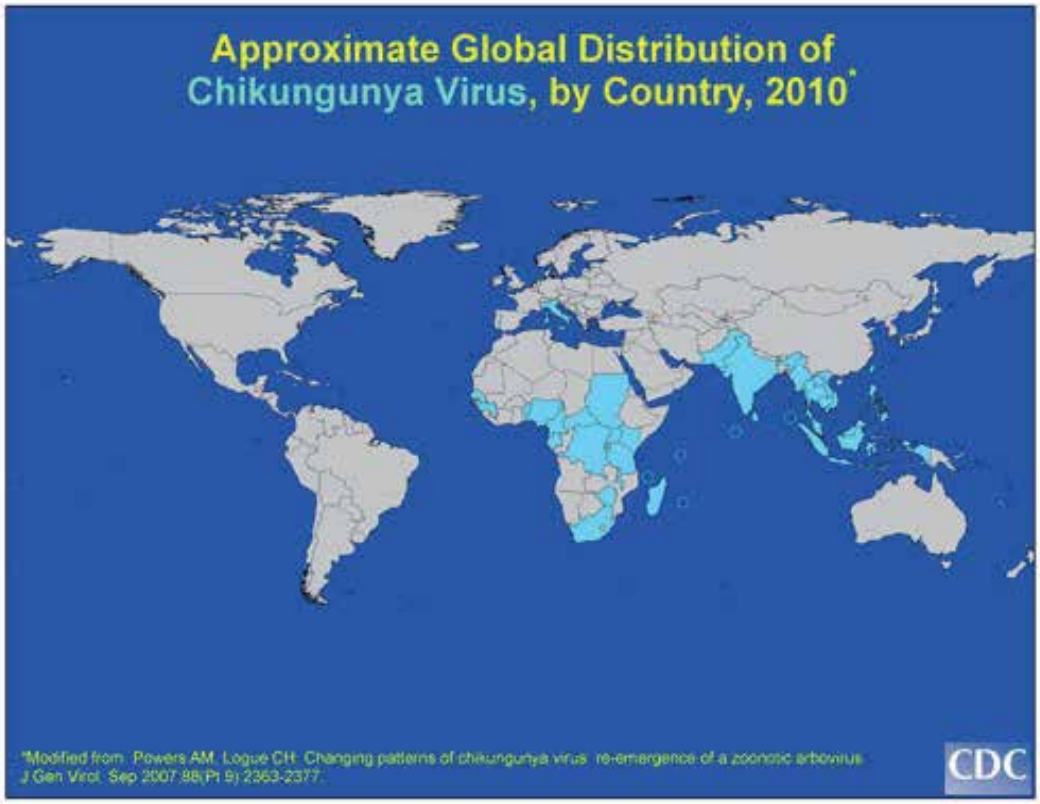


Fig. 1. Geographical distribution of CHIKV shown in the most recent map coming from the CDC's Traveler's Health website (http://wwwn.cdc.gov/travel/default.aspx).

9. Phylogenesis

Three lineages of CHIKV, with distinct genotypic and antigenic characteristics, have been identified. Isolates that caused the 2004-06 Indian Ocean outbreak form a distinct cluster within the large eastern and central Africa phylogenetic group, in addition to the Asian and west African phylogenetic groups (Powers et al., 2000; Schuffenecker et al., 2006). Phylogenetic analysis of CHIKV strains circulating in *A. Albopicus*-humans transmission cycles, obtained during outbreaks, have identified the independent acquisition of a common mutation in E1 glycoprotein (E1gp), namely A226V, in strains isolated from different geographic regions (Schuffenecker et al., 2006; de Lambellerie et al., 2008). This mutation, together with M269V, D284E mutations of E1 CHIKV glycoprotein have been described as molecular signatures of the Indian Ocean outbreak (Arankalle et al., 2007; Tsetsarkin et al., 2007; Vazeille at al., 2007). In particular, the A226V mutation, which was absent in the strains isolated during the initial phases of the outbreak in Réunion, appeared in >90% of the isolates after Dicember 2005. This change could be related to virus adaptation to the mosquito vector species. Together with the lack of herd immunity, this might explain the abrupt and escalating nature of the Reunion outbreak. Has been clearly demonstrated that the A226V mutation is able to increase viral fitness in the *Aedes albopictus* vector (Tsetsarkin

et al., 2007; Vazeille et al., 2007), that, in turn, may expand the potential for CHIKV to diffuse to the Americas and Europe, due to the widespread distribution of this vector, in particular in Italy (Knudsen, 1995). In a previous paper we characterized 7 viral isolates (5 imported and 2 autochthonous cases), with respect to the molecular signatures of the Indian Ocean Outbreak in E1, particularly the A226V mutation. Imported cases included 3 returning from Mauritius in 2006 and 2 returning from India in 2006 and 2007, respectively; the autochthonous cases occurred during the 2007 Italian outbreak (Bordi et al., 2008). CHIKV sequences of a 1013 bp fragment of E1 gene (nucleotide positions 10145-11158, respect to the reference strain S27) have been analyzed (Fig.2).

All 7 isolates carried the M269V and D284E Indian Ocean signatures while the A226V mutation was present in all the isolates imported from Mauritius, in the autochthonous cases from the Italian outbreak and in the isolate imported from India in 2007, but was absent in the case imported from India in 2006.

Our findings indicated that, during 2006 and 2007, multiple strains have been imported to Italy from countries where explosive Chikungunya outbreaks were ongoing. All the strains isolated in Italy, both imported and autochthonous, displayed two molecular signatures of

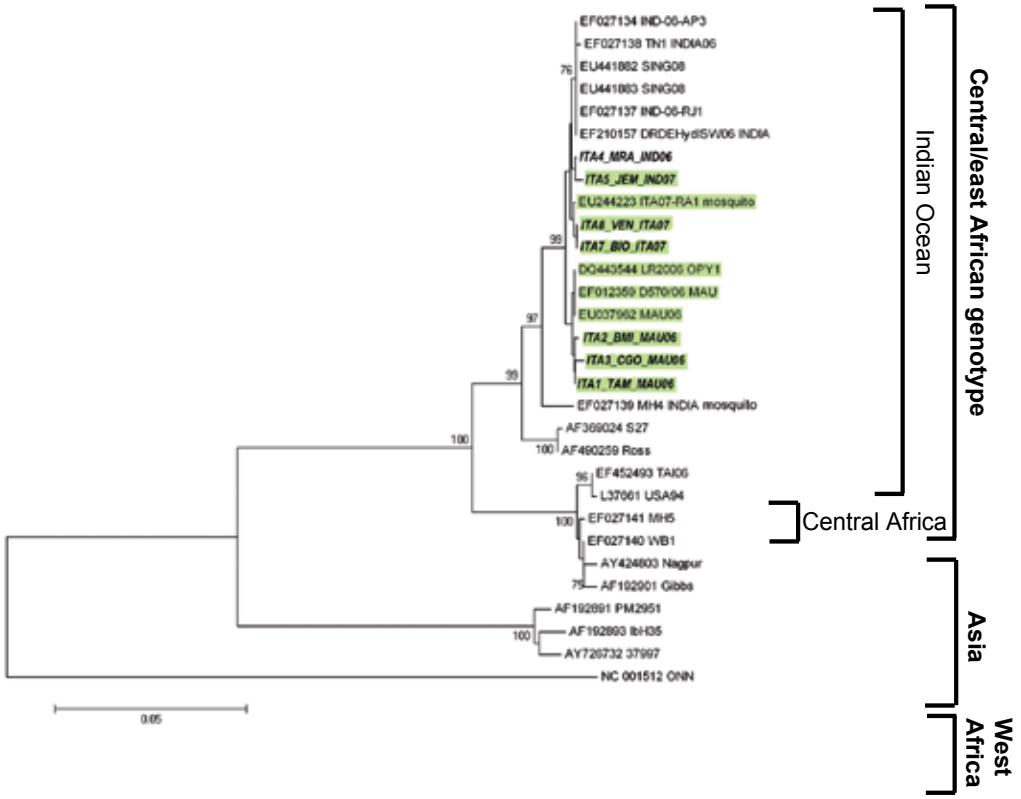


Fig. 2. Phylogenetic tree of CHIKV strains performed on partial E1 gene
CHIKV sequences of a 1013 bp fragment of E1 gene (nucleotide positions 10145-11158, respect to the reference strain S27) have been analyzed. The strains isolated from human cases in Italy are in bold (Bordi et al., Clin Infect Dis, 2008)

the Indian Ocean outbreak (M269V and D284E). Concerning the A226V mutation, this was present in all imported and autochthonous cases, with the exception of the isolate imported from the Indian subcontinent in 2006. The absence of this mutation in the isolate imported in 2006 from India was in agreement with published data (Arankalle et al., 2007), and with available GenBank sequence data, indicating that the virus strains circulating in India in 2006 lacked this mutation.
The presence of A226V in the isolate imported from India in July 2007 and in the isolates from the 2007 Italian outbreak (originating from a case imported from India) supports the view that the virus envelope sequence of strains from India changed over time, acquiring after 2006 the E1 mutation associated with enhanced fitness in *Aedes albopictus*. So it appears that the acquisition and fixation of the A226V mutation may be a common pathway of chikungunya explosion in epidemic areas, in a parallel interplay with the mosquito vector dynamics. Noteworthy, the outbreak in Singapore, where the A226V mutation was absent, has been rapidly controlled.

10. Immune-pathogenesis

Given the expanding geographic range of CHIKV and its potential to rapidly cause large scale epidemics, it has become important to understand the immune and pathogenic mechanisms active during CHIKV infections in order to guide the development of targeted and effective control and treatment strategies.
In a review the possible interactions of the immune system with the different stages of the CHIKV life cycle have been discussed (Kam et al., 2009). The first encounter of CHIKV with human host is intradermal inoculation by the mosquito: replication of the virus starts at the site of inoculation. Different resident cell types are present in this location, including keratinocytes, dermal dendritic cells (DCs), Langerhans cells (LCs), and dermal macrophages, cells involved in the innate immune response.
The innate immune response is the first barrier against viruses, being able to inhibit viral replication through cytolytic and non-cytolytic mechanisms. IFN system plays an important role in limiting virus spread at an early stage of infection. *In vitro* growth of all tested alphaviruses can be greatly suppressed by the antiviral effects of Interferon-α/β (IFN-α/β) when it is added to cells prior to infection, and, more specifically, CHIKV replication is significantly influenced by type I and II IFNs (Courderc et al., 2008; Schilte et al., 2010; Sourisseau et al., 2007). The finding that aberrant Type I interferon signalling in mice led to severe forms of CHIKF (Couderc et al., 2008) further highlighted important roles cytokines play in the pathology of CHIKV infection. Moreover, in a very recent study Wauquier and colleague demonstrated that CHIKV infection in humans elicit strong innate immunity involving the production of numerous proinflammatory mediators. Interestingly, high levels of Interferon IFN-α were consistently found. Production of interleukin (IL) 4, IL-10, and IFN-γ suggested the engagement of the adaptive immunity. This was confirmed by flow cytometry of circulating T lymphocytes that showed a CD8+ T lymphocyte response in the early stages of the disease, and a CD4+ T lymphocyte mediated response in the later stages (Wauquier et al., 2011).
It was already known that skin cell fibroblasts were susceptible to CHIKV infection (Sourisseau et al.,2007); recently has also been demonstrated that CHIKV antigens could be detected *in vivo* in the monocytes of acutely infected patients (Her et al, 2010). CHIKV

interactions with monocytes, and with other blood leukocytes, induced a robust and rapid innate immune response with the production of specific chemokines and cytokines. In particular, high levels of IFN-α were rapidly produced after CHIKV incubation with monocytes. The identification of monocytes during the early phase of CHIKV infection *in vivo* is significant as infected monocyte/macrophage cells have been detected in the synovial tissues of chronically CHIKV-infected patients, and these cells may behave as the vehicles for virus dissemination. This may explain the persistence of joint symptoms despite the short duration of viraemia (Her et al., 2010).

Since the A226V mutation has been associated with enhanced replication and fitness of CHIKV in *A. albopictus* vector and has also been shown to modulate cholesterol requirement for infection of insect cells (Tsetsarkin et al., 2007), in a recent paper we investigated the possible involvement of A226V mutation in enhancing human pathogenesis in non vector hosts, by testing the replication competence in primate cell cultures of two isolates, differing for the presence or absence of this mutation (Bordi et al., 2011). We observed that the presence of A226V mutation did not influence the replication kinetics on primate cells. Moreover, the time course of appearance of cytopathic effect (CPE) and of cells immunostained with CHIKV-specific antiserum, was very similar for both the isolates, as well as the shape of the virus-positive multicellular foci, thus suggesting a similar mechanism of spread of the virus in the infected cell cultures.

In addition, we considered the possibility that the A226V mutation could be associated with partial resistance to the inhibitory action of IFN-α in classical experiments of inhibition of virus replication. Surprisingly, the A226V-carrying strain was more susceptible to the antiviral action of recombinant IFN-α. (Fig.3)

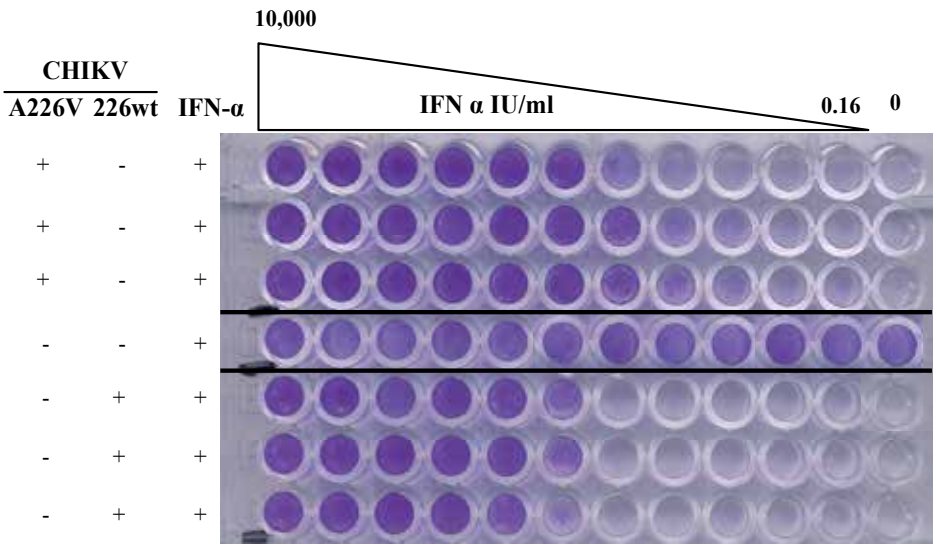


Fig. 3. Dose-dependent reduction of viral CPE by recombinant IFN-α.

In vitro experiments of inhibition of virus replication by recombinant IFN-α on Vero E6 cells showing a dose-dependent reduction of CPE for both isolates: the A226V, carrying isolate and the wt (Bordi et al., New Microbiol, 2011).

Overall, our result did not support the concept that A226V mutation confers a replicative advantage in primate cell cultures, neither supported the possibility that partial resistance to the inhibitory action of IFN-α could account for the explosive spread of the mutated strain in the human population in the countries where this mutation had occurred. However, the possibility that the interplay between the virus and the innate defence system may act at different levels of the virus/host interaction is to be taken into consideration, by exploring, for instance, other steps of the IFN response activation.

At the moment, understanding CHIKV immuno-biology is still in its infancy and there is a long way to go before answers related to the interaction between virus and host immunity will be obtained. These will certainly be important in designing novel antiviral control strategies against the spread of CHIKV infection.

11. References

Akahata W., Yang Z.Y., Andersen H., et al. A VLP vaccine for epidemic Chikungunya virus protects nonhuman primates against infection. Nat Med, 2010; 16: 334-338

Arankalle V.A., Shrivastava S., Cherian S., et al. Genetic divergence of Chikungunya viruses in India (1963-2006) with special reference to the 2005-2006 explosive epidemic. J Gen Virol, 2007; 88: 1967-1976

Barksby H.E., Lea S.R, Preshaw P.M., et al. The expanding family of interleukin-1 cytokines and their role in destructive inflammatory disorders. Clin Exp Immunol, 2007; 149: 217-225

Beesoon S., Funkhouser E., Kotea N., Spielman A., et al. Chikungunya fever, Mauritius, 2006. Emerg Infect Dis, 2008; 14: 337-338

Beltrame A., Angheben A., Bisoffi Z., et al. Imported Chikungunya infection, Italy. Emerg Infect Dis, 2007; 13: 1264-1266

Blackburn N.K., Besselaar T.G. & Gibson G. Antigenic relationship between chikungunya virus strains and o'nyong nyong virus using monoclonal antibodies. Res Virol, 1995; 146: 69–73

Bordi L., Carletti F., Castilletti C. et al. Presence of the A226V mutation in autochthonous and imported Italian chikungunya virus strains. Clin Infect Dis, 2008; 47: 428-429

Bordi L., Meschi S., Selleri M., et al. Chikungunya virus isolates with/without A226V mutation show different sensitivity to IFN-a, but similar replication kinetics in non human primate cells. New Microbiol, 2011; 34: 87-91

Brighton S.W., Prozesky O.W. & de la Harpe A.L. Chikungunya virus infection. A retrospective study of 107 cases. S Afr Med J, 1983; 63: 313–315

Brighton S.W. Chloroquine phosphate treatment of chronic Chikungunya arthritis: an open pilot study. S Afr Med J, 1984; 66: 217-218

Briolant S., Garin D., Scaramozzino N. et al. In vitro inhibition of Chikungunya and Semliki Forest viruses replication by antiviral compounds: synergistic effect of interferon-alpha and ribavirin combination. Antiviral Res, 2004; 61: 111-117

Carletti F., Bordi L., Chiappini R., et al. Rapid detection and quantification of Chikungunya virus by a one-step reverse transcription polymerase chain reaction real-time assay. Am J Trop Med Hyg, 2007; 77: 521-524.

Centers for Disease Control and Prevention (CDC).Chikungunya fever diagnosed among international travelers--United States, 2005-2006. MMWR Morb Mortal Wkly Rep., 2006; 55: 1040-2.

Centers for Disease Control and Prevention (CDC). Update: chikungunya fever diagnosed among international travelers--United States, 2006. MMWR Morb Mortal Wkly Rep., 2007; 56:276-7.

Charrel R.N., de Lamballerie X. & Raoult D. Chikungunya outbreaks--the globalization of vector-borne diseases. N Engl J Med, 2007; 356: 769-771

Charrel R. & de Lamballerie X. Chikungunya in north-eastern Italy: a consequence of seasonal synchronicity. Euro Surveill, 2008; 13: pii: 8003

Chatterjee S.N., Chakravarti S.K., Mitra A.C. et al. Virological investigation of cases with neurological complications during the outbreak of haemorrhagic fever in Calcutta. J Indian Med Assoc, 1965; 45: 314-316

Chatterjee S.N. & Sarkar J.K. Electron microscopic studies of suckling mouse brain cells infected with Chikungunya virus. Indian. J Exp Biol, 1965; 3: 227-234

Cordel H., Quatresous I., Paquet C. et al. Imported cases of chikungunya in metropolitan France, April 2005 - February 2006. Euro Surveill, 2006; 11: E060420.3

Couderc T., Chrétien F., Schilte C. et al. A mouse model for Chikungunya: young age and inefficient Type-I interferon signalling are risk factors for severe disease. Plos Pathogen, 2008; 4: e29

Couderc T., Khandoudi N., Grandadam M. et al. Prophylaxis and therapy for Chikungunya virus infection. J Infect Dis, 2009; 200: 516-523

Cui F., Raymond M. & Qiao C.L. . Insecticide resistance in vector mosquitoes in China. Pest Manag Sci, 2006; 62: 1013-1022

D'Ortenzio E., Grandadam M., Balleydier E. et al. Sporadic cases of chikungunya, Reunion Island, August 2009. Euro Surveill, 2009; 14: pii: 19324

D'Ortenzio E., Grandadam M., Balleydier E. et al. A226V strains of Chikungunya virus, Réunion Island, 2010. Emerg Infect Dis, 2011; 17: 309-311

de Lambellerie X., Leroy E., Charrel R.N. et al. Chikungunya virus adapts to tiger mosquito via evolutionary convergence: a sign of things to come? Virol J, 2008; 5: 33

de Lamballerie X., Boisson V., Reynier J.C. et al. On chikungunya acute infection and chloroquine treatment. Vector Borne Zoonotic Dis, 2008; 8: 837-839

Depoortere E., & Coulombier D. Chikungunya risk assessment for Europe: recommendations for action. Euro Surveill, 2006; 11: E060511.2

Dessain S.K., Adekar S.P., & Berry J.D. Exploring the native human antibody repertoire to create antiviral therapeutics. Curr Top Microbiol Immunol, 2008; 317: 155–183

Eckels K.H. Harrison V.R. & Hetrick .FM. Chikungunya virus vaccine prepared by Tween-ether extraction. Appl Microbiol, 1970; 19: 321-325

Edelman R., Tacket C.O., Wasserman S.S. et al. Phase II safety and immunogenicity study of live Chikungunya virus vaccine TSI-GSD-218. Am J Trop Med Hyg, 2000; 62: 681-685

Enserink M. Infectious diseases. Massive outbreak draws fresh attention to little-known virus. Science, 2006; 311: 1085

Fourie E.D. & Morrison J.G. Rheumatoid arthritic syndrome after chikungunya fever. S Afr Med J, 1979; 56: 130–132

Fusco F.M., Puro V., Di Caro A. et al. Cases of Chikungunya fever in Italy in travellers returning from the Indian Ocean and risk of introduction of the disease to Italy. Infez Med, 2006; 14: 238-245

Fusco F.M. et al. (2010). Chikungunya fever, a re-emerging disease, In: *Tropical and Emerging Infectious Diseases*, Maltezou Helen C. and Achilleas Gikas, 93-110, ISBN: 978-81-308-0389-0

Gubler D.J., Reiter P., Ebi K.L. et al. Climate variability and change in the United States: potential impacts on vector- and rodent-borne diseases. Environ Health Perspect, 2001; 109 Suppl 2: 223-233

Harrison V.R., Binn L.N. & Randall R. Comparative immunogenicities of chikungunya vaccines prepared in avian and mammalian tissues. Am J Trop Med Hyg, 1967; 16: 786-791

Harrison V.R, Eckels K.H., Bartelloni P.J. et al. Production and evaluation of a formalin-killed Chikungunya vaccine. J Immunol, 1971; 107: 643-647

Hearn H.J. Jr. & Rainey C.T. Cross-protection in animals infected with Group A arboviruses. J Immunol, 1963; 90: 720-724

Her Z., Malleret B., Chan M. et al. Active Infection of Human Blood Monocytes by Chikungunya Virus Triggers an Innate Immune Response. J Immunol, 2010; 184: 5903-5913

Higgs S. The 2005–2006 chikungunya epidemic in the Indian Ocean. Vector Borne Zoonotic Dis 2006; 6: 115–116

Hochedez P., Jaureguiberry S., Debruyne M. et al. Chikungunya infection in travelers. Emerg Infect Dis, 2006; 12: 1565–1567

Hochedez P., Hausfater P., Jaureguiberry S. et al. Cases of chikungunya fever imported from the islands of the South West Indian Ocean to Paris, France: 80 cases in France. Euro Surveill, 2007; 12: [Epub ahead of print]

Inoue S., Morita K., Matias R.R. et al. Distribution of three arbovirus antibodies among monkeys (*Macaca fascicularis*) in the Philippines. J Med Primatol, 2003; 32: 89–94

Jacups S.P., Whelan P.I. & Currie B.J. Ross River virus and Barmah Forest virus infections: a review of history, ecology, and predictive models, with implications for tropical northern Australia. Vector Borne Zoonotic Dis, 2008; 8: 283-297

Jain M., Rai S. & Chakravarti A. Chikungunya: a review. Trop Doc, 2008; 38: 70-72

Jeandel P., Josse R. & Durand J.P. Exotic viral arthritis: role of alphavirus. Med Trop, 2004; 64: 81-88 (in French)

Josseran L., Paquet C., Zehgnoun A. et al. Chikungunya disease outbreak, Reunion Island. Emerg Infect Dis, 2006; 12: 1994-1995

Jupp P.G., McIntosh B.M., Dos Santos I. et al. Laboratory vector studies on six mosquito and one tick species with chikungunya virus. Trans R Soc Trop Med Hyg, 1981; 75: 15-19

Jupp P.G. & McIntosh B.M. *Aedes furcifer* and other mosquitoes as vectors of chikungunya virus at Mica, northeastern Transvaal, South Africa. J Am Mosq Control Assoc, 1990; 6: 415-420

Kam Y.W., K.S. Ong E., Laurent R et al. Immuno-biology of Chikungunya and implications for disease intervention. Microbes Infect, 2009; 11: 1186-1196

Kennedy A.C., Fleming J. & Solomon L. Chikungunya viral arthropathy: a clinical description. J Rheumatol, 1980; 7: 231-236

Kenney J.L., Volk S.M., Pandya J., et al. Stability of RNA virus attenuation approaches. Vaccine, 2011; 29: 2230-4.

Khan A.H., Morita K., Parquet Md Mdel C. et al. Complete nucleotide sequence of Chikungunya virus and evidence for an internal polyadenylation site. Gen Virol, 2002; 83, 3075-3084

Knudsen A.B. Global distribution and continuing spread of *Aedes albopictus*. Parassitologia, 1995; 37: 91-97

Labadie K., Larcher T., Joubert C. et al. Chikungunya disease in nonhuman primates involves long-term viral persistence in macrophages. J Clin Invest, 2010; 120: 894-906

Lakshmi V., Neeraja M., Subbalaxmi M.V. et al. Clinical features and molecular diagnosis of Chikungunya fever from South India. Clin Infect Dis, 2008; 46: 1436-1442

Lam S.K., Chua K.B., Hooi P.S. et al. Chikungunya infection—an emerging disease in Malaysia. Southeast Asian J Trop Med Public Health , 2001; 32: 447-451

Lebrun G., Chadda K., Reboux A.H. et al. Guillain-Barré syndrome after chikungunya infection. Emerg Infect Dis, 2009; 15: 495-496

Lemant J., Boisson V., Winer A. et al. Serious acute Chikungunya virus infection requiring intensive care during the Reunion Island outbreak in 2005-2006. Crit Care Med, 2008; 36: 2536-2541

Leo Y.S., Chow A.L.P., Tan L.K. et al. Chikungunya Outbreak, Singapore, 2008. Emerg Infect Dis, 2009 15: 836-837

Levitt N.H., Ramsburg H.H., Hasty S.E. et al. Development of an attenuated strain of chikungunya virus for use in vaccine production. Vaccine, 1986; 4: 157-162

Lidbury B.A., Rulli N.E., Suhrbier A. et al. Macrophage-derived proinflammatory factors contribute to the development of arthritis and myositis after infection with an arthrogenic alphavirus. J Infect Dis, 2008; 197: 1585-1593

Litzba N., Schuffenecker I., Zeller H. et al.Evaluation of the first commercial chikungunya virus indirect immunofluorescence test. J Virol Methods, 2008; 149: 175-179

Mahalingam S., Meanger J., Foster P.S. et al. The viral manipulation of the host cellular and immune environments to enhance propagation and survival: a focus on RNA viruses. J Leukoc Biol, 2002; 72: 429-439

Mahendradas P., Ranganna S.K., Shetty R. et al. Ocular manifestations associated with chikungunya. Ophthalmol, 2008; 115: 287-291

Mallilankaraman K., Shedlock D.J., Bao H. et al. A DNA vaccine against chikungunya virus is protective in mice and induces neutralizing antibodies in mice and nonhuman primates. PLoS Negl Trop Dis, 2011; 5: e928

Mavalankar D., Shastri P. & Raman P. Chikungunya epidemic in India: a major public-health disaster. Lancet Infect Dis, 2007; 7: 306-307

Mavalankar D., Shastri P., Bandyopadhyay T. et al. Increased mortality rate associated with Chikungunya epidemic, Ahmedabad, India. Emerg Infect Dis, 2008; 14: 412-415

Muthumani K., Lankaraman K.M., Laddy D.J. et al. Immunogenicity of novel consensus-based DNA vaccines against Chikungunya virus. Vaccine, 2008; 26: 5128-5134

Ng L.F.P., Chow A., Sun Y.J. et al. IL-1b, IL-6,and RANTES as biomarkers of Chikungunya severity. PLoS One, 2009; 4: e4261

Ozden S., Huerre M., Riviere J.P. et al. Human muscle satellite cells as targets of Chikungunya virus infection. PLoS One, 2007; 2: e527

Panning M., Grywna K., van Esbroeck, M. et al. Chikungunya fever in travelers returning to Europe from the Indian Ocean region, 2006. Emerg Infect Dis, 2008; 14: 416-422

Parola P., de Lamballerie X., Jourdan J. et al. Novel Chikungunya virus variant in travellers returning from Indian Ocean islands. Emerg Infect Dis, 2006; 12: 1493-1499

Pedersen C.E, Robinson D.M., Cole F.E. Isolation of the vaccine strain of Venezuelan equine encephalomyelitis virus from mosquitoes in Louisiana. Am J Epidemiol, 1972; 95:490–496

Pfeffer M., Hanus I., Löscher T. et al. Chikungunya fever in two German tourists returning from the Maldives, September, 2009. Euro Surveill, 2010; 15: pii: 19531

Pialoux G., Gaüzère B.A., Jauréguiberry S. et al. Chikungunya, an epidemic arbovirosis. Lancet Infect Dis, 2007; 7: 319-327

Pile J.C., Henchal E.A., Christopher G.W. et al. Chikungunya in a North American traveler. J Travel Med, 1999; 6: 137–139

Pistone T., Ezzedine K., Schuffenecker I., et al. An imported case of Chikungunya fever from Madagascar: use of the sentinel traveller for detecting emerging arboviral infections in tropical and European countries. Travel Med Infect Dis, 2009;7: 52-4.

Plante K., Wang E., Partidos C.D., et al. Novel Chikungunya Vaccine Candidate with an IRES-Based Attenuation and Host Range Alteration Mechanism. PLoS Pathog, 2011; 7 : e1002142.

Poletti P., Messeri G., Ajelli M. et al. Transmission potential of chikungunya virus and control measures: the case of Italy. PLoS One, 2011; 6: e18860

Powers A.M., Brault A.C., Tesh R.B. et al. Re-emergence of chikungunya and o'nyong-nyong viruses: evidence for distinct geographical lineages and distant evolutionary relationships. J Gen Virol, 2000; 81: 471-479

Precious S.W. Webb H.E. & Bowen E.T.W. Isolation and persistence of Chikungunya virus in cultures of mouse brain cells. J Gen Virol, 1974; 23: 271-279

Quatresous I. E-alert 27 January: chikungunya outbreak in Reunion, a French overseas department. Euro Surveill 2006; 11: E060202.1

Ravi V. Re-emergence of chikungunya virus in India. Indian J Med Microbiol, 2006; 24: 83-84

Ravichandran R. & Manian M. Ribavirin therapy for Chikungunya arthritis. J Infect Dev Ctries, 2008; 2: 140-142.

Receveur M., Ezzedine K., Pistone T. et al. Chikungunya infection in a French traveller returning from the Maldives, October, 2009. Euro Surveill, 2010; 15: 19494

Reiter P., Fontenille D. & Paupy C. Aedes albopictus as an epidemic vector of chikungunya virus: another emerging problem? Lancet Infect Dis, 2006; 6: 463-464

Rezza G., Nicoletti L., Angelini R. et al. CHIKV study group. Infection with Chikungunya virus in Italy: an outbreak in a temperate region. Lancet, 2007; 370: 1840-1846

Robinson M.C. An epidemic of virus disease in Southern Province, Tanganyika Territory, in 1952-1953. I. Clinical features. Trans Royal Society Trop Med Hyg, 1955; 49:28-32

Sam I.C. & AbuBakar S. Chikungunya virus infection. Med J Malaysia 2006; 61: 264–269

Saxena S., Singh M., Mishra N et al. Resurgence of chikungunya virus in India: an emerging threat. Euro Surveill, 2006; 11: E060810.2

Schilte C., Couderc T., Chretien F. et al. Type I IFN controls chikungunya virus via its action on non-hematopoietic cells. J Exp Med, 2010; 207: 429-442

Schuffenecker I., Iteman I., Michault A. et al. Genome microevolution of Chikungunya viruses causing the Indian Ocean outbreak. Plos Medicine, 2006; 3: 1058-1070

Sourisseau M., Schilte C., Casartelli N. et al. Characterization of reemerging chikungunya virus. PLoS Pathogen, 2007; 3: e89

Strauss J.H., Strauss E.M. The alphaviruses: gene expression, replication and evolution. Microbiol Rev, 1994; 58: 491-562

Taubitz W., Cramer J.P., Kapaun A. et al. Chikungunya fever in travelers: clinical presentation and course. Clin Infect Dis, 2007; 45: e1-4

Tsetsarkin K.A., Vanlandingham D.L., McGee C.E. et al. A Single Mutation in Chikungunya Virus Affects Vector Specificity and Epidemic Potential. PLoS Pathog, 2007; 3: 1895-1906

Vazeille M., Moutailler S., Coudrier D. et al. Two Chikungunya Isolates from the Outbreak of La Reunion (Indian Ocean) Exhibit Different Patterns of Infection in the Mosquito, Aedes albopictus. PLoS One, 2007; 2: 1-9

Wang E., Volkova E., Adams A.P. et al. Chimeric alphavirus vaccine candidates for chikungunya. Vaccine, 2008; 26: 5030-5039

Wang D., Suhrbier A., Penn-Nicholson A. et al. A complex adenovirus vaccine against chikungunya virus provides complete protection against viraemia and arthritis. Vaccine, 2011; 29: 2803-2809

Wang E., Kim D.Y., Weaver S.C., et al. Chimeric Chikungunya Viruses are Nonpathogenic in Highly Sensitive Mouse Models, but Efficiently Induce a Protective Immune Response. J Virol, 2011 Jun 22. [Epub ahead of print]

Warter L., Lee C.Y., Thiagarajan R. et al. Chikungunya virus envelope-specific human monoclonal antibodies with broad neutralization potency. J Immunol, 2011; 186: 3258-3264

Wauquier N., Becquart P., Nkoghe D. et al. The acute phase of chikungunya virus infection in humans is associated with strong innate immunity and T CD8 cell activation. J Infect Dis, 2011; 204: 115-123

White A., Berman S. & Lowenthal J.P. Comparative immunogenicities of Chikungunya vaccines propagated in monkey kidney monolayers and chick embryo suspension cultures. Appl Microbiol, 1972; 23: 951-952

Wielanek A.C., Monredon J.D., Amrani M.E. et al. Guillain-Barré syndrome complicating a Chikungunya virus infection. Neurology, 2007; 69: 2105-2107

Wolfe N.D., Kilbourn A.M., Karesh W.B. et al. Sylvatic transmission of arboviruses among Bornean orangutans. Am J Trop Med Hyg, 2001; 64: 310-316

Yergolkar P., Tandale B., Arankalle V. et al. Chikungunya outbreaks caused by African genotype, India. Emerg Infect Dis, 2006; 12: 1580–1583

Zeller H.G. Dengue, arbovirus and migrations in the Indian Ocean. Bull Soc Pathol Exot, 1998; 91: 56-60

Ziegler S.A., Lu L.,. da Rosa A.P. et al. An animal model for studying the pathogenesis of Chikungunya virus infection. Am J Trop Med Hyg, 2008; 79: 133-139

Part 2

Tropical Diseases Due to Protozoa and Helminths

10

Malaria Chemoprophylaxis for the International Traveler, Current Options and Future Possibilities

Ross Parker and Kevin Leary
Uniformed Services University of the Health Sciences
USA

1. Introduction

Malaria remains the most important parasitic disease in the world, causing approximately 250 million infections annually and one million deaths, mostly in African children. International travelers are at risk of developing malaria when visiting endemic regions, and account for an estimated 30,000 cases of malaria annually (World Health Organization, 2011). The parasite is transmitted by the female Anopheles mosquito and is caused by four protozoa of the *Plasmodium* genus *(P. falciparum, P. vivax, P. ovale* and *P. malaria)*. *P. falciparum* causes the most significant disease burden with the highest morbidity and mortality. In addition to mosquito control and avoidance measures, chemoprophylaxis remains a critical component for preventing malaria infection in non-immune travelers.

Addressing malaria chemoprophylaxis for the international traveler can be challenging. In addition to patient-specific factors, the provider must consider a wide array of other variables, such as the predicted risk of malaria associated with the destination, type and duration of exposure during the trip as well, as the profile of the drug being prescribed. Seasonal, geographic, and climate are among the environmental variables that should be addressed to appreciate risk of malaria transmission. There can be upwards of a 200-fold difference in relative risk of contracting malaria depending on geographic variables for the international traveler, with sub-Saharan Africa conferring the greatest risk (Leder et al., 2004; Freedman, 2008). The traveler's accommodations, anticipated understanding and adherence to mosquito avoidance and control measures, chemoprophylaxis and access to appropriate medical care contribute to the risk of morbidity and mortality associated with malaria.

Patient-specific variables can also present challenges to the provider. Pregnant, nursing and pediatric travelers present unique considerations when determining the most appropriate chemoprophylactic regimen. Pregnant patients incur a much higher risk of mortality and morbidity from malaria than non-pregnant travelers, and require extensive counselling on the risks and benefits of proposed travel to areas at risk of transmission. Emerging parasite drug resistance patterns, side-effect profiles, both long and short term, contraindications and poor adherence are additional challenges that need to be considered when selecting an appropriate antimalarial chemoprophylactic agent. In addition, how to address chemoprophylaxis in long-term travelers, generally defined as travel greater than six-

months in duration, can be very difficult as consensus guidelines in this population are not available (Chen et al., 2006).
It has been over ten years since the U.S. Food and Drug Administration has approved an antimalarial chemoprophylactic drug. Lack of market incentive, increasing difficulty in the design and execution of clinical trials, as well as the changing ethical environment after Declaration of Helsinki 2000 have contributed to the lag in continued development for the malaria chemoprophylaxis indication (Dow et al., 2008).

2. Education

Before travel, counseling the individual on the specific risks in the areas they may be visiting is an essential part of trip preparation. When counseling the traveler prior to visiting an endemic area, they must be made aware of the route of transmission of malaria, associated symptoms, variable incubation periods prior to symptom onset, when to seek medical aid, and the risks of contracting the disease, including death, especially in high-risk populations. They need to be aware that recent immigrants to non-malaria endemic areas returning to their home of origin to visit friends and relatives (VFR's) are at high risk for contracting malaria as acquired immunity is not long lasting (Centers for Disease Control and Prevention, 2012). Travelers should be counseled on proper personal protective measures including mosquito bite avoidance, especially during the peak transmission periods of evening and nighttime hours, mechanical and chemical barrier protection, vector control, and the appropriate use and importance of chemoprophylaxis.
Malaria can be effectively treated if suspected and recognized early and appropriate medical intervention is made within a timely manner. Time to symptom onset from initial exposure can vary, ranging as early as 7 days following a mosquito bite to several months or greater following departure from an endemic region. The diagnosis of malaria is a medical emergency since time to definitive treatment is a critical factor in determining clinical outcome. For these reasons, travelers should be counseled to seek medical care as soon as possible if they have any symptoms that may be related to malaria. The clinical presentation of malaria consists of a nonspecific, flu-like illness manifested by fever, chills, malaise, anorexia and headache. In cases of severe illness, altered mental status, seizures, respiratory disease (ARDS) and coma may be present (CDC, 2012).
Availability of medical care while traveling should be explored prior to travel. There may be rare instances where the chemoprophylaxis regimen is suboptimal or the traveler does not agree to medically advised chemoprophylaxis. In cases when the traveler develops clinical symptoms consistent with malaria and does not have timely access to medical care and definitive parasitological diagnosis, presumptive, self-administered therapy may be considered (WHO, 2010; CDC, 2012). When prescribing presumptive self-treatment, the CDC recommends a consecutive 3-day course of either atovaquone-proquanil or artemether-lumefantrine. One should never use the same drug for treatment that had been prescribed for prophylaxis. It should be stressed to the traveler that even though presumptive treatment may be available, they should seek medical care as soon as possible.

3. Personal protective measures

Several measures can be taken by the traveler while in endemic areas to reduce the risk of mosquito bites, thus reducing the risk of contracting malaria. The *Anopheles* mosquito only

feeds at night, making the hours between dusk and dawn those that the traveler must be most vigilant for vector avoidance and mosquito control measures. Staying indoors, sleeping in screened-structures, and using mosquito nets during peak feeding times are all effective and relatively simple ways to reduce transmission of malaria. Other protective measures including clothing that minimizes exposed skin, eliminating mosquito breeding sites, and using appropriate repellents/insecticides on skin and clothing, should be discussed with the traveler as well (Chen et al., 2006; CDC, 2012).

A systematic literature review concluded that environmental management programs were highly effective at reducing the morbidity and mortality associated with malaria, and if educated properly travelers can reduce their risk significantly through these personal and environmental protective measures (Keiser et al., 2005).

Insecticides such as permethrin can be used as a spray to kill mosquitoes on contact, or can be used to impregnate clothes and mosquito nets for long-term protection. A 2003 randomized-controlled trial in sub-Saharan Africa showed a reduction in all-cause child mortality by 15-33% with the use of permethrin treated bed nets and curtains, and a 1995 study of permethrin impregnated uniforms in Columbian soldiers showed a decrease in incidence of malaria from 14% to 3% (Phillips-Howard & Nahlen et al., 2003; Soto & Medina et al., 1995), indicating mosquito avoidance and control measures can be highly effective in preventing malaria transmission.

Repellents prevent arthropod bites via alterations to sensorial organs. There are several different commercially available repellents including DEET (*N*,*N*-diethly-3-methyl-benzamide), picaridin (2-(2-hydroxyethyl)-1-piperidinecarboxylic acid 1-methylpropyl ester), oil of lemon eucalyptus (*para*-menthane-3,8-diol), and IR3535 (3-[*N*-butyl-*N*-acetyl]-aminopropionic acid, ethyl ester). The efficacy and duration of repellents vary considerably among products and species of mosquito (Zielinski-Gutierrez et al., 2012). Several studies have suggested DEET and picaridin to be the most efficacious and long lasting (Fradin & Day, 2002; Trigg, 1996; Govere et al., 2000; Badolo et al., 2004). Both DEET and picaridin demonstrate efficacy between five and seven hours after application, with variations in efficacy and duration of effectiveness related to repellent concentration, humidity, temperature, perspiration, exposure to water, and abrasion (Zielinski-Gutierrez et al., 2012). There seems to be a ceiling effect with DEET at concentrations above 50%, where higher concentrations do not offer additional benefit.

DEET, at concentrations up to 50%, can be used on children over two months of age. Children less than two months should be protected with a child carrier covered with a mosquito net. Beyond labeled precautions, the U.S. Environmental Protection Agency (EPA) and CDC do not recommend additional warnings for repellents in children > 2 months, pregnant or lactating women (Zielinski-Gutierrez et al., 2012). Like physical barriers, repellents and insecticides are only effective if used properly and consistently, thus ensuring the traveler is aware of proper use before departing is an essential part of pre-trip counseling.

4. Chemoprophylaxis

Educating travelers on the clinical indications, as well as proper use and risks of chemoprophylaxis is an important part of pre-travel counseling. Patients should be told of the options available for the area they are traveling based on CDC recommendations, and when clinically indicated, an appropriate chemoprophylaxis should be chosen and

prescribed based on the patient's medical history, tolerability of side effects, compliance, and known resistance in the area (Table 1). Resistance to antimalarial drugs is growing, and is a major public health concern (WHO, 2010). Resistance of *P. falciparum* to chloroquine, the most widely available and least expensive chemoprophylaxis agent, is now widespread, except in a few limited areas of the Caribbean, Central and South America, and a few countries in the Middle East. Resistance to mefloquine is spreading and has been confirmed in areas of SE Asia including along the borders of Burma and China, Laos and Burma, Thailand and Burma, Thailand and Cambodia, and in southern Vietnam (CDC, 2012).

4.1 Chloroquine

Chloroquine is a 4-aminoquinoline oral antimalarial agent first introduced in the 1940's. It has good bioavailability, is rapidly absorbed and appreciably concentrated in tissues such as the liver, spleen, and to a lesser extent in the CNS (WHO, 2010). Its plasmodicidal activity is thought to be related to its interaction with malarial DNA, specifically haem detoxification (Castelli et al., 2010; WHO, 2010). Chloroquine is dosed once weekly and is effective against the erythrocytic stages of sensitive plasmodium species.

Chloroquine has long shown its efficacy against malaria, and was a cornerstone of treatment until growing resistance became a problem in the 1980's (Castelli et al., 2010). *P. falciparum* resistance to chloroquine is widespread, thus making it an acceptable choice only in chloroquine sensitive areas. There is some evidence of mutations making non-falciparum strains resistant, with resistance of *P. vivax* to chloroquine reported in areas of Papua New Guinea, West Papua, Guyana, Vanuatu, Myanmar, Indonesia, and India (WHO, 2010; Kain et al., 2001; Davis et al., 2003).

Chloroquine has a generally mild side effect profile with the most common events being nausea, headache, blurred vision, insomnia, and pruritis (Castelli et al., 2010). Serious side effects, although rare, include myopathy, hepatitis, hearing loss, Stevens-Johnson Syndrome, seizures, and irreversible retinopathy (WHO, 2010). Retinopathy is usually seen after 100g cumulative dose, which is equivalent to what a long-term traveler may ingest in 5-6 years of weekly dosing (Chen et al., 2006). Chloroquine-induced retinopathy is rare in patients taking malaria prophylaxis and is more frequently seen in the higher doses administered for the treatment of rheumatoid arthritis (CDC, 2012). In a large (N=2701) trial of peace corps volunteers undergoing malaria prophylaxis it was found that chloroquine was better tolerated and had fewer serious side effects than mefloquine or doxycycline, however prophylaxis in general was not tolerated well with 9% reporting severe events and 23% at some point changing their prophylactic medication (Korhonen et al., 2007).

Chloroquine is considered safe for use in children and pregnancy, however strict adherence to weight-based dosing must be adhered to for children since serious adverse events have been reported in children receiving as little as 1 gram of chloroquine (Chen et al., 2006). While chloroquine is safe for breast-feeding mothers, the infant should receive separate prophylaxis as the amount of chloroquine secreted in breast milk is not sufficient for protection.

Chloroquine is available in 500mg tablets, which is equivalent to 300mg chloroquine base. Dosing is done weekly starting 2 weeks before travel into an endemic area and for 4 weeks after leaving the area. Pediatric dosing is 5mg/kg base, never to exceed adult dosing.

While generally considered a safe and efficacious drug, the growing resistance to chloroquine is making it a choice only available in limited areas of the world. However,

because it is one of two current drugs considered safe in pregnant women and children, and because it is fairly well tolerated, it will remain a viable choice for prophylaxis if resistance patterns are taken into account when prescribing.

4.2 Primaquine

Primaquine phosphate is an oral antimalarial agent first approved by the FDA in 1952. The mechanism of action is not well understood, but its plasmodicidal activity is thought to be related to disruption of the parasitic electron transport chain (Castelli et al., 2010). It has a short half-life of approximately seven hours, thus requiring daily dosing. Before the approval of primaquine, there was no available treatment of relapsing malaria because anti-malarial drugs available at the time were only effective against the erythrocytic stages of Plasmodium species (Shanks et al., 2001). Primaquine's approval was important because it is effective against both the erythrocytic and exoerythocytic stages of Plasmodium species, making it an effective choice for *P.vivax, P. ovale, or P. falciparum* (WHO, 2010; Shanks, Kain et al., 2001). However, it is only FDA-approved for the treatment of vivax malaria, but has long been used for treatment off-label for other species and is the drug of choice for terminal prophylaxis in travelers at risk for relapsing malaria (Castelli et al., 2010; Hill et al., 2006).

Multiple clinical trials have shown the efficacy of primaquine against both vivax and falciparum malaria (Shanks et al., 2001). In two placebo controlled trials on the island of New Guinea it was shown that primaquine had an efficacy of 93 - 95% against *P. falciparum* and 88 - 90% against *P. vivax* (Baird et al., 2001; Fryauff et al., 1995). In two placebo controlled trials done in Columbia good efficacy was also seen, with an overall efficacy of 89% against *P. falciparum* and 88% against *P. Vivax* (Soto et al., 1998, 1999). While good efficacy has been seen in the past, there is emerging evidence of increasing resistance to *P. Vivax* strains in some areas of Oceana, South East Asia, and South America (Baird, 2009).

Primaquine is a well-tolerated medication with the most common side effects being nausea, vomiting, and abdominal cramps (Fryauff et al., 1995). It was shown to have better tolerability than chloroquine in Irian Jaya transmigrates, and in a retrospective study of travelers to Ethiopia it had favorable tolerability compared to mefloquine and doxycycline (Schwartz & Regev-Yochay, 1999; Baird et al., 2001). Severe hemolytic anemia can occur in patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency and should be avoided in any patient with this enzymopathy. All patients taking primaquine should be evaluated for G6PD deficiency prior to receiving this drug (Hill et al., 2006).

Off-label dosing recommendations are 30mg base per day for 14 days for terminal prophylaxis, and 30mg per day 1-2 days before travel and continued for 7 days after travel for prophylaxis (CDC, 2012). It should be taken with food to limit side effects. Dosing for children is 0.5 mg/kg base per day. It has been shown safe in studies up to one year with no labeled restrictions on duration of use (Fryauff et al., 1995; Chen et al., 2006). Primaquine is contraindicated in pregnant women, making prevention or treatment of malaria in areas with *P.vivax* difficult in this population. If used as a primary prophylaxis, it negates the need for terminal prophylaxis, however, if another primary chemoprophylaxis is chosen, and relapsing malaria is a concern, it makes a good choice for terminal prophylaxis.

4.3 Mefloquine

Mefloquine hydrochloride is a methanol-quinoline oral antimalarial agent whose mechanism of action is not completely understood, but is thought to be similar to quinine

(Castelli et al., 2010). It acts as a blood schizonticide, making it highly effective against the erythrocytic stages of Plasmodium species, however it does not have any exoerythrocytic activity. Mefloquine has a long half-life, with the average being 21 days, thus only requiring once-weekly dosing. Mefloquine is effective against chloroquine resistant Plasmodium species, however there are some areas in SE Asia with known mefloquine resistance. Of note, mefloquine has been found to have serious neuropsychiatric adverse events, which limit its usefulness in certain populations (Castelli et al., 2010; Croft & Garner, 2008).

Mefloquine efficacy has been shown to be greater than 90% in multiple clinical trials (Kain, Shanks et al., 2001), the longest of which was in Peace-Corps volunteers in Africa during the early 1990's. In addition, a review of mefloquine trials found that it did prevent malaria in chloroquine resistant areas (Croft & Garner, 2000). Although an effective medication, the endemic risk of malaria, current resistance patterns, the drug's side effect profile and the patient's medical and psychiatric history should be carefully considered before prescribing mefloquine (Croft & Garner, 2000).

The mefloquine label lists many side effects, the most common of which are nausea, vomiting, diarrhea, and abdominal pain. However, it also states that mefloquine can cause serious mental problems including anxiety, paranoia, hallucinations, and suicidal thoughts. These psychiatric problems may lead to prophylaxis discontinuation, and should be considered when choosing the appropriate chemoprophylactic regimen. The high rate of side effects associated with mefloquine has been shown in several clinical trials including a randomized, double blind controlled study of 623 travelers. This study found that mefloquine had the highest rate of neuropsychiatric adverse events at 37%, with the highest proportion of the events in women. A retrospective study of 4240 patients taking malaria prophylaxis completed in 2009 also showed that mefloquine had the highest incidence of neuropsychiatric events among antimalarials, and that there were 22 deaths including 5 suicides associated with normal doses of the drug (Jacquerioz & Croft, 2009). There also have been clinical trials showing that mefloquine has a higher rate of discontinuation than either placebo (3.3% for mefloquine overall) or atovaquone and proguanil (5% vs. 3.9% of atovaquone and proguanil) due to GI upset, dizziness, and neuropsychological events (Kain et al., 2001; Hogh et al., 2000). Additionally, Mefloquine has been linked with an increased risk of seizures and cardiac arrhythmias, and a 2008 FDA post marketing review associated pneumonitis and eosinophilic pneumonia with the use of mefloquine (FDA, 2008).

Mefloquine is a safe choice for children and women. It is available in 250mg tablets, with the dose being 1 pill weekly for adults and for children over 45kg. Mefloquine should be taken with food or water. Prophylactic therapy should begin ≥ 2 weeks before travel to endemic areas, and must continue four weeks after leaving the area. The effectiveness of mefloquine against resistant species of plasmodium still makes it a good choice for travelers going to chloroquine resistant regions such as Africa, and the fact that it is safe in children and pregnant women make it a much more versatile drug. However, the neuropsychiatric adverse events and other side effects should be taken into account when choosing it as a prophylactic medication.

4.4 Doxycycline

Doxycycline is a broad-spectrum antibiotic derived from oxytetracycline that acts on the 30S ribosome subunit thus disrupting protein synthesis, and has activity against not only bacteria, but several parasitic diseases as well. It has a short half-life, necessitating daily

dosing and can be used as a malarial chemoprophylaxis in areas with known chloroquine and mefloquine resistance (Kain et al., 2001). It does not have activity against the exoerythorcytic stages of malaria making it a less efficacious choice for areas of the world with endemic *P. vivax* and *P. ovale.*

Several studies have shown the efficacy of prophylactic doxycycline in the prevention of malaria. In a double-blind, placebo controlled study doxycycline had 99% efficacy in the prevention of malaria in soldiers in Irian Jaya, Indonesia (Ohrt et al., 1997). In a separate trial investigating the prophylactic efficacy of azithromycin vs. doxycycline in 213 adult volunteers in a malaria endemic area it was found that daily doxycycline had an efficacy of 92.6% vs. 82.7% for daily azithromycin (Andersen et al., 1998). In addition, doxycycline was well tolerated in both of these studies.

While generally well tolerated, the most common side effects of doxycycline are primarily gastrointestinal including nausea, vomiting, diarrhea, glossitis, dysphagia, and rare instances of esophagitis and esophageal ulceration (Castelli et al., 2010). Doxycycline is a photosensitizing agent, and thus care must be taken to prevent over exposure to the sun while on this medication. Doxycycline also increases the risk for vaginal candidiasis by inhibiting the growth of natural vaginal flora. In a randomized, double-blind, placebo controlled trial of 623 non-immune travelers requiring short-term malaria chemoprophylaxis, doxycycline demonstrated a comparable side-effect profile to atovaquone/proquanal (Schlagenhauf et al., 2003). Importantly however, this study used doxycycline monohydrate, the more expensive form of the drug that has a favorable gastrointestinal side-effect profile. The findings of this study should not be extrapolated to the use of doxycycline hyclate or the non-enteric coated forms of doxycycline, where tolerability would likely be less favorable and might affect adherence to this chemoprophylactic drug.

The dosing for adults is 100mg daily. It is contraindicated in children under 8 years of age due to the risk of permanent discoloration of the teeth, and in pregnant or breast-feeding women due to the risk of toxicity to the fetus/infant (CDC, 2012). The recommended dosing for children ≥ 8 years of age is 2.2 mg/kg up to 100mg/day (CDC, 2012). Treatment should start one to two days before and last for 28 days after leaving an endemic area. Doxycycline should be taken with copious water to reduce the risk of esophagitis and may be taken with food to reduce GI side effects, although caution should be taken to avoid concomitant administration of antacids, magnesium salts, or bismuth subsalicylate as these may decrease intestinal absorption. The traveler should remain upright for at least 30 minutes following administration to reduce the risk of esophageal irritation or ulceration.

4.5 Atovaquone and proguanil hydrochloride

Atovaquone and proguanil is a combination antimalarial that works by inhibiting parasite mitochondrial electron transport and parasitic DNA synthesis by inhibiting dihydrofolate reductase. Proguanil significantly enhances the ability of atovaquone to inhibit parasitic mitochondrial electron transport (Kain et al., 2001; McKeage & Scott, 2003; Srivastava & Vaidya, 1999).

Atovaquone/proguanil is a once daily medication that is effective against both the erythrocytic and exoerythorcytic stages of malaria making this an acceptable choice not only for *P. falciprum,* but also *P. vivax and P. ovale,* both of which have hepatic life cycles. The efficacy of atovaquone as a causal prophylaxis was seen in a small volunteer challenge study

in which it was shown that parasites were eliminated prior to the establishment of erythrocytic infection, thus supporting causal efficacy (Shapiro et al., 1999). Atovaquone/proquanal has demonstrated efficacy for malaria prophylaxis in areas with predominantly *P. vivax* (Soto et al., 2006).

Atovaquone/proguanil's suppressive prophylaxis effectiveness has been shown in several clinical trials, three of which were among semi-immune populations in Gabon, Kenya, and Zambia and conducted as double-blinded, randomized, and placebo controlled trials, where overall efficacy of 98% in preventing malaria was observed in this population (Lell et al., 1998; Shanks et al., 1998; Sukwa et al., 1999; Kain et al., 2001). In two other large studies, in which non-immune travelers to malaria endemic regions were randomized to receive atovaquone/proguanil, mefloquine, or chloroquine plus proguanil, none of the patients in the atovaquone/proguanil arms who completed the study developed malaria (Hogh et al., 2000; Overbosch et al., 2001). It was also shown to be 100% effective against *P. falciparum* in non-immune Columbian soldiers (Soto et al., 2006). In a study of non-immune Indonesian immigrants, atovaquone/proguanil was 93% effective at preventing *P. falciparum* and 84% effective at preventing *P.vivax* (Ling et al., 2002).

The causal prophylactic activity of atovaquone/proguanil results in required dosing for only 1-week post exposure vs. up to 4 weeks with antimalarials that only have activity against the erythrocytic stage of the parasite, which may contribute to better adherence by travelers than some other malaria prophylaxis (Kain et al., 2001). In addition, the discontinuation rate due to side effects of the medication was found to be lower in atovaquone/proguanil then either mefloquine or chloroquine/proguanil (1.2%, vs. 5.0% and 0.2% vs. 2.0%, respectively) (Hogh et al., 2000; Overbosch et al., 2001). In a randomized control study, atovaquone/proguanil had a relatively low incidence of reported side effects compared to other available chemoprophylactic agents (Schlagenhauf et al., 2003). The most serious side effects of atovaquone/proguanil, although rare, include Steven-Johnson Syndrome, and a transient elevation of liver enzymes, while the most common were headache, abdominal pain, cough, diarrhea, and myalgias (McKeage & Scott, 2003).

Atovaquone/proguanil tablets are available in two formulations, adult and pediatric with the adult tablet containing 250mg of atovaquone and 100mg of proguanil, and the pediatric tablet containing 62.5 mg and 25mg of atovaquone and proguanil, respectively. Adult dosing is 1 adult tablet daily for 1-2 days before travel into an endemic area, daily throughout the stay, and then for 7 days once out of the endemic area. Dosing in the pediatric population is the same as for adults, except the number of pediatric tablets given daily is weight based. Atovaquone is highly a lipophilic compound in which the rate and extent of absorption is increased when taken with dietary fat, thus atovaquone/proguanil should be taken with food or a milky drink. While the safety of atovaquone/proguanil in the adult and pediatric populations has been shown through clinical trials, the safety during pregnancy is unknown and is contraindicated in this population. In addition, this medication should be avoided in mother's breastfeeding infants under 5kg and should not be used in patients with severe renal impairment (creatinine clearance < 30 ml/min).

While there are some case reports of resistance, in general atovaquone/proguanil is an efficacious, well-tolerated medication that should be considered first line in chloroquine and mefloquine resistant areas. The cost is higher than with other antimalarial chemoprophylactic drugs, which may limit its use in certain circumstances. Once atovaquone/proguanil becomes generic, this drug may be a more affordable option for malaria chemoprophylaxis in the future.

	Atovaquone/	Doxycycline	Primaquine	Mefloquine	Chloroquine
Adult Dose, scheduling	250mg/150mg once daily, 1-2 days before entering endemic region and 7 days after last exposure	100 mg once daily, 1-2 days before entering endemic region and 4 weeks after last exposure	30 mg base once daily, 1-2 days before entering endemic region and 7 days after last exposure	250 mg once weekly, 2 weeks before entering endemic region and 4 weeks after last exposure	500 mg (300mg base) once weekly, begin 1-2 weeks before entering endemic region and continue 4 weeks after last exposure
Pediatric	Yes for children 5 kg, weight based dose (only FDA-approved for children 11kg (see text)	Contraindicated < 8 years of age	Yes, weight based dose	Yes, for children >5kg and > 6 months, see text for children 5 kg or younger than 6 months of age	Yes, 5mg/kg base once weekly up to adult dose
Pregnancy	Insufficient data, not recommended	Contraindicated	Contraindicated	Yes	Yes
Limits on	None	4 months	None	None	None
Advantages	Very well tolerated, convenient, good efficacy	Inexpensive, relatively well tolerated, good efficacy, available worldwide	Inexpensive, relatively well tolerated, effective against all malaria species	Inexpensive, convenient, effective against all malaria species	Inexpensive, relatively well tolerated, convenient
Disadvantages	Expensive, contraindicated for poor renal function	Photosensitivity, increased risk for vaginal yeast infections, inconvenient	G6PD testing required before administration	Growing resistance, poorly tolerated, neurotoxicity, contraindicated with active or recent depression and other psychiatric disorders	Wide spread resistance, only useful in limited areas of the world
Comments	May become more affordable once generic.	May protect against rickettsial infections and leptospirosis as well as malaria	Not US FDA approved for chemoprophylaxis, dosing based on CDC recommendations	Contraindicated in those allergic to quinine/quinidine, cardiac conduction abnormalities	May worsen psoriasis

Table 1. Malaria Prophylaxis Options

5. Special populations

Children and pregnant women are at the highest risk for severe malaria when traveling to endemic areas and increased vigilance should be taken when dealing with these populations. It should be recommend that travel to endemic areas with a risk of transmission be avoided by these populations if possible, however if patients insist, the provider should stress that the traveler or the parents of the traveler insure that both personnel protective measures and chemoprophylaxis are strictly adhered to.

5.1 Pregnancy

Contracting malaria while pregnant puts the mother at an increased risk for adverse outcomes. Malaria infection during pregnancy has been associated with premature labor, abortion and stillbirth. The traveler should be counseled that the diagnosis of malaria in pregnancy may be difficult due to relatively low parasitemia at clinical presentation. A very high degree of suspicion should be taken when a pregnant women presents with fever in an endemic area, as missing the diagnosis could have grave consequences (McGready et al., 2004). Appropriate precautions should be followed including mosquito avoidance and control measures discussed previously as well as chemoprophylaxis when clinically indicated. Reviewing label-specific information and current CDC recommendations should be adhered to. There are no published data indicating elevated risks with the use of DEET in pregnant or lactating women, and current U.S. Environmental Protection Agency and CDC sources do not advise additional precautions for using FDA-approved insect repellants in this population (Koren et al., 2003; Zielinski-Gutierrez et al., 2012). Chemoprophylaxis in pregnancy is limited, as both doxycycline and primaquine are contraindicated, and atovoquone/proguanil is not currently recommended due to lack of safety data from clinical studies. Medications considered safe during pregnancy include chloroquine and mefloquine. While data in the past have only recommended mefloquine in the last half of pregnancy, current recommendations state that there is no evidence of adverse outcome if taken in any of the three trimesters of pregnancy when no other option is available (CDC, 2012).

5.2 Children

Children are at risk of malaria and the associated complications while traveling to endemic areas, and should have the same personal protective measures as adults. DEET has been shown to be safe and effective and is recommended for use by both the CDC and the American Academy of Pediatrics (AAP) for all children over 2 months of age at concentrations between 10-30% based on duration of protection required (Koren et al., 2003; AAP, 2009, 2011; CDC, 2012). Chemoprophylaxis for children is not as restricted as for pregnant women, with contraindications including doxycyline usage in children under 8 and primaquine usage in G6PD deficient patients. Atovaquone/proguanil is FDA approved for children greater than 11kg but recommended for off-label use by the CDC and AAP for children >5 kg. Mefloquine is only FDA approved for children > 5 kg and older than 6 months of age, but when necessary recommend off-label for children < 5kg and any age (AAP, 2009; CDC, 2012). Parents of very young infants should be counseled to avoid areas endemic for malaria given the risk of severe disease in this population. Adherence to personal protective measures and chemoprophylaxis if often poor in children, and thus it must be stressed to the traveling parent the importance of these precautions.

6. Existing challenges and future possibilities

As outlined in this chapter, there are only five available options for malaria chemoprophylaxis in the United States, one of which, primaquine, is not FDA approved for this indication. Emerging resistance, traveler intolerance, non-adherence, side effects and contraindications to existing options warrant development of newer agents for future chemoprophylactic use.

Resistance to existing drugs is well documented. Wide spread chloroquine resistance has led to the use of this drug in only very limited areas of the world, and mefloquine resistance continues to emerge. Doxycycline resistance has not been established and there is no well accepted definition or validated approach to measuring primaquine resistance (Baird, 2009). Primaquine tolerant or refractory strains of *P. vivax* have been well described, notably the Chesson strain from New Guinea (Collins & Jeffery, 1996). As resistance continues to emerge among existing options for the prevention of malaria, the importance of developing new and effective chemoprophylactic drugs for the international traveler becomes critical.

There are very few candidates currently being developed for malaria chemoprophylaxis, and the last FDA approval for this indication, atovoquone/proquanil, was over ten years ago in 2000. Doxycycline and mefloquine received FDA approval for malaria chemoprophylaxis in the preceding 11 years prior to the atovoquone/proquanil approval. The current lack of development of drugs for this indication is unprecedented and is cause for great concern.

Many reasons have been postulated for the lack of candidates in the developmental pipeline for the chemoprophylaxis indication. Obvious barriers are the lack of market incentive for this indication, although it is clear that the number of international non-immune travelers visiting endemic malarious regions has increased substantially in the last few decades, with case fatality rates ranging from 1-3% for falciparum malaria (Chen & Keystone, 2005). One challenge that has been attributed to stalled development in this area is the 5th Amendment to the Declaration of Helsinki (DH2000). First adopted by the World Medical Association (WMA) in 1964, the Declaration of Helsinki was an attempt to formally identify core ethical principles and guidelines for physicians and others involved in the design, execution and oversight of clinical research (Carlson et al., 2004). Principles relating to the use of placebo in clinical trials, post-trial access to investigational drugs, and social benefit as defined in DH2000, have presented challenges to existing models and development strategies for antimalarial chemoprophylactic drugs. Strategies to address these challenges in clinical development have been proposed (Dow et al., 2008).

Azithromycin, piperaquine, and tafenoquine have all been suggested as possible future chemoprophylactic drugs (Dow et al., 2008). Azithromycin has shown some promise, however future study is needed with combination agents to clarify its role for this indication (Chen & Keystone, 2005).

Tafenoquine, an 8-aminquinolone is a synthetic analogue of primaquine. First developed by the Walter Reed Army Institute of Research as WR238605 or etaquine, tafenoquine has a very long elimination half-life of approximately 14 days allowing weekly dosing. This novel compound shows promise for causal chemoprophylaxis against *P. falciparum* and *P. vivax*, as well as radical cure of *P. vivax.* It has been shown in rhesus monkeys to have 10x higher potency than primaquine for causal prophylaxis, and has demonstrated greater activity against blood and liver-stage parasites *in vitro.* (Cooper et al., 1994; Shanks et al., 2001).

In phase I studies of tafenoquine the drug is well tolerated with single doses up to 600mg and with chronic dosing (6 months) at 200mg weekly following a load of 200mg daily for 3 days (Brueckner et al., 1998; Leary et al., 2009) with no dose-limiting adverse events. The most common side effects observed are gastrointestinal, including heartburn, nausea and gas, usually associated with higher (>200mg) dosing. The most significant toxicity associated with tafenoquine is the potential to induce hemolysis in G6PD deficient individuals and methemoglobinemia (Crockett & Kain, 2007). GlaxoSmithKline, in partnership with Medicines for Malaria Venture, are undertaking an ascending-dose safety study of tafenoquine in G6PD heterozygous patients to identify the maximum safe dose in this population (MMV, 2011).

There have been several placebo-controlled trials evaluating tafenoquine as a causal antimalarial chemoprophylactic drug. In a study of non and semi-immune Thai soldiers, tafenoquine was administered monthly at 400mg, following a 1200mg loading dose (given as 400mg/day for 3 days) for a total of 6 months and demonstrated a protective efficacy 96% for *P. vivax* and 100% for multidrug-resistant *P. falciparum* (Walsh et al., 2004).

In a dose ranging prophylactic trial of tafenoquine to prevent *P. falciparum* in Gabon semi-immunes, which included children, doses of 25mg, 50mg, 100mg or 200mg/day for three consecutive days were tested. Tafenoquine was given one week following treatment with halofantrine. Subjects were actively followed for positive blood smears at day 56 and day 77 following tafenoquine dosing. Notwithstanding the 25mg dose, which uniformly failed, tafenoquine demonstrated 100% protective efficacy (PE) at all doses of 50mg or greater at day 56 and had PE of 80%, 93% and 100% at day 77 for doses of 50mg, 100mg and 200mg, respectively (Lell et al., 2000).

In a 13-week prophylactic trial in Kenyan semi-immune adults, tafenoquine was evaluated at weekly doses of 200mg and 400mg followed by a 600mg and 1200mg 3-day load, respectively. A third group consisted of a loading dose (1200mg load), followed by weekly placebo and a fourth group received placebo only throughout the 13-week period. The 200mg and 400mg weekly doses demonstrated PE of 91% and 93%, respectively. The loading dose only group had a PE of 80% (Shanks et al., 2001).

A large randomized, non-placebo controlled trial of non-immune Australian soldiers evaluated tafenoquine, dosed at 200mg weekly for 6 months following a loading dose (200mg/day for 3 days) while deployed to East Timor. Study participants were randomized 3:1 to receive either tafenoquine (n=492) or mefloquine (n=162) throughout their deployment. Although treatment-emergent adverse events were similar between the two groups and tafenoquine was well-tolerated during the study, there were no cases of malaria in any group. Exposure to malaria could not be assessed and therefore efficacy was not established (Nasveld et al., 2010).

In a subset of subjects (n=74) in the East Timor study who received tafenoquine, detailed safety assessments were conducted which detected vortex keratopathy. This finding had no effect on visual acuity and was fully resolved within one year following cessation of therapy (Nasveld et al., 2010). More extensive clinical ophthalmic evaluation of tafenoquine in a subsequent phase 1 safety trial further supports the vortex keratopathy seen with tafenoquine is not clinically significant (Leary et al., 2009).

Tafenoquine shows promise as a causal prophylactic drug and is currently being developed for a radical cure indication for *P. vivax* through a joint collaboration between GlaxoSmithKline and Medicines for Malaria Venture (MMV, 2011).

7. Conclusion

Addressing malaria chemoprophylaxis for the international traveler can be challenging and should be done within the context of a comprehensive medical evaluation well before visiting the endemic region. Malaria is a life-threatening illness and requires a multidimensional, individually tailored approach to ensure the most appropriate measures, including non-drug strategies, are taken to prevent infection if the traveler is exposed. Existing chemoprophylactic drugs offer effective options for the international traveler, however emerging resistance, side effect profiles and contraindications limit use in many circumstances. Future effort is needed to identify and develop new effective and safe options for malaria chemoprophylaxis.

The views expressed in this chapter are those of the authors and do not necessarily reflect the official policy or position of the Department of the Navy, Army or Air Force, the Department of Defense, nor the U.S. Government

8. References

AAP (2009). American Academy of Pediatrics Red Book.

AAP. (2011). "American Academy of Pediatrics." Retrieved July 10, 2011, 2011, from http://www.aap.org/.

Andersen, S. L., A. J. Oloo, et al. (1998). "Successful double-blinded, randomized, placebo-controlled field trial of azithromycin and doxycycline as prophylaxis for malaria in western Kenya." Clin Infect Dis 26(1): 146-150.

Badolo, A., E. Ilboudo-Sanogo, et al. (2004). "Evaluation of the sensitivity of Aedes aegypti and Anopheles gambiae complex mosquitoes to two insect repellents: DEET and KBR 3023." Trop Med Int Health 9(3): 330-334.

Baird, J. K. (2009). "Resistance to Therapies for Infection by *Plasmodium vivax*." Clinical Microbiology Reviews 22(3): 508-534.

Baird, J. K., M. D. Lacy, et al. (2001). "Randomized, parallel placebo-controlled trial of primaquine for malaria prophylaxis in Papua, Indonesia." Clin Infect Dis 33(12): 1990-1997.

Brueckner, R. P., K. C. Lasseter, et al. (1998). "First-Time-In-Humans Safety and Pharmacokinetics of WR 238605, a New Antimalarial." American Journal of Tropical Medicine and Hygiene 58(5): 645-649.

Carlson, R. V., K. M. Boyd, et al. (2004). "The revision of the Declaration of Helsinki: past, present and future." British Journal of Clinical Pharmacology 57(6): 695-713.

Castelli, F., S. Odolini, et al. (2010). "Malaria prophylaxis: A comprehensive review." Pharmaceuticals 3(10): 3212-3239.

CDC (2012). Infectious Disease Related to Travel, Malaria. Atlanta, U.S. Department of Health and Human Services, Public Health Service.

Chen, L. H. and J. S. Keystone (2005). "New Strategies for the Prevention of Malaria in Travelers." Infectious Disease Clinics of North America 19: 185-210.

Chen, L. H., M. E. Wilson, et al. (2006). "Prevention of malaria in long-term travelers." Journal of the American Medical Association 296(18): 2234-2244.

Collins, W. E. and G. M. Jeffery (1996). "Primaquine Resistance in *Plasmodium Vivax*." American Journal of Tropical Medicine and Hygiene 55(3): 243-249.

Cooper, R. D., W. K. Milhous, et al. (1994). "The efficacy of WR238605 against the blood stages of a chloroquine resistant strain of Plasmodium vivax." Trans R Soc Trop Med Hyg 88(6): 691-692.

Crockett, M. and K. C. Kain (2007). "Tafenoquine: a promising new antimalarial agent." Expert Opininion on Investigational Drugs 16(5): 705-715.

Croft, A. M. and P. Garner (2000). "Mefloquine for preventing malaria in non-immune adult travellers." Cochrane Database Syst Rev(2): CD000138.

Croft, A. M. and P. Garner (2008). "WITHDRAWN: Mefloquine for preventing malaria in non-immune adult travellers." Cochrane Database Syst Rev(1): CD000138.

Davis, T. M., D. A. Syed, et al. (2003). "Toxicity related to chloroquine treatment of resistant vivax malaria." Ann Pharmacother 37(4): 526-529.

Dow, G. S., A. J. Magill, et al. (2008). "Clinical development of new prophylactic antimalarial drugs after the 5th Amendment to the Declaration of Helsinki." Ther Clin Risk Manag 4(4): 803-819.

FDA. (2008). "Postmarketing Reviews - Volume 1, Number 4, Summer 2008." Retrieved June 25th, 2011, from http://www.fda.gov/Drugs/DrugSafety/DrugSafetyNewsletter/ucm120613.htm.

Fradin, M. S. and J. F. Day (2002). "Comparative efficacy of insect repellents against mosquito bites." N Engl J Med 347(1): 13-18.

Freedman, D. O. (2008). "Clinical practice. Malaria prevention in short-term travelers." N Engl J Med 359(6): 603-612.

Fryauff, D. J., J. K. Baird, et al. (1995). "Randomised placebo-controlled trial of primaquine for prophylaxis of falciparum and vivax malaria." Lancet 346(8984): 1190-1193.

Govere, J., D. N. Durrheim, et al. (2000). "Efficacy of three insect repellents against the malaria vector Anopheles arabiensis." Med Vet Entomol 14(4): 441-444.

Hill, D. R., J. K. Baird, et al. (2006). "Primaquine: report from CDC expert meeting on malaria chemoprophylaxis I." Am J Trop Med Hyg 75(3): 402-415.

Hogh, B., P. D. Clarke, et al. (2000). "Atovaquone-proguanil versus chloroquine-proguanil for malaria prophylaxis in non-immune travellers: a randomised, double-blind study. Malarone International Study Team." Lancet 356(9245): 1888-1894.

Jacquerioz, F. A. and A. M. Croft (2009). "Drugs for preventing malaria in travellers." Cochrane Database Syst Rev(4): CD006491.

Kain, K. C., G. D. Shanks, et al. (2001). "Malaria chemoprophylaxis in the age of drug resistance. I. Currently recommended drug regimens." Clin Infect Dis 33(2): 226-234.

Keiser, J., B. H. Singer, et al. (2005). "Reducing the burden of malaria in different eco-epidemiological settings with environmental management: a systematic review." Lancet Infect Dis 5(11): 695-708.

Koren, G., D. Matsui, et al. (2003). "DEET-based insect repellents: safety implications for children and pregnant and lactating women." CMAJ 169(3): 209-212.

Korhonen, C., K. Peterson, et al. (2007). "Self-reported adverse events associated with antimalarial chemoprophylaxis in peace corps volunteers." Am J Prev Med 33(3): 194-199.

Leary, K. J., M. A. Riel, et al. (2009). "A Randomized, Double-Blind, Safety and Tolerability Study to Assess the Ophthalmic and Renal Effects of Tafenoquine 200mg Weekly

versus Placebo for 6 Months in Healthy Volunteers." American Journal of Tropical Medicine and Hygiene 81(2): 356-362.

Leder, K., J. Black, et al. (2004). "Malaria in Travelers: A Review of the GeoSentinel Surveillance Network." Clin Infect Dis 39: 1104-1112.

Lell, B., J. F. Faucher, et al. (2000). "Malaria chemoprophylaxis with tafenoquine: a randomised study." Lancet 355: 2041-2045.

Lell, B., D. Luckner, et al. (1998). "Randomised placebo-controlled study of atovaquone plus proguanil for malaria prophylaxis in children." Lancet 351(9104): 709-713.

Ling, J., J. K. Baird, et al. (2002). "Randomized, placebo-controlled trial of atovaquone/proguanil for the prevention of Plasmodium falciparum or Plasmodium vivax malaria among migrants to Papua, Indonesia." Clin Infect Dis 35(7): 825-833.

McGready, R., E. A. Ashley, et al. (2004). "Malaria and the pregnant traveller." Travel Med Infect Dis 2(3-4): 127-142.

McKeage, K. and L. Scott (2003). "Atovaquone/proguanil: a review of its use for the prophylaxis of Plasmodium falciparum malaria." Drugs 63(6): 597-623.

MMV. (2011). "MMV Interactive Science Portfolio: Tafenoquine." Retrieved July 5th, 2011, 2011, from www.mmv.org/research-development/science-portfolio.

Nasveld, P. E., M. D. Edstein, et al. (2010). "Randomized, Double-Blind Study of the Safety, Tolerability, and Efficacy of Tafenoquine versus Mefloquine for Malaria Prophylaxis in Nonimmune Subjects." Antimicrob Agents Chemother 54(2): 792-798.

Ohrt, C., T. L. Richie, et al. (1997). "Mefloquine compared with doxycycline for the prophylaxis of malaria in Indonesian soldiers. A randomized, double-blind, placebo-controlled trial." Ann Intern Med 126(12): 963-972.

Overbosch, D., H. Schilthuis, et al. (2001). "Atovaquone-proguanil versus mefloquine for malaria prophylaxis in nonimmune travelers: results from a randomized, double-blind study." Clin Infect Dis 33(7): 1015-1021.

Phillips-Howard, P. A., B. L. Nahlen, et al. (2003). "The efficacy of permethrin-treated bed nets on child mortality and morbidity in western Kenya I. Development of infrastructure and description of study site." Am J Trop Med Hyg 68(4 Suppl): 3-9.

Schlagenhauf, P., A. Tschopp, et al. (2003). "Tolerability of malaria chemoprophylaxis in non-immune travellers to sub-Saharan Africa: multicentre, randomised, double blind, four arm study." BMJ 327(7423): 1078.

Schwartz, E. and G. Regev-Yochay (1999). "Primaquine as prophylaxis for malaria for nonimmune travelers: A comparison with mefloquine and doxycycline." Clin Infect Dis 29(6): 1502-1506.

Shanks, G. D., D. M. Gordon, et al. (1998). "Efficacy and safety of atovaquone/proguanil as suppressive prophylaxis for Plasmodium falciparum malaria." Clin Infect Dis 27(3): 494-499.

Shanks, G. D., K. C. Kain, et al. (2001). "Malaria chemoprophylaxis in the age of drug resistance. II. Drugs that may be available in the future." Clin Infect Dis 33(3): 381-385.

Shanks, G. D., A. J. Oloo, et al. (2001). "A New Primaquine Analogue, Tafenoquine (WR 238605), for Prophylaxis against *Plasmodium falciparum* Malaria." Clin Infect Dis 33: 1968-1974.

Shapiro, T. A., C. D. Ranasinha, et al. (1999). "Prophylactic activity of atovaquone against Plasmodium falciparum in humans." Am J Trop Med Hyg 60(5): 831-836.

Soto, J., F. Medina, et al. (1995). "Efficacy of permethrin-impregnated uniforms in the prevention of malaria and leishmaniasis in Colombian soldiers." Clin Infect Dis 21(3): 599-602.

Soto, J., J. Toledo, et al. (2006). "Randomized, double-blind, placebo-controlled study of Malarone for malaria prophylaxis in non-immune Colombian soldiers." Am J Trop Med Hyg 75(3): 430-433.

Soto, J., J. Toledo, et al. (1998). "Primaquine prophylaxis against malaria in nonimmune Colombian soldiers: efficacy and toxicity. A randomized, double-blind, placebo-controlled trial." Ann Intern Med 129(3): 241-244.

Soto, J., J. Toledo, et al. (1999). "Double-blind, randomized, placebo-controlled assessment of chloroquine/primaquine prophylaxis for malaria in nonimmune Colombian soldiers." Clin Infect Dis 29(1): 199-201.

Srivastava, I. K. and A. B. Vaidya (1999). "A mechanism for the synergistic antimalarial action of atovaquone and proguanil." Antimicrob Agents Chemother 43(6): 1334-1339.

Sukwa, T. Y., M. Mulenga, et al. (1999). "A randomized, double-blind, placebo-controlled field trial to determine the efficacy and safety of Malarone (atovaquone/proguanil) for the prophylaxis of malaria in Zambia." Am J Trop Med Hyg 60(4): 521-525.

Trigg, J. K. (1996). "Evaluation of a eucalyptus-based repellent against Anopheles spp. in Tanzania." J Am Mosq Control Assoc 12(2 Pt 1): 243-246.

Walsh, D. S., C. Eamsila, et al. (2004). "Efficacy of Monthly Tafenoquine for Prophylaxis of *Plasmodium vivax* and Multidrug-Resistant *P. falciparum* Malaria." The Journal of Infectious Diseases 190: 1456-1463.

WHO (2010). Guidelines for the treatment of malaria. Geneva, Switzerland, World Health Organization.

WHO. (2011). "World Health Organization Website." Retrieved July 13th, 2011, from http://www.who.int/malaria/en/.

Zielinski-Gutierrez, E., R. A. Wirtz, et al. (2012). Protection against Mosquitoes, Ticks, & Other Insects & Arthropods (Chapter 2, The Pre-Travel Consultation). The Yellow Book: CDC Health Information for International Travel 2012.

11

Effects of Irrigated Rice Fields and Seasonality on *Plasmodium* Transmission in West Africa, Particularly in Central Côte d'Ivoire

Benjamin G. Koudou[1,2,3], Marcel Tanner[4,5] and Juerg Utzinger[4,5]
[1]*Vector Group, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool*
[2]*Centre Suisse de Recherches Scientifiques, Abidjan,*
[3]*University of Abobo-Adjame´, Abidjan,*
[4]*Department of Epidemiology and Public Health,*
Swiss Tropical and Public Health Institute, Basel,
[5]*University of Basel, Basel,*
[1]*UK*
[2,3]*Côte d'Ivoire*
[4,5]*Switzerland*

1. Introduction

The transmission of the parasites that cause human malaria is influenced by myriad environmental factors, including changes in agricultural practices, deforestation, and water-resources development and management (Ijumba and Lindsay, 2001; Ijumba et al., 2002; Keiser et al., 2005; Guerra et al., 2006), climatic factors such as rainfall, humidity and temperature (Reiter, 2008), and various cultural, economic, political and social factors, including health-seeking behaviour, urbanization, armed conflict and war (Esse et al., 2008; Baragatti et al., 2009). Drug resistance in the causative parasites and insecticide resistance in the mosquito vectors are also important factors that now influence malaria transmission (Reiter, 2008). Each year, in many parts of Africa, the local populations of anopheline mosquitoes build up rapidly and peak shortly after the onset of the rainy season (Mbogo et al., 1995). In two studies on the relationships between mosquito abundance, malaria transmission and rainfall in West Africa, 70%–90% of the children investigated were found infected with *Plasmodium spp*. after the rainy season (Bonnet et al., 2002; Koudou et al., 2009). It is particularly during and at the end of the rainy season that malaria becomes one of the leading causes of mortality and health-seeking at dispensaries and hospitals in this region (Rey et al., 1987). Not only season but also changing patterns of agriculture, particularly irrigated rice farming, influence malaria transmission in Africa (Ijumba & Lindsay, 2001; Diuk-Wasser et al., 2007; Sogoba et al., 2007).

Additionally, malaria transmission, *Plasmodium* prevalence rates, the proportion of presumptive and clinically-confirmed malaria episodes have been studied in two villages of central Côte d'Ivoire: one with irrigated rice farming (Zatta) and one without (Tiémélékro) (Koudou et al., 2009). Due to a farmers' conflict over land and socio-political issues, irrigated rice farming was interrupted in Zatta in 2003. The goal of this contribution to a book chapter is to evaluate the relationship between *Plasmodium* transmission, seasonality and agriculture practices.

2. Methods

2.1 Study sites

The study described here was carried out in the villages of Tiemelekro (geographical coordinates: 6˚500 N, -4˚170 W) and Zatta (6˚880 N, -5˚390 W), located in central Côte d'Ivoire (Figure 1). A detailed description of Tiemelekro, including climatic conditions, current health care delivery structures and key demographic and socioeconomic indicators, has been presented recently (Girardin et al., 2004). Zatta is located 7 km north-west of Yamoussoukro, the capital city of Côte d'Ivoire. The mean annual temperature in this village is 26.5˚C and the mean annual precipitation is 1280 mm. There is a long rainy season between April and July and a shorter one in October/November. A dispensary, run by two local nurses, is located in Zatta and also covers nearby settlements. Two small dams were constructed in this village in the mid-1970s. Since 1997, a very large irrigated rice field has been cultivated on an estimated surface area of 36 ha, in close proximity to human habitations. However, due to unstable socio-political conditions and a farmers' conflict over land, rice irrigation was interrupted in 2000 and again in 2003/2004.

Living conditions and several of the investigated household characteristics are comparable between the two study villages. For example, similar proportions of houses utilized iron-corrugated sheets as roofing material (93.8% in Tiemelekro vs. 92.9% in Zatta), and had running water at home (74.1% vs. 65.4%). On the other hand, improved sanitation facilities were less prominent in Tiemelekro than in Zatta (17.0% vs. 47.6%). With regard to personal protective measures against mosquito bites, the proportion of people sleeping under a bednet was similarly low in both villages (8.4–11.2%), whereas use of fumigating coils was much more pronounced in Zatta (47.3%) when compared to Tiemelekro (9.1%).

2.1.1 Rainfall data collection

The Ivorian "Societe d'Exploitation et de Developpement Aeroportuaire et Meteorologique" (SODEXAM) holds rainfall data for the study area, from 1971 onwards. For the present study, monthly rainfall data from 2002 to 2005 were extracted from the society's records.

2.1.2 Adult mosquitos' collection

Overall, 13 entomological surveys were carried out: seven in the long rainy seasons (in the April and June of 2002, the April, May, June and July of 2003, and the May of 2005), and six in dry seasons (in the February and August of 2002, 2003 and 2005). Adult mosquitoes were collected by means of human-bait night catches. The surveys in 2002 and 2005 were each conducted between 18.00 and 06.00 hours, both inside and outside sentinel houses. Each of the six surveys carried out in 2003, however, covered shorter time periods (from 22:00 to 06:00 hours) and the collectors were only stationed inside the sentinel house because of the unstable socio–political situation at the time. Overall, 96, 48 and 32 night catches were carried out in 2002, 2003 and 2005, respectively. No surveys could be undertaken in 2004, as it was then considered too dangerous to reach the study villages.

2.1.3 Laboratory procedures

Adult mosquitoes were brought to a laboratory and processed. Firstly, the physiological age of each female *Anopheles* and the corresponding parity rate (i.e. the proportion of female mosquitoes that had laid eggs at least once) were determined by dissection of ovaries and

examination of tracheoles (Detinova, 1962). For quality control, a random sample of 10% of the mosquitoes investigated were re-examined by a senior technician. Secondly, a proportion of *An. gambiae* female having laid eggs at least once was checked for *P. falciparum* infection, in an ELISA for detecting the parasite's circumsporozoite protein (Beier et al.,

Fig. 1. Vegetation mapping of Côte-d'Ivoire presenting both study sites (Zatta and Tiemelekro) located in the central part of the country.

1988). Thirdly, some of the females belonging to the *An. gambiae* complex were identified to species level, in a PCR-based assay (Scott et al., 1993). Finally, some of the mosquitoes belonging to the *An. funestus* group were further identified using an assay based on a multiplex PCR (Koekemoer et al., 2002; Cohuet et al., 2003).

2.1.4 Clinical and Parasitological surveys

Repeated cross-sectional surveys were carried out in the study villages to assess malaria parasitaemia and clinical malaria in children aged ≤15 years. The first survey was done in June 2002. In 2003, two surveys were carried out in Zatta and three in Tiémélékro. The research team first worked in the primary schools and all children aged between 7 and 15 years from randomly selected classes were invited for a finger prick blood sample. Next, mothers and caregivers of under 7-year-old children were invited to accompany their children to a designated community location where a blood sample was taken from each child.

Thick and thin blood films were prepared on microscope slides. The slides were air-dried prior to transfer to a nearby laboratory where they were stained with Giemsa for 45 min. The slides were examined by the same experienced laboratory technician throughout the study under a microscope at high magnification. *Plasmodium* species and gametocytes were identified and counted against 200 leucocytes. When less than 10 parasites were found, reading was continued for a total of 500 leucocytes. Parasitaemia was expressed by the number of parasites per µl of blood, assuming for a standard count of 8000 leucocytes/µl blood. For quality control, 10% of the slides were randomly selected and re-examined by a second senior technician.

In our study, fever was defined when an individual had an axillary temperature >37.5 ◦C. Clinical malariawas defined as fever plus parasitaemia (Smith et al., 1994). Particular emphasis was placed on clinical cases with a parasitaemia >5000 parasites/_l blood. The latter threshold has been chosen after comparing the proportions of fever cases and asymptomatic carriers for different classes of parasite density (Gaye et al., 1989). Subjects with malaria-related symptoms (e.g. headache) plus axillary temperature >37.5 ◦C were given artesunate plus amodiaquine (the respective first-line antimalarial treatment at the time of the study) and paracetamole.

2.1.5 Ethical issues

The study protocol was approved by the institutional research commission of the Centre Suisse de Recherches Scientifiques (Abidjan, Côte d'Ivoire). Ethical clearance was obtained from the Ivorian Ministry of Public Health and National Malaria Control Programme. People who acted as bait and collectors in the mosquito collections were all volunteers and signed informed consent forms. During the study, Patients with malaria-related symptoms who presented at the dispensaries and mosquitoes' collectors were treated and protected for free against malaria by artesunate–amodiaquine chemoprophylaxis (artesunate–amodiaquine being the recommended, first-line treatment for malaria in Côte d'Ivoire at the time of the present study) and all mosquitoes' collectors were immunized against yellow fever. The heads of household in both study sites were informed and the parents or legal guardians of participating children signed a written informed consent sheet.

3. Results

3.1 Species composition of *An. gambiae* complex and *An. funestus* group

A total of 110 mosquitoes were identified to species level by PCR: 60 from Tiémélékro and 50 from Zatta. Within *Anopheles* spp. morphologically identified as *An. gambiae* complex, 100% were *An. gambiae s. s.* With regard to the *An. funestus* group, it consisted of 100% *An. funestus s. s.*

3.2 Effects of agricultural practices (irrigated rice fields & vegetable farming) on *Plasmodium* transmission

Comparison between years revealed that the biting rate of *An. gambiae s.l.* in Zatta decreased several-fold from 49.3 bites per person per night (b/p/n) in 2002 to 7.9 b/p/n in 2003 (likelihood ratio test (LRT=1072.66; P<0.001). In Tiemelekro, the biting rates recorded in 2002 and 2003 remained fairly constant. These observations were paralleled by a marked decrease in the infective rate of *An. gambiae s.l.* in Zatta (4.6–1.2%), and an increase in Tiemelekro (3.1–7.6%). Meanwhile, the entomological inoculation rate (EIR) of *An. gambiae s.l.* decreased 21-fold in Zatta, from 789 to 38 infective bites per person per year (ib/p/y), whereas it remained high in Tiemelekro (233 vs. 342 ib/p/y). In Zatta, the return to irrigated rice farming in January 2005 was paralleled by a significant increase of the EIR ranging from 38 infective bites per person per year (ib/p/y) in 2003 to 295 ib/p/y in 2005. In Tiémélékro high EIRs were found in 2003 (342 ib/p/y) and 2005 (572 ib/p/y).

3.3 Effects of agricultural practices (irrigated rice fields & vegetable farming) on *Plasmodium* prevalence and clinical malaria cases

3.3.1 Irrigated rice fields and *Plasmodium* prevalence

In both villages, the peak prevalence of *P. falciparum* was generally observed in children aged 3-6 years. There were three exceptions: in Tiémélékro, the peak prevalence of *P. falciparum* during the May 2005 survey was found in the youngest age group (≤ 2 years), whereas in Zatta, the highest prevalence in the baseline survey (June 2002) and the second last survey (May 2005) was observed in children aged 7-15 years.

In June 2002, similarly high *P. falciparum* prevalence rates were observed in Zatta (85.4%) and Tiémélékro (86.1%). In Zatta, a significant decrease in the mean *P. falciparum* prevalence rate occurred from 2002 to 2003 (58.4%; $\chi^2 = 42.33$, degree of freedom (df) = 1; $P < 0.001$). There was a significant increase from 2003 to 2005 (66.0%; $\chi^2 = 14.78$, df = 1, $P = 0.012$). In Tiémélékro, the *P. falciparum* prevalence rate in June 2003 (78.2%) was significantly lower than during the June 2002 survey ($\chi^2 = 4.92$, df = 1; $P = 0.027$). The annual *P. falciparum* prevalence rate decreased significantly from 2003 (70.7%) to 2005 (60.4%; $\chi^2 = 17.27$, df = 1; $P < 0.001$).

3.3.2 Fever cases and asymptomatic carriers, stratified by parasite density

Table 1 shows how many of the children examined with parasitaemia in the 2003 surveys were either asymptomatic carriers or presented with a fever. There was a strong seasonal variation in the proportion of fever cases among individuals with parasitaemia. In Zatta, for example, the proportion of fever cases among *Plasmodium*-positive individuals was significantly higher towards the end of the rainy season (August) when compared to the dry season (March) (22.1% **versus** 9.9%; $\chi^2 = 9.90$, df = 1; $P = 0.002$). In Tiémélékro, considerably higher frequencies of fever cases among *Plasmodium*-positive individuals were recorded during the peak rainy

season in June (27.3%) and towards the end of the rainy season in August (25.5%) when compared to the dry season in March (15.9%; $P < 0.05$ for both comparisons).
In Zatta, all individuals with a high level of parasitaemia (≥ 5000 parasites/µl blood) presented with fever, accounting for a highly significant difference between the proportion of asymptomatic carriers and fever cases in this parasitaemia class ($P < 0.001$). Similarly, there was a highly significant association between the fever cases and high parasitaemia in the three surveys carried out in 2003 in Tiémélékro ($P < 0.05$). No statistically significant difference was found in children with lower parasitaemias (1000-5000 parasites/µl of blood), neither in Zatta (March: $\chi^2 = 1.53$; df = 1; $P = 0.068$ and August: $\chi^2 = 0.116$; df = 1; $P = 0.733$) nor in Tiémélékro (March: $\chi^2 = 0.18$; df = 1; $P = 0.671$, June: $\chi^2 = 2.23$; df = 1; $P = 0.135$ and August: $\chi^2 = 0.001$; df = 1; $P = 0.973$).

3.3.3 Annual variation of presumptive cases and malaria transmission

In Zatta, 966, 812, 693 and 884 presumptive cases were recorded in 2002, 2003, 2004 and 2005, respectively. The annual number of presumptive malaria cases decreased significantly by 15.1% and 14.7%, respectively, from 2002 to 2003 (IRR = 0.841, $P < 0.001$) and from 2003 to 2004 (IRR = 0.853, $P = 0.002$). An opposite trend was observed from 2004 to 2005; the number of presumptive malaria cases increased significantly by 27.5% (IRR = 1.276, $P < 0.001$). The monthly number of presumptive cases was not related to the monthly number of infective bites per person (IRR = 0.994, $P = 0.827$).

Date of survey	*P. falciparum* parasitaemia (parasites/µl blood)	Tiémélékro		Zatta	
		No. (%) of asymptomatic carriers	No. (%) of children with fever	No. (%) of asymptomatic carriers	No. (%) of children with fever
March 2003	< 1000	69 (54.8%)	13 (10.3%)	135 (73.4%)	9 (4.2%)
	1000-5000	37 (29.4%)	6 (4.8%)	57 (26.8%)	9 (4.2%)
	≥ 5000	0 (0)	1 (0.8%)	0 (0)	3 (1.4%)
	Total	106 (84.1%)	20 (15.9%)	192 (90.1%)	21 (9.9%)
June 2003[a]	< 1000	86 (52.1%)	34 (20.6%)	n.a.	n.a.
	1000-5000	32 (19.4%)	7 (4.2%)	n.a.	n.a.
	≥ 5000	2 (1.2%)	4 (2.4%)	n.a.	n.a.
	Total	120 (72.7%)	45 (27.3%)	n.a.	n.a.
August 2003	< 1000	99 (50.5%)	22 (11.2%)	78 (57.4%)	12 (8.8%)
	1000-5000	46 (23.5%)	16 (8.2%)	28 (20.6%)	7 (5.1%)
	≥ 5000	1 (0.5%)	12 (6.1%)	0 (0)	11 (8.1%)
	Total	146 (74.5%)	50 (25.5%)	106 (77.9%)	30 (22.1%)
Overall 2003 (number of positive children/ number of total children)		54.0% (372/689)	16.7% (115/689)	49.8% (298/598)	8.5% (51/598)

n.a.: not assessed
[a] No survey carried out in June 2003 in Zatta due to unstable sociopolitical situation

Table 1. Number (%) of children infected with *P. falciparum* who were asymptomatic carriers or presented with fever, stratified by different levels of parasitaemia, in the two study villages of Tiémélékro and Zatta, central Côte d'Ivoire.

In Tiémélékro, the yearly numbers of presumptive malaria cases were 2089, 1858, 1655 and 1541. Thus, we observed significant decreases in the yearly number of presumptive cases by 11.1% from 2002 to 2003 (IRR = 0.889, $P < 0.001$), 9.0% from 2003 to 2004 (IRR = 0.910, $P = 0.005$) and 8.9% from 2004 to 2005 (IRR = 0.911, $P = 0.008$).
As in the case of Zatta, the monthly number of presumptive cases was not related to the monthly number of infective bites per person (IRR = 1.007; $P = 0.776$).

3.4 Effects of seasonality on *Plasmodium* Transmission

Tables 2 and 3 summarise the mean biting rate, infection rate and the entomological inoculation rate (EIR) of *An. gambiae* and *An. funestus* in the two study villages in 2002, 2003 and 2005.

3.4.1 Relationship between season and biting and infection rates

In Zatta, significantly higher *An. gambiae s. s.* biting rates were recorded in the dry seasons of 2002 and 2005 when irrigated rice farming was practiced, compared to the dry season of 2003 when irrigated rice farming was interrupted (LRT comparing 2002 with 2003: 13.79, LRT comparing 2005 with 2003: 20.50; both $P < 0.001$). In 2003, there was no seasonal difference in the biting rate of *An. gambiae s. s.* (LRT = 0.13; $P = 0.900$) and *An. funestus s. s.* (LRT = 0.17, $P = 0.879$). In Tiémélékro, in 2002 (LRT = 1.84; $P = 0.069$) and 2005 (LRT = 0.56; $P = 0.455$), there were no significant differences in *An. gambiae s. s.* biting rates between the dry and the rainy season. In 2003, the biting rate was significantly higher in the long rainy season (LRT = 3.87, $P < 0.001$). Regarding *An. funestus s. s.* biting rates, those recorded in the dry season of 2002 (LRT = 6.15) and 2003 (LRT = 4.50) were significantly higher than those recorded in the rainy season (both $P < 0.001$). The difference in the biting rates between the dry and rainy season in 2005 also showed statistical significance (LRT = 3.26; $P = 0.031$).

3.4.2 Relationship between season and *Plasmodium* transmission

In both villages, higher EIRs of *An. gambiae s. s.* were usually recorded in the rainy season. For example, in Zatta, the EIR of *An. gambiae s. s.* recorded in the rainy seasons of 2002 and 2005 were 458 and 365 infective bites per person per season (ib/p/s), respectively. In 2003, when irrigated rice farming was interrupted in Zatta, *P. falciparum* transmission by *An. gambiae s. s.* and *An. funestus s. s.* only occurred during the rainy season. In Tiémélékro, in the rainy seasons of 2003 and 2005, the number of infective bites recorded for *An. gambiae s. s.* (357 and 208 ib/p/s, respectively) were 3-14 times higher than in the dry seasons (25 and 77 ib/p/s in the respective years). In 2002, in contrast, the EIR of *An. gambiae s. s.* recorded in the dry season was 2.5 times higher than the one recorded in the rainy season.
The highest EIRs of *An. funestus s. s.* were usually noted during the dry season. In Tiémélékro, this species was the primary *P. falciparum* transmitter during the dry season of 2005 when 207 ib/p/s were recorded. With regard to infection rates, with the exception of the 2005 infection rate of *An. funestus s. s.* recorded in Tiémélékro ($\chi^2 = 4.47$, $P = 0.035$), no significant differences were observed between seasons, neither for *An. gambiae s. s.* nor for *An. funestus s. s.* in any village.

Malaria vector	Entomolo-gical parameter	Dry season		Rainy season		χ^2 or LRT	P value
		Mean (n)	95% CI	Mean (n)	95% CI		
An. gambiae							
2002[a]	Biting rate	38.7	36.3-41.1	59.9	56.9-68.2	3.79	<0.001
	Infection rate	4.8 (1,120)	3.6-6.1	4.2 (978)	3.0-5.5	0.33	0.564
	Parity rate	40.4 (673)	36.7-44.1	31.1 (1,157)	28.4-33.8	16.28	<0.001
	Total EIR	338	-	458	-		
2003[b]	Biting rate	7.1	5.4-9.3	8.3	5.0-11.6	0.13	0.900
	Infection rate	0.0 (65)	0.0-0.2	1.7 (176)	0.0-3.6	1.12	0.290
	Parity rate	61.9 (63)	49.8-74.2	36.7 (139)	28.6-44.8	11.16	<0.001
	Total EIR	0	-	26	-		
2005[c]	Biting rate	18.3	11.8-24.7	58.6	23.8-93.4	20.50	<0.001
	Infection rate	2.3 (127)	0.2-5.8	3.4 (136)	1.7-5.2	0.37	0.542
	Parity rate	52.9 (194)	45.2-60.6	46.2 (199)	40.6-52.0	1.71	0.191
	Total EIR	77		365			
An. funestus							
2002[a]	Biting rate	0.0	0.0-0.2	0.0	0.0	0	0
	Infection rate	0.0 (0)	0.0	0.0 (0)	0.0	0	0
	Parity rate	0.0 (0)	0.0	0.0 (0)	0.0	0	0
	Total EIR	0		0			
2003[b]	Biting rate	1.4	0.7-2.4	1.2	0.6-2.1	0.17	0.879
	Infection rate	0.0 (18)	0.0-0.2	2.3 (44)	0.0-6.8	0.42	0.519
	Parity rate	0.0 (9)	0.0-0.2	58.3 (12)	25.6-91.1	7.88	0.005
	Total EIR	0	-	5	-		
2005[c]	Biting rate	2.7	1.1-4.4	0.6	0.0-1.5	0.61	0.435
	Infection rate	8.3 (8)	0.0-28.4	0.0 (3)	0.0	0.87	0.824
	Parity rate	70.9	24.3-84.3	60.0	0.0-100.0	0.58	0.216
	Total EIR	41		0			0

In brackets are the number of malaria vectors analyzed; LRT (likelihood ratio test)
[a]Irrigated rice farming performed in a synchronized manner
[b]Interruption of rice cultivation
[c]Irrigated rice farming performed in a synchronized manner

Table 2. Monthly average biting rate, infection rate, parity rate and entomological inoculation rate (EIR) of *An. gambiae* and *An. funestus* during the dry season and the rainy season in 2002, 2003 and 2005 in Zatta, central Côte d'Ivoire

Malaria vector	Entomological parameter	Dry season		Rainy season		χ^2 or LRT	P value
		Mean (n)	95% CI	Mean (n)	95% CI		
An. gambiae							
2002*	Biting rate	19.6	18.1-21.1	12.6	11.2-14.1	1.84	0.069
	Infection rate	4.1 (268)	1.7-6.5	2.6 (531)	1.3-4.0	1.27	0.260
	Parity rate	72.5 (240)	66.8-78.2	52.4 (597)	48.4-56.4	28.34	<0.001
	Total EIR	146	-	60	-		
2003**	Biting rate	5.2	3.8-7.0	24.7	21.6-28.3	3.87	<0.001
	Infection rate	2.6 (35)	0.0-8.7	7.9 (467)	5.4-10.4	1.19	0.274
	Parity rate	59.0 (39)	42.8-75.1	58.6 (449)	54.0-63.1	0.002	0.961
	Total EIR	25	-	357	-		
2005**	Biting rate	3.9	2.1-5.7	16.7	9.1-24.4	10.56	<0.001
	Infection rate	10.9 (52)	3.7-17.3	6.8 (163)	2.6-10.6	1.47	0.226
	Parity rate	48.3 (103)	33.3-63.4	81.1 (201)	75.5-86.7	30.19	<0.001
	Total EIR	77	-	208	-		
An. funestus							
2002	Biting rate	5.0	4.1-5.9	0.7	0.4-1.1	6.15	<0.001
	Infection rate	3.1 (97)	0.0-6.6	7.7 (26)	0.0-18.7	1.11	0.292
	Parity rate	65.4 (185)	58.5-72.3	50.0 (22)	27.3-72.7	2.02	0.155
	Total EIR	28	-	9	-		
2003	Biting rate	8.4	6.6-10.5	4.0	2.9-5.4	4.50	<0.001
	Infection rate	3.6 (55)	0.0-8.7	9.1 (66)	1.9-16.2	1.45	0.229
	Parity rate	75.0 (60)	63.7-86.3	69.0 (42)	54.4-83.6	0.44	0.507
	Total EIR	55	-	67	-		
2005	Biting rate	29.9	20.9-38.9	1.0	0.6-1.9	3.26	0.031
	Infection rate	3.8 (131)	1.2-5.6	17.6 (11)	0.0-37.8	4.47	0.035
	Parity rate	65.1 (203)	50.6-73.2	91.7 (12)	73.3-100.0	3.61	0.057
	Total EIR	207	-	32			

In brackets are the number of malaria vectors analyzed; LRT (likelihood ratio test)
*Vegetable farming is performed intensively with 2 production cycles per year
**Vegetable farming is performed intensively with 1 production cycle per year

Table 3. Monthly average biting rate, infection rate, parity rate and entomological inoculation rate (EIR) of *An. gambiae* and *An. funestus* during the dry season and the rainy season in 2002, 2003 and 2005 in Tiémélékro, central Côte d'Ivoire

4. Discussion

The interruption of irrigated rice farming due to a farmers' dispute over land property rights, coupled with an unstable socio-political situation in the face of the 2002-2004 armed conflict (Betsi et al., 2006; Fürst et al., 2009) offered a unique opportunity to study the dynamics of malaria transmission. Our analyses complement previous publications

(Girardin et al., 2004; Koudou et al., 2005, 2007, 2009), now with an explicit focus on the effect of seasonality on malaria transmission under changing agro-ecological conditions. The following points are offered for discussion.

Firstly, biting rates of *An. gambiae* in both villages were usually significantly higher in the rainy season than in the dry season. When irrigated rice farming was interrupted in Zatta in 2003, much lower biting rates were observed than in the preceding year and in 2005, but there were no seasonal differences. Hence, the interruption of irrigated rice farming appeared to have hidden the effect of season on *An. gambiae* biting rate. These findings are in agreement with previous investigations in the humid savannah of Côte d'Ivoire: in an area characterised by intensive agriculture, the biting rate of *An. gambiae* increased significantly a few weeks after the beginning of the rainy season, whereas it decreased and became lowest towards the end of the dry season (Doannio et al., 2006). Moreover, the blunting of seasonal differences in biting rates due to changing patterns in irrigated rice farming has been documented previously for the savannahs of Senegal (Faye et al., 1993) and Mali (Dolo et al., 2004). In contrast to *An. gambiae* with the highest biting rates usually observed in the rainy season, the highest biting rates of *An. funestus* were consistently recorded in the dry season regardless of the prevailing agricultural activity. Moreover, interruption of irrigated rice farming in Zatta showed no effect.

Secondly, with the only exception of a significantly higher infection rate of *An. funesuts* in Tiémélékro in the rainy season compared to the dry season of 2005, infection rates of both *An. gambiae* and *An. funestus* showed no clear seasonal patterns. Different results were reported from Dielmo, a holoendemic area in Senegal, where the infection rate of malaria vectors showed considerable seasonal variation (Fontenille et al., 1997). The observations made in Senegal corroborated previous findings obtained in the savannah area in the north of Côte d'Ivoire (Dossou-Yovo et al., 1995), and other findings documenting a high infection rate of *An. funestus* at the beginning of the dry season in an irrigated rice area compared to a non-irrigated rice farming area (Dossou-Yovo, 2000). It should be noted, however that the mean annual infection rate of *An. gambiae* in Zatta was significantly higher when irrigated rice farming was in place (in 2002 and 2005) compared to a year with interrupted irrigated rice farming (Koudou et al., 2005).

Thirdly, the influence of changing patterns of irrigated rice farming on the *An. gambiae*-specific EIR in Zatta has been discussed elsewhere (Koudou et al., 2005, 2007). In brief, interruption of irrigated rice farming resulted in several-fold lower EIRs compared to normal years. Here, we now document that seasonal patterns of transmission remained. Indeed, considerably higher EIRs were observed for *An. gambiae* in the rainy season compared to the dry season. Of note, the EIR of *An. gambia* in the dry season of 2003 in Zatta dropped to zero. In Tiémélékro, high EIRs were recorded throughout the study period for *An. gambiae* and, in general, EIRs were higher in the rainy season compared to the dry season. *An. funestus* seemed to play an important role in the transmission of malaria, particularly in the dry season. Our results therefore confirm previous observations made elsewhere in the northern savannah of Côte d'Ivoire (Dossou-Yovo, 2000) and in southern Cameroon (Bonnet et al., 2002). Whilst *An. gambiae* was the key *P. falciparum* transmitter mainly during the rainy season, *An. funestus* was the main vector species during the dry season. It is interesting to note that a previous study focusing on climatic models for suitable malaria transmission in Africa, based on monthly rainfall and temperature data, concluded that an average of 80 mm rainfall per month, for at least 3-5 months, is a minimum to ascertain stable malaria transmission (Craig et al., 1999). Usually, a rapid rise in the

An. gambiae population at the beginning of the short rainy season was followed by an increase in the EIR (Bonnet *et al*., 2002).
With regard to *An. funestus*, the highest EIRs were usually observed during the dry season. Indeed, *An. funestus* is often abundant and has high EIR during dry season compared to the rainy season (Fontenille et al., 1997; Manga et al., 1997). *An. funestus* was identified as the main malaria vector in the Guinean climatic region, in East Africa and Madagascar (Robert et al., 1985; Severini et al., 1990). As shown in our study, despite the presence of irrigated rice field, there is a great variability in the annual EIR values and seasonality would seem to play a key role (Mabaso et al., 2007).
Finally, an important finding of our study is that in Zatta, where irrigated rice farming was interrupted in 2003/2004, *Plasmodium* prevalence rates and the number of presumptive malaria cases decreased. This observation is corroborated by a significant decrease in the EIR from 2002 to 2003 (Koudou et al., 2005) and a significant increase from 2003 to 2005 (Koudou et al., 2007). This study demonstrated also that irrigated rice cultivation is associated with elevated malaria prevalence rates, as well as high numbers of presumptive malaria cases, as seen in Burundi (Coosemans, 1985), Kenya (Githeko et al., 1993) and Madagascar (Marrama et al., 1995). However, research carried out in Tanzania showed that irrigated rice farming was not associated with a higher risk of malaria. One important reason for this observation is that farmers engaged in irrigated rice have the opportunity to gain some extra money, part of which is spent for protective measures against malaria. A reduced risk of malaria despite enhanced rice production has been termed 'paddies paradox' (Ijumba & Lindsay, 2001).

5. Conclusion

In conclusion, analyses of our entomological data revealed that malaria transmission in two different agro-ecological settings of central Côte d'Ivoire is very high, but there are clear seasonal patterns. Whilst the interruption of irrigated rice farming in one of the two study villages resulted in a highly significant reduction in the EIR, seasonal patterns of transmission remained. Hence, even in intensive agriculture areas, the effect of season on malaria transmission must be taken into consideration for the design of integrated interventions and their monitoring.
Additionally, in Zatta, from 2002 to 2003, the highly significant reduction in the annual EIR was paralleled by a significant reduction in the *Plasmodium* prevalence rate, and the proportions of presumptive and clinically-confirmed malaria cases. Once irrigated rice farming was resumed, there was an increase in entomological and parasitological parameters of malaria. In Tiémélékro, despite the significant increase in the EIR from the year 2002 to 2005 (Koudou et al., 2005, 2007), malaria prevalence rates, and the presumptive and clinical malaria cases decreased. Hence, the reduction of malaria transmission in endemic areas does not necessary reduce the incidence of clinical malaria episodes (Charlwood et al., 1998), highlighting the complex relationship between these parameters.

6. Acknowledgements

Our thanks are addressed to the local authorities and villagers of Tiémélékro and Zatta, and the district health officers of Dimbokro and Yamoussoukro, for their commitment in the present study. This investigation received financial support from the 'Fonds Ivoiro Suisse de

Développement Economique et Sociale' (FISDES) and the Swiss Federal Commission for Fellowships for Foreign Students (CFBEE). While the field works were ongoing Profs Benjamin Koudou and Marcel Tanner were partially supported by the National Centre of Competence in Research (NCCR) North-South, integrated programme #4 (IP4) entitled "Health and Well-being". JU acknowledges financial support from the Swiss National Science Foundation (project no. PPOOB--102883 and PPOOB--119129).

7. References

Baragatti, M., Fournet, F., Henry, M. C., Assi, S., Ouedraogo, H., Rogier, C. & Salem, G. (2009). Social and environmental malaria risk factors in urban areas of Ouagadougou, Burkina Faso. *Malaria Journal*, 8, 13.

Betsi, N. A., Koudou, B. G., Cissé, G., Tschannen, A. B., Pignol, A. M., Ouattara, Y., Madougou, Z., Tanner, M. & Utzinger, J. (2006). Effect of an armed conflict on human resources and health systems in Côte d'Ivoire: prevention of and care for people with HIV/AIDS. *AIDS Care*, 18, 356-365.

Beier, M. S., Schwartz, I. K., Beier, J. C., Perkins, P. V., Onyango, F., Koros J. K., Campbell, G. H., Andrysiak, P.M. & Brandling-Bennet, A. D. (1988). Identification of malaria species by ELISA in sporozoite and oocyst infected *Anopheles* from western Kenya. *American Journal of Tropical Medicine and Hygiene*, 39, 323-327.

Bonnet, S., Paul, R. E. I., Gouagna, C, Safeukui, I., Meunier, J. Y., Gounoue, R. & Boudin, C. (2002). Level and dynamics of malaria transmission and morbidity in an equatorial area of South Cameroon. *Tropical Medicine and International Health*, 7, 249-256.

Charlwood, J.D., Smith, T., Lyimo, A., Kitua, Y., Masanja, H., Booth, M., Alonso, P.L. & Tanner, M. (1998). Incidence of Plasmodium falciparum infection in infants in relation to exposure to sporozoïte-infected Anophelines. Am. J. Trop. Med. Hyg. 59, 243-251.

Cohuet, A., Simard, F., Toto, F., J. C., Kengne, P., Coetzee, M. & Fontenille D. (2003). Species identification within the *Anopheles funestus* group of malaria vectors in Cameroon and evidence for a new species. *American Journal of Tropical Medicine and Hygiene*, 69, 200-205.

Coosemans, M.H. (1985). Comparison of malarial endemicity in a rice-growing area and a cotton-growing area of the Rusizi Plain, Burundi. Ann. Soc. Belge Med. Trop. 65, 187-200.

Craig, M. H., Snow, R. W. & le Sueur, D. (1999). African climatic model of malaria transmission based on monthly rainfall and temperature. *Parasitology Today*, 15, 105-111.

Detinova, T. S. (1962). Age-grouping methods in Diptera of medical importance, with special reference to some vectors of malaria. World Health Organization, Geneva, 220p.

Diuk-Wasser, M. A., Touré, M. B., Dolo, G., Bagayoko, M., Sogoba, N., Sissoko, I., Traoré, S. F. & Taylor, C. E. (2007). Effect of rice cultivation patterns on malaria vector abundance in rice-growing villages in Mali. *American Journal of Tropical Medicine and Hygiene*, 76, 869-874.

Doannio, J. M. C., Dossou-Yovo, J., Diarrassouba, S., Rakotondraibe, M. E., Chauvancy, G. & Rivière, F. (2006). Comparaison de la composition spécifique et de la dynamique des populations de moustiques dans deux villages du centre de la Côte d'Ivoire,

avec et sans périmetre de riziculture irriguée. *Bulletin de la Société de Pathologie Exotique*, 99, 1-3.

Dolo, G., Briët, O. J. T., Dao, A., Traoré, S. F., Bouaré, M., Sogoba, N., Niaré, O., Bagayogo, M., Sangaré, D., Teuscher, T. & Touré, Y. T. (2004). Malaria transmission in relation to rice cultivation in the irrigated Sahel of Mali. *Acta Tropica*, 89, 147-159.

Dossou-Yovo, J. (2000). Etude éthologique des moustiques vecteurs du paludisme en rapport avec les aspects parasitologiques de la transmission du *Plasmodium* dans la région de Bouaké. Thèse de doctorat d'Etat en entomologie médicale. Université de Cocody, Abidjan, Côte d'Ivoire.

Dossou-Yovo, J., Doannio, J., Rivière, F. & Chauvancy, G. (1995). Malaria in Côte d'Ivoire wet Savannah region: the entomological inputs. *Tropical Medicine and Parasitology*, 46, 263-269.

Essé, C., Utzinger, J., Tschannen, A. B., Raso, G, Pfeiffer, C., Granado, S., Koudou, B. G., N'Goran, E. K., Cissé, G., Girardin, O, Tanner, M. & Obrist, B. (2008). Social and cultural aspects of 'malaria' and its control in central Côte d'Ivoire. *Malaria Journal*, 7, 224.

Faye, O., Fontenille, D., Hervé, J. P., Diack, P. A., Diallo, S. & Mouchet, J. (1993). Malaria in the sahelian region of Senegal. 1. Entomological transmission findings. *Annales de la Société Belge de Médecine Tropicale*, 73, 21-30.

Fontenille, D., Lochouarn, L., Diagne, N., Sockhna, C., Lemasson, J. J., Diatta, M., Konaté, L., Faye, F., Rogier, C. & Trape, J. F. (1997). High annual and seasonal variations in malaria transmission by anophelines and vector species composition in Dielmo, a holoendemic area in Senegal. *American Journal of Tropical Medicine and Hygiene*, 56, 247-253.

Fürst, T., Raso, G., Acka, C. A., Tschannen, A. B., N'Goran, E. K. & Utzinger, J. (2009). Dynamics of socioeconomic risk factors for neglected tropical diseases and malaria in an armed conflict. *PLoS Neglected Tropical Diseases*, 3, e513.

Gaye, O., Bah, L., Faye, F. & Baudon, C. (1989). Une étude de la morbidité palustre en milieu rural et urbain au Sénégal. *Med. Trop.* 49, 59-62.

Girardin, O., Dao, D., Koudou, B. G., Essé, C., Cissé, G., Yao, T., N'Goran, E. K., Tschannen, A. B., Bordmann, G., Lehmann, B., Nsabiman, C., Keiser J., Killeen, G. F., Singer, B. H, Tanner, M. & Utzinger, J. (2004). Opportunities and limiting factors of intensive vegetable farming in malaria endemic Côte d'Ivoire. *Acta Tropica* 89, 109-123.

Githeko, A.K., Service, M.W., MBogo, C.M., Atieli, F.K. & Jumao, F.O. (1993). Plasmodium falciparum sporozoite and entomological inoculation rates at Ahero rice irrigation scheme and the Miwani sugar-belt in Western Kenya. Ann. Trop. Med. Parasitol. 87, 379-391.

Guerra, C. A., Snow, R. W. & Hay, S. I. (2006). A global assessment of closed forests, deforestation and malaria risk. *Annals of Tropical Medicine and Parasitology*, 100, 189-204.

Ijumba, J. N. & Lindsay, S. W. (2001). Impact of irrigation on malaria in Africa: paddies paradox. *Medical and Veterinary Entomology*, 15, 1-11.

Ijumba, J. N., Mosha, F. W. & Lindsay, S. W. (2002). Malaria transmission risk variations derived from different agricultural practices in an irrigated area of northern Tanzania. *Medical and Veterinary Entomology*, 16, 28-38.

Keiser, J., Castro, M. C., Maltese, M. F., Bos, R., Tanner, M., Singer, B. H. & Utzinger, J. (2005). Effect of irrigation and large dams on the burden of malaria on a global and regional scale. *American Journal of Tropical Medicine and Hygiene*, 72, 392-406.

Koekemoer, L. L., Kamau, L, Hunt, R. H. & Coetzee, M. (2002). A cocktail polymerase chain reaction assay to identify members of the *Anopheles funestus* (Diptera: Culicidae) group. *American Journal of Tropical Medicine and Hygiene*, 66, 804-811.

Koudou, B. G., Adja, A. M., Matthys, B., Cissé, G, Koné, M., Tanner, M. & Utzinger, J. (2007). Pratiques agricoles et dynamique de la transmission du paludisme dans deux différentes zones eco-épidemiologiques, au centre de la Côte d'Ivoire. *Bulletin de la Société de Pathologie Exotiques*, 100, 124-126.

Koudou, B. G., Tano, Y., Doumbia, M., Nsanzabana, C., Cissé, G., Girardin, O., Dao, D., N'Goran, E. K., Vounatsou, P., Bordmann, G., Keiser, J., Tanner, M. & Utzinger, J. (2005). Malaria transmission dynamics in central Côte d'Ivoire: the influence of changing patterns of irrigated rice agriculture. *Medical and Veterinary Entomology* 19, 27-37.

Koudou, B. G., Tano, Y., Keiser, J., Vounatsou, P., Girardin, O., Klero, K., Koné, M., N'Goran, E. K., Cissé, G., Tanner, M. & Utzinger, J. (2009). Effect of agricultural activities on prevalence rates, and clinical and presumptive malaria episodes in central Côte d'Ivoire. *Acta Tropica*, 111, 268-274.

Mabaso, M. L., Craig, M., Ross, A., Smith, T. (2007). Environmental predictors of the seasonality of malaria transmission in Africa: the challenge. *American Journal of Tropical Medicine and Hygiene*, 76, 33-38.

Manga, L., Toto, J. C., Le Goff, G. & Brunhes, J. (1997). The bionomics of *Anopheles funestus* and its role in malaria transmission in a forest area of southern Cameroon. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 91, 232-233.

Marrama, L., Rajaonarivelo, E., Laventure, S. & Rabison, P. (1995). Anopheles funestus et la riziculture sur les plateaux de Madagascar. *Cahiers Santé*, 5, 415-419.

Mbogo, C. N. M., Snow, R. W., Khamala, C. P. M, Kabiru, E. W., Ouma, J. H., Githure, J. L., Marsh, K. & Beier, J. C. (1995). Relationships between *Plasmodium falciparum* transmission by vector populations and the incidence of severe disease at nine sites on the Kenyan coast. *American Journal of Tropical Medicine and Hygiene*, 52, 201-206.

Reiter, P. (2008). Climate change and mosquito-borne disease: knowing the horse before hitching the cart. *Revue Scientifique et Technique*, 27, 383-398.

Robert, V., Gazin, P., Boudin, C., Molez, J. F., Ouédraogo, V. & Carnevale, P. (1985). La transmission du paludisme en zone de savane arborée et en zone rizicole des environs de Bobo-Dioulasso, Burkina Faso. *Annales de la Société Belge de Médecine Tropicale*, 65 (Suppl. 2), 201-214.

Scott, J. A., Brogdon, W. G. & Collins, F. H. (1993). Identification of single specimens of the *Anopheles gambiae* complex by the polymerase chain reaction. *American Journal of Tropical Medicine and Hygiene*, 49, 520-529.

Severini, C., Fontenille, D. & Ramiakajato, M. R. (1990). Importance d'*An. funestus* dans la transmission du paludisme au hameau de Mahitsy, à Tananarive, Madagascar. *Bulletin de la Société Pathologie Exotique*, 83, 114-116.

Smith, T., Armstrong-Schellenberg, J. & Hayes, R. (1994). Attributable fraction estimates and case definitions for malaria in endemic areas. *Stat. Med*. 13, 2345-2358.

Sogoba, N., Doumbia, S., Vounatsou, P., Bagayoko, M. M., Dolo, G., Traoré, S. F., Maïga, H. M., Touré, Y. T. & Smith, T. (2007). Malaria transmission dynamics in Niono, Mali: the effect of the irrigation systems. *Acta Tropica*, 101, 232-240.

12

Toxoplasmosis: Advances and Vaccine Perspectives

Oscar Bruna-Romero[1], Dulcilene Mayrink de Oliveira[1]
and Valter Ferreira de Andrade-Neto[2,*]
[1]Department of Microbiology, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais – UFMG, Belo Horizonte-MG
[2]Department of Microbiology and Parasitology, Centro de Biociências, Universidade Federal do Rio Grande do Norte – UFRN, Natal-RN
Brazil

1. Introduction

Toxoplasma gondii was first identified more than 100 years ago in the tissues of birds and mammals. In 1908 Nicolle and Manceoux described it for the first time in the gundi (*Ctenodactylus gundi*), a North African rodent, in tachyzoite forms. At the same time, Splendore in Brazil, identified the parasite in rabbit tissues. Due to its bow-like shape (Greek: *Toxo* = Arc) the genus was named *Toxoplasma*. However, only in the 1970's was the complete life cycle known and the parasite recognized as a coccidian parasite (member of the phylum Apicomplexa). It is ubiquitous throughout the world and estimated to infect approximately half of the world's population. It is characterized by a polarized cell structure and two unique apical secretory organelles called micronemes and rhoptries.

Toxoplasma has a complex life cycle consisting of a sexual cycle in its feline definitive hosts and an asexual cycle in its intermediate hosts. The latter, including humans, can be infected by ingestion of oocysts shed in cat feces. Unlike most other Apicomplexan parasites, *Toxoplasma* can be transmitted between intermediate hosts by either vertical (via placenta) or horizontal (carnivorism) transmission.

Toxoplasma parasite is found in intermediate hosts in two interconvertable stages: bradyzoites and tachyzoites. Bradyzoites, a dormant form, are slow-growing, transmissible and encysted. Infections with bradyzoite-containing cysts occur upon ingestion of undercooked meat. The wall of these cysts is digested inside the host stomach and the released bradyzoites, which are resistant to gastric peptidases, subsequently invade the small intestine. There, they convert into tachyzoites, the rapidly growing, disease-causing form that can infect most nucleated cells, replicate inside a parasitophorous vacuole, egress, and then infect neighboring cells. These tachyzoites activate a potent host immune response that eliminates most of the parasites. Some tachyzoites, however, escape destruction and convert back into bradyzoites. In the absence of an adequate immune response, tachyzoites

* Corresponding Author

will grow unabated and cause tissue destruction, which can be severe and even fatal. However, the inflammatory immune response induced by tachyzoites can cause immune-mediated tissue destruction. Therefore, a subtle balance between inducing and evading the immune response is crucial for *Toxoplasma* to establish a chronic infection.

The success of *Toxoplasma* as a widespread pathogen is due to the ease in which it can be transmitted between intermediate hosts. Humans do not play a major role in transmission; consequently, pathogenesis in humans is the indirect result of adaptations to infection in other hosts and treatment of human infections is unlikely to lead to the spread of drug resistance.

Once inside a host, the parasite develops powerful tools to modulate its host cell and develop into a chronic infection that can evade the host's immune system as well as all known anti-toxoplasmatic drugs. The ability of the parasite to replicate within a host cell, evade immune responses and undergo bradyzoite development requires the parasite to effectively modulate its host.

Toxoplasmosis remains a major health concern in pregnancy, where it causes severe birth defects or miscarriage, and in immunocompromised hosts. Thus, new toxoplasmosis control strategies are needed. The development of effective human and veterinary vaccines against toxoplasmosis is a relevant goal for Public Health (Gazzinelli *et al*. 1996; Pifer and Yarovinsky 2011). Even if new therapeutic drugs, with less hypersensitivity and toxicity-related events, are developed, not only for acute *T. gondii* infection but also for the currently untreatable latent bradyzoite form of the parasite, a prophylactic vaccine against the disease would still be the best option from the financial, epidemiological, and social points of view. A vaccine would decrease the enormous costs of diagnosis/treatment, the premature loss of lives, the extensive rates of dissemination as well as the social impact of the disease. One major fact that suggests the possibility of vaccination against toxoplasmosis is that primary infection with the *T. gondii* parasite elicits protective immunity against re-infection in most individuals.

2. Mechanisms of protective immunity against toxoplasmosis

Immune responses during the early stages of *T. gondii* infection are characterized by activation of innate mechanisms mediated by macrophages and dendritic cells (DC) (Gazzinelli *et al*. 1996; Pifer and Yarovinsky 2011). These cells are activated in mice (not yet known how in humans) after parasite internalization, by engagement of endosomal toll-like receptor 11 (and probably others) with tachyzoite products, which drives subsequent production of interleukin-12 (IL-12) and tumor necrosis factor alpha (TNF-α). In turn, IL-12 activates natural killer (NK) cells (Denkers *et al*. 1993) to secrete gamma interferon (IFN-γ) (Gazzinelli *et al*. 1994), which then acts as stimulus for T-cell activation and, in synergy with TNF-α, mediates killing of tachyzoites by macrophages through enhanced production of free oxygen radicals and nitric oxide (NO).

Acquired immunity against *T. gondii* develops afterward, and is characterized by strong $CD4^+$ and $CD8^+$ T cell activity (Gazzinelli *et al*. 1992). The cytokine IFN-γ continues to be central in resistance to the parasite during the successive acute and chronic stages of infection, driving the differentiation of $CD4^+$ T lymphocytes specific for parasite antigens to a helper T cell type (Th1) cytokine profile. More important, the newly generated $CD8^+$ T

cells become crucial to control parasite replication, not only by serving as additional sources of IFN-γ but also by developing cytotoxic activity against infected cells, eliminating parasite factories and thus preventing reactivation of infection (Denkers *et al*. 1993; Denkers and Gazzinelli 1998; Bhopale 2003). Whether B cells also play a role in protection against this parasite is not clear, but studies have generated indirect evidences that IgG antibodies may be important for protection (Kang *et al*. 2000). B cell-deficient mice have shown increased susceptibility to brain inflammatory pathology in chronic infections with the parasite, despite presenting similar levels of serum and tissue pro-inflammatory cytokines, such as IFN-γ. Furthermore, adoptive transfer of polyclonal anti- *T. gondii* IgG antibodies to these mice prevented both pathology and mortality.

3. Major toxoplasma vaccines and candidates studied to date

To reproduce what the immune system does naturally to protect hosts against *T. gondii* infection (and re-infection), researchers have attempted several strategies for vaccination. These include the use of whole parasites (attenuated in different ways), soluble parasite antigens, recombinant purified proteins (subunit vaccines) or recombinant live vectors that express heterologous antigen(s) within host organisms (figure 1). Currently, some of these tools are also being used in combination, as part of prime-boost immunization protocols. Below is a review of current's state of the art of most of these technologies.

3.1 Whole-parasite attenuated vaccines

Sporulated oocysts (sporozoite-containing cysts) from the environment or tissue cysts (bradyzoite-containing cysts) from infected animals are the two major sources of infection with *T. gondii* (figure 2). However, vaccine candidates that include sporozoites or sporozoite antigens have traditionally been less studied because of the ease of access to bradyzoites and tachyzoites, e.g. using animal brain cysts or acutely infected animal peritoneal lavage/cell cultures, respectively. As a result, the first *T. gondii* whole-parasite experimental vaccines were mainly based on attenuated tachyzoites/bradyzoites, in particular those generated by inactivation or irradiation. Inactive parasites were used for immunization of experimental animals from 1956 (Cutchins and Warren 1956) to 1972 (Krahenbuhl *et al*. 1972) with not much success. In contrast, gamma-irradiated *T. gondii* tachyzoites were successfully tested as experimental vaccines in 1975 (Seah and Hucal 1975), in part after taking the idea from the pioneering irradiated-sporozoite malaria vaccines, which were initially tested in the 1960s and 70s (Nussenzweig *et al*. 1967; Gwadz *et al*. 1979). In the 1975 report, all animals inoculated with highly irradiated *T. gondii* parasites survived, were free of tissue cysts and were solidly protected against a subsequent rechallenge. Later, a few reports (Dubey *et al*. 1996; Omata *et al*. 1996; Dubey *et al*. 1998) have also used irradiated sporozoites (under the form of sporulated oocysts) to vaccinate mice, cats and pigs against toxoplasmosis, but in contrast to tachyzoites, results were not very encouraging, though some protection was also observed.

Other attempts to induce protection against toxoplasmosis with whole-parasite vaccines included the use of live attenuated parasites (tachyzoites) such as the S-48, the cps1-1, the temperature-sensitive TS-4, the MIC1-3 knock-out or the non-replicative Δrps13 strains (McLeod *et al*. 1988; Hakim *et al*. 1991; Buxton 1993; Gigley *et al*. 2009; Lu *et al*. 2009; Hutson

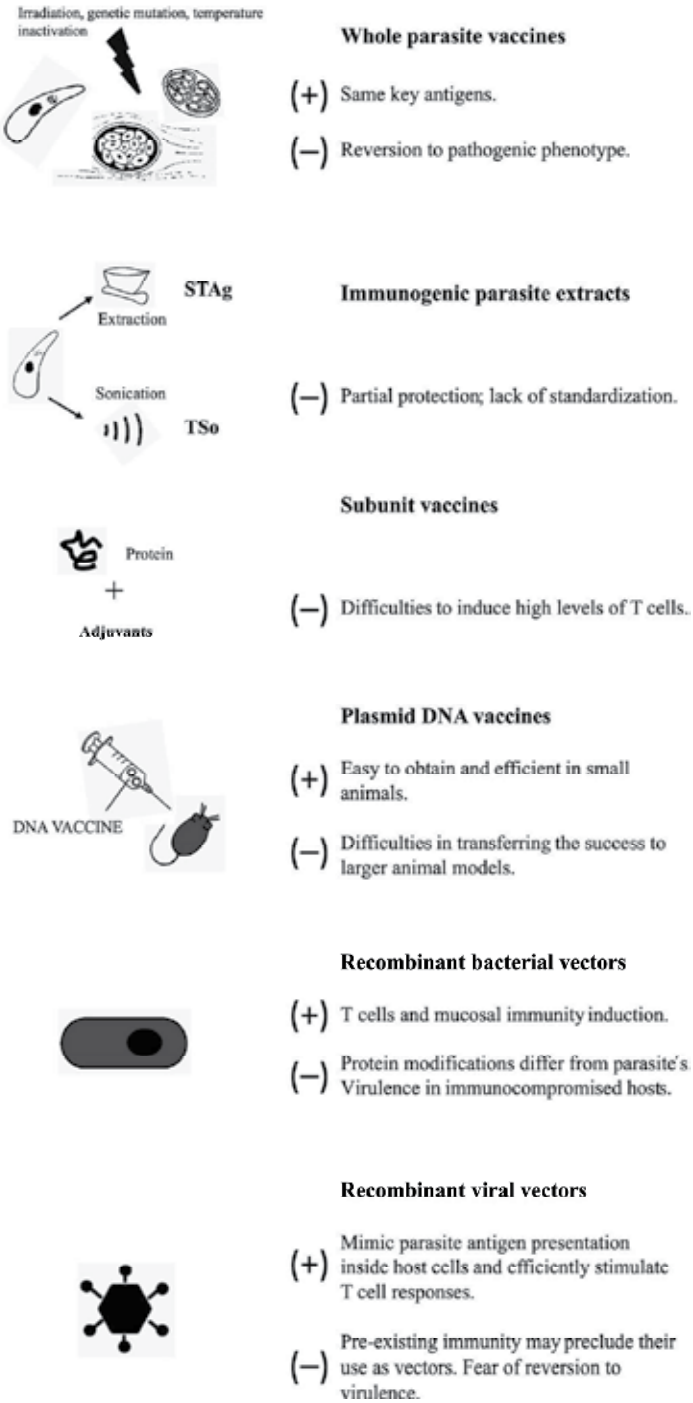


Fig. 1. Potential advantages (+) and concerns (-) of the major vaccination strategies used to immunize hosts against *T. gondii* infection. Abbreviations: STAg, Soluble Tachyzoite Antigen; TSo, Tachyzoites Sonicate.

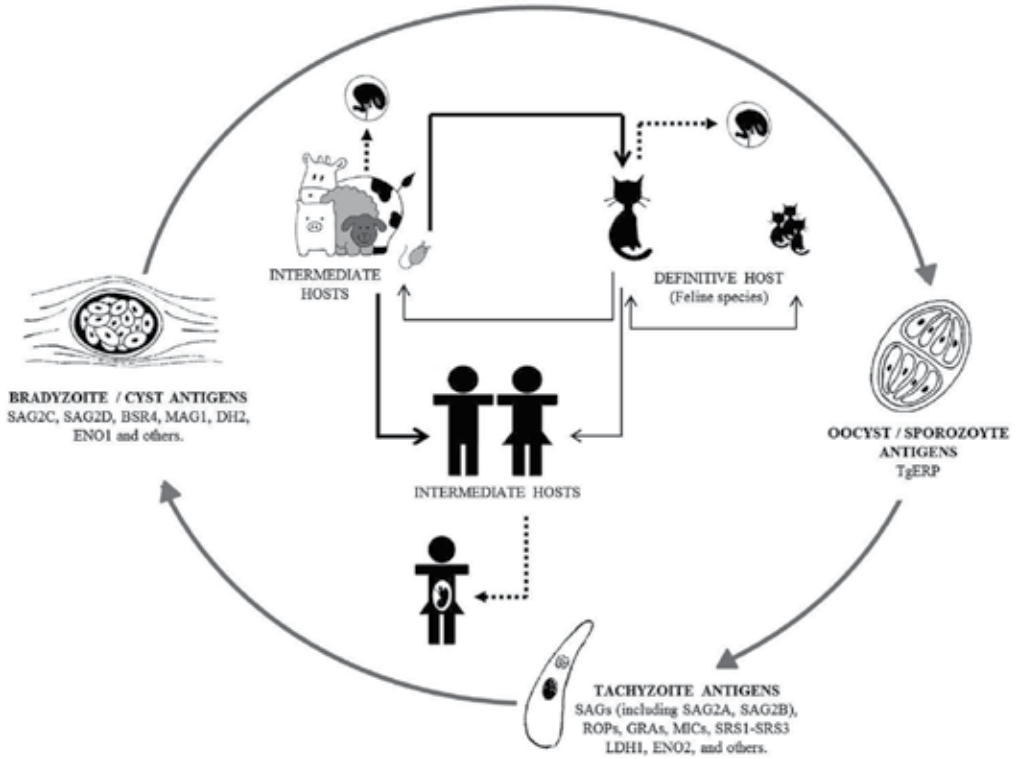


Fig. 2. Major *T. gondii* antigens identified to date in the different stages of the parasite´s life cycle and major routes of parasite transmission. Thin black arrow = horizontal transmission via oocysts; thick black arrow = horizontal transmission via tissue cysts; dotted arrows = vertical transmission via tachyzoites. Abbreviations: SAG, surface antigen; ROP, rhoptry protein; GRA, dense granules; MIC, microneme protein; SRS, SAG-related sequences; BSR, bradyzoite-specific recombinant; MAG, matrix antigen; LDH, lactate dehydrogenase; ENO, enolase; TgERP0, *T. gondii* embryogenesis-related protein.

et al. 2010; Mevelec *et al*. 2010). The only vaccine commercialized for veterinary purposes, Ovilis®Toxovax (Intervet/Schering-Plough Animal Health, UK), based on the incomplete parasite S-48 strain (not able to generate either tissue cysts or oocysts), began to be marketed in New Zealand and the United Kingdom in 1988 to control miscarriages provoked by *T. gondii* in sheep. Reduction in fetal loss and in formation of cysts in the meat used for consumption has been reported. This vaccine seems to reduce infection in sheep, which in free-range grazing are constantly exposed to oocyst contamination.

Interestingly, up to date, the most recent and technologically advanced recombinant vaccine formulations have reached, at best, the same levels of protective immunity induced by whole-parasite vaccines. Three main reasons may be responsible for that difference: (i) true protective antigens (or more plausibly antigen combinations) of the parasite have not yet been identified, (ii) while the whole organism and the recombinant vaccines contain the same antigenic sequences, the process by which the recombinant products are generated result in the loss of crucial features that are key for protein's immunogenicity (Crampton and Vanniasinkam 2007) or, finally, (iii) gamma-irradiated or otherwise attenuated parasites

maintain metabolic functions, retain the capacity to invade mammalian cells, present antigens to the host's immune system and elicit cellular immunity and cytokine responses in a highly similar way to natural infection (Hiramoto *et al*. 2002), and exogenous recombinant antigens do not.

However, even though protection has been repeatedly demonstrated after immunization with whole-parasite vaccines, real concerns also exist regarding the use of this type of vaccines, in particular for uses other than veterinary immunization. The major fear is that attenuated parasites could revert to the pathogenic phenotype. For this reason, studies towards developing a human vaccine have focused on parasite extracts or recombinant technologies that use defined immunodominant antigens and delivery strategies.

3.2 Immunogenic parasite extracts

Identification and functional characterization of proteins of the tachyzoite stage of *T. gondii* has been the focus of extensive research, because antigens within this stage are presented to the immune system effectively during natural infection, forcing the parasite to enter (in less than two weeks) into the latent bradyzoite stage seeking for protection. This strategy results in physical parasite shielding by encystation and different, and much lower, antigen availability for the immune system.

The soluble tachyzoite antigen extract (STAg) was the first protein blend identified as source of protective products, before wide-scale proteomic analyses were available (Denkers *et al*. 1993; Yap *et al*. 1998). Protection with STAg is only partial, even when the very efficient CpG oligodeoxynucleotides are used as adjuvants (Yin *et al*. 2007). Similar partial protection was also induced by the *T. gondii* sonicate of tachyzoites (TSo), even when mixed with cholera toxin (CT, a mucosal adjuvant) for oral administration (Bourguin *et al*. 1991; Bourguin *et al*. 1993).

One of the reasons why immunogenic parasite extracts render non-protective immunity may be the diversification of immune responses amongst all the different antigens (immunodominant or not) present in those extracts. Additionally, the extraction process (in the case of STAg) may have eliminated some of the innate immunity activators, namely TLR agonists, present in the whole parasite. Current proteomic analyses (high-throughput 2-dimensional electrophoresis combined with mass spectrometry) have identified nine novel vaccine candidates within STAg (Ma *et al*. 2009) and we should see some of these promising antigens being tested *in vivo* as recombinant subunit or vectorised vaccines in the near future.

3.3 Individual antigens identified and used as subunit vaccines

Three major particularities characterize the difficulties found in the development of recombinant vaccines against toxoplasmosis; these are: (i) *T. gondii* is an unicellular protozoan parasite formed by thousands of different proteins, glycoproteins, lipoproteins, and other types of molecules that can become feasible candidate antigens for a vaccine, (ii) the parasite evolves into several different stages during its life cycle, with some of those stages (cysts) particularly protected against the action of the immune system and, in addition, the antigens of one stage may not be effective vaccines for subsequent stages, and (iii) numerous strains of the parasite coexist in nature, most of them with significant differences in antigenic sequences, pathogenicity and physiological behavior within the host. Thus, protection induced against one strain may not be either cross-reactive or sufficient to prevent infection by other strain(s).

Apprehension in using attenuated whole parasites for human (and in many countries also animal) vaccination has lead research´s efforts to the development of safer vaccines by means of the identification and subsequent experimental administration of individual antigens. In principle, for an efficient immunization against *T. gondii*, the best antigens to use in vaccines should be those that are excreted/secreted (ESA) from the parasite stages that invade the host, since those have been reported as the most relevant targets of immune responses during natural infection, which, in contrast to what is seen in other diseases, controls the parasite´s ability to spread and multiply.

The surface of the tachyzoite stage was the initial source of antigens tested as vaccines because of its accessibility. The abundant surface antigen 1 (SAG-1) was the initial and most widely studied tachyzoite antigen. Multiple other antigens (see figure 2) of the tachyzoite and bradyzoite stages of the parasite subsequently entered the vaccine development pipeline, and even sporozoite-specific antigens have recently begun to be identified in mice and humans (Hill *et al*. 2011). The use of all these antigens has been carefully reviewed by Jongert *et al*. (Jongert *et al*. 2009).

In brief, up-to-date a few bradyzoite antigens, such as the abundant BAG-1, BRAD-1, CST-1, SAG4A, SRS-9, BSR-4, or the bradyzoite/tachyzoite expressed protein MAG-1 and, innumerable tachyzoite antigens, including surface antigens (SAGs), dense granule (GRAs), rhoptries (ROPs) and microneme (MICs) antigens have been identified and used as vaccine candidates with relative success and mostly in small experimental animal models.

In humans, major T-cell antigens have yet to be identified (Boothroyd 2009) although recent tests using predicted CD8 epitopes derived from the most relevant mouse antigens could identify several reactive peptides presented by HLA-A02, A03 and B07 human major histocompatibility complex (MHC) molecules (Tan *et al*. 2010; Cong *et al*. 2011). Candidate proteins include 2 surface antigens (SAG1, and SUSA1, a surface marker specific to the slow-growing, bradyzoite form of *T. gondii*) and 7 secreted proteins (GRA2, GRA3, GRA6, GRA7, ROP2, ROP16, ROP18).

Recombinant proteins were the first and most obvious tools to induce immunity against *T. gondii* and antigens produced in bacteria or yeast cells were the first finely characterized molecules inoculated into experimental animals (Jongert *et al*. 2009). However, immunization with pure proteins did not show much success in terms of induction of protective immunity. Synthetic peptides encompassing antigenic epitopes were also tested as vaccine candidates (Duquesne *et al*. 1991), but with the same unfortunate fate. New combinations of antigens/adjuvants (specially the new generation of innate-stimulating adjuvants) aiming at inducing more adequate and stronger Th1/CTL responses are the paths to follow in order to improve the results obtained to date with these types of experimental vaccines.

3.4 Genetic vaccines: DNA molecules and live vectors

Proteins are excellent inducers of antibodies, but have some difficulties to induce high levels of T lymphocytes. Genetic vaccines, on the contrary, are highly efficient to induce antigen-specific T lymphocytes. This group of vaccines represents a number of novel technologies that involve direct delivery of genes encoding antigens of interest to host cells, which then serve as antigen factories and immune-related processing plants for the resulting products. The intracellular presence of these products facilitates further induction of antigen-specific cellular immune responses by means of the easier antigen presentation in association with MHC class I molecules, which efficiently primes $CD8^+$ T lymphocyte responses. These novel

vaccine technologies have therefore being used in clinical trials against a variety of pathogens for which this cellular immune profile renders protection (Barouch 2006).

Immunization with DNA vaccines (almost exclusively bacterial plasmids because of the easy construction and multiplication, see Table 1) has traditionally been the first choice for experimental genetic vaccination against toxoplasmosis (Jongert *et al*. 2009). However, despite their successful application in many preclinical disease models, one of the most significant hurdles of DNA vaccine development has been transferring the success of inducing protective immunity in small animal models to larger animal models. The low potency of DNA vaccines in primates has so far precluded the development of most human health programs beyond Phase I clinical trials (Ulmer *et al*. 2006; Abdulhaqq and Weiner 2008). The reasons for the failure of DNA vaccines to induce potent immune responses in humans have not yet been elucidated. However, it is reasonable to assume that the low levels of overall antigen production, the inefficient cellular delivery of DNA plasmids and the insufficient stimulation of the innate immune system may be the major causes responsible for the lack of efficiency (Ulmer *et al*. 2006).

Vaccine candidate	Mouse strain	*T. gondii* chalange strain	Outcome of vaccination	References
pGRA4 + liposome, im	C57BL/6	ME49 tissue cysts, oral	Partial protection	Chen *et al*., 2009
	BALB/c	RH tachyzoites, ip	No protection	Chen *et al*., 2009
pSAG1 + pIL-18, im	BALB/c	RH tachyzoites, ip	Protection	Liu *et al*., 2010
pSAG1, im	BALB/c	RH tachyzoites, ip	Partial protection	Liu *et al*., 2010
pSAG1, im	BALB/c	RH tachyzoites, ip	No protection	Hoseinian Khosroshahi *et al*., 2011
pROP2, im	BALB/c	RH tachyzoites, ip	No protection	Hoseinian Khosroshahi *et al*., 2011
pSAG1 + pROP2, im	BALB/c	RH tachyzoites, ip	Partial protection	Hoseinian Khosroshahi *et al*., 2011
pMIC3, im	BALB/c	RH tachyzoites, ip	Partial protection	Fang *et al*. 2009
pSCA/MIC3, im	BALB/c	RH tachyzoites, ip	Partial protection	Fang *et al*. 2009
pMIC3 + pGM-CSF, im	CBA/J	76K tissue cysts, oral	Protection	Ismael *et al*. 2003; Ismael *et al*., 2009
pMIC3, sc	Kunming	RH tachyzoites, ip	Protection	Xiang *et al*. 2009
pT-ME (multi-epitope DNA), im	BALB/c	RH tachyzoites, ip	Protection	Liu *et al*., 2009
	C57BL/6	RH tachyzoites, ip	Protection	Liu *et al*., 2009
pEC2 (multi-epitope DNA), im	BALB/c	Prugniaud tissue cysts, oral	Partial protection	Rosenberg *et al*., 2009
	BALB/c	Trousseau tissue cysts, oral	No protection	Rosenberg *et al*., 2009
pEC3 (multi-epitope DNA), im	BALB/c	Prugniaud tissue cysts, oral	Partial protection	Rosenberg *et al*., 2009
	BALB/c	Trousseau tissue cysts, oral	No protection	Rosenberg *et al*., 2009
pSAG1-MIC4, in	BALB/c	RH tachyzoites, ip	Partial protection	Wang *et al*., 2009
pSAG1-MIC4 + pCTA2/B, in	BALB/c	RH tachyzoites, ip	Protection	Wang *et al*., 2009

Abbreviations: p, plasmid; im, intramuscular; ip, intraperitoneal; sc, subcutaneous; in, intranasal; pSCA, suicidal vector based on the SFV replicon; T-ME, $SAG1_{238-256}$, $SAG1_{281-320}$, $GRA1_{170-193}$, $GRA4_{331-345}$, $GRA4_{229-245}$, $GRA2_{171-185}$; EC2, MIC2-MIC3-SAG1; EC3, GRA3-GRA7-M2AP; CTA2/B, Cholera toxin A2 and B subunits.

Table 1. Major DNA vaccine candidates recently developed and tested against *T. gondii* infection

In order to increase the immunogenicity of DNA vaccines in large animal models, various methods have been tested including: (i) improvements in the design of the plasmid, e.g. by adding antigen-ubiquitination signals for improved immunoproteasome degradation and antigen presentation (Ishii *et al*. 2006), (ii) delivering multiple antigens at the same time (Beghetto *et al*. 2005; Mevelec *et al*. 2005; Jongert *et al*. 2007; Xue *et al*. 2008; Qu *et al*. 2009; Wang *et al*. 2009; Hoseinian Khosroshahi *et al*. 2011), (iii) using chemical adjuvants or immunomodulatory molecules formulated into microparticles or liposomes (van Drunen Littel-van den Hurk *et al*. 2004; Greenland and Letvin 2007), and (iv) using plasmid molecules as part of prime-boost immunization regimes (Doria-Rose and Haigwood 2003; Dunachie and Hill 2003; Dondji *et al*. 2005; Shang *et al*. 2009). However, at present, the low immunogenicity of DNA vaccines has forced researchers to find alternative immunization vectors, and recombinant bacterial or viral vectors, which carry and express DNA sequences into the host organisms more efficiently, have gradually substituted bacterial plasmids for experimental vaccination studies.

The use of bacteria as vehicles for genetic vaccination is an attractive and simple idea that derives from a number of intrinsic properties of the system. Live bacteria that contain recombinant plasmids encoding heterologous antigens of other pathogens have the potential of being oral delivery vectors for DNA vaccines in animal industry (Grillot-Courvalin *et al*. 1999; Grillot-Courvalin *et al*. 2002). In Table 2. we show two studies conducted by Qu *et al*. in which ICR mice were orally immunized with DNA vaccines encoding SAG1 and/or MIC3 antigens delivered by an attenuated *S. typhimurium* strain (Dam^- and $PhoP^-$) at different doses, and challenged with 500 tachyzoites of *T. gondii* RH strain. Those studies show that oral administration of the attenuated bacteria could induce humoral and cellular immune responses, although they just elicited partial protection of animals (a maximum of 20% improvement in survival rate). Thus, new vectors and constructs have to be tested to consider this methodology as an applicable option.

Vaccine candidate	Mouse strain	*T. gondii* chalange strain	Outcome of vaccination	References
rPRV/SAG1, im	BALB/c	RH tachyzoites, ip	Partial protection	Liu *et al*., 2008
pSAG1 (prime) + rPRV/SAG1 (boost), im	BALB/c	RH tachyzoites, ip	Protection	Shang *et al*., 2009
BV-G/SAG1, im	BALB/c	RH tachyzoites, ip	Protection	Fang *et al*., 2010
rFLU/SAG2 (prime), in + rAd/SAG2 (boost), sc	BALB/c	P-Br tissue cysts, oral	Protection	Machado *et al*., 2010
S. typhimurium/pSAG1, oral	ICR	RH tachyzoites, ip	Partial protection	Qu *et al*., 2008
S. typhimurium/pSAG1-MIC3, oral	ICR	RH tachyzoites, ip	Partial protection	Qu *et al*., 2009

Abbreviations: p, plasmid; im, intramuscular; ip, intraperitoneal; sc, subcutaneous; in, intranasal; rPRV: recombinant pseudorabies virus; BV-G: recombinant baculovirus; rFLU: recombinant influenza A; rAd: recombinant adenovirus.

Table 2. Selected examples of live attenuated vectors expressing *Toxoplasma gondii* antigens currently in tests.

Viral vectors exhibit many advantages for the development of a vaccine against toxoplasmosis. In summary, viral vectors express foreign antigens directly inside host cells very efficiently; as a result they present antigen fragments in association with MHC molecules more proficiently and, subsequently, they better stimulate the required anti-toxoplasma T cell responses (Th1 and CTL) because they act as natural adjuvants and stimulate intracellular innate immunity receptors. In addition, they can be administrated through the natural route of infection, such as via nasal mucosa, and they are able to induce effective and long lasting immune responses.

Our group has tested adenoviruses and influenza viruses as feasible vaccine vectors against toxoplasmosis (Caetano *et al*. 2006; Machado *et al*. 2010; Mendes *et al*. 2011) and they have shown significant improvement in comparison with naked plasmid vaccines. For those studies we have focused on possible formulations and immunization protocols using *T. gondii* surface antigens (SAGs). These molecules are involved in host cell attachment and invasion, and their sequences are conserved among different strains of *T. gondii*, sharing a high degree of homology even between type I (pathogenic and lethal in mice) and type II/III strains (cystogenic). However, these favorable traits do not ensure that these antigens will end up displaying sufficient protective capacities, but the proofs-of-principle obtained with their use will surely be maintained for any other antigens that display better protective properties.

In Caetano *et al*. (Caetano *et al*. 2006), we generated three recombinant adenoviruses encoding genetically modified SAG1, SAG2 and SAG3, without the 3'-end GPI anchoring motifs to ensure secretion and subsequent induction of combined Th/CTL immune responses. BALB/c mice received rAd/SAG1, rAd/SAG2, rAd/SAG3, or a combination of the three viruses (rAdMIX) and were challenged with 100 live tachyzoites of the *T. gondii* RH strain or with 20 cysts of the P-Br strain. Adenovirus immunization elicited potent antibody responses against each protein and displayed a significant bias toward a Th1 profile. When comparing the three recombinant viruses, rAd/SAG2 was the most efficient in eliciting antigen-specific antibodies. A significant reduction in cysts loads in the brain was observed in animals challenged with the P-Br strain. Vaccination with a mixture of all viruses promoted the highest level of inhibition of cyst formation, about 80%. However, no protection was observed against tachyzoites of the highly virulent RH strain (Caetano *et al*. 2006).

In the study by Machado *et al*. (2010), we generated a recombinant Influenza A vector encoding SAG2 of *T. gondii* and explored an original heterologous prime-boost immunization protocol using influenza virus (rFLU/SAG2) and a recombinant adenovirus (rAd/SAG2). Influenza A viruses are promising but currently under-explored vectors, which display some advantageous features to be used as live recombinant vaccines, such as the ability to infect and activate antigen presenting cells as well as to present high immunogenicity at mucosal and systemic levels (Rocha *et al*. 2004; Machado *et al*. 2010). BALB/c mice primed with an intranasal rFLU/SAG2 dose and boosted with a subcutaneous rAd/SAG2 dose elicited both humoral and cellular immune responses specific for SAG2. Moreover, when immunized animals were challenged with the cystogenic P-Br strain of *T. gondii*, they displayed up to 85% of reduction in parasite burden. These results demonstrate the potential use of recombinant influenza and adenoviruses in vaccination protocols to protect against oral challenge with *T. gondii* (Machado *et al*. 2010), although there is room for improvement.

Literature shows that, for other diseases, there is a good reproducibility of results when transferring experimental results obtained by immunization with some viral vectors (in particular adenoviruses) from small experimental animals to larger animal models or

humans. We expect this to be also true for the experimental vaccines generated against toxoplasmosis, so that we can see some encouraging results in the near future.

4. Conclusion

Recombinant subunit vaccines (proteins in adjuvants, DNA vaccines and recombinant live vectors) are the present trends for the development of a vaccine against Toxoplasmosis. A myriad of parasite antigens have been described and researchers are testing them in many animal models of the disease. It is our belief that, more than the description of new parasite antigens that could be used in a final vaccine formulation, one of the major issues for the next future is to develop and test highly antigenic formulations using currently known antigens. Developing this type of formulations requires a deep knowledge of the immune system's antigen processing and presentation pathways, proficiency in the use of molecular biology techniques to adapt the parasite antigen sequences to enter those pathways, and using the new generation of adjuvants and delivery vectors in a manner that can best stimulate the pretended anti-parasite Th1 cellular (and probably humoral) immune responses. The options and combinations are so broad, and yet untested, that several years of research will be needed before we can decide which combination will be more adequate [antigen(s) + adjuvant(s) + vector(s)] or what will be the most efficient immunization protocol (single dose, multiple dose, homologous or heterologous prime-boost, etc.).
Finally, we would like to call attention to the fact that a possible result of the immunization/protection tests may actually be the achievement of a cost-effective vaccine that may be suitable for large-scale production and use. Then, one key question will arise for the future, regarding the correct use of that vaccine. In principle, the vaccine should be applied to animals, because preventing oocyst shedding by cats and tissue cyst formation in meat-producing animals should have great impact on both environmental contamination and public health. But this intervention could pose a risk because of the loss of herd immunity against the parasite and the resurgence of a different profile of *Toxoplasma*-related pathologies because of the primo-infection of non-vaccinated adults traveling to other countries or regions instead of kids or young adults being infected at its home places. To solve this, a possibility would be the universal vaccination of all children against toxoplasmosis, although this might end up being not feasible in practice or even might not be considered as a priority. Researchers should include these topics amongst those to be discussed in the forthcoming years within the field of vaccine development against toxoplasmosis.

5. References

Abdulhaqq, S. A. and D. B. Weiner (2008). "DNA vaccines: developing new strategies to enhance immune responses." *Immunol Res* 42(1-3): 219-232.

Andre, F. E., R. Booy, et al. (2008). "Vaccination greatly reduces disease, disability, death and inequity worldwide." *Bull World Health Organ* 86(2): 140-146.

Barouch, D. H. (2006). "Rational design of gene-based vaccines." *J Pathol* 208(2): 283-289.

Beghetto, E., H. V. Nielsen, et al. (2005). "A combination of antigenic regions of Toxoplasma gondii microneme proteins induces protective immunity against oral infection with parasite cysts." *J Infect Dis* 191(4): 637-645.

Bhopale, G. M. (2003). "Development of a vaccine for toxoplasmosis: current status." *Microbes Infect* 5(5): 457-462.

Boothroyd, J. C. (2009). "Toxoplasma gondii: 25 years and 25 major advances for the field." *Int J Parasitol* 39(8): 935-946.

Bourguin, I., T. Chardes, et al. (1993). "Oral immunization with Toxoplasma gondii antigens in association with cholera toxin induces enhanced protective and cell-mediated immunity in C57BL/6 mice." *Infect Immun* 61(5): 2082-2088.

Bourguin, I., T. Chardes, et al. (1991). "Amplification of the secretory IgA response to Toxoplasma gondii using cholera toxin." *FEMS Microbiol Lett* 65(3): 265-271.

Buxton, D. (1993). "Toxoplasmosis: the first commercial vaccine." *Parasitol Today* 9(9): 335-337.

Caetano, B. C., O. Bruna-Romero, et al. (2006). "Vaccination with replication-deficient recombinant adenoviruses encoding the main surface antigens of toxoplasma gondii induces immune response and protection against infection in mice." *Hum Gene Ther* 17(4): 415-426.

Chen, R., S. H. Lu, et al. (2009). "Protective effect of DNA-mediated immunization with liposome-encapsulated GRA4 against infection of Toxoplasma gondii." *J Zhejiang Univ Sci B* 10(7): 512-521.

Cong, H., E. J. Mui, et al. (2011). "Towards an immunosense vaccine to prevent toxoplasmosis: protective Toxoplasma gondii epitopes restricted by HLA-A*0201." *Vaccine* 29(4): 754-762.

Crampton, A. and T. Vanniasinkam (2007). "Parasite vaccines: the new generation." *Infect Genet Evol* 7(5): 664-673.

Cutchins, E. C. and J. Warren (1956). "Immunity patterns in the guinea pig following Toxoplasma infection and vaccination with killed Toxoplasma." *Am J Trop Med Hyg* 5(2): 197-209.

Denkers, E. Y. and R. T. Gazzinelli (1998). "Regulation and function of T-cell-mediated immunity during Toxoplasma gondii infection." *Clin Microbiol Rev* 11(4): 569-588.

Denkers, E. Y., R. T. Gazzinelli, et al. (1993). "Bone marrow macrophages process exogenous Toxoplasma gondii polypeptides for recognition by parasite-specific cytolytic T lymphocytes." *J Immunol* 150(2): 517-526.

Denkers, E. Y., R. T. Gazzinelli, et al. (1993). "Emergence of NK1.1+ cells as effectors of IFN-gamma dependent immunity to Toxoplasma gondii in MHC class I-deficient mice." *J Exp Med* 178(5): 1465-1472.

Dondji, B., E. Perez-Jimenez, et al. (2005). "Heterologous prime-boost vaccination with the LACK antigen protects against murine visceral leishmaniasis." *Infect Immun* 73(8): 5286-5289.

Doria-Rose, N. A. and N. L. Haigwood (2003). "DNA vaccine strategies: candidates for immune modulation and immunization regimens." *Methods* 31(3): 207-216.

Dubey, J. P., M. C. Jenkins, et al. (1996). "Killing of Toxoplasma gondii oocysts by irradiation and protective immunity induced by vaccination with irradiated oocysts." *J Parasitol* 82(5): 724-727.

Dubey, J. P., J. K. Lunney, et al. (1998). "Immunity to toxoplasmosis in pigs fed irradiated Toxoplasma gondii oocysts." *J Parasitol* 84(4): 749-752.

Dunachie, S. J. and A. V. Hill (2003). "Prime-boost strategies for malaria vaccine development." *J Exp Biol* 206(Pt 21): 3771-3779.

Duquesne, V., C. Auriault, et al. (1991). "Identification of T cell epitopes within a 23-kD antigen (P24) of Toxoplasma gondii." *Clin Exp Immunol* 84(3): 527-534.

Fang, R., H. Feng, et al. (2010). "Construction and immunogenicity of pseudotype baculovirus expressing Toxoplasma gondii SAG1 protein in BALB/c mice model." *Vaccine* 28(7): 1803-1807.

Fang, R., H. Nie, et al. (2009). "Protective immune response in BALB/c mice induced by a suicidal DNA vaccine of the MIC3 gene of Toxoplasma gondii." *Vet Parasitol* 164(2-4): 134-140.

Gazzinelli, R., Y. Xu, et al. (1992). "Simultaneous depletion of CD4+ and CD8+ T lymphocytes is required to reactivate chronic infection with Toxoplasma gondii." *J Immunol* 149(1): 175-180.

Gazzinelli, R. T., D. Amichay, et al. (1996). "Role of macrophage-derived cytokines in the induction and regulation of cell-mediated immunity to Toxoplasma gondii." *Curr Top Microbiol Immunol* 219: 127-139.

Gazzinelli, R. T., M. Wysocka, et al. (1994). "Parasite-induced IL-12 stimulates early IFN-gamma synthesis and resistance during acute infection with Toxoplasma gondii." *J Immunol* 153(6): 2533-2543.

Gigley, J. P., B. A. Fox, et al. (2009). "Long-term immunity to lethal acute or chronic type II Toxoplasma gondii infection is effectively induced in genetically susceptible C57BL/6 mice by immunization with an attenuated type I vaccine strain." *Infect Immun* 77(12): 5380-5388.

Greenland, J. R. and N. L. Letvin (2007). "Chemical adjuvants for plasmid DNA vaccines." *Vaccine* 25(19): 3731-3741.

Grillot-Courvalin, C., S. Goussard, et al. (1999). "Bacteria as gene delivery vectors for mammalian cells." *Curr Opin Biotechnol* 10(5): 477-481.

Grillot-Courvalin, C., S. Goussard, et al. (2002). "Wild-type intracellular bacteria deliver DNA into mammalian cells." *Cell Microbiol* 4(3): 177-186.

Gwadz, R. W., A. H. Cochrane, et al. (1979). "Preliminary studies on vaccination of rhesus monkeys with irradiated sporozoites of Plasmodium knowlesi and characterization of surface antigens of these parasites." *Bull World Health Organ* 57 Suppl 1: 165-173.

Hakim, F. T., R. T. Gazzinelli, et al. (1991). "CD8+ T cells from mice vaccinated against Toxoplasma gondii are cytotoxic for parasite-infected or antigen-pulsed host cells." *J Immunol* 147(7): 2310-2316.

Hill, D., C. Coss, et al. (2011). "Identification of a sporozoite-specific antigen from Toxoplasma gondii." *J Parasitol* 97(2): 328-337.

Hiramoto, R. M., A. J. Galisteo, et al. (2002). "200 Gy sterilised Toxoplasma gondii tachyzoites maintain metabolic functions and mammalian cell invasion, eliciting cellular immunity and cytokine response similar to natural infection in mice." *Vaccine* 20(16): 2072-2081.

Hoseinian Khosroshahi, K., F. Ghaffarifar, et al. (2011). "Evaluation of the immune response induced by DNA vaccine cocktail expressing complete SAG1 and ROP2 genes against toxoplasmosis." *Vaccine* 29(4): 778-783.

Hutson, S. L., E. Mui, et al. (2010). "T. gondii RP promoters & knockdown reveal molecular pathways associated with proliferation and cell-cycle arrest." *PLoS One* 5(11): e14057.

Ishii, K., H. Hisaeda, et al. (2006). "The involvement of immunoproteasomes in induction of MHC class I-restricted immunity targeting Toxoplasma SAG1." *Microbes Infect* 8(4): 1045-1053.

Ismael, A. B., D. Hedhli, et al. (2009). "Further analysis of protection induced by the MIC3 DNA vaccine against T. gondii: CD4 and CD8 T cells are the major effectors of the MIC3 DNA vaccine-induced protection, both Lectin-like and EGF-like domains of MIC3 conferred protection." *Vaccine* 27(22): 2959-2966.

Ismael, A. B., D. Sekkai, et al. (2003). "The MIC3 gene of Toxoplasma gondii is a novel potent vaccine candidate against toxoplasmosis." *Infect Immun* 71(11): 6222-6228.

Jongert, E., S. de Craeye, et al. (2007). "GRA7 provides protective immunity in cocktail DNA vaccines against Toxoplasma gondii." *Parasite Immunol* 29(9): 445-453.

Jongert, E., C. W. Roberts, et al. (2009). "Vaccines against Toxoplasma gondii: challenges and opportunities." *Mem Inst Oswaldo Cruz* 104(2): 252-266.

Kang, H., J. S. Remington, et al. (2000). "Decreased resistance of B cell-deficient mice to infection with Toxoplasma gondii despite unimpaired expression of IFN-gamma, TNF-alpha, and inducible nitric oxide synthase." *J Immunol* 164(5): 2629-2634.

Krahenbuhl, J. L., J. Ruskin, et al. (1972). "The use of killed vaccines in immunization against an intracellular parasite: Toxoplasma gondii." *J Immunol* 108(2): 425-431.

Liu, Q., S. Gao, et al. (2008). "A recombinant pseudorabies virus expressing TgSAG1 protects against challenge with the virulent Toxoplasma gondii RH strain and pseudorabies in BALB/c mice." *Microbes Infect* 10(12-13): 1355-1362.

Liu, Q., L. Shang, et al. (2010). "The protective effect of a Toxoplasma gondii SAG1 plasmid DNA vaccine in mice is enhanced with IL-18." *Res Vet Sci* 89(1): 93-97.

Liu, S., L. Shi, et al. (2009). "Evaluation of protective effect of multi-epitope DNA vaccine encoding six antigen segments of Toxoplasma gondii in mice." *Parasitol Res* 105(1): 267-274.

Lu, F., S. Huang, et al. (2009). "The temperature-sensitive mutants of Toxoplasma gondii and ocular toxoplasmosis." *Vaccine* 27(4): 573-580.

Ma, G. Y., J. Z. Zhang, et al. (2009). "Toxoplasma gondii: proteomic analysis of antigenicity of soluble tachyzoite antigen." *Exp Parasitol* 122(1): 41-46.

Machado, A. V., B. C. Caetano, et al. (2010). "Prime and boost immunization with influenza and adenovirus encoding the Toxoplasma gondii surface antigen 2 (SAG2) induces strong protective immunity." *Vaccine* 28(18): 3247-3256.

McLeod, R., J. K. Frenkel, et al. (1988). "Subcutaneous and intestinal vaccination with tachyzoites of Toxoplasma gondii and acquisition of immunity to peroral and congenital toxoplasma challenge." *J Immunol* 140(5): 1632-1637.

Mendes, E. A., B. C. Caetano, et al. (2011). "MyD88-dependent protective immunity elicited by adenovirus 5 expressing the surface antigen 1 from Toxoplasma gondii is mediated by CD8(+) T lymphocytes." *Vaccine* 29(27): 4476-4484.

Mevelec, M. N., D. Bout, et al. (2005). "Evaluation of protective effect of DNA vaccination with genes encoding antigens GRA4 and SAG1 associated with GM-CSF plasmid,

against acute, chronical and congenital toxoplasmosis in mice." *Vaccine* 23(36): 4489-4499.

Mevelec, M. N., C. Ducournau, et al. (2010). "Mic1-3 Knockout Toxoplasma gondii is a good candidate for a vaccine against T. gondii-induced abortion in sheep." *Vet Res* 41(4): 49.

Nussenzweig, R. S., J. Vanderberg, et al. (1967). "Protective immunity produced by the injection of x-irradiated sporozoites of plasmodium berghei." *Nature* 216(5111): 160-162.

Omata, Y., Y. Aihara, et al. (1996). "Toxoplasma gondii: experimental infection in cats vaccinated with 60Co-irradiated tachyzoites." *Vet Parasitol* 65(3-4): 173-183.

Pifer, R. and F. Yarovinsky (2011). "Innate responses to Toxoplasma gondii in mice and humans." *Trends Parasitol*.

Qu, D., S. Wang, et al. (2008). "Protective effect of a DNA vaccine delivered in attenuated Salmonella typhimurium against Toxoplasma gondii infection in mice." *Vaccine* 26(35): 4541-4548.

Qu, D., H. Yu, et al. (2009). "Induction of protective immunity by multiantigenic DNA vaccine delivered in attenuated Salmonella typhimurium against Toxoplasma gondii infection in mice." *Vet Parasitol* 166(3-4): 220-227.

Rocha, C. D., B. C. Caetano, et al. (2004). "Recombinant viruses as tools to induce protective cellular immunity against infectious diseases." *Int Microbiol* 7(2): 83-94.

Rosenberg, C., S. De Craeye, et al. (2009). "Induction of partial protection against infection with Toxoplasma gondii genotype II by DNA vaccination with recombinant chimeric tachyzoite antigens." *Vaccine* 27(18): 2489-2498.

Seah, S. K. and G. Hucal (1975). "The use of irradiated vaccine in immunization against experimental murine toxoplasmosis." *Can J Microbiol* 21(9): 1379-1385.

Shang, L., Q. Liu, et al. (2009). "Protection in mice immunized with a heterologous prime-boost regime using DNA and recombinant pseudorabies expressing TgSAG1 against Toxoplasma gondii challenge." *Vaccine* 27(21): 2741-2745.

Tan, T. G., E. Mui, et al. (2010). "Identification of T. gondii epitopes, adjuvants, and host genetic factors that influence protection of mice and humans." *Vaccine* 28(23): 3977-3989.

Ulmer, J. B., B. Wahren, et al. (2006). "Gene-based vaccines: recent technical and clinical advances." *Trends Mol Med* 12(5): 216-222.

van Drunen Littel-van den Hurk, S., S. L. Babiuk, et al. (2004). "Strategies for improved formulation and delivery of DNA vaccines to veterinary target species." *Immunol Rev* 199: 113-125.

Wang, H., S. He, et al. (2009). "Toxoplasma gondii: protective effect of an intranasal SAG1 and MIC4 DNA vaccine in mice." *Exp Parasitol* 122(3): 226-232.

Xiang, W., Z. Qiong, et al. (2009). "The location of invasion-related protein MIC3 of Toxoplasma gondii and protective effect of its DNA vaccine in mice." *Vet Parasitol* 166(1-2): 1-7.

Xue, M., S. He, et al. (2008). "Evaluation of the immune response elicited by multi-antigenic DNA vaccine expressing SAG1, ROP2 and GRA2 against Toxoplasma gondii." *Parasitol Int* 57(4): 424-429.

Yap, G. S., T. Scharton-Kersten, et al. (1998). "Partially protective vaccination permits the development of latency in a normally virulent strain of Toxoplasma gondii." *Infect Immun* 66(9): 4382-4388.

Yin, G. R., X. L. Meng, et al. (2007). "[Intranasal immunization with mucosal complex vaccine protects mice against Toxoplasma gondii]." *Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi* 25(4): 290-294.

13

Screening of the Prevalence of Antibodies to the Tick *Hyalomma lusitanicum* in a Province of Northern Spain

Consuelo Giménez Pardo and Lourdes Lledó García
Alcalá University
Spain

1. Introduction

In the recent decades it has been found the occurrence of a large number of hitherto unknown or undervalued pathogens, and they present a risk to health and human welfare. Almost all incidents caused by emerging pathogens have been attached to zoonotic agents, which expanded its host range and are capable of breaching the species barrier (Zeier et al., 2005). Besides the known diseases, new ones emerge or reemerge due to a variety of socioeconomic, health and environmental questions. This increase in communicable diseases has serious implications for public health and animal health (Chomel, 1998; Daszak et al., 2000, Cleaveland et al., 2001, Simpson, 2002; Daszak & Cunningham, 2003; Zeier et al., 2005; Cunningham, 2005; Blancou et al., 2005, Gibbs, 2005; Gortázar et al., 2007).

Emerging zoonoses are also a public health problem, the biggest threat to animal welfare, environmental quality and conservation of biodiversity (Daszak et al., 2000; Cunningham, 2005, Briones et al., 2002). The expected increase contact between humans and wildlife, caused by anthropogenic interference in the ecosystem, increase the emergence of pathogens originating in wildlife cycles, which can cross-infect man and animals (Bengis et al., 2004). Thus, wildlife is a constant source of "zoonotic pool" that plays a fundamental role in human exposure to infectious agents against novel animal (Morse, 1995).

In the recent past, the diseases of wild animals have been important only when they threatened livestock or human health (Daszak et al., 2000), but outbreaks in endangered species have led to them having more significant consideration. Currently, these diseases are booming, especially in the space where interaction occurs between wildlife and farm animals, including an increase in contact between them and the man (Simpson, 2002; Gortázar et al., 2007). Arthropod-borne diseases represent the most common zoonosis in relation to wildlife in the northern hemisphere, especially the Old World (Lindgren et al., 2000), so infectious agents are diverse and constantly growing, so their relationship would be endless (Bueno et al., 2009).

Wild animals and arthropod vectors play also important roles in the exposure of humans and domestic animals to animal-borne pathogens (Morse, 1995). Contact between humans and wild animals may occur when people venture into the latter's ecosystems, such contact in foreseen as a future generator of cross infestation, but the knowledge of ectoparasites, with special mention to ticks and their hosts' reservoirs that are located in many areas of

world are unstudied. Ticks are vectors of disease have a wide range of pathogens (bacteria, rickettsia, viruses, protozoa and nematodes), affecting both domestic and wild animals, and humans with a zoonotic character. Ticks are considered as one of the most efficient arthropod vector role (Hillyard, 1996, Wall & Shearer, 1997).

In fact, it's known that ticks are vectors of transmission of a number of human viruses that causes Tick-borne meningoencephalitis, Colorado tick fever and Crimean-Congo hemorrhagic fever, among others, bacteria (*Rickettsia* spp, *Anaplasma phagocytophilum*, *Borrelia burgdorferi* and *Francisella turalensis*, among others) (Lopez-Vélez & Molina, 2005; Toledo et al., 2009) and protozoan pathogens including parasites (Lledó et al., 2010) and control of ticks and tick-borne diseases is a major component of animal health programes for the protection of livestock. Over the ixodid tick species which are often found on humans exposed to infested vegetation *Amblyomma* species, *Dermacentor* spp, *Haemaphysalis* spp, *Ixodes* spp and *Hyalomma* spp are found (Estrada- Peña et al., 1999).

In this sense, Genus *Hyalomma* is a phylogenetically young group of ixodid ticks. As proposed Kolonin (2009), domestication and the development of cattle-breeding stimulated the evolution and biological progress of this group. These transformations continue to this day, as is apparent from the great number of intraspecific forms. *Hyalomma* ticks (Figure 1) are medium to large sized, with prominent mouthparts. Most species are 3 hosts, but there are also 1 and 2 hosts. Some species of this genus can use 1, 2 or 3 hosts to develop according to the host they found. The life cycle can last between 3-4 months and more than a year, depending on species and climate. The nymphs and adults stay overwinter in cracks and crevices between the stones of walls and barns, or uncultivated grasslands. Adults are found throughout the year, although the parasite load is higher in spring and summer, parasitizing deer and wild boar. Larvae and nymphs parasitize rabbits and are more prevalent in spring. Though this genus is usually restricted to the Mediterranean region, one of the species, *Hyalomma lusitanicum,* Koch 1844 (*Ixodoidea: Ixodida*) has a widespread distribution in some regions of Southern Spain, from which is introduced by wild animals (Encinas-Grandes, 1986).

Fig. 1. Adult of Hyalomma lusitanicum

This ixodid tick is also located in the Burgos province (north western Spain) (Cordero del Campillo et al., 1994), in areas mainly rural, though recreational activities attracting non-residents have increased in recent years (Figure 2). This tick is not the most prevalent tick in this area.

In this studied area, in the northern sector, the winters are cold and humid and the summers are cold. Vegetation under Atlantic influences consists in oakwood and beechwood, with brushwood. In the southern sector is submediterranean and shows similar winter but hottest summers. Vegetation consists in gall-oak groes and holm-oak wood, being brush scarce (Roman et al., 1996; Dominguez, 2004). Mean summer temperatures in this area range between 16 and 20ºC, while mean winter temperatures range between 2 and 5ºC. Rainfall is usually high in winter at some 900-1100 mm/year. Altitude is ranging between 600-800 meters on the plateau. Climatic differences among different areas of Spain are responsible of both, the diversity of tick species and the circulation of tick-borne pathogens.

Fig. 2. Landscape of the studied area

While ticks have a clear geographical distribution in relation to climate, temperature, humidity and like attitude, climate change and global warming have influenced the geographical distribution of ticks. So, some groups as Experts from the International Scientific Working Group (ISW-TBE) on Central European encephalitis transmitted by ticks (Tick-Borne Encephalitis) warn of the first detection of these arthropods in areas above 1,500 m above sea level.

In Spain, it looks to be *Ixodes ricinus* the most abundant and widespread tick in the Basque Country (Northern Spain) (Barandika et al., 2006) and some authors (Toledo et al., 2009) have observed that one of the most abundant species in Central regions of Spain in terms of infection and tick abundance is *Hyalomma lusitanicum.* However, as happens with other ectoparasites and their host-reservoirs, are located in many areas of Spain that are unstudied or missstudied.

In this sense, the genus *Hyalomma* is one of the vectors for *Theileria annulata* that causes Mediterranean theileriosis, and produces considerable economic losses in cattle (Viseras & Garcia Fernandez., 1999) though in terms of public health, this tick is considered as not anthropophilic.

Tick bites are generally painless and many people may not even notice the bite and may never find the tick if it falls off. The majority of individuals with tick bites develop no symptoms, and many do not remember getting bitten. The direct damage caused by ticks depends on the number, species and location of the parasites. However, the most harmful effects on animal and public health are derived from indirect vector character (Hillyard, 1996, Wall & Shearer, 1997; Encinas et al., 1999, Sonenshine et al., 2002). Damage can be

divided, according to the scope of the consequences, cutaneous and systemic. We can point to the inflammatory reaction in the fixation point, which causes itching, scratching, excoriations and self-harm. The reaction can spread awareness of the antigenic components of saliva, causing even anaphylactic shock. The bites often become infected with pyogenic agents such as *Staphylococcus aureus*, not ruling out the occurrence of myiasis (Wall & Shearer, 1997; Encinas et al., 1999, Wall, 2007).Systemic effects include tick paralysis is caused by a neurotoxin secreted by the females of some species, producing a neuromuscular blockade (Sonenshine et al., 2002). *Ixodes ricinus* and *Haemaphysalis punctata* are two species in our area involved in the process of paralysis (Hillyard, 1996, Wall & Shearer, 1997; Encinas et al., 1999). The mechanical transmission of pathogens from sepsis occurs in tick infestation in lambs or calves (Kettle, 1995), not neglecting the effects of blood loss. In this sense, a female has been eating packed up to 4 g of blood, so intense in parasites are common anemias (Encinas et al., 1999).

When ticks bite at time of the attachment they inoculate saliva and occasionally, a neurotoxin secreted at the time of attachment. Saliva in feeding ticks is rich in several biochemical components including various enzymes (Sauer et al., 1995; Giménez-Pardo & Martínez-Grueiro, 2008). Immunogenic and pathogenic proteins enter in the mammalian host during feeding via the tick salivary gland (Kaufman 1989). Their saliva secretions during bites are capable to produce toxicoses and allergic reactions, and in animals it's known that ticks are capable to induce a high humoral immune response (Perez-Sanchez et al., 1992). In human tick attachment are brief and sometimes, are immature forms which introduce a low quantity of antigens which are not enough to induce a good humoral response.

In this paper we i) study the preliminary humoral response in humans, employing antigens from male and female *Hyalomma lusitanicum* tick as a previous work to know if this tick could in a future be capable to bite humans in rural regions being implicated in the transmission of human pathogens and ii) observe differences between males and females in the response that could induce when they bite humans. For this, it was assayed a panel of human sera of different days post bitten by indirect enzyme-linked immunoabsorbent assay technique. This study was carried out in Burgos province (Spain) and results are compared with those obtained from general population and without history of tick bites**.**

2. Material and methods

2.1 Parasites

465 unfed adult ticks (males and females) were collected from vegetation in spring and each one was identified by binocular lens. Males (190) were separated from females (275) and processed individually. Specimens were immersed in 70% alcohol for ten minutes rinsed for 30 seconds in Milli-Q water and dried in a filter paper. Each ixodid tick was transferred in PBS-saline (10mM PBS pH 7.2, 146mM NaCl) in a Potter-Elvejem on an ice bath and homogenized. Extracts were removed in eppendorf tubes and centrifuged for 1min at 500 rpm to deposit cuticle fragments and tissue rests. Supernatants were collected and centrifuged in new eppendorf at 14000 rpm for 5 min, filtered through a 0.22μm filter (Millipore) and the resultant one were again filtered through a 0.22μm filter. This whole tick extract was collected and the protein concentration determined using the technique of Bradford (Bradford, 1976) and subsequently adjusted to 1mg/ml for females and 0.635 mg/ml for males using PBS-saline.

2.2 Human sera

The study was carried out on two population groups: sera from people who had been bitten by ticks and serum samples from the general population with no history of tick bites as control group. For the first group, 42 samples of human serum from 23 patients bitten by ticks were randomly collected from patients to the Burgos Health Centres (Figure 2), and the following information was recorded for each person: age, sex, occupation and area of residence. A serum sample was withdrawn from all bitten patients at the time of consultation and the patients were asked to return to provide another sample at 15-20 days later. All residents living in the study area are people related to livestock, or have pets in their care. They are people with an epidemiological history of tick bite, but without identification of the same. For the second group, serum from 97 people was obtained from the general population presented to healthcare centres for reasons unrelated to infectious diseases. All samples were aliquoted and conserved at -20ºC until use. The survey was performed with the consent of the subjects included in the study, and in compliance with the ethical standards of Alcalá de Henares University´s Comitte on Human experimentation, as well as with the Helsinki Declaration of 1975 as revised in 2004.

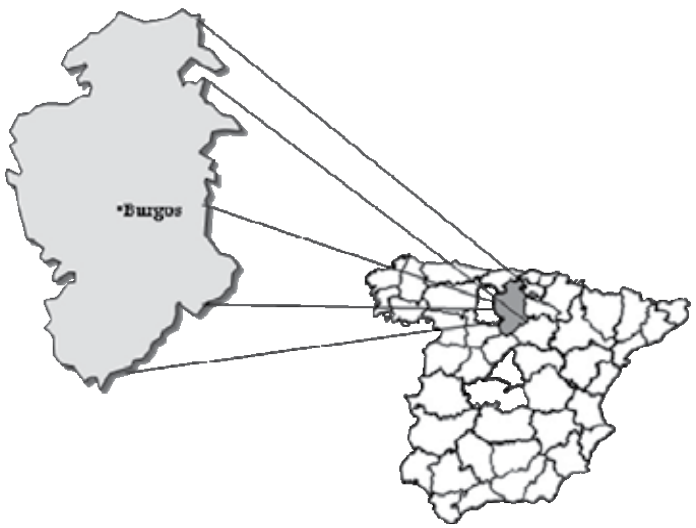


Fig. 3. Location in the Spain map the Burgos province

2.3 ELISA reactions

An indirect ELISA technique was used to detect anti-ticks Abs (IgG, IgM, IgE) in the samples. As antigens were used protein extracts from both males and females of *H. lusitanicum*. ELISA plates were coated with 1µg of antigen per well diluted in 0.1M carbonate/bicarbonate buffer, pH 9.6 and incubated at 4ºC overnight. It was realized three washes in 0.05% PBS-Tween 20. After incubation with 0.05% PBS-Tween 20-1% casein for 1h at 37º and the subsequent washes, 100µl per well of human sera diluted at 1/50 for IgG, 1/20 for IgM and 1/10 for IgE in 0.05% PBS-Tween-20 and incubated for 1h at 37ºC was added. After the subsequent washes, peroxidase-labeled anti-human immunoglobulin antibodies were also diluted in 0.05% PBS-Tween at 1/4000 for both anti-IgG (CalBiochem),

anti-IgM (CalBiochem) and 1/500 for anti-IgE (CalBiochem), incubated for 1h at 37ºC. ABTS (2-2´-Azino bis(3-Ethylbenzhia-zoline-6-sulfonic acid) and H_2O_2 were used as substrates. Reactions were stopped after with 3N sulphuric acid and results were read on a spectrophotometer at 405nm. On each ELISA plate were included negative controls sera obtained from people that never have been bitten by any tick. Experiences were done in triplicate (or more depending on the sera volume). The sample sera were considered positive when their optical density surpased a treshold calculated as the mean optical density of the negative control sera plus three times the standard deviation (mean OD+3δ). The same parameters were employed when it was referred to the general population.

3. Results

Twenty three of the patients presented at the Burgos Health Services for tick bites were asymptomatic at the time of sampling. Of these patients, 12 were males and 11 were females (Table 1).

	Age-groups (years)		
	0-10	**11-59**	**60-97**
Males (%)	2 (8.69)	4 (17.39)	6 (26.08)
Females (%)	2 (8.69)	8 (34.78)	1 (4.34)

Table 1. Characteristics of the patients attended for tick bites in this study

Nine patients were bled once, 13 patients were bled two times and 1 patient was bled in three occasions. All patients were bitten by unknown tick species and in 9 of them the serum samples were withdrawn more than 21 days after the tick bite (Table 2). There was no relation between the tick bite and the age, profession or place where the patient lived.

Of the patients studied, 4 had Abs (IgG) against both *H. lusitanicum* male and female antigens and two of them reacted only with male but not with female whole tick antigen as indicated in Table 2. Two sera showed Abs (IgG) against female but not male *H. lusitanicum* antigens.

Because ticks could have been removed from people in the first 48h post-attachment and even in the case of an effective transmission, the patients could have been bled before the synthesis of the anti-tick Abs have occurred, low values of IgM were found. Afterall, sera from three patients were considered positive against female antigens.

Serum from one patient was positive against male antigens of *Hyalomma lusitanicum.* Two patients showed IgM positive as only response and this fact indicate a possible acute case of *H. lusitanicum* bitten (see in Table 2).

Antibodies to IgE were analyzed in those sera that were positive against both male and female antigens, but we have not observed response to anti-IgE, neither male nor female *H. lusitanicum* antigens.

Relative to the general unexposed population, none of the sera studied were positive against female antigens of *H. lusitanicum* though when sera were assayed against male antigens, one serum was positive (see in Table 3).

Patient (n°)	Sera (n°)	Days (p.b)[1]	IgG (X±SE)		IgM (X±SE)	
			Female (Ag)	Males(Ag)	Females(Ag)	Males(Ag)
1	**5**		1.235±0.046	**2.194±0.144**	0.140±0.014	0.112±0.031
2	**6**		0.856±0.021	0.785±0.074	0.086±0.002	0.163±0.043
	7		0.975±0.077	1.098±0.207	0.079±0.022	0.165±0.022
3	**8**		1.270±0.094	1.224± 0.161	0.059±0.009	0.148±0.002
4	**9**		**1.760±0.058**	**1.903±0.165**	0.102±0.017	0.122±0.028
5	**17a**	17	1.044±0.015	0.928±0.133	0.106±0.009	0.144±0.038
	17b		0.816±0.040	0.499±0.062	0.100±0.026	0.057±0.027
6	**23a**		0.946±0.113	1.199±0.132	**0.399±0.025**	0.023±0.002
	23b	25	1.291±0.201	1.268±0.111	**0.350±0.033**	0.144±0.046
7	**36a**		0.724±0.067	0.445±0.095	0.060±0.001	0.050±0.007
	36b	11	1.442±0.124	1.229±0.024	0.046±0.010	0.134±0.019
	50	20	1.033±0.061	0.516±0.036	0.050±0.012	0.121±0.021
8	**80**		1.450±0.050	1.359±0.072	0.068±0.012	0.166±0.015
	79		1.443±0.190	1.250±0.138	0.107±0.004	0.187±0.005
9	**81**		1.161±0.093	0.674±0.066	0.023±0.004	0.150±0.016
	82		0.404±0.042	0	0.062±0.004	0.175±0.031
10	**84**		0.926±0.112	0.707±0.166	0.041±0.010	0.173±0.042
	83		1.241±0.011	0	0.040±0.004	0.171±0.039
11	**189**		1.256±0.187	1.040±0.140	0.100±0.011	0.166±0.041
	190	22	1.378±0.184	1.136±0.031	0.099±0.004	0.166±0.040
12	**270**		0.743±0.042	0.714±0.120	0.137±0.002	0.226±0.039
	271	33	0.872±0.009	0.626±0.176	0.146±0.016	0.238±0.025
13	**272**		1.289±0.161	1.164±0.038	0.068±0.004	0.060±0.007
	273	25	1.057±0.074	0.766±0.072	0.077±0.010	0.097±0.009
14	**279**		**1.991±0.018**	**2.139±0.031**	0.150±0.010	0.228±0.017
	280	30	**2.152±0.029**	**1.767±0.029**	0.119±0.009	0.290±0.032
15	**258**		**1.730±0.047**	**1.876±0.205**	0.091±0.009	0.256±0.032
	259	13	**1.750±0.051**	**1.496±0.150**	0.107±0.007	0.259±0.045
16	**290**		**1.625±0.095**	**1.936±0.088**	0.136±0.011	**0.371±0.025**
	291	27	**1.628±0.070**	**1.864±0.101**	0.114±0.007	**0.316±0.020**
17	**293**		1.283±0.033	0.931±0.078	0.029±0.0007	0.055±0.002
18	**303**		0.854±0.073	0.324±0.047	0.089±0.014	0.159±0.009
	304	18	0.883±0.074	0.517±0.046	0.055±0.007	0.156±0.018
19	**308**		1.531±0.070	1.104±0.070	0.030±0.012	0.134±0.006
	309	20	1.309±0.098	0.833±0.045	0.054±0.008	0.110±0.025
20	**313**		0.974±0.019	0.310±0.026	**0.149±0.010**	0.109±0.015
21	**314**		1.589±0.241	0.512±0.044	0.101±0.014	0.077±0.010
	315	24	**1.891±0.055**	0.493±0.007	0.120±0.010	0.116±0.026
22	**388**		0.624±0.054	0.309±0.037	0.100±0.005	0.111±0.009
	389	22	0.664±0.070	0.088±0.026	0.063±0.007	0.127±0.021
23	**354**		1.072±0.239	1.096±0.026	0.121±0.014	0.195±0.014
	355	25	**1.711±0.054**	0.251±0.077	**0.237±0.028**	0.214±0.009
Control sera (n=8)			0.996±0.149	0.782±0.098	0.086±0.013	0.149±0.021

Table 2. Reactivity of the human sera from the patients bitten by unspecific ticks, against the *Hyalomma lusitanicum* (male or female) whole antigens.

Sera (nº) (n=3)	IgG (X±SE)		Sera (nº) (n=3)	IgG (X±SE)	
	Females	Males		Females	Males
1	1.036±0.096	0.608±0.015	**49**	0.650±0.047	0.966±0.058
2	0.897±0.149	0.895±0.048	**50**	0.585±0.072	0.967±0.036
3	0.465±0.014	1.140±0.053	**51**	0.276±0.013	**1.75±0.025**
4	0.377±0.016	0.896±0.015	**52**	0.296±0.030	0.763±0.031
5	0.887±0.096	0.643±0.100	**53**	0.479±0.046	0.570±0.053
6	1.031±0.077	0.802±0.055	**54**	0.346±0.014	0.700±0.176
7	0.279±0.043	0.593±0.088	**55**	0.299±0.005	0.676±0.022
8	0.915±0.055	0.898±0.038	**56**	0.817±0.015	0.605±0.127
9	0.630±0.043	0.425±0.045	**58**	0.343±0.021	1.225±0.067
10	0.544±0.024	0.650±0.088	**59**	0.415±0.098	0.525±0.037
11	0.378±0.056	0.802±0.065	**60**	0.349±0.042	0.519±0.123
12	0.311±0.008	0.606±0.060	**61**	0.434±0.010	0.492±0.035
13	0.447±0.061	0.554±0.025	**62**	0.430±0.012	1.023±0.008
14	0.117±0.024	0.176±0.009	**63**	0.325±0.041	0.585±0.103
15	0.386±0.017	0.884±0.060	**64**	1.040±0.024	1.156±0.193
16	0.778±0.093	0.527±0.103	**65**	0.824±0.140	0.685±0.015
17	1.019±0.114	0.613±0.038	**66**	0.424±0.012	0.722±0.094
18	0.938±0.045	0.666±0.030	**67**	0.498±0.066	1.476±0.055
19	0.234±0.007	0.314±0.017	**68**	1.077±0.081	1.004±0.082
20	0.485±0.089	0.614±0.055	**69**	0.927±0.068	0.800±0.172
22	0.671±0.048	0.466±0.045	**70**	0.234±0.042	0.461±0.107
24	0.473±0.055	0.211±0.026	**71**	0.323±0.014	0.891±0.200
26	0.383±0.055	0.250±0.058	**72**	0.876±0.030	0.446±0.130
28	0.389±0.041	0.555±0.082	**73**	0.524±0.060	ND
29	0.475±0.009	0.167±0.032	**74**	0.897±0.100	1.182±0.304
30	0.676±0.051	0.306±0.067	**76**	0.786±0.016	0.516±0.045
31	0.437±0.005	0.810±0.025	**77**	0.888±0.074	0.922±0.036
32	0.892±0.038	0.206±0.060	**79**	0.345±0.027	0.856±0.035
33	0.943±0.017	0.148±0.053	**80**	0.904±0.017	1.408±0.144
34	1.003±0.109	0.957±0.164	**81**	0.670±0.017	0.826±0.076
35	0.160±0.057	0.499±0.046	**82**	0.340±0.062	0.866±0.085
36	0.382±0.016	0.943±0.076	**83**	0.398±0.042	0.985±0.074
37	0.989±0.045	1.093±0.077	**84**	0.786±0.016	0.578±0.032
38	0.728±0.025	1.400±0.080	**85**	0.425±0.010	ND
39	0.224±0.055	1.306±0.080	**86**	0.212±0.007	0.777±0.060
40	0.752±0.067	0.970±0.136	**87**	0.322±0.061	0.671±0.016
41	0.699±0.018	0.506±0.052	**89**	1.013±0.117	0.926±0.103
42	0.556±0.019	1.043±0.228	**90**	0.408±0.091	0.950±0.176
43	0.238±0.013	0.928±0.155	**91**	0.649±0.062	0.946±0.066
44	0.389±0.029	1.012±0.147	**92**	0.891±0.016	0.966±0.117
45	0.894±0.084	1.184±0.065	**93**	0.574±0.084	1.054±0.056
46	0.785±0.089	1.320±0.077	**94**	0.218±0.057	0.658±0.035
47	0.476±0.011	1.382±0.120	**95**	0.609±0.055	1.053±0.127
48	0.528±0.033	0.857±0.106	**96**	0.438±0.055	1.462±0.250
Control sera ♀ (n=16)	0.570±0.045		**Control sera ♂ (n=18)**		0.780±0.194

Table 3. Results of the general population antibodies (IgG) against *Hyalomma lusitanicum* male and female antigens

4. Conclusion

Burgos is a province of the inner north western region of the Iberian Peninsula. Its climate is continental with cold dry winters and mild summers. Animal husbandry is a very important economical source and livestock parasitism of ticks is common in the region. *Hyalomma* spp. ticks are distributed in Africa, the Mediterranean climatic zone of southern Europe, and in Asia.

It is known that ticks are important pets and livestock transmitting tick-borrne diseases. In this sense, though humans are not the preferred hosts of *Hyalomma* ticks and are infrequently bitten in comparison to livestock, sporadic infection of people is usually caused by *Hyalomma* ticks.

In fact, Crimea-Congo Haemorrhagic Fever transmitted by *Hyalomma* spp occurs sporadically throughout many areas of Africa, Asia and Europe, but can cause mortality (Estrada-Peña & Jongejan., 1999). Recently a new group of spotted fever has been isolated from *Hyalomma marginatum marginatum* ticks in Morocco (Beati et al., 1997). In Europe have been detected genotipically similar organisms in Portugal (Beati et al., 1995), Croatia (Punda-Polic et al., 2002), Corsica (Matsumoto et al., 2004), Germany (Rumer et al., 2011) and in Spain (Fernández-Soto et al., 2003).

Hyalomma lusitanicum is called perinneal specie which is present on cattle year around. These parasitize domestic and wild animals and birds, and are usually abundant in semi-arid zones. Its distribution reflects peaks in May-June and October that corresponds to the periods of maximum activity of adults (Habela et al., 1999), but in winter specimens (males and females) remain fixed on their host without feeding (Yousfi-Monod & Aeschliman., 1986). Adult *Hyalomma* actively run out from their resting sites when a host approaches. Cattle, rabbits, hares and deer which are the hosts of *Hyalomma lusitanicum* are well represented in the studied region.

The frequency with which different tick species bite humans varies significantly from one zone to another and much it depends on the likelihood of humans entering their biotope (human contact with ticks for professional and recreational activities) and the tick affinity for humans. Climatic changes could be probable implicated in the northern establishment of ticks, but perhaps would be more dependent on the introduction of adult females on wild and domestic ruminants due to the uncontrolled movement of livestock as have been proposed recently Rumer et al., (2011) in which the only documented *Hyalomma* spp. tick in Germany was found on a human in the southern part of the country (Lake Constance area) in May 2006, but they did not ruled out the tick transportation from Spain.

However it is not easy to detect that a person has been bitten by a tick, because people may confuse the bite ticks or other arthropods that might be no elicit antibody response.

Ticks can inoculate a variety of active molecules during feeding that can block pain, reduce inflammation and suppress or modulate innate and specific acquired immune defences (Brossard & Wikel., 2004). But the duration of the tick attachment may be insufficient to allow for adequate amount of saliva to elicit a detectable antibody response. Sometimes happens that is necessary several tick exposures before the antibody response will be strongly enough to be detected by ELISA. Ticks deposit saliva at the site of their attachment

to a host in order to inhibit haemostasis, inflammation and innate and adaptive immune responses but ticks are able to modulate their host's local haemostatic reactions (Carvalho et al., 2010)

As happens with other ticks in which salivary gland proteins are immunogenic (Sanders et al., 1996), our results have shown that *H. lusitanicum* has proteins (antigens) that stimulate the production of immunoglobulins in humans as well as the finding of a significant high prevalence in bitten patients by ticks respect to the control group (see in Tables 1 and 2). It would be very interesting to conduct an epidemiological study, in those sera that have positive results, if at any time have had a history of febrile illness of unknown etiology with or without rash.

As occurs with other genus or species of ticks capable to induce high responses, *H. lusitanicum* could share antigens with other ticks. It is easy to found that antigens can cross react with the antigens of a closely related species. This fact has been observed in several studies that have realized cross-resistance studies between *Dermacentor andersoni* and *Dermacentor variabilis* (McTier et al., 1981), *Hyalomma anatolicum anatolicum* and *Boophilus microplus* (Parmar et al., 1996), *H. a. anatolicum, Hyalomma dromedari* and *Boophilus microplus* (Kumar & Kondal., 1999) as well as between a series of other tick species (Brown & Askenase., 1984; Jaansen van Vuuren et al., 1992). In fact, the homology among several tick species suggests that possibly common antigen(s) may be suitable for a vaccine against some different ticks.

By other hand, though ticks are well known blood suckers, blood sources between males and females seem to be different and gene expressions in fedding males and females are also different (Aljamali et al., 2009). In fact the male blood meals may be digested and nutrients can be used for spermatogenesis. The host blood meal is necessary for egg production in female ticks (Sanders et al., 1996). As occurs with partially fed female *Ixodes ricinus* (Linnaeus, 1758), female and male *Amblyomma variegatum* (Fabricius, 1794) and *Rhipicephalus appendiculatus* Neumann, 1901 in which have been observed that exist species- and sex-specific differences in the effects of tick salivary gland antigens on human lymphocyte proliferation, our results, as can be seen in Table III, have demonstrated that exist differences in the human antibodies response against male and female *H. lusitanicum* antigens. This fact makes that both could be considered as susceptible to bite humans.

Since for each tick-borne disease there may be one or several vectors (Lane, 1994), perhaps this ixodid could be implicated as a vector susceptible of parasitizing humans. In this sense, other studies would in a future provide us about the frequency and which life cycle stages of this tick can infest humans as well as its role in the transmission of human pathogens as happened with those studies in which *Dermacentor marginatus* was recently demonstrated to be the vector in the transmission of *Rickettsia slovaca*, that causes the TIBOLA/DEBONEL disease in humans (Rehacek, 1984; Lakos., 1997; Raoult et al., 1997; Ibarra et al., 2006).

Until date, each tick species has preferred environmental conditions and biotopes that determine the geographical distribution of the ticks and the risk areas fro tick borne diseases, but day to day more research studies are going on in order to elucidate a higher diversity of ixodid tick species infesting humans potentially transmisors of underdiagnosed diseases.

In this sense, we consider it would be very interesting to educate primary care physicians in these areas, to be able to identify ticks and how clinicians should deal with patients who have been bitten by ticks, because it would be very interesting to discover potential transmitters of both old and new diseases. It is necessary to develop studies in which ticks removed from the patients must be directly preserved in ethanol and identified because the knowledge about tick species that are susceptible of parasitizing humans is essential for assessing the risks for people who become infected, we can detect pathogens and design measures to prevent infection.

5. Acknowledgment

We want to give thanks to Helga Barrios Vidal and Gema Cervantes Uría for their grateful help in developing this study.

6. References

Aljamali ,M.N.; Ramakrishnan, V.G.; Weng, H.; Tucker, J.S.; Sauer, J.R & Essenberg R.C. (2009). Microarray analysis of gene expression changes in feeding female and male lone star ticks, *Amblyomma americanum* (L). *Archieves of Insect Biochemistry and Physiology*, Vol. 71, Nº 4 (August), pp. 236-253, ISSN 0739-4462.

Barandika, J.F.; Berriatua, E.; Barral, M.; Juste, R.A.; Anda, P & García-Pérez, A.L. (2006). Risk factors associated with ixodid ticks species distributions in the Basque region in Spain. *Medical and Veterinary Entomology*, Vol. 20, Nº 2 (June), pp 177-188, ISSN 0269-283X.

Beati, L.; Kelly, P.J.; Matthewman, L.A.; Mason, P.R. & Raoult, D. (1995). Prevalence of rickettsia-like organisms and spotted fever group rickettsiae inticks (*Acari: Ixodidae*) from Zimbabwe. *Journal of Medical Entomology*, Vol. 32, Nº 6 (November), pp. 787-792, ISSN 0022-2585.

Beati, L.; Meskini, M.; Thiers, B. & Raoult, D. (1997). *Rickettsia aeschlimann*ii sp. nov., a new spotted fever group rickettsia associated with *Hyalomma marginatum ticks*. *International Journal of Systematic Bacteriology*, Vol. 47, Nº 2 (April), pp. 548-554, ISSN 0020-7713.

Bengis, R.G.; Leighton, F,A.; Fischer, J.R.; Artois, M.; Mörner, T.& Tate, C.M. (2004). The role of wildlife in emerging and re-emerging zoonoses. *Revue Scientifique Technique (International Office of Epizootics)*, Vol. 23, Nº 2, pp. 497-511, ISSN 0253-1933.

Blancou, J.; Chomel, B.B.; Belotto, A. & Meslin, F.X. (2005). Emerging or re-emerging bacterial zoonoses: facts of emergence, surveillance and control. *Veterinary Research*, Vol. 36, pp. 507-522, ISSN 0928-4249.

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram of protein-dye binding. *Analytical Biochemistry*, Vol. 72, Nº 1-2 (May), pp. 248-254, ISSN 0003-2697.

Briones, V.; Téllez, S.; Ballesteros, C.; González, S.; Bordes, I.; Domínguez L *et al.* (2002). Enfermedades transmisibles de los mamíferos silvestres. *Quercus*, Vol. 198, pp. 22-27, ISSN 0212-0054.

Brossard, M. & Wikel, S. K. (2004). Tick immunobiology. *Parasitology*, Vol. 129, Suppl 1, pp. 161-176, ISSN 0031-1820.

Brown, S.J & Askenase, P.W. (1984). Analysis of host components mediating immune resistance to ticks, p.1040-1050. In: D. A Griffiths and C. E Browman (eds). *Acarology VI*, Vol 2. Ellis Horwood, Chichester, ISBN 9780130054890.

Bueno, R; Moreno, J.; Oltra, M.T. & Jiménez, R. (2009). Artrópodos con interés vectorial en la salud pública en España. *Revista Española de Salud Pública*, Vol. 83, Nº 2, pp. 201-214, ISSN: 2173-9110 .

Carvalho, W. A.; Maruyama, S. R.; Franzin, A. M.; Abatepaulo, A. R.; Anderson, J.M.; Ferreira, B. R.; Ribeiro, J.M.; Moré, D.D.; Augusto Mendes Maia, A.; Valenzuela, J. G.; Garcia, G. R. & de Miranda Santos, I. K. (2010). *Rhipicephalus* (Boophilus) *microplus*: clotting time in tick-infested skin varies according to local inflammation and gene expression patterns in tick salivary glands. *Experimental Parasitology*, Vol. 124, Nº 4 (April), pp. 428-435, ISSN 0014-4894.

Chomel, B. (1998). New emerging zoonoses: a challenge and an opportunity for the veterinary profession. *Comparative Immunology, Microbiology and Infectious Diseases*, Vol. 21, Nº 1 (March), pp. 1-14, ISSN 0147-9571.

Cleaveland, S.; Laurenso, M. K. & Taylor, H. (2001). Diseases of human and their domestic mammals: pathogen characteristics, host range and the risk of emergence. *Philosophical Transactionc Royal Society Londondon. Series B, Biological Sciences.*, Vol. 356, Nº 1411 (July), pp. 991-999, ISSN 0080-4622.

Cordero del Campillo, M.; Castañón Ordóñez, L. & Reguera Feo, A. (1994). *Índice-Catálogo de Zooparásitos Ibéricos*, p. 650. Universidad de León, Spain, ISBN 9788477194033, León.

Cunningham, A.A. (2005). A walk on the wild side emerging wildlife diseases. *British Medical Journal*, Vol. 331, Nº 7527 (November), pp. 1214-1215, ISSN 2007-9999.

Daszak, P.; Cunningham, A. A. & Hyatt, A. D. (2000). Emerging infectious diseases of wildlife threats to biodiversity and human health. *Science*, Vol. 287, Nº 5452 (January), pp. 443-449, ISSN 0036-8075.

Daszak, P. & Cunningham, A. A. (2003). Anthropogenic change, biodiversity loss, and a new agenda for emerging diseases. *Journal of Parasitology*, Vol. 89 Suppl (April), pp. 37-41, ISSN 0022-3395.

Domínguez, G. (2004). North Spain (Burgos) wild mammals ectoparasites. *Parasite*, Vol. 11, pp. 262-272, ISSN 1776-1042.

Encinas-Grandes, A. (1986). Ticks of the province of Salamanca (Central/NW/Spain). Prevalence and parasitization Intensity in dogs and domestic ungulates. *Annales de Parasitologie Humaine et Comparée*, Vol. 61, Nº1 (January), pp. 95-107, ISSN 0003-4150.

Encinas, A.; Oleaga, A. & Pérez, R. (1999). Garrapatas duras. En: Cordero del Campillo *et al.*, editores. *Parasitología Veterinaria.*, McGraw-Hill Interamericana; pp. 420-429, ISBN 9788448602369, Madrid.

Estrada-Peña, A. & Jongejan, F. (1999). Ticks feeding on humans: a review of records on human biting *Ixodoidea* with special reference to pathogen transmission. *Experimental and Applied Acarology,* Vol. 23, Nº 9 (September), pp. 685-715, ISSN 0168-8162.

Fernández-Soto, P.; Encinas-Grandes, A. & Pérez-Sánchez, R. (2003). *Rickettsia aeschlimannii* in Spain: molecular evidence in *Hyalomma marginatum* and five other tick species that feed on humans. *Emerging and Infectious Diseases*, Vol. 9, Nº 7 (July), pp. 889-890, ISSN 1080-6040.

Gibbs, E. P.J. (2005). Emerging zoonotic epidemics in the interconnected global community. *Veterinary Records*, Vol. 26, Nº 22 (November), pp. 673-679, ISSN 0042-4900.

Giménez-Pardo, C. & Martínez-Grueiro, M. M. (2008). Some hydrolase activities from the tick *Hyalomma lusitanicum* Koch, 1844 (*Ixodoidea*: *Ixodida*). *Parasite*, Vol. 15, Nº 4 (December), pp. 589-593, ISSN 1776-1042.

Gortázar, C.; Ferroglio, E.; Höfle, U.; Frölich, K.; Vicente, J. (2007). Diseases shared between wildlife and livestock: a European perspective. *European Journal of Wildlife Research*, Vol. 53, Nº 4 (April), pp. 241-256, ISSN 1612-4642.

Habela, M.; Rol, J. A.; Antón, J. M.; Pena, J.; Cordero, E.; van Ham, I. & Jorge, E. (1999). Epidemiology of mediterranean theileriosis in Extremadura region, Spain. *Parassitologia,* Vol. 41, Suppl 1 (September), pp. 47-51, ISSN 0048-2951.

Hillyard, P. D. (1996). Ticks of north-west Europe. Synopses of the British fauna. Ed. Barnes KRS & Crothers JH: The Linnean Society of London and the Estuarine and Coastal Sciences Association, ISBN 978-1851532575, London.

Ibarra, V.; Oteo, J. A.; Portillo, A, Santibáñez, S.; Blanco, J. R.; Metola, L.; Eiros, J. M.; . &, Sanz, M. (2006). *Rickettsia slovaca* infection: DEBONEL/TIBOLA. *Annals of the New York Academy of Science.* Vol. 1078 (October), pp. 206-214, ISSN 0077-8923.

Jansen van Vuuren, A.M.; Crause, J.C.; Vershoor, J.A.; Spickett, A.M. & Neitz, A.N. (1992). The identification of a shared immunogen present in the salivary glands and gut of ixodid and argasid ticks. *Experimental and Applied Acarology* Vol. 15, Nº 3, pp. 205-210, ISSN 0168-8162.

Kaufman W. (1989). Tick-host interaction: A synthesis of current concepts *Parasitology Today* Vol. 5, Nº 2, pp. 47-56. ISSN 0169-4758.

Kettle, D.S. (1995). Medical and veterinary entomology. 2th ed. Cambridge: CAB International, ISBN : 0-851-98968-3.

Kolonin, G.V. (2009). Fauna of Ixodid ticks of the world (*Acari, Ixodidae*). Moscow, ISSN 0132-8077.

Kumar, S. & Kondal, J.K. (1999): Lack of immunological cross-reactivity of 36 kDa secretory salivary glands antigen of *Hyalomma anatolicum anatolicum* with *Hyalomma*

dromedary and *Boophilus microplus* ticks. *Zentralblatt für Veterinärmedizin. Reihe* B. Vol. 46, Nº 6 (august), pp. 405-410, ISSN 0514-7166.

Lane, S. L. (1994). Competence of ticks as vectors of microbial agents with an emphasis on *Borrellia burgdorferi,* p. 45-67. In: Sonen Shine, DE, Mather, TN. (eds), *Ecological dynamics of tick-borne zoonoses*, Oxford University Press, ISBN 978-0195073133, New York.

Lakos, A. (1997). Tick-borne lymphadenopathy, a new rickettsial disease. *The Lance*t. Vol. 350, Nº 4 (October), pp. 1006, ISSN 0140-6736.

Lindgren, E.; Talleklint, L.; Polfeldt, T. (2000). Impact of climate change on the northern latitude limit and population density of the disease transmitting European tick *Ixodes ricinus*. *Environmental Health Perspectives*, Vol. 108, Nº 2 (February), pp. 119-123, ISSN 0091-6765.

Lledó, L.; Giménez-Pardo, C.; Domínguez-Peñafiel, G; Sousa, R.; Gegundez-Cámara, M.I.; Casado, N. & Criado-Fornelio, A. (2010). Molecular detection of Hemoprotozoa and *Rickettsiae* sp in arthropods from wild animals in the Burgos province, Spain. *Vector-Borne and Zoonotic Diseases*, Vol. 10, Nº 8 (October), pp. 735-738, ISSN 1530-3667.

López-Vélez, R. & Molina, R. (2005). Cambio climático en España y riesgo de enfermedades infecciosas y parasitarias transmitidas por artrópodos y roedores. *Revista Española de Salud Pública*, Vol. 79, Nº 2, pp. 177-190, ISSN 1135-5727.

Matsumoto, K.; Parola, P.; Brouqui, P. & Raoult,D. (2004). *Rickettsia aeschlimannii* in *Hyalomma* ticks from Corsica. *European Journal of Clinical Microbiology and Infectious Diseases*, Vol. 23, Nº 9 (August), pp. 732-734, ISSN 0934-9723.

McTier, T.L.; George, T.E. & Benett, S.N. (1981). Resistance and cross-resistance of guinea pigs to *Dermacentor andersoni* Stiles, *D. variabilis* (Say), *Amblyomma americanum* (Linnaeus) and *Ixodes scapularis* (Say). *Journal of Parasitology*, Vol. 67, Nº 6 (December), pp. 813-832, ISSN: 1937-2345.

Morse, S. S. (1995). Factors in the emergence of infectious diseases. *Emerging Infectious Diseases,* Vol. 1, No. 1 (January-March), pp. 7-15, ISSN 1080-6040.

Parmar, A.; Grewal A.S. & Dhillon P. (1996). Immunological cross-reactivity between salivary gland proteins of *Hyalomma anatolicum anatolicum* and *Boophilus microplus* ticks. *Veterinary Immunology and Immunopathology,* Vol.1, Nº 3-4 (June), pp. 345-352, ISSN 0165-2427.

Pérez-Sánchez, R.; Oleaga-Pérez, A. & Encinas-Grandes, A. (1992). Analysis of the specificity of the salivary antigens of *Ornithodoros erraticus* for the purpose of serological detection of swine farms harbouring the parasite. *Parasite Immunology*, Vol. 14, Nº 2 (March), pp. 201-216, ISSN 0141-9838.

Punda-Polic, V.; Petrovec, M.; Trilar, T.; Duh, D.; Bradaric, N.; Klismanic, Z. & Basic-Zupanc, T. (2002). Detection and identification of spotted fever group rickettsiae in ticks collected in southern Croatia. *Experimental and Applied Acarology*, Vol. 28, Nº 1-4, pp. 169-176, ISSN 0168-8162.

Raoult, D.; Berbis, P.; Roux, V.; Xu, W. & Maurin, M. (1997). A new tick-transmitted disease due to *Rickettsia slovaca*. *The Lancet*, Vol. 350, Nº 12 (July), pp. 112-113, ISSN 0140-6736.

Rehacek, J. (1984). *Rickettsia slovaca*, the organism and its ecology. *Acta Scientiarum Naturalium Academiae Scientiarum Bohemoslovacae* ,Vol. 18, No 2 pp. 1-50, ISSN 0032-8758.

Rolníková, T.; Kazimírová, M.; Buc, M. (2003). Modulation of human lymphocyte proliferation by salivary gland extracts of ixodid ticks (*Acari: Ixodidae*): effect of feeding stage and sex. *Folia Parasitologica*, Vol. 50, Nº 4, pp. 305-12, ISSN 0015-5683.

Román, J; Román, L.; Ansola, L.M;, Palma, C. & Ventosa, R. (1996). Atlas de las aves nidificantes en la provincia de Burgos. Ed. Aldecoa, ISBN 978-84-7009-485-9, Burgos.

Rumer, L.; Graser, E.; Hillebrand, T.; Talaska, T.; Dautel, H.; Mediannikov, O.; Roy-Chowdhury, P.; Sheshukova, O.; Donoso Mantke, O. & Niedrig, M. (2011). *Rickettsia aeschlimannii* in *Hyalomma marginatum* ticks, Germany. *Emerging Infectious Diseases* , Vol. 17, Nº 2 (February), pp. 325-325, ISSN 1080-6040.

Sanders, M,L.; Scott, A. L.; Glass, G.E. & Schwartz, B.S. (1996). Salivary gland changes and host antibody responses associated with feeding of male lone star ticks (*Acari:Ixodidae*). *Journal of Medical Entomology,* Vol. 33, Nº 4 (July), pp. 628-34, ISSN: 0022-2585.

Sauer, J.R.; McSwain, J.L.; Bowman, A.S. &. Essenberg, R.C. (1995). Tick salivary gland physiology. *Annual Review of Entomology*, Vol. 40, (January), pp. 245-267, ISSN: 0066-4170.

Simpson, V. R. (2002). Wild animals as reservoirs of infectious diseases in the UK. *The Veterinary Journal*, Vol. 163, Nº 2 (March), pp. 128-146, ISSN: 1090-0233.

Sonenshine, D.; Lane, R. S. & Nicholson, W. L. (2002). Ticks (Ixodida). In: Mullen G & Durden L, editors. *Medical and Veterinary Entomology*, Elsevier Science, pp. 517-557, ISBN 978-0125104517, Orlando.

Toledo, A.; Olmeda, S.; Escudero, R.; Jado,I.; Valcárcel, F.; Casado-Nistal,M.; Rodríguez-Vargas, M.; Gil, H. & Anda, P. (2009). Tick-borne zoonotic bacteria in ticks collected from central Spain. *American Journal of Tropical Medicine and Hygiene*, Vol. 81, Nº 1 (July), pp. 67-74, ISSN 0002-9637.

Valenzuela, J. G.(2004). Exploring tick saliva: from biochemistryto"sialomes" and functional genomics. *Parasitology*. Vol. 129, Suppl, pp. S83-S94, ISSN 0031-1820.

Viseras, J. & García-Fernández, P. (1999). Studies on theileriosis in Southern Spain. *Parassitologia*, Vol. 41, Suppl 1 (September), pp. 111-115, ISSN 0048-2951.

Wall, R. & Shearer, D. (1997). *Veterinary Entomology: Arthropods Ectoparasites of Veterinary importance*. 1th edition, Chapman & Hall, ISBN 978-0412615108, London.

Wall, R. (2007). Ectoparasites: Future challenges in a changing world. *Veterinary Parasitology*, Vol. 148, Nº 1 (August), pp. 62-74, ISSN 0304-4017.

Yousfi-Monod, R. & Aeschlimann, A. (1986). Studies on ticks (Acarina: Ixodidae), parasites of cattle in west Algeria. I. Systematic survey and seasonal activity. *Annales de parasitologie humaine et comparee,* Vol. 61, Nº 3, pp. 341-358, ISSN 0003-4150.

Zeier, M.; Handermann, M.; Bahr, U.; Rensch, B.; Müller, S.; Kehm, R, et al. (2005). New ecological aspect of hantavirus infection: A change of paradigm and challenge of prevention. *Virus Genes*, Vol. 3, Nº 2 (March), pp. 157-180, ISSN 0920-8569.

14

Amoebiasis in the Tropics: Epidemiology and Pathogenesis

A. Samie[1], A. ElBakri[2] and Ra'ed AbuOdeh[2]
[1]*Molecular Parasitology and Opportunistic Infection Program, Department of Microbiology, University of Venda, Private Bag X5050 Thohoyandou 0950*
[2]*Medical Laboratory Technology Department, College of Health Sciences, University of Sharjah, Sharjah,*
[1]*South Africa*
[2]*United Arab Emirates*

1. Introduction

Entamoeba histolytica is a protozoan parasite that causes amebic dysentery and liver abscess. The disease is common in tropical regions of the world where hygiene and sanitation is often approximate. The epidemiology of *E. histolytica* has been studied around the world. However, there is a dearth of comprehensive literature on the epidemiology of this pathogen as well as its pathogenicity in the tropical and underdeveloped regions of the world where the disease is actually more common. Epidemiological figures in many endemic tropical countries are often overestimated because of inaccurate identification. Accurate data on the prevalence of the pathogenic strain(s) of *E. histolytica* in those regions will allow for the effective cure of patients with anti-amoebic drugs thus preventing the development of resistant types and reducing management costs.

With the advents of HIV and AIDS, several organisms have been identified as potential opportunistic pathogens. However, it is not clear whether amoebiasis is an opportunistic infection or not. Up to date, very little data has been published on the occurrence of *E. histolytica* in relation to HIV and AIDS. In developed countries amebiasis tends to be more common in older patients and occurs mostly among men who have sex with men or in institutions. However, in tropical regions, the epidemiology of amoebiasis is completely different and is more common among the general population and particularly among patients attending health care centers with diarrhea. Therefore, it is important to understand the epidemiology of this pathogen in tropical areas where it is responsible for most morbidity and mortality.

The recent reclassification of *E. histolytica* into different species now including the pathogenic *Entamoeba histolytica* and the non pathogenic *Entamoeba dispar* and *Entamoeba moshkovskii* has further added to the complexity of the epidemiology of amoebiasis since these three species cannot be differentiated by microscopy that is the most commonly used diagnostic method particularly in tropical countries where resources are limited, but can only be differentiated by the use of molecular methods such as the polymerase chain reaction based methodologies. Recent development of simpler but more sensitive methods

such as the Loop-Mediated Isothermal Amplification (LAMP) should improve the understanding of the epidemiology of this disease.

Over the past few years we have studied the epidemiology of *E. histolytica* in African countries (Cameroon, Zimbabwe, and South Africa). In the present chapter, we review these and other studies conducted in the African continent as well as other tropical regions in the light of new and more specific and sensitive molecular methods. The pathogenesis mechanism of amoebiasis is still not clear and recently differences in population levels of *E. histolytica* strains isolated from asymptomatic and symptomatic individuals have been shown to exist. One of the factors believed to be the determinant of the various clinical presentations of the disease is the organism's virulence. The different methodologies used for the detection and epidemiology of amoebiasis will be reviewed as well as the role of *E. histolytica* in HIV disease. Recent advances on the pathogenesis and control of amoebiasis will also be reviewed.

Amoebiasis caused by the protozoan parasite *E. histolytica* was first recognized as a deadly disease by Hippocrates who described a patient with fever and dysentery (460 to 377 B.C.). With the application of a number of new molecular biology-based techniques, tremendous advances have been made in our knowledge of the diagnosis, natural history, and epidemiology of amoebiasis. Amoebiasis remains an important health problem in tropical countries where sanitation infrastructure and health are often inadequate (Ximénez et al., 2009). Clinical features of amoebiasis range from asymptomatic colonization to amoebic colitis (dysentery or diarrhea) and invasive extraintestinal amoebiasis, which is manifested most commonly in the form of liver abscesses (Fotedar et al., 2007). Current WHO estimates of 40-50 million cases of amoebic colitis and amoebic liver abscess (ALA) and up to 100,000 deaths annually, place amoebiasis second only to malaria in mortality (Stanley 2003; Ravdin 2005; WHO/PAHO/UNESCO 1997). Global statistics on the prevalence of *E. histolytica* infection indicates that 90% of individuals remain asymptomatic while the other 10% develop clinically overt disease (Jackson et al, 1985; Haque et al., 1999). Although all the deaths could be due to invasive *E. histolytica* infections, the value for the prevalence of *E. histolytica* is an overestimate since it dates from before the separation of the pathogen *E. histolytica* from the non-pathogen *E. dispar* (Diamond & Clark, 1993). Recently however, *Entamoeba moshkovskii*, a morphologically identical species, has been detected in individuals inhabiting endemic areas of amoebiasis (Ali et al., 2003, Fotedar et al., 2008, Khairnar et al., 2007, Parija & Khairnar, 2005) and could be contributing to the prevalence figures. Thus, the reclassification of *E. histolytica* into the three morphologically identical yet genetically different species has further added to the complexity of the epidemiology of amoebiasis since they cannot be differentiated by microscopy that is the most commonly used diagnostic method particularly in tropical countries where resources are limited. Furthermore, the worldwide prevalence of these species has not been specifically estimated. Thus, obtaining accurate species prevalence data remains a priority as there are gaps in our knowledge for many geographic regions of the tropics.

Although only a minority of *E. histolytica* infections - one in every four asymptomatic intestinally infected individuals - progress to development of clinical symptoms (Gathiram and Jackson, 1987; Blessmann et al., 2003; Haque et al., 2006), the exact basis for this difference remains mostly unsolved. This might be partly due to the differences in the pathogenic potential of the infecting strains (Burch et al., 1991) and/or the parasite genotype

(Ali et al., 2007) or due to the variability of the host immune response against amoebic invasion (Mortimer and Chadee, 2010).
The disease mechanism and the exact prevalence and incidence of infection caused by *E. histolytica* are still unknown. The epidemiological data available for endemic countries however, albeit sporadic, is based mostly on the microscopic identification of the *E. histolytica/E. dispar/E. moshkovskii* complex, often inaccurately reported as "*E. histolytica*". To date many highly sensitive and specific techniques such as enzyme-linked immuno-sorbent assays (ELISA) and polymerase chain reaction (PCR) have been developed for the accurate identification and detection of *E. histolytica* in various clinical samples (Ackers, 2002). It is anticipated that these molecular tools will allow us to reconstruct a more reliable picture of the true epidemiology of the disease mainly in endemic regions of the world and to better our understanding of the role of the parasite and/or host factors that determine the disease outcome.

2. Biology of *Entamoeba histolytica*

Entamoeba histolytica trophozoites (Figure 1) live and multiply indefinitely within the mucosa of the large intestine feeding normally on starches and mucous secretions and interacting metabolically with the host's gut bacteria. However, such trophozoites commonly initiate tissue invasion when they hydrolyze mucosal cells and absorb the predigested products in order to meet their dietary provisions. Filopodia (tiny cytoplasmic extensions) that form from the surface of their trophozoites are believed to play a role in the pathogenicity of certain strains. Examples of functions related to pathogenesis include: endocytosis and/or pinocytosis, exocytosis, tissue penetration, cytotoxic substances release or contact cytolysis of host cells. Other host factors that may also influence the invasiveness of *E. histolytica* are the oxidation-reduction potential and gut contents pH both of which are largely influenced by the overall nutritional state of the host.
Once the parasites invade the intestinal wall, they reach the submucosa and the underlying blood vessels. From there, trophozoites travel in the blood to sites such as the liver, lungs or skin. These parasite forms are now considered to be dead-end course since they cannot leave the host and cause infection in others. Encystation occurs in the intestinal lumen, and cyst formation is complete when four nuclei are present. These infective cysts are passed into the environment in human feces and are resistant to a variety of physical conditions. On occasions, trophozoites may exit in the stool, but they cannot survive outside the human host. The signals leading to encystations or excystation are poorly understood, but findings in the reptilian parasite *Entamoeba invadens* suggest that ligation of a surface galactose-binding lectin on the surface of the parasite might be the one trigger for encystations (Stanley, 2003; Eichinger, 2001). Also, several previous proteomic and transcriptomic studies have shown that a few dozens of Rab genes/proteins are involved in important biological processes, such as stress response, virulence, and pathogenesis, and stage conversion (Picazarri et al., 2008; Chatterjee et al., 2009; Novick and Zerial, 1997; Stenmark, 2009; Nozaki and Nakada-Tsukui, 2006). EhRab11A was reported to be recruited to the cell surface by iron or serum starvation, and was suggested to be involved in encystation (McGugan and Temesvari, 2003). In contrast, EhRab11B is involved in cysteine protease secretion, and its overexpression enhanced the secretion of cysteine protease (Mitra et al., 2007; Nozaki and Nakada-Tsukuia, 2006).

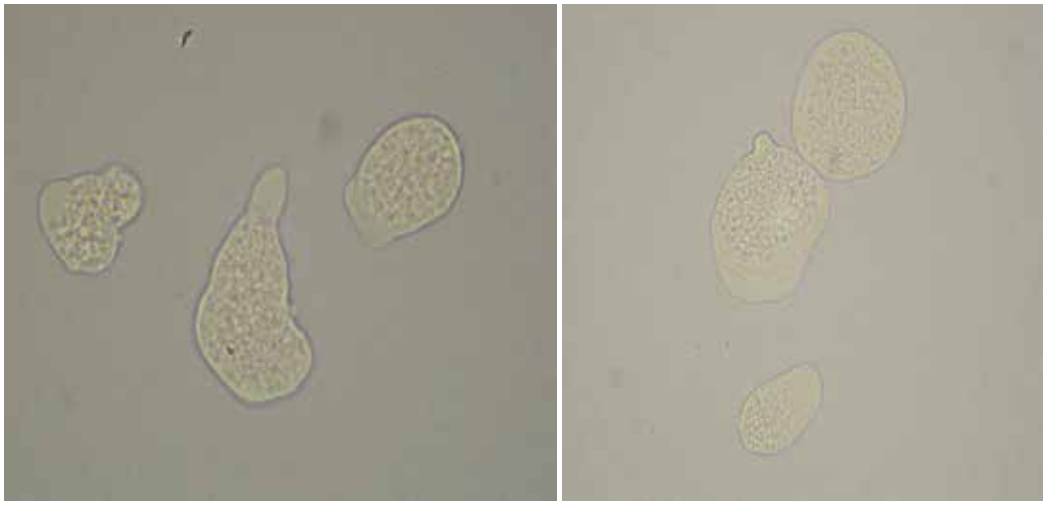


Fig. 1. *Entamoeba histolytica* trophozoites observed under the microscope stain with methylene blue (Observe that the cells did not accept the stain since they were still alive at the time the picture was taken) (Photos by Samie A)

The life cycle of *E. histolytica* is simple and consists of an infective cyst stage (10 to 15 µm in diameter) and a multiplying trophozoite stage (10 to 60 µm in diameter). Like other protozoa, *E. histolytica* appears incapable of de novo purine synthesis. Biochemical analysis has indicated that glutathione is not present. For this reason, *E. histolytica* is different from higher eukaryotes. It also uses pyrophosphate instead of ATP (McLaughlin and Aley, 1985). Mature cysts in the large intestine leave the host in large numbers and remain viable and infective in a moist, cool environment for at least 12 days. In water, cysts can live for up to 30 days. Nonetheless, they are rapidly killed by desiccation, and temperatures below 5°C and above 40°C. Mature cysts are also resistant to chlorine levels normally used to disinfect water. When swallowed, cysts pass through the stomach unharmed. In the small intestine, where conditions are alkaline and as a result of nuclear division, eight motile trophozoites are produced. These motile trophozoites then settle in the large intestine lumen, where they divide by binary fission and feed on host cells, bacteria and food particle (Figure 2). This is the first chance of the parasite making contact with the mucosa.

The organisms' biochemistry and metabolism have been reviewed by McLaughlin and Aley (1985). It has many hydrolytic enzymes, including phosphatases, glycosidases, proteinases, and an RNAse. Major metabolic end products are carbon dioxide, ethanol and acetate. *E. histolytica* is more of a metabolic opportunist which is able to exploit oxygen when it is present in the environment. Glucose is metabolized via the Embden-Meyerhof pathway exclusively, and fructose phosphate is phosphorylated, prior to lysis, by enzymatic reactions unique to *Entamoeba* spp. Pyruvate is converted mostly to ethanol, even in the presence of oxygen, via coenzyme-A, and pyruvate oxidase. Terminal electron transfers are accomplished with ferredoxinlike iron-sulphur proteins, a trait that may contribute to the efficacy of metronidazole in treatment. Similar metabolic traits in *Trichomonas vaginalis* and *Giardia lamblia* also are metronidazole targets. Mitogen Activated Protein Kinases (MAPK) – a group of proline directed serine/threonine kinases

(Bardwell, 2006) - regulate a number of different cellular processes such as proliferation, and response to a variety of environmental stresses like osmotic stress, heat shock and hypoxia (Junttila, 2008). The existence of MAPK homologues has been documented in certain parasitic protozoa. For instance ERK1 and ERK2 homologues of *Giardia lamblia* have been shown to play a critical role in trophozoite differentiation into cysts (Ellis et al., 2003), Pfmap2, a MAPK homologue in *Plasmodium falciparum* is essential for the completion of the asexual phase of the parasite lifecycle (Dorin-Semblat et al., 2006) and *Leishmania major* MAPK homologues exhibit an increased phosphotransferase activity in response to pH and temperature shift (Morales et al., 2007). On the other hand, *E. histolytica* has been shown to possess a single homologue of a typical MAPK gene (EhMAPK). Activation of EhMAPK in *E. histolytica* has been found to be associated with stress survival such as heat shock and oxidative stress response (Ghosh et al, 2010).

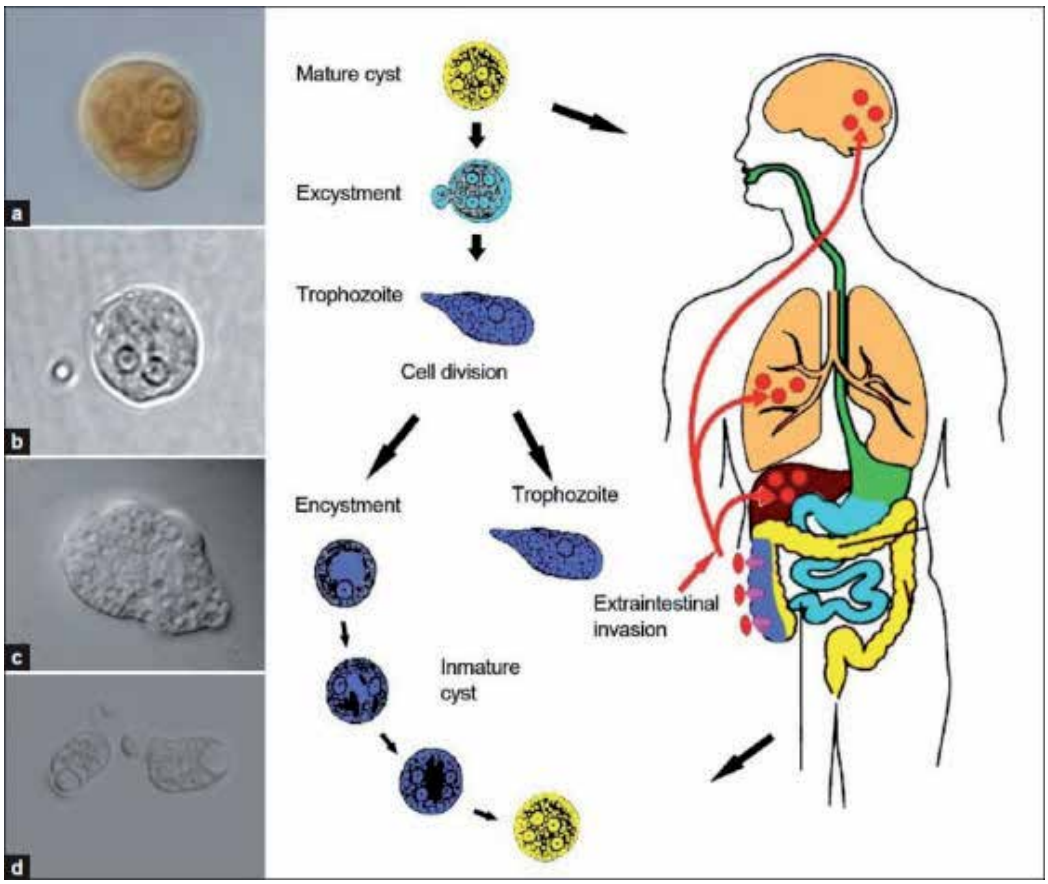


Fig. 2. Life cycle of *E. histolytica*/*E. dispar*. a) Mature cyst stained with 4% Lugol solution (100× magnification). b) Mature cyst without staining (100×). c) Trophozoite observed with differential interference contrast (DIC) (100×). d) Trophozoites of *E. histolytica* species with phagocyted erythrocytes (DIC 40×). Obtained with permission from Ximenez et al (2011).

3. Epidemiology of amoebiasis and its occurrence in the era of HIV and AIDS

The epidemiology of amoebiasis around the world is complicated by the existence of three different forms that are morphological identical but genetically distinct and include *E. histolytica* which is a known pathogen, *E. dispar* and *E. moshkovskii* which are non pathogens (Ali et al., 2008). This is particularly relevant to the African continent as well as many other developing countries in the world, including Latin American and Asian countries, where there is lack of specific diagnostic tools. According to some studies conducted in some African countries (Alonzo et al., 1993; Molback et al., 1994; Njoya et al., 1999; Roche et al., 1999) from 6% to 75% of the population carry the parasite. These studies were conducted using microscopic examination giving a general idea on the distribution of the disease in the population. Such results require confirmation by techniques that clearly differentiate *E. histolytica* from *E. dispar*, which is not pathogenic. Countries in Central and Latin America where the parasite displays endemic behavior include Mexico, Brazil, and Ecuador. In Mexico for example, the incidence rate of intestinal amoebiasis from 1995 to 2000 was reported to be between 1000 and 5000 cases/100,000 inhabitants annually. Incidence values from 2002 to 2006 were 1128.8 to 615.85/100,000 inhabitants per year. As in other developing countries, those under 15 years of age were the most frequently affected group, with a notable increase in children aged 5–9 (Ximenez, 2009). In Aracaju, Brazil, Lawson et al (2004) demonstrated *E. histolytica* in 1% of cases whereas *E. dispar* was found in 13% of the cases. Whilst in Pernambuco state, northeastern Brazil *E. dispar* was found in 74.19% of culture positive samples using the PCR method, no *E. histolytica* was reported (Pinheiro *et al*., 2004). In a remote area of Ecuador, Gatti et al (2002) using isoenzyme analysis reported an 18.9% infection rate with *E. histolytica* while 70.3% were infected with *E. dispar*. In the Indian subcontinent, the prevalence of intestinal amoebiasis among hospitalized patients was found to be around 11.7% using microscopy. However, using molecular biology tools such as PCR, *E. histolytica* was shown to be in 3.5% of those infected (Khairnar et al., 2007). In another endemic country such as Bangladesh and using ELISA antigen detection kits, *E. histolytica* prevalence was found to be 4.2% among children living in the urban slums of Dhaka (Haque et al. 2006). Many studies have been conducted in different parts of the world, (Ghosh et al., 2000) but the region most concerned by this problem (Africa) remains unexplored. Thus, the epidemiology of amoebiasis still remains very uncertain particularly in this part of the world.

Following the HIV/AIDS pandemic, numerous studies demonstrated that intestinal parasites such as *Cryptosporidium* sp, Microsporidia sp, *Isospora belli*, and *Cyclospora cayetenensis* were frequently associated with episodes of severe and often fatal diarrhea in both industrialized and poor countries. There have been controversies around the impact of HIV on the occurrence of amebiasis. However, recent data have shown an increase in the occurrence of *E. histolytica* among HIV patients in countries such as Japan, Mexico, Taiwan, and South Africa (Moran et al., 2005; Hung et al., 2008; Samie et al., 2009; Watanabe et al., 2011). With the hall mark of HIV infection being the depletion of CD4+ T cells count (below 200 cells/μl) and the progressive decline of the mucosal immunologic defense mechanisms, HIV/AIDS patients become prone to life-threatening gastrointestinal manifestations such as diarrhea (Stark et al., 2009). Table 1 provides a summary of the prevalence studies reporting *E. histolytica* and/or *E. histolytica*/*E. dispar* infections in HIV positive individuals in different countries. The association of *E. histolytica* infections with HIV positive individuals in some studies is not clear-cut. In a Mexican study no clear association between *E. histolytica* and

HIV has been noted. In this study, the prevalence of *E. histolytica* in HIV/AIDS patients was 25.3% compared to 18.4% in a control HIV-group (Moran et al., 2005). Other studies in South American countries have shown no obvious association. However, a significant association between high levels of serum anti-*E. histolytica* antibodies and the presence of *E. histolytica* in the stool has been noted in studies from both Vietnam (Blessman et al., 2006) and Africa (Stauffer et al., 2006). In a South African study in the Vhembe district in the northern part of the country, a positive association between *E. histolytica* infection and HIV-positive individuals has been indicated. Among the HIV-positive individuals, those with CD4+ count less than 200 cells/μl, were relatively more likely to be seropositive for *E. histolytica* (Samie et al., 2010). In a Chinese study, a higher seroprevalence of *E. histolytica* infections was also found in HIV-infected patients (Chen et al, 2007). Furthermore, two studies conducted in Taiwan revealed a positive association as well (Hung et al., 2005; Tsai et al., 2006).

Country	**Prevalence of *Entamoeba species***	**Reference**
Cuba	1.5% (*E. histolytica/dispar*)	Escobedo, A. A. 1999
Bogota, Colombia	*13%* (*E. histolytica*)	Florez et al., 2003
San Pedro Sula, Honduras	*5.8%*(*E. histolytica*)	Lindo et al., 1998
Venezuela (Zulia state)	*10.8%*(*E. histolytica*)	Rivero et al., 2009
Brazil	3.3% and 1% (*E. histolytica/ dispar* before and after HAART)	Bachur et al., 2008
Mexico	25.3% in HIV+ and 18.5% in HIV-contacts (*E. histolytica*)	Moran et al., 2005
Tajikistan	25.9% (*E. histolytica/dispar* non HIV)	Matthys et al., 2011
Northern India	*7.7% (E. histolytica)*	Prasad et al., 2000
Taiwan	*5.8%* (*E. histolytica* in HIV patients)	Hung et al., 2008
Bangladesh	2.1% vs. 1.4% in diarrhea and control (*E. histolytica*)	Haque et al., 2009
India (Kolkata)	*3.6% (E. histolytica)*	Mukherjee et al., 2010
Sydney, Australia	*3.2% (E. histolytica/E. dispar)*	Stark et al., 2007
Mazandaran province, Iran	1.6% (*E. histolytica*)	Daryani et al., 2009
Uganda	1.4% (*E. histolytica*)	Brink et al., 2002
Ethiopia	10.3% (*E. histolytica*)	Hailemariam et al., 2004
Dakar, Senegal	5.1% (*E. histolytica*)	Gassama et al., 2001
South Africa	12.4% (*E. histolytica*)	Samie et al., 2006

Table 1. Global prevalence of *E. histolytica* in HIV-infected and non-infected persons.

Over the past decade, there has been an increasingly reported risk of amebiasis in East Asian countries like Japan, Taiwan and South Korea particularly among men who have sex with men (MSM) probably due to oral-anal sexual contact (Hung et al., 2008; Watanabe et al., 2011). In Japan, *E. histolytica* often occur in institutions of mentally retarded individuals where outbreacks of amebiasis have been described with the prevalence rate and positive serology rate as high as 38.2% and 67.1%, respectively (Nishise et al., 2010) and has been

occurring more often in HIV positive patients (Watanabe et al., 2011). In addition to HIV/AIDS, the increasing use of organ transplants and other immunosuppressed conditions such as neutropenia have been considered important risk factor for invasive amoebiasis in many countries. In Colombia for example, a study of organ transplant patients revealed that about 24.7% had detectable antiamoebic antibodies (Reyes et al., 2006) whereas in another study 14.3% neutropenic patients were found to have antiamoebic antibodies (Cardona et al., 2004).

Certain risk behaviors, such as homosexual relations and practicing oro-anal sex, can exacerbate the possibility of acquiring *E. histolytica* infections as well as other intestinal parasites such as *Cryptosporidium* spp., where the symptomatic pictures are more severe than those of immunocompetent individuals (Tatiana et al., 2008; Hung 2008). A recent study in Vietnam had indicated that socio-economic and personal hygiene factors determined infection with *E. histolytica*, rather than exposure to human and animal excreta in agricultural activities (Pham duc et al., 2011). In a study in Bangladesh, it was shown that wet environment is not the only factor that affects the detection curve of *E. histolytica*, but anti-Carbohydrate Recognition Domain IgA level in the gut is another determining factor for its occurrence in a closed population (Haque et al., 2006). Although, numerous seroprevalence studies suggest that HIV/AIDS individuals are at a higher risk of *E. histolytica* infections and are therefore more likely to develop symptomatic infections or severe forms of the disease, modest data exist to support these findings and further research is needed to confirm this hypothesis.

4. Diagnosis of amoebiasis

Amoebiasis diagnosis rests on the demonstration of *E. histolytica* trophozoites or cysts in stool or colonic mucosa of patients. For many years a direct smear examined either as a wet mount or fixed and stained was done by microscopic examination of stool. Repeated stool sample examinations (at least three) may be needed. The presence of haematophagous amoebic trophozoites in a stool sample has always suggested *E. histolytica* infections (Gonzalez-Ruiz, A. et al 1994). Nonetheless, the specificity of this finding was further reduced when it was demonstrated that in some patients *E. dispar* also contains RBCs (Fotedar et al., 2007). Also, in view of the high frequency of *E. dispar* in many areas, dysentery due to entities such as shigellosis and campylobacter will probably be misdiagnosed as amoebic colitis if microscopy is the sole diagnostic criteria (Stanley 2003). However, in the absence of haematophagous trophozoites, the sensitivity of microscopy is limited by its ability to distinguish between samples infected with *E. histolytica* and the morphologically identical *E. dispar* and *E. moshkovskii*. Confusion between *E. histolytica*, other non-pathogenic amoeba and white blood cells such as macrophages and polymorphonuclear cells in feces frequently result in the overdiagnosis of amoebiasis. Delays in the processing of stool samples affect the sensitivity of light microscopy, which under the best circumstances is only 60% of that of the stool culture method followed by isoenzyme analysis (Krogstad et al., 1978).

Stool culture technique followed by isoenzyme analysis has been considered as the "gold standard" for many years. This method has been used to distinguish between *E. histolytica* and *E. dispar*. For more details on the culture technique the reader is advised to consult reference (Clark and Diamond, 2002). Culture of *E. histolytica* can be performed from fecal specimens, rectal biopsy specimens, or liver abscess aspirates. However, the process usually

takes between 1-4 weeks to perform and requires sophisticated laboratory equipment making it not feasible as a routine procedure especially in the developing world where *E. histolytica* is rampant. The rate of success of *E. histolytica* culture in reference laboratories has been reported to be between 50 and 70%. Moreover, isoenzyme (zymodeme) analysis is labor intensive, costly and often produces false-negative results for many microscopy positive stool specimens (Strachan et al., 1988).

Serological methods may be useful diagnostically to detect infections with *E. histolytica* in developed countries where infections are not as common as in endemic developing nations (Ohnishi et al., 1997). In developing countries individuals are constantly exposed to *E. histolytica* making serological tests unable to definitively distinguish past from current infections (Caballero et al., 1994). Amoebic serology is highly sensitive and specific for the diagnosis of ALA (Zengzhu et al., 1999). Conversely, a study of asymptomatic individuals living in an *E. histolytica* endemic area of Vietnam revealed that about 83% of those infected had detectable anti-amoebic antibodies (Blessmann et al., 2002). Several assays for the detection of antibodies to *E. histolytica* infections have been developed (Table 2). These include: indirect hemagglutination (IHA), latex agglutination, immunoelectrophoresis, counterimmunoelectrophoresis (CIE), the amebic gel diffusion test, immunodiffusion, complement fixation, indirect immunofluorescence assay (IFA), and enzyme-linked immunosorbent assay (ELISA). With the exception of ELISA, all the other tests have been either costly to perform (Complement fixation), less sensitive and nonspecific (IHA and Latex agglutination test), time consuming (immunodiffusion) or requires skills in culture and antigen preparation (IFA) (Fotedar et al., 2007).

Serological Assay	**Sensitivity (%)**	**Specificity (%)**	**Reference(s)**
IHA	100[a], 99	90.9-100[a] , 99.8	Pillai et al., 1999; Hira et al., 2001
Novagnost *Entamoeba* IgG	>95	>95	Manufacturer's recommendation
I.H.A. Amoebiasis	93	97.5	Robert et al., 1990
Amebiasis Serology microplate ELISA	95	97	Manufacturer's recommendation
RIDASCREEN *Entamoeba* (IgG detection)	100, 97.7-100 (100)	95.6, 97.4 (100)	Manufacturer's recommendation; Knappik et al., 2005

Table 2. List of some of the commercially available antibody assays used for the diagnosis of amoebiasis.

ELISA is a reliable, easy to perform and rapid method for the diagnosis of *E. histolytica* infections especially in developing countries. It has been used widely for the study of the epidemiology and diagnosis of symptomatic amoebiasis (intestinal and/or extraintestinal). An ELISA to detect antibodies to *E. histolytica* has been shown to be 97.9% sensitive and 94.8% specific for detection of *E. histolytica* antibodies in ALA patients in a non endemic country (Hira et al., 2001). Unlike IgG, immunoglobulin M (IgM) is short lived and does not

remain in the serum for longer periods making it a very useful marker for the detection of present or current *E. histolytica* infections. An ELISA for the detection of serum IgM antibodies to the amoebic Gal or GalNAc-inhibitable adherence lectin has been reported. In this study, conducted in Egypt, anti-lectin IgM antibodies in the serum were detected in 45% of patients who had been suffering from acute colitis for <1 week (Abd-Alla et al., 1998). Since there is no cross-reaction with other non-*E. histolytica* parasites (Goncalves et al., 2004), the use of ELISA thus seems to be an excellent choice for the routine laboratory diagnosis as well as the surveillance and control of amoebiasis in the developing world.

The newer methods available to distinguish between *E. dispar* and *E. histolytica* have thrown into question the commonly accepted figure of 500 million infections worldwide suggesting that the actual number may be closer to 50 million. PCR and monoclonal antibody techniques are now available to distinguish between these three species in fresh and preserved stool samples, including those with mixed infections. Several investigators have developed ELISAs that detect antigens in fresh stool samples with sensitivity closer to that of stool culture methods and PCR. These ELISAs are usually easy and rapid to perform. Copro-antigen based ELISA kits specific for *E. histolytica* exploit monoclonal antibodies against the Gal/GalNAc-specific lectin of *E. histolytica* (*E. histolytica* II; TechLab, Blacksburg, VA) or against serine-rich antigen of *E. histolytica* (Optimum S kit; Merlin Diagnostika, Bornheim-Hersel, Germany). Other ELISA kits include the Entamoeba CELISA PATH kit (Cellabs, Brookvale, Australia) and the ProSpecT EIA (Remel Inc.; previously manufactured by Alexon-Trend, Inc., Sunnyvale, CA) (Fotedar et al., 2007). The early nineties of the 20th century have witnessed the introduction by TechLab of an ELISA kit for the specific detection of *E. histolytica* in feces. This antigen detection test captures and detects the parasite's Gal/GalNAc lectin in stool samples. It can also be used for the detection of the lectin antigen in the serum and liver abscesses in patients with invasive intestinal amoebiasis and ALA (Haque et al., 2000). However, the diagnosis of ALA normally relies on the identification of liver lesions and positive anti-*E. histolytica* serology. Yet neither provides conclusive results for ALA. The Gal/GalNAc lectin is conserved and highly immunogenic, and because of the epitopic differences in the lectins of *E. histolytica* and *E. dispar*, the test enables specific identification *E. histolytica* (Haque et al., 1993; Mirelman 1997). Because of some disadvantages observed with the TechLab ELISA kit, a newer more sensitive and specific version, TechLab *E. histolytica* II kit, was produced. This second – generation *E. histolytica* II kit has demonstrated good sensitivities and specificities when compared to real-time PCR (71 to 79% and 96 to 100%, respectively) (Roy et al., 2005; Visser et al., 2006). Other studies however, have reported a lesser sensitivity (14.3%) and specificity (98.4%) in comparison to stool culture and isoenzyme analysis (Gatti et al., 2002). Cross reactivity is another concern with the use of the assay, since it seems that *E. dispar* positive samples by means of PCR may sometimes give false-positive outcomes (Furrows et al., 2004). Accordingly, accurate detection of *E. histolytica*, *E. dispar* and *E. moshkovskii* could be helpful for diagnostic and epidemiological studies in places where it is impractical and expensive to use molecular assays and where amoebiasis is most prevalent, such as in the developing countries. An antigen detection kit for the specific identification of *E. dispar* and *E. moshkovskii* is yet to be developed.

Several PCR-based techniques that amplify and detect *E. histolytica* DNA are currently used for the clinical and epidemiological studies in non-endemic rich countries (Acuna-Soto et al.,

1993; Katzwinkel-Wladarsch et al., 1994; Calderaro et al., 2006; Hamzah et al., 2006). The sensitivity and specificity of PCR-based methods for the diagnosis of *E. histolytica* infection approach those of stool culture followed by isoenzyme analysis. PCR methods can be used to detect *E. histolytica* in stool, tissues and liver lesion aspirates. Of all the different gene targets used to identify *E. histolytica*, the small-subunit rRNA gene (18SrDNA) is believed to be more sensitive than the best antigen detection method used and performs equally well compared to stool culture (Mirelman et al., 1997).

Several groups have developed a variety of excellent conventional PCR assays, targeting different genes, for the direct detection and differentiation of *E. histolytica*, *E. dispar*, and *E. moshkovskii* DNA in clinical specimens such as stool and liver abscess samples (Tanyuksel and Petri Jr., 2003; Paul et al., 2007). Of all the targeted genes, assays amplifying the 18SrDNA genes are the ones in wide use as they are present in multiple copies on extrachromosomal plasmids thus making them easily detectable than single copy genes (Battacharya et al., 1989). Other gene targets used in PCR to study the epidemiology of *E. histolytica* include: the serine-rich *E. histolytica* protein (SREPH) gene (Stanley et al., 1990), cysteine proteinases gene and actin genes (Freitas et al., 2004). The SREHP is also used to study the genotypes of *E. histolytica* in human populations. However, it is now being replaced by the use of PCR amplification of tRNA gene-linked short tandem repeats which in addition to providing details of the epidemiology of *E. histolytica*, it also provides a tool to predict the outcome of the infection (Ali et al., 2005).

A nested multiplex PCR was developed by many groups. This method has the added advantage of increasing the sensitivity and specificity of the test whilst simultaneously detecting and differentiating *E. histolytica* and *E. dispar* from DNA extracted from microscopy-positive stool specimens (Evangelopoulos et al., 2000; Hung et al., 2005; Nunez et al., 2001). A nested PCR method for the identification of *E. moshkovskii* in fecal samples was developed as a nested 18S rDNA PCR followed by restriction endonuclease digestion (Ali et al., 2003). The method exhibited a high sensitivity and specificity (100%).

Real time PCR is another type of PCR which is more sensitive than the conventional PCR. It is faster than the conventional PCR and characterized by the elimination of gel analysis and other post-PCR analysis, thus reducing the risk of contamination and cost (Klein 2002). However, its application in developing countries is limited to research only. Real-time PCR allows specific detection of the PCR product by binding to one or two fluorescence-labeled probes during PCR, thereby enabling continuous monitoring of the PCR product formation throughout the reaction. Furthermore, real-time PCR is a quantitative method and allows the determination of the number of parasites in various samples (Fotedar et al., 2007). Despite being used for the successful identification of *E. histolytica*, *E. dispar* and *E. moshkovskii,* the various PCR methods use is still confined to research institutes in the developing world where amoebiasis is endemic. PCR-based methods application in routine clinical diagnostic laboratories in low income societies is hindered by difficulties such as cost, and time to perform the test.

A new platform for the detection of pathogens has been developed known as loop-mediated isothermal amplification (LAMP) and was developed in 2000 by Notomi and colleagues. This method uses a set of two specifically designed inner primers and two outer primers that recognize six distinct regions of the targeted DNA. The reaction is performed under isothermal conditions and simple incubators, such as a water bath or heat block, are

adequate for the specific amplification of the desired genetic material. Considering these advantages, the LAMP assay could be a useful and valuable diagnostic tool particularly in developing countries where most of the infections are common as well as in hospital laboratories. Recently this method was developed specifically for the detection of *E. histolytica* (Liang et al., 2009). The efficiency of the developed method was compared to that of existing PCR methodology and was similar in terms of sensitivity and specificity. This method needs further evaluations to be used in local conditions in Africa in order to improve the understanding of amebiasis in the continent as well as elsewhere.

5. Pathogenicity of amoebiasis

E. histolytica causes intestinal and extraintestinal amoebiasis based on the site of infection. Though most infections do not harm the host (asymptomatic infections), establishment in the colonic mucosa via the Galactose/N-acetyl Galactosamine inhibitable lectin (Gal-lectin) is a pre-requisite for the disease (Chadee et al., 1987). Pathogenic forms of the parasite are known to secrete enzymes that facilitate their invasion into the mucosa and sub-mucosa causing deep-flask shaped ulcers (Figure 3), and in some cases entering the circulation and reaching internal organs like the liver, lungs, skin, etc. The disease in the colon is the most common with acute diarrhoea and dysentery accounting for 90% of the clinical amoebiasis cases (Espinosa-Cantellano and Martínez-Palomo, 2000) and only 1% involve the liver (Haque et al., 2003) (Figure 4).

5.1 Asymptomatic colonization

Asymptomatic infections are characterized by the parasite living in perfect harmony within the host. *E. histolytica* trophozoites have developed elusive tactics to prevent them from being purged from the host.. By modulating signals by intestinal epithelial cells (IEC), trophozoites direct anti-inflammatory host responses leading to a tolerogenic/hyporesponsive immune state favourable to their survival (Kammanadiminti and Chadee, 2006). Furthermore, products secreted by non-pathogenic *E. histolytica* strains normally disrupt and suppress NFκB signaling and as a result diminish pro-inflammatory responses normally detrimental to the parasite (Artis, 2008). Interleukin 10 (IL-10), an anti-inflammatory cytokine, has been shown to play a significant role in maintaining this hyporesponsive state. On the other hand, a deficiency of IL-10 more often than not predisposes the host to develop the clinical amoebiasis (Hamano et al., 2006)

5.2 Intestinal amoebiasis

After an incubation period of 1-4 weeks, the parasite invade the colonic mucosa, producing characteristic ulcerative lesions and a profuse bloody diarrhea (amoebic dysentery). Amoebic invasion through the mucosa and into the submucosa is the hallmark of amoebic colitis. Contact of the trophozoites via the Gal/GalNAc lectin triggers a signaling cascade initiating the death of the host cell through different mechanisms such as phagocytosis, cytotoxicity and caspase activation instigating the invasive (intestinal and/or extra-intestinal) stages of the disease. Other molecules involved in the disease process include: a serine-rich *E. histolytica* protein (SREHP), amoebapores, and cysteine proteases (Boettner at al., 2002; Mortimer and Chadee, 2010). Activation of damaging inflammatory and non-

inflammatory responses following contact of the trophozoites to the gut wall induces a massive neutrophil infiltration across the epithelium into the underlying tissues resulting in weakening of epithelial cells and the mucous layer and allowing trophozoites to invade the intestinal epithelium and disseminating to other bodily sites (Ackers and Mirelman, 2006). The ulcers formed may be generalized involving the whole length of the large intestine or they may be localized in the ileo-caecal or sigmoido-rectal regions. Ulcers are normally disconnected with sizes varying from pin-head size to more than 2.5 cm in diameter. They may be deep or superficial. Base of the deep ulcers is generally formed by the muscularis layer. Nonetheless, superficial ulcers do not extend beyond the muscularis layer. A large number of fatalities results from perforated colons with concomitant peritonitis. *E. histolytica* also causes amoebomas. These are pseudotumoural lesions, whose formation is associated with necrosis, inflammation and oedema of the mucosa and submucosa of the colon. These granulomatous masses may obstruct the bowel.

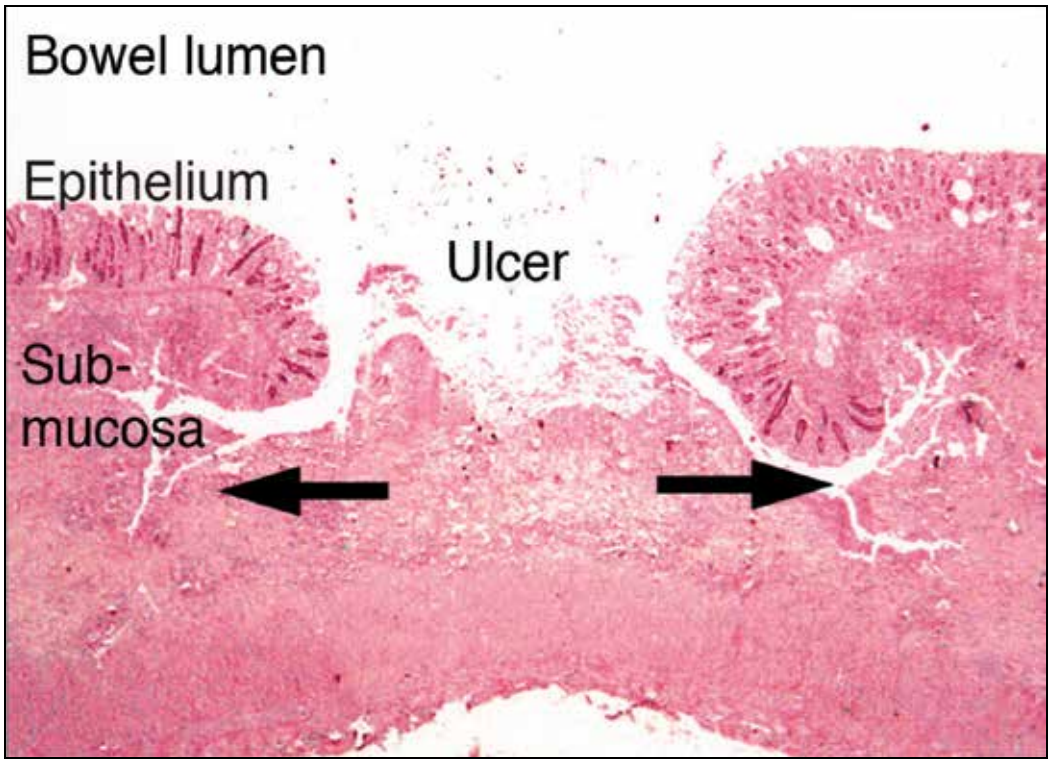


Fig. 3. "Flask-shaped" ulcer of invasive intestinal amebiasis (hematoxylin-eosin, original magnification ×50). Source: **Pritt B S , Clark C G Mayo Clin Proc. 2008;83:1154-1160: Mayo Clinic Proceedings**

While the serine rich *E. histolytica* protein (SREHP) have been shown to promote adhesion of the trophozoites to host cells, cysteine proteases (CP), are known for their virulence in other protozoa as well as in tumour metastasis. Five *E. histolytica* proteins (EhCP1, 2, 3, 5 and 112) have been identified. All are alleged to play a role in the destruction of host cells, phagocytosis, together with the recruitment of neutrophils and macrophages and the induction of intestinal

inflammation (Mortimer and Chadee, 2010). Moreover, EhCP5 has also been shown to perform a variety of functions such as evasion of the host complement and immune system by preventing the activation of the classical complement system via the inactivation of IgG and the degradation of IgA (Laughlin and Temesvari, 2005).

Equally important in the pathogenesis and virulence of *E. histolytica* is the role of the phagosome-associated proteins. Many have been identified and their function in endocytosis and pathogenesis has been established. Examples include: EhRacA, EhRacG, EhPAK, actin and several Rab7-related GTPases (Laughlin and Temesvari, 2005). Cytokines such as IL-1β, IL-1α, IL-8 and TNF-α are suspected of aggravating the disease process and driving the immunopathogenesis mechanism (Kammanadiminti et al., 2003). Although neutrophils are known to cause intestinal tissue damage they are nevertheless critical for controlling the infection. Nonetheless, host and/or parasite factors normally play a role in determining whether the parasite is cleared or the disease becomes established (Asgharpour et al., 2005).

Although most intestinal invasions heal following an acute inflammatory response, *E. histolytica* evades destruction in a modest number of individuals and a chronic state is established. This chronic state is associated with the development of a non-protective adaptive immune response. Human data, in vitro and in vivo models support a paradigm that Th1 responses in the gut clear *E. histolytica*, while Th2 responses through the production of IL-4 are anti-protective, likely through suppressing IFN-γ. It is not yet clear what signals drive an anti-protective Th2 immune response instead of an effective protective Th1 response towards the infection. Evidence suggesting that genetics, the MHC restriction, nutrition and bacterial flora might play a role in directing the immune response towards *E. histolytica* infection exists. For example, the MHC class II allele DQBl*0601 was reported to be associated with resistance to *E. histolytica* (Mortimer and Chadee, 2010). Susceptibility to ALA has been found to be associated with HLA-DR3 and complotype SC01 in some Mexican populations; this association is not seen for amoebic colitis or asymptomatic colonization with *E. histolytica* (Stanley, 2003).

5.3 Extraintestinal amoebiasis

About 5% individuals with intestinal amoebiasis, 1-3 months after the disappearance of the dysenteric attack, develop extraintestinal amoebiasis. Once in the blood, the parasite uses many different strategies to avoid elimination by the host and reaches other sites in the body (such as the liver, lungs, brain, etc). The most common extraintestinal site affected by the parasite is the liver and an Amoebic liver abscess (ALA) is its most common manifestation, predominantly seen in adult males. This chronic stage of ALA is characterized by defective cell-mediated immunity and the suppression of T cells and their defective proliferative responses (Campbell et al., 1999). *E. histolytica* trophozoites reaching the liver create their unique abscesses, which are well circumscribed regions of cytolysed liver cells, liquefied cells, and cellular debris. The lesions are surrounded by connective tissue enclosing few inflammatory cells and trophozoites. Parenchymal cells adjacent to the lesion are often unaffected. However, lysis of neutrophils by *E. histolytica* trophozoites might release mediators that lead to the death of liver cells, and extend damage to hepatocytes not in direct contact with the parasite. Studies have shown that in ALA in mice, most hepatocytes die from apoptosis, but necrosis is also present. In ALA from humans, the small numbers of amoebas relative to the size of the abscess suggests that *E. histolytica* can kill hepatocytes

without direct contact (Stanley 2003). From the liver, *E. histolytica* trophozoites may enter into the general circulation and reach other organs (Figure 4).

Fig. 4. Amoebic Liver abscess. Gross specimen of liver tissue with an abscess (white) that formed due to infection of the organ with *Entamoeba histolytica*. Source: http://www.sciencephoto.com/media/250248/enlarge

6. Role of genetic characteristics of the infecting strains in the pathogenesis of amoebiasis

The outcome of an infection may depend on several factors among which the genetic characteristics of the specific pathogen have been identified as an important one. Few polymorphic genetic loci have been identified and targeted to aid in the study of the population structure of *E. histolytica* strains and their possible relationships with the parasite's virulence and disease outcome (Clark, 2006; Paul et al., 2007). Examples of these genetic markers include protein coding genes (serine – rich *E. histolytica* protein, [SREHP] and Chitinase) and non-coding DNA (Strain Specific Gene and tRNA gene linked short tandem repeats [STR]) of PCR-amplified genes (Haghighi et al., 2003; Samie et al., 2008). In a study in Bangladesh, the tRNA-linked STR genotyping system has provided evidence that the parasite genome does influence the outcome of infection. tRNA-linked STR genotyping was also behind the recent observation of differences between parasite genotypes in the intestine and the liver abscess of same patients (Ali et al., 2007). Few studies, albeit inconclusive, using the polymorphic SREHP marker have indicated that certain SREHP profiles might be responsible for the presentation of intestinal amoebic symptoms (Ayehkumi et al., 2001; Samie et al., 2008). Yet, all studies with SREHP marker did support previous findings of extensive genetic diversity among *E. histolytica* isolates from the same

geographic origin (Ayeh-kumi et al., 2001; Simonishvili et al., 2005; Samie et al., 2008; Tanyuksel et al., 2008). Thus, it seems that the parasite genotype does play a role in the outcome of infection in humans thus linking parasite diversity and virulence. Other approaches, such as SNP identification coupled with microarray-based analysis of gene expression or proteomic comparisons among parasites will be needed to identify the actual genes responsible for these results and to help us understand the mechanism of parasite virulence and pathogenesis (Ali et al., 2008).

7. Conclusions

Up to date, there are still large gaps in our knowledge of species prevalence rates in different regions of the world particularly in the African continent where very few studies are being conducted using molecular methods. In order to address this limitation, there is need to implement species-specific diagnosis of *E. histolytica*, *E. dispar* and *E. moshkovskii*, particularly in countries where these organisms are endemic. Based on the limited information available to date it appears that molecular and genomic studies are still needed combined to molecular epidemiology studies in order to advance our understanding of amoebiasis. The currently available genome sequence is very useful in better understanding the biology of the parasite, however, *E. histolytica* strains from Africa still need to have the genome sequenced. Comparative genomics will probably allow the understanding of the pathogenicity of some strains of *E. histolytica* compared to non-pathogenic strains as well as better understanding of *E. dispar* in relation to *E. histolytica*. Further collaborations between scientists from developed countries and those from developing countries is essential in answering questions on the epidemiology, pathogenesis and biochemistry of *E. histolytica* which is the causing agent of amoebiasis.

8. References

Abd-Alla MD, and Ravdin JI (2002). Diagnosis of amebic colitis by antigen capture ELISA in patients presenting with acute amebic diarrhea in Cairo Egypt. Trop Med Int Health 7: 365--370.

Abd-Alla MD, Jackson TG and Ravdin JI (1998). Serum IgM antibody response to the galactose-inhibitable adherence lectin of Entameoba histolytica. Am J Trop Med Hyg 59: 431-434.

Ackers JP (2000). The diagnostic implications of the separation of *Entamoeba histolytica* and *Entamoeba dispar*. J Biosci 27: 573-578.

Acuna-Soto R, Samuelson J, De Girolami P, Zarate L, Millan-Velasco F, Schoolnick G, Wirth D (1993) Application of the polymerase chain reaction to the epidemiology of pathogenic and nonpathogenic *Entamoeba histolytica*. Am J Trop Med Hyg 48: 58-70.

Ali IKM, Hossain MB, Roy S, Ayeh-Kumi PF, Petri WA, Jr, Haque R, Clark CG (2003). *Entamoeba moshkovskii* infections in children, Bangladesh. Emerg Infect Dis 9:580–584.

Ali IKM, Mondal U, Roy S, Haque R, Petri WA, Jr, Clark CG (2007). Evidence for a link between parasite genotype and outcome of infection with *Entamoeba histolytica*. J Clin Microbiol 45: 285–289.

Ali IK, Zaki M and ClarkCG (2005). Use of PCR amplification of tRNA gene-linked short tandem repeats for genotyping *Entamoeba histolytica*. J Clin Microbiol 43: 5842-5847.

Ali IK, Clark CG, Petri Jr. WA (2008). Molecular epidemiology of amoebiasis. Infect Genet Evol 8: 698-707.

Ali IK, Mondal U, Roy S, Haque R, Petri Jr.WA, Clark CG (2007). Evidence for a link between parasite genotype and outcome of infection with *Entamoeba histolytica*. J Clin Microbiol 45: 285-289.

Ali IK, Solaymani-Mohammadi S, Akhter J, Roy S, Gorrini C, Calderaro A, Parker SK, Haque R, Petri Jr.WA, Clark CG (2008). Tissue invasion by *Entamoeba histolytica*: evidence of genetic selection and/or DNA reorganization events in organ tropism. PLoS Negl Trop Dis 2: e219.

Alonzo E, Alonzo V, Forino D, Ioli A (1993). Parasitological research conducted between 1982 and 1991 in a sample of Zinvie (Benin) residents. Med Trop 53(3):331-335.

Artis D (2008). Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut. Nat Rev Immunol 8:411–420.

Asgharpour A, Gilchrist C, Baba D, Hamano S, Houpt E (2005). Resistance to intestinal *Entamoeba histolytica* infection is conferred by innate immunity and Gr-1+ Cells. Infect Immun 73: 4522–4529.

Ayeh-Kumi PF, Ali IM, Lockhart LA, Gilchrist CA, Petri Jr, WA, Haque R (2001). *Entamoeba histolytica*: genetic diversity of clinical isolates from Bangladesh as demonstrated by polymorphisms in the serine-rich gene. Exp Parasitol 99(2):80-88.

Bachur TP, Vale JM, Coêlho IC, Queiroz TR, Chaves Cde S (2008). Enteric parasitic infections in HIV/AIDS patients before and after the highly active antiretroviral therapy. Braz J Infect Dis. 12(2):115-122.

Bardwell L (2006) Mechanisms of MAPK signalling specificity. Biochem Soc Trans 34: 837–841.

Bhattacharya S, Bhattacharya A, Diamond LS and Soldo AT(1989). Circular DNA of Entamoeba histolytica encodes ribosomal RNA. J Protozool 36:455-458.

Blessman J, Le Van A, and Tannich E (2006). Epidemiology and treatment of amoebiasis in Hue, Vietnam. Arch Med Res 37: 270-272.

Blessmann J, Ali IKM, Nu PA, Dinh BT, Viet TQ, Van AL, Clark CG, Tannich E (2003). Longitudinal study of intestinal *Entamoeba histolytica* infections in asymptomatic adult carriers. J Clin Microbiol. 41:4745–50.

Blessmann J, Buss H, Nu PA, Dinh BT, Ngo QT, Van AL, Alla MD, Jackson TF, Ravdin JI, and Tannich E (2002). Real-time PCR for detection and differentiation of *Entamoeba histolytica* and *Entamoeba dispar* in fecal samples. J Clin Microbiol 40:4413-4417.

Blessmann J, Van Linh P, Nu PA, Thi HD, Muller-Myhsok B, Buss H, and Tannich E (2002). Epidemiology of amebiasis in a region of high incidence of amebic liver abscess in central Vietnam. Am J Trop Med Hyg 66:578-583.

Boettner DR, Hutson C, Petri Jr. WA (2002). Galactose/N-acetylgalactoseamine lectin: the coordinator of host cell killing. J Bio 6(Suppl.3):553-557.

Brink AK, Mahe C, Watera C, Lugada E, Gilks C, Whitworth J, and French N (2002). Diarrhoea, CD4 counts and enteric infections in a community-based cohort of HIV-infected adults in Uganda. J Infect 45:99-106.

Burch DJ, Li E, Reed S, Jackson TF, Stanley SL Jr (1991). Isolation of strain-specific Entamoeba histolytica cDNA clone. J Clin Microbiol 29(4):696-701.

Caballero-Salcedo A, Viveros-RogelM, Salvatierra B, Tapia-Conyer R, Sepulveda-Amor J, Gutierrez G, and Ortiz-Ortiz L (1994). Seroepidemiology of amebiasis in Mexico. Am J Trop Med Hyg 50:412-419.

Calderaro A, Gorrini C, Bommezzadri S, Piccolo G, Dettori G, and Chezzi C (2006). *Entamoeba histolytica* and *Entamoeba dispar*: comparison of two PCR assays for diagnosis in a non-endemic setting. Trans R Soc Trop Med Hyg 100:450-457.

Campbell D, Gaucher D, Chadee K (1999). Serum from *Entamoeba histolytica*-infected gerbils selectively suppresses T cell proliferation by inhibiting Interleukin-2 production. J Infect Dis 179:1495–1501.

Cardona AF, Cambariza JF, Reveiz L, Ospina EG, Poveda CM, Ruiz CA, Ramos P, Aponte DM (2004). Caracterısticas clınicas y microbiologicas de la colitis neutropenica en adultos con neoplasias hematologicas del Instituto Nacional de Cancerologıa de Bogota, D.C. (Colombia). Enferm Infecc Microbiol Clin 22: 462–466.

Chadee K, Petri WA Jr, Innes DJ, Ravdin JI (1987). Rat and human colonic mucins bind to and inhibit adherence lectin of *Entamoeba histolytica*. J Clin Invest 80(5):1245-54.

Chatterjee A, Ghosh SK, Jang K, Bullitt E, Moore L, Robbins PW and Samuelson J (2009). Evidence for a "wattle and daub" model of the cyst wall of *Entamoeba*. PLoS Pathogens 5 : e1000498.

Chen Y, Zhang Y, Yang B, Qi T, Lu H, Cheng X, and Tachibana H (2007). Seroprevalence of *Entamoeba histolytica* infection in HIV-infected patients in China. Am J Trop Med Hyg 77: 825-828.

Clark CG and Diamond LS (2002). Methods for the cultivation of luminal parasitic protists of clinical importance. Clin Microbiol Rev 15:329-341.

Clark CG (2006). Methods for the investigation of diversity in *Entamoeba histolytica*. Arch Med Res 37(2):258-262.

Daryani A, Sharif M, Meigouni M, Mahmoudi FB, Rafiei A, Gholami Sh, Khalilian A, Gohardehi Sh, Mirabi AM (2009). Prevalence of intestinal parasites and profile of CD4+ counts in HIV+/AIDS people in north of Iran. Pak J Biol Sci. 12(18):1277-1281.

Diamond LS, Clark CG (1993). A redescription of *Entamoeba histolytica* Schaudinn, 1903 (Emended Walker, 1911) separating it from Entamoeba dispar Brumpt, 1925. J Eukaryot Microbiol 40:340–344.

Dorin-Semblat D, Quashie N, Halbert J, Sicard A, Doerig C, Peat E, Ranford-Cartwright L, Doerig C (2007) Functional characterization of both MAP kinases of the human malaria parasite *Plasmodium falciparum* by reverse genetics. Mol Microbiol 65: 1170–1180.

Eichinger D (2001). A role for a galactose lectin and its ligands during encystment of *Entamoeba*. J Eukaryot Microbiol 48: 17-21.

Ellis JG, Davila M, Chakrabarti R (2003). Potential involvement of extracellular signal-regulated kinase 1 and 2 in encystation of a primitive eukaryote, *Giardia lamblia*. Stage-specific activation and intracellular localization. J Biol Chem 278: 1936–1945.

Escobedo AA and Nunez FA (1999). Prevalence of intestinal parasites in Cuban acquired immunodeficiency syndrome (AIDS) patients. Acta Trop 72:125-130.

Espinosa-Cantellano M, Martínez-Palomo A (2000). Pathogenesis of Intestinal Amebiasis: From Molecules to Disease. Clin Microbiol Rev 13(2): 318-331.

Evangelopoulos A, Spanakos G, Patsoula E and Vakalis N (2000). A nested, multiplex, PCR assay for the simultaneous detection and differentiation of *Entamoeba histolytica* and *Entamoeba dispar* in faeces. Ann. Trop. Med. Parasitol. 94:233-240.

Evangelopoulos A, Spanakos G, Patsoula E, Vakalis N, and Legakis NA (2000). A nested, multiplex, PCR assay for the simultaneous detection and differentiation of *Entamoeba histolytica* and Entamoeba dispar in faeces. Ann Trop Med Parasitol 94:233-240.

Florez AC, Garcia DA, Moncada L, Beltran M (2003). Prevalence of Microsporidia and other intestinal parasites in patients with HIV infection, Bogota, 2001. Biomedica 23(3):274-82.

Fotedar R, Stark D, Beebe N, Marriott D, Ellis J, Harkness J (2007). Laboratory diagnostic techniques for *Entamoeba* species. Clin Microbiol Rev. 20(3):511-32.

Fotedar R, Stark D, Marriott D, Ellis J, Harkness J (2008). *Entamoeba moshkovskii* infections in Sydney, Australia. Eur J Clin Microbiol Infect Dis. 27:133–7.

Freitas MA, Vianna EN, Martins ES, Silva EF, Pesquero JL and Gomes MA (2004). A single step duplex PCR to distinguish *Entamoeba histolytica* from *Entamoeba dispar*. Parasitology 128:625-628.

Fujishima T, Nishise S, Ichihara M, Kobayashi S, Takeuchi T (2010). Difficulties in the treatment of intestinal amoebiasis in mentally disabled individuals at a rehabilitation institution for the intellectually impaired in Japan. Chemotherapy. 56(4):348-52.

Furrows SJ, Moody AH and Chiodini PL (2004). Comparison of PCR and antigen detection methods for diagnosis of Entamoeba histolytica infection. J Clin Pathol 57:264-266.

Gassama A, Sow PS, Fall F, Camara P, Gueye-N'diaye A, Seng R, Samb B, M'Boup S, Aidara-Kane A (2001). Ordinary and opportunistic enteropathogens associated with diarrhea in Senegalese adults in relation to human immunodeficiency virus serostatus. Int J Infect Dis 5(4):192-8.

Gathiram V, Jackson TFHG (1987). A longitudinal study of asymptomatic carriers of pathogenic zymodemes of *Entamoeba histolytica*. S Afr Med J 72:669–72.

Gatti S, Swierczynski G, Robinson F, Anselmi M, Corrales J, Moreira J, Montalvo G, Bruno A, Maserati R, Bisoffi Z, and Scaglia M (2002). Amebic infections due to the Entamoeba histolytica-Entamoeba dispar complex: a study of the incidence in a remote rural area of Ecuador. Am J Trop Med Hyg 67:123-127.

Ghosh S, Frisardi m, Rminez-Avila L, Descoteaux S, sturm-Ramirez K, Newton-Sanchez OC, Santos-precado JI, Ganguly C, Lohia A, Reed S, and Samuelson J (2000). Molecular

epidemiology of *Entamoeba* spp.: Evidence of a bottleneck (Demographic Sweep) and Transcontinental Spread of Diploid Parasites.J Clin Microbiol 38(10) : 3815-3821.

Goncalves ML, da Silva VL, de Andrade CM, Reinhard K, da Rocha GC, Le Bailly M, Bouchet F, FerreiraLF, and Araujo A (2004). Amoebiasis distribution in the past: first steps using an immunoassay technique. Trans R Soc Trop Med Hyg 98:88-91.

González-Ruiz A, Haque R, Aguirre A, Castañón G, Hall A, Guhl F, Ruiz-Palacios G, Miles MA, Warhurst DC (1994). Value of microscopy in the diagnosis of dysentery associated with invasive Entamoeba histolytica. J Clin Pathol 47: 236-239.

Haghighi A, Kobayashi S, Takeuchi T, Thammapalerd N, Nozaki T (2003). Geographic diversity among genotypes of *Entamoeba histolytica* field isolates. J Clin Microbiol 41(8): 3748-56.

Haileeyesus A and Beyene P (2009). Intstinal protozoan infections amonh HIV positive persons with and without antiretroviral treatment (ART) in selected ART centres in Adama, Afar and Dire-Dawa, Ethiopia. Ethiop. J Health Dev 23(2): 133-140.

Hailemariam G, Kassu A, Abebe G, Abate E, Damte D, Mekonnen E and Ota F (2004). Intestinal parasitic infections in HIV/AIDS and HIV seronegative individuals in a teaching hospital, Ethiopia. Jpn J Infect Dis 57:41-43.

Hamano S, Asgharpour A, Stroup SE, Wynn TA, Leiter EH, Houpt E (2006). Resistance of C57BL/6 mice to amoebiasis is mediated by nonhemopoietic cells but requires hemopoietic IL-10 production. J Immunol 177: 1208–1213.

Hamzah Z, Petmitr S, Mungthin M, Leelayoova S and Chavalitshewinkoon-Petmitr P (2006). Differential detection of *Entamoeba histolytica*, *Entamoeba dispar*, and *Entamoeba moshkovskii* by a single-round PCR assay. J Clin Microbiol 44:3196-3200.

Haque R, Mondal D, Duggal P, Kabir M, Roy S, Farr BM, Sack RB, Petri WA., Jr (2006). *Entamoeba histolytica* infection in children and protection from subsequent amebiasis. Infect Immun 74:904–9.

Haque R, Mondal D, Duggal P, Kabir M, Roy S, Farr BM, Sack RB, Petri WA Jr (2006). *Entamoeba histolytica* Infection in Children and Protection from Subsequent Amebiasis. Infect Immunity 74 (2): 904-909.

Haque R, Mondal D, Karim A, Molla IH, Rahim A, Faruque AS, Ahmad N, Kirkpatrick BD, Houpt E, Snider C, Petri WA Jr (2009). Prospective case-control study of the association between common enteric protozoal parasites and diarrhea in Bangladesh. Clin Infect Dis 48(9):1191-7.

Haque R, Mondal D, Duggal P, Kabir M, Roy S, Farr BM, Sack RB, Petri WA Jr (2006). *Entamoeba histolytica* infection in children and protection from subsequent amebiasis. Infect Immun. 74(2):904-9.

Haque R, Hutson CD, Hughes M, Houpt E, Petri Jr WA (2003). Amoebiasis. N Engl J Med 348: 1565-1573.

Haque R, Ali IKM, and Petri, Jr WA (1999). Prevalence and immune response of *Entamoeba histolytica* infection in preschool children in Bangladesh. Am J Trop Med Hyg 60:1031-1034.

Haque R, Kress K, Wood S, Jackson TFGH, Lyerly D, Wilkins T, and Petri, Jr.WA (1993). Diagnosis of pathogenic *Entamoeba histolytica* infection using a stool ELISA based on monoclonal antibodies to the galactose specific adhesin. J Infect Dis 167:247-249.

Haque R, Mollah NU, Ali IKM, Alam K, Eubank A, Lyerly D, and Petri Jr.WA (2000). Diagnosis of amebic liver abscess and intestinal infection with the TechLab *Entamoeba histolytica* II antigen detection and antibody tests. J Clin Microbiol 38:3235-3239.

Hira PR, Iqbal J, Al-Ali F, Philip R, Grover S, D'Almeida E and Al-Eneizi AA (2001). Invasive amebiasis: challenges in diagnosis in a non-endemic country (Kuwait). Am J Trop Med Hyg 65:341-345.

Hung CC, Ji DD, Sun HY, Lee YT, Hsu SY, Chang SY, Wu CH, Chan YH, Hsiao CF, Liu WC, Colebunders R (2008). Increased risk for *Entamoeba histolytica* infection and invasive amebiasis in HIV seropositive men who have sex with men in Taiwan. PLoS Negl Trop Dis 2(2):e175.

Hung CC, Ji DD, Sun HY, Lee YT, Hsu SY, Ya TL, Shui YH, Sui YC, Cheng HW, Yun HC, Chin FH, Wen CL, Robert C (2008). Increased risk for *Entamoeba histolytica* infection and invasive amoebiasis in HIV seropositive men who have sex with men in Taiwan. PLOS Negl Trop Dis 2(2):e175.

Hung CC, Deng HI, Hsiao WH, Hsieh SM, Hsiao CF, Chen MY, Chang SC and Su KE (2005) Invasive amoebiasis is an emerging parasitic infection in patients with HIV infection. Archives of Internal Medicine, 165: 409-415.

Jackson TF, Gathiram V, Simjee AE (1985). Seroepidemiological study of antibody responses to the zymodemes of *Entamoeba histolytica*. Lancet. 1(8431):716-9.

Junttila MR, Li SP, Westermarck J (2008). Phosphatase-mediated crosstalk between MAPK signaling pathways in the regulation of cell survival. Faseb J 22: 954–965.

Kammanadiminti SJ, Mann BJ, Dutil L, Chadee K (2003). Regulation of toll-like receptor-2 expression by the Gal-lectin of *Entamoeba histolytica*. The FASEB 8: 155–7.

Kammanadiminti SJ, Chadee K (2006). Suppression of NF-κB Activation by *Entamoeba histolytica* in intestinal epithelial cells is mediated by heat shock protein 27. J Biol Chem 281:26112–26120.

Katzwinkel-Wladarsch S, Loscher T and Rinder H (1994). Direct amplification and differentiation of pathogenic and nonpathogenic *Entamoeba histolytica* DNA from stool specimens. Am J Trop Med Hyg 51: 115-118.

Khairnar K, Parija SC, Palaniappan R (2007). Diagnosis of intestinal amoebiasis by using nested polymerase chain reaction-restriction fragment length polymorphism assay. J Gastroenterol 42:631–640.

Klein D (2002). Quantification using real-time PCR technology: applications and limitations. Trends Mol Med 8:257-260.

Knappik M, Borner U, and Jelinek T (2005). Sensitivity and specificity of a new commercial enzyme-linked immunoassay kit for detecting Entamoeba

histolytica IgG antibodies in serum samples. Eur J Clin Microbiol Infect Dis 24:701-703.

Krogstad DJ, Spencer HC Jr, Healy GR, Gleason NN, Sexton DJ, Herron CA (1978) Amoebiasis: epidemiologic studies in the United States, 1971-1974. Ann Intern Med 88: 89-97.

Kumiko Nakada-Tsukuia, Yumiko Saito-Nakanoa, Afzal Husaina and Tomoyoshi Nozaki T (2010). Conservation and function of Rab small GTPases in *Entamoeba*: Annotation of *E. invadens* Rab and its use for the understanding of *Entamoeba* biology. Exp Parasitol 126(3): 337-347.

Laughlin RC, Temesvari LA (2005). Cellular and molecular mechanisms that underlie *Entamoeba histolytica* pathogenesis: prospects for intervention. Expert Reviews in Molecular Medicine 7: 1-19.

Lawson LL, Bailey JW, Beeching NJ, Gurgel RG and Cuevas LE (2004). The stool examination reports amoeba cysts: should you treat in the face of over diagnosis and lack of specificity of light microscopy? Trop Doctor 34(1):28-30

Liang SY, Chan YH, Hsia KT, Lee JL, Kuo MC, Hwa KY, Chan CW, Chiang TY, Chen JS, Wu FT, Ji DD (2009). Development of loop-mediated isothermal amplification assay for detection of *Entamoeba histolytica*. J Clin Microbiol 47(6):1892-5.

Lindo JF, Dubon JM, Ager AL, De Gourville EM, Solo-Gabriele H, Klaskala WI, Baum VK, and Palmer CJ (1998). Intestinal parasitic infections in human immunodeficiency virus (HIV)-positive and HIV-negative individuals in San Pedro Sula, Honduras. Am J Trop Med Hyg 58:431-435.

Matthys B, Bobieva M, Karimova G, Mengliboeva Z, Jean-Richard V, Hoimnazarova M, Kurbonova M, Lohourignon LK, Utzinger J, Wyss K (2011). Prevalence and risk factors of helminths and intestinal protozoa infections among children from primary schools in western Tajikistan. Parasit Vectors 4(1):195.

McGugan Jr GC and Temesvari LA (2003). Characterization of a Rab11-like GTPase, EhRab11, of *Entamoeba histolytica*. Mol Biochem Parasitol 129 : 137–146.

McLaughlin J, and Aley S (1985). The biochemistry and functional morphology of the Entamoeba. J Protozool 32:221-240.

Mirelman D, Nuchamowitz Y, and Stolarsky T (1997). Comparison of use of enzyme-linked immunosorbent assay-based kits and PCR amplification of rRNA genes for simultaneous detection of *Entamoeba histolytica* and *E. dispar*. J Clin Microbiol 35:2405-2407.

Mitra BN, Saito-Nakano Y, Nakada-Tsukui K, Sato D and Nozaki T (2007). Rab11B small GTPase regulates secretion of cysteine proteases in the enteric protozoan parasite *Entamoeba histolytica*, Cell Microbiol 9 :2112–2125.

Molbak K, Wested N, Hojlyng N, Scheutz F, Gottschau A, Aaby P, da Silva AP (1994). The etiology of early childhood diarrhea : a community study from Guinea-Bissau.J Infect Dis 169 (3) :581-7.

Morales MA, Renaud O, Faigle W, Shorte SL, Spath GF (2007). Over-expression of Leishmania major MAP kinases reveals stage-specific induction of phosphotransferase activity. Int J Parasitol 37: 1187–1199.

Moran P, Ramos F, Ramiro M, Curiel O, González E, Valadez A, Gómez A, García G, Melendro EI, Ximénez C (2005). *Entamoeba histolytica* and/or *Entamoeba dispar*: infection frequency in HIV+/AIDS patients in Mexico City. Exp Parasitol 110(3):331-4.

Moran P, Ramos F, Ramiro M, Curiel O, Gonzalez E, Valadez A, Gomez A, Garcia G, Melendro EI and Ximenez C (2005). Infection by human immunodeficiency virus-1 is not a risk factor for amoebiasis. American Journal of Tropical Medicine and Hygiene, 73: 296-300.

Mortimer L, Chadee K (2010). The immunopathogenesis of *Entamoeba histolytica*. Exp Parasitol 126(3):336-80.

Mortimer L, Chadee K (2010). The immunopathogenesis of *Entamoeba histolytica*. Exp Parasitol 126 (3): 366-80.

Mukherjee AK, Das K, Bhattacharya MK, Nozaki T, Ganguly S (2010). Trend of *Entamoeba histolytica* infestation in Kolkata. Gut Pathog. 2(1):12.

Nishise S, Fujishima T, Kobayashi S, Otani K, Nishise Y, Takeda H, Kawata S (2010). Mass infection with *Entamoeba histolytica* in a Japanese institution for individuals with mental retardation: epidemiology and control measures. Ann Trop Med Parasitol. 104(5):383-90.

Njoya O, Njitoyap Ndam EC, Ngoue V, Ngonde Sende C (1999). Amoebic liver abscesses in Yaounde. Cahiers sante. 9(2):119-22.

Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N and Hase (2000). Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 28E63.

Novick and M. Zerial (1997). The diversity of Rab proteins in vesicle transport, Current Opinion in Cell Biology 9 (1997), pp. 496–504.

Nozaki and Nakada-Tsukui (2006). Nozaki T and Nakada-Tsukui K (2006). Membrane trafficking as a virulence mechanism of the enteric protozoan parasite *Entamoeba histolytica*, Parasitology Research 98: 179–183.

Nunez YO, Fernandez MA, Torres-Nunez D, Silva JA, Montano I, Maestre JL, and Fonte L (2001). Multiplex polymerase chain reaction amplification and differentiation of *Entamoeba histolytica* and *Entamoeba dispar* DNA from stool samples. Am J Trop Med Hyg 64:293-297.

Ohnishi K, and Murata M (1997). Present characteristics of symptomatic amebiasis due to *Entamoeba histolytica* in the east-southeast area of Tokyo. Epidemiol. Infect. 119:363-367.

Parija SC, Khairnar K (2005). *Entamoeba moshkovskii* and *Entamoeba dispar*-associated infections in Pondicherry, India. J Health Popul Nutr 23:292–295.

Paul J, Srivastava S, Bhattacharya S (2007). Molecular methods for diagnosis of *Entamoeba histolytica* in a clinical setting: An overview. Exp Parasitol 116: 35-43.

Pham Duc P, Nguyen-Viet H, Hattendorf J, Zinsstag J, Dac Cam P, Odermatt P (2011). Risk factors for *Entamoeba histolytica* infection in an agricultural community in Hanam province, Vietnam. Parasit Vectors. 4:102.

Picazarri K, Nakada-Tsukui K and Nozaki T (2008). Autophagy during proliferation and encystation in the protozoan parasite *Entamoeba invadens*, Infect Immun 76: 278–288.

Pillai DR, Keystone JS, Sheppard DC, MacLean JD, MacPherson DW, and Kain KC (1999). *Entamoeba histolytica* and *Entamoeba dispar*: epidemiology and comparison of diagnostic methods in a setting of non endemicity. Clin Infect Dis 29:1315-1318.

Pinheiro SM, Carneiro RM, Aca IS, Irmao JI, Morais MAJr, Coimbra MR and Carvalho LB Jr 2(004). Determination of the prevalence of *Entamoeba histolytica* and *E. dispar* in the pernambuco state of northeastern Brazil by a polymerase chain reaction. AmJ Trop Med Hyg 70(2):221-4.

Prasad KN, Nag VL, Dhole TN, and Ayyagari A (2000). Identification of enteric pathogens in HIV-positive patients with diarrhoea in northern India. J Health Popul Nutr 18:23-26.

Ravdin JI, Stauffer WM (2005). *Entamoeba histolytica* (amoebiasis). In: Mandell GL, Bennett JE, Dolin R, eds. Principles and Practice of Infectious Diseases, vol. 2. Ed., Phiadelphia: Churchill Livingstone; 2005: 3097-3111.

Reyes P, Cortes JA, Potdevin G, Urdaneta AM, Rosales J, Cuervo SI, Bermudez D, Arroyo PA (2006). Infecciones en pacientes con trasplante de me´dula o´ sea en el tro´ pico. Infection 10: 101.

Rivero Z, Bracho A, Calchi M, Díaz I, Acurero E, Maldonado A, Chourio G, Arráiz N, Corzo G (2009). [Detection and differentiation of *Entamoeba histolytica* and *Entamoeba dispar* by polymerase chain reaction in a community in Zulia State, Venezuela]. Cad Saude Publica. 25(1):151-9.

Robert R, Mahaza C, Bernard C, Buffard C, and Senet JM (1990). Evaluation of a new bicolored latex agglutination test for immunological diagnosis of hepatic amoebiasis. J Clin Microbiol 28:1422-1424.

Roche J, Benito A (1999). Prevalence of intestinal parasite infections with special reference to *Entamoeba histolytica* on the island of Bioko (Equatorial Guinea). Am J Trop Med Hyg 60(2):257-62.

Roy S, Kabir M, Mondal D, Ali IK, Petri Jr. WA and Haque R (2005). Real-time-PCR assay for diagnosis of *Entamoeba histolytica* infection. Clin. Microbiol. 43:2168-2172.

Samie A, Barrett LJ, Bessong PO, Ramalivhana JN, Mavhandu LG, Njayou M, and Gurreant RL (2010). Seroprevalence of *Entamoeba histolytica* in the context of HIV and AIDS: the case of the Vhembe district, in South Africa's Limpopo province. Ann Trop Med Parasitol 104 (1): 55-63.

Samie A, Obi CL, Bessong PO, Houpt E, Stroup S, Njayou M, Sabeta C, Mduluza T, Guerrant RL (2008). *Entamoeba histolytica*: genetic diversity of African strains based on the polymorphism of the serine-rich protein gene. Exp Parasitol 118(3): 354-61.

Sardar Ghosh A, Ray D, Dutta S, Raha S (2010). EhMAPK, the Mitogen-Activated Protein Kinase from Entamoeba histolytica Is Associated with Cell Survival. PLoS ONE 5(10): e13291. doi:10.1371/journal.pone.0013291.

Simonishvili S, Tsanava S, Sanadze K, Chlikadze R, Miskalishvili A, Lomkatsi N, Imnadze P, Petri Jr.WA, Trapaidze N (2005). *Entamoeba histolytica*: the serine-rich gene polymorphism-based genetic variability of clinical isolates from Georgia. Exp Parasitol 110(3):313-7.

Stanley SL Jr (2003). Amoebiasis. Lancet. 361(9362):1025-1034.

Stanley S LJr., Becker A, Kunz-Jenkins C, Foster L and Li E (1990). Cloning and expression of a membrane antigen of *Entamoeba histolytica* possessing multiple tandem repeats. Proc Natl Acad Sci USA 87:4976-4980.

Stanley SL (2003). Amoebiasis. Lancet 361, 1025-34.

Stark D, Barratt JLN, van Hal S, Marriott D, Harkness J, and Ellis JT (2009). Clinical significance of enteric protozoa in the immunosuppressed human population. Clin Microbiol Rev 22(4): 634-650.

Stark D, Fotedar R, van Hal S, Beebe N, Marriott D, Ellis TG and Harkness J (2007). Prevalence of enteric protozoa in human immunodeficiency virus (HIV)-positive and HIV-negative men who have sex with men from Sydney, Australia. Am J Trop Med Hyg 76:549-552.

Stauffer W, Abd-Alla M and Ravdin JI (2006). Prevalence and incidence of Entamoeba histolytica in South Africa and Egypt. Arch Med Res 37: 266-269.

Stenmark H (2009). Rab GTPases as coordinators of vesicle traffic, Nature Reviews Molecular Cell Biology 10:513–525.

Strachan WD, Chiodini PL, Spice WM, Moody AH and Ackers JP (1988). Immunological differentiation of pathogenic and nonpathogenic isolates of E. histolytica. Lancet i:561-563.

Tanyuksel M., Petri Jr.WA (2003). Laboratory diagnosis of amoebiasis. Clin Microbiol Rev 16: 713-729.

Tanyuksel M, Ulukanligil M, Yilmaz H, Guclu Z, Araz RE, Mert G, Koru O, Petri Jr WA. (2008). Genetic variability of the serine-rich gene of the *Entamoeba histolytica* in clinical isolates from Turkey. Turk J Med Scdi 38(3): 239-244.

Bachur TP, Vale JM, Coêlho IC, Queiroz TR, Chaves Cde S (2008). Enteric parasitic infections in HIV/AIDS patients before and after the highly active antiretroviral therapy. Braz J Infect Dis 12(2):115-122.

Tsai JJ, Sun HY, Tsai KS, Chang SY, Hsieh SM, Hsiao CF, Yen JH, Hung CC, Chang SC (2006). Higher seroprevalence of Entamoeba histolytica infection is associated with human immunodeficiency virus type 1 infection in Taiwan. Am J Trop Med Hyg 74(6):1016-9.

Visser LG, Verweij JJ, Van Esbroeck M, Edeling WM, Clerinx J, Polderman AM (2006). Diagnostic methods for differentiation of Entamoeba histolytica and Entamoeba dispar in carriers: performance and clinical implications in a non-endemic setting. Int J Med Microbiol 296:397-403.

WHO (1997). "WHO/PAHO/UNESCO report. A consultation with experts on amoebiasis. Mexico City, Mexico 28-29 January 1997. Epidemiological Bulletin 18 (1): 13-14.

Ximénez C, Morán P, Rojas L, Valadez A, Gómez A, Ramiro M, Cerritos R, González E, Hernández E, and Oswaldo P (2011). Novelties on Amoebiasis: A Neglected Tropical Disease. J Glob Infect Dis 3(2): 166–174.

Ximénez C, Morán P, Rojas L, Valadez A, Gómez A (2009). Reassessment of the epidemiology of amebiasis: state of the art. Infect Genet Evol 9(6):1023-32.

Zengzhu G, Bracha R, Nuchamowitz Y, Cheng W and Mirelman D (1999). Analysis by enzyme-linked immunosorbent assay and PCR of human liver abscess aspirates from patients in China for *Entamoeba histolytica*. J Clin Microbiol 37:3034-3036.

15

Retrospective Analysis of Leishmaniasis in Central Tunisia: An Update on Emerging Epidemiological Trends

Akila Fathallah Mili[1,3], Fatma Saghrouni[1], Zeineb BenSaid[2], Yusr Saadi- BenAoun[3], Ikram Guizani[3] and Moncef BenSaid[1]
[1]*Laboratory of Parasitology, Farhat Hached Hospital, Sousse*
[2]*Service of Dermatology, Farhat Hached Hospital, Sousse*
[3]*Laboratory of Parasitic Epidemiology and Ecology (LEEP) - LR00SP04, Institut Pasteur, Tunis*
Tunisia

1. Introduction

This study aimed at describing the spatio-temporal distribution of leishmaniasis in patients who have sought diagnosis in the laboratory of Parasitology of Farhat Hached hospital, Sousse, Tunisia, across the 1986-2010 period in order to: **i)** highlight important features and trends of leishmaniasis and its epidemiology; **ii)** and to assess whether the activity of the unit reflects the situation of the disease at the national level and whether it could constitute an indicator of public health relevance.

2. Current situation of leishmaniasis in Tunisia

Tunisia is located in Northern Africa, bordering the Mediterranean sea between Algeria and Libya. Its climate is of the hot temperate Mediterranean type. Bioclimatic zones range from humid in the north to saharian in the extreme south (Figure 1).
The country is divided into 24 governorates, each composed of a variable number of "delegations"; and each delegation is subdivided into localities also named "imadas" (Figure 2).
Four forms of leishmaniasis are known to occur in Tunisia: i) the sporadic cutaneous leishmaniasis (SCL); ii) the chronic cutaneous leishmaniasis (CCL); iii) the zoonotic cutaneous leishmaniasis (ZCL); iv) the visceral leishmaniasis (VL).

2.1 The sporadic cutaneous leishmaniasis (SCL)

This form is caused by dermotropic zymodemes of *Leishmania infantum*, mainly the zymodeme MON 24 and to a lesser extent MON 1 (Aoun et al., 2000, 2008; Ben Ismail et al, 1986, 1992; Gramiccia et al., 1991; Haouas et al., 2007; Kallel et al., 2005, 2008b). The dog is supposed to be the reservoir as it is the case for the viscerotropic zymodemes and *Phlebotomus perfiliewi* as the phletotomine vector. But other vectors can not be excluded; following an outbreak of SCL in the locality of Oued Souani, le Kef governorate, *P. langeroni* was found infected by *L. infantum* (Guerbouj et al., 2007).

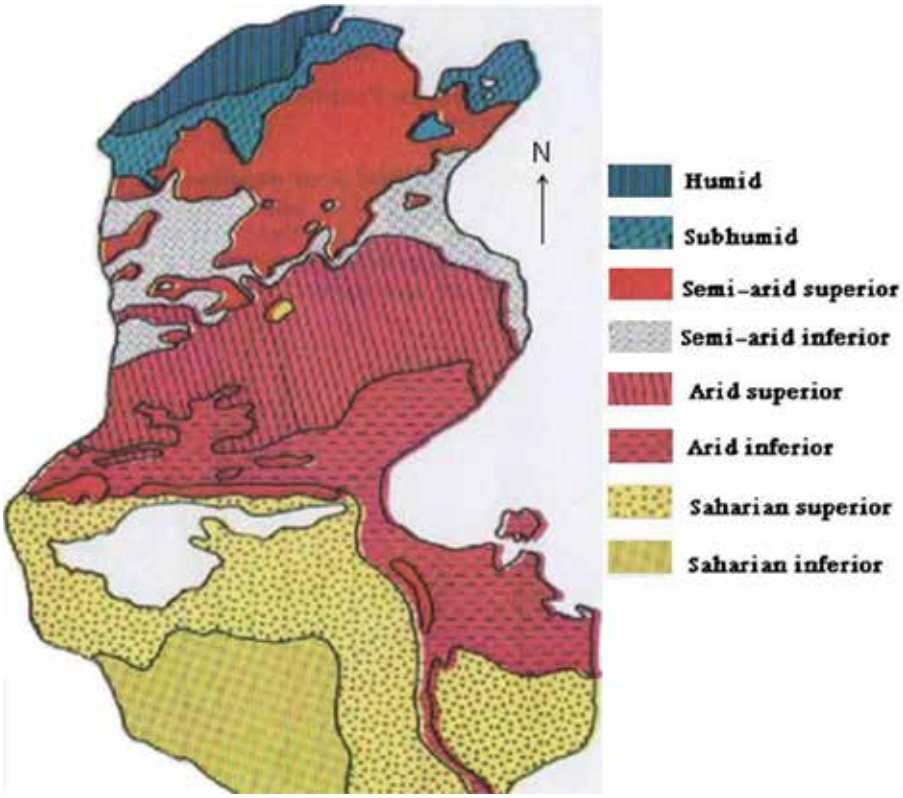


Fig. 1. Map of Tunisia showing the distribution of bioclimatic zones.

SCL was first described in 1917 in a patient from Sakiet Sidi Youssef (Le Kef governorate) located next to the Algerian frontier (Nicolle & Blanc, 1917), and again in 1945 (Chadli et al., 1968; Vermeil, 1956). Since 1945, the disease has sporadically been reported with, however, an incidence that gradually increased from a median of one case /year between 1945 and 1955 to 22 cases /year in the 1990s and 59 cases/year in the 2000s (Table 1; Anonymous ; Ben Abda et al., 2009; Ben Said et al., 2006; Ben Ismail & Ben Rachid, 1989; Ben Rachid et al., 1983, 1992; Chadli et al., 1968 ; Chaffai et al., 1988; Ladjimi & Lakhoua, 1955 ; Vermeil, 1956). SCL has long been supposed to be confined to the humid and subhumid bioclimatic areas north to the "dorsale" or "tunisian Ridge" (the eastern extension of the Atlas mountains), in rural areas where its distribution overlaps with that of VL (Ben Ismail et al., 1989; Ben Rachid et al., 1983; Ben Rachid & Ben Ismail, 1989; Chadli et al., 1986 ; Vermeil, 1956). However, over the last three decades, many cases originating from central Tunisia governorates (Monastir, Sousse, Mahdia and Kairouan) and referred to our laboratory were found to be very suggestive of SCL on the basis of epidemiological, clinical and parasitological criteria. Typed strains obtained from some of these patients proved indeed to be *L. infantum* (Ben Said et al., 2006). These data were very indicative of SCL spread towards the south of the country. These findings were further confirmed by additional reports (Aoun et al, 2000, 2008; Ben Abda et al., 2009; Kallel et al., 2008a, 2008b). The revised SCL distribution is shown in figure 3. SCL usually occurs sporadically within no particular season. However, over the last few years, local outbreaks were reported like that of Sidi Bourouis in Siliana governorate, which involved more than 30 patients over a short period (Bel Hadj et al., 2003).

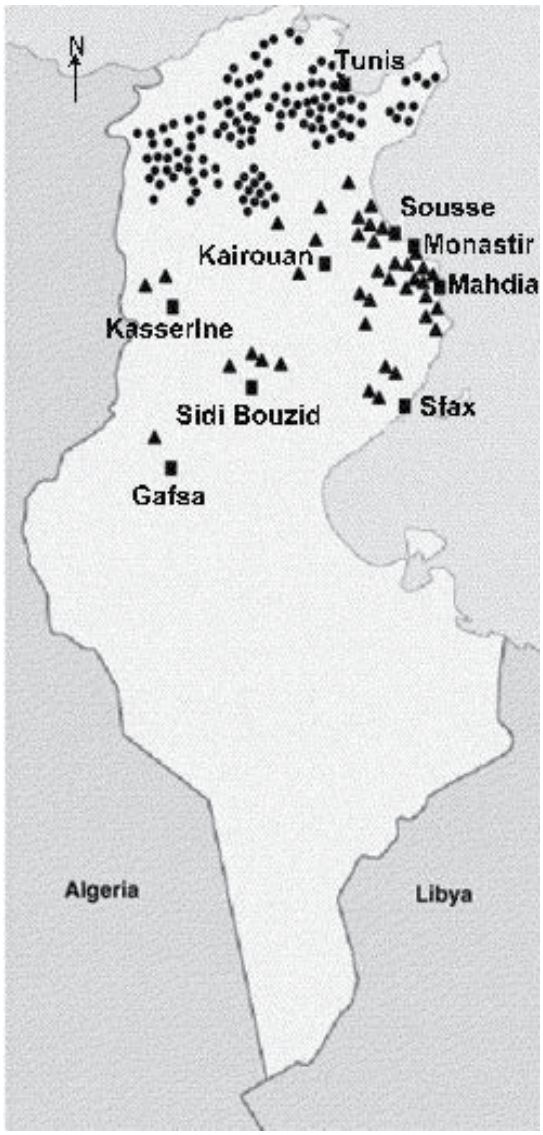
Fig. 2. Map of Tunisia showing its subdivision into 24 governorates (Grand Tunis is composed of 4 governorates).

Years	Nb of cases	Median
1945-55	10	1.0
1956-67	17	1.4
1968-78	84	8.4
1979-89	151	15.1
1990-99	217	21.7
2000-10	652	59.3
Total	1131	17.1

Table 1. Number of recorded sporadic cutaneous leishmaniasis cases since 1945.

Clinically, SCL is characterized in more than 90 % of patients by a small single ulcerated or lupoid lesion of the face that often lasts longer than one year and up to three years (Bel Hadj et al., 1996; Ben Ismail & Ben Rachid, 1989; Chaffai et al, 1988; Masmoudi et al., 2007). On a parasitological level, amastigotes of *L. infantum* are smaller than those of *L. major*, usually

less than 3μ , and promastigotes are difficult to maintain on NNN medium (Aoun et al., 2000, 2003).



•: corresponds to the classical distribution of the cases; ▲: illustrates the emerging distribution of cases.

Fig. 3. Updated distribution of sporadic cutaneous leishmaniasis in Tunisia.

2.2 The chronic cutaneous leishmaniasis (CCL)

Formerly known as anthroponotic CL, CCL sporadically occurs in micro-foci located in the south-eastern presaharian and saharian areas of Tunisia. Its distribution is rural, sub-urban and urban; and cases are reported from houses, farms, and even from troglodytes of Tataouine (Tataouine governorate) and Matmata (Gabès governorate), with a median

annual incidence of 10 cases /year (Figure 4; Ben Ismail & Ben Rachid, 1989; Ben Rachid et al., 1983, 1992; Chadli et al., 1968). CCL was first reported in 1957 and nearly nothing was known about the disease before this date. In 1979, an outbreak involving 47 individuals arose in Tataouine; and the epidemiological investigation, which included the isoenzymatic typing of strains isolated from the patients, led to the identification of the parasite as the MON 8 zymodeme of *L. tropica*, named *L. killicki,* a species previously described in Kenya, Namibia, Yemen and, more recently in Algeria and Libya (Harrat et al., 2009; Rioux et al., 1986; Sang et al., 1994).

Fig. 4. Actual distribution of chronic cutaneous leishmaniasis.

Surprisingly, over the last decade, cases of CCL were reported in patients originating from areas where this form has never been previously reported, the first case being in a child from Meknassy in Sidi Bouzid governorate (Haouas et al., 2005). Later, additional cases were reported from Kairouan, Gafsa and Siliana governorates, very far from the classical foci of Tataouine (Aoun et al., 2008; Ben Abda et al., 2009; Bouratbine et al., 2005).

Hence the actual distribution and incidence of CCL need further investigations and obviously should be revised.
L. killicki is transmitted by *Phlebotomus sergenti*, but the reservoir is still debated. Median age of patients suffering of CCL is 21 years (Ben Abda et al., 2009; Ben Ismail & Ben Rachid, 1989). Clinically, CCL most often presents as single or very few lesions on the face or limbs, that are dry, extensive, and chronic, lasting for up to six years (Ben Abda et al., 2009; Ben Ismail & Ben Rachid., 1989; Chaffai et al., 1988; Masmoudi et al., 2007).

2.3 The zoonotic cutaneous leishmaniasis (ZCL)

ZCL is by far the most frequent and the most widely distributed form of CL in Tunisia where it constitutes a major public health problem. It is endemo-epidemic in extended areas of central and southern Tunisia (Figure 5). It is caused by *L. major* and transmitted by the zoo-anthropophilic *Phlebotomus papatasi* sandfly which is mainly encountered and caught in and around the rodent burrows, and less in and around human habitations (Ben Ismail et al., 1987b, 1987c; Ben Rachid et al., 1992; Ghrab et al., 2006; Helal et al., 1987). The reservoirs are rodents of the *Psammomys* and *Meriones* genera. The main one is *P. obesus*, a prolific diurnal rodent that is very abundant in arid and subsaharian areas. Its feeding requirements consist exclusively of chenopodiaceae (*Salicornia*, *Salsola*, *Atriplex*) that mainly grow in sandy, humid and salty soils unsuitable for agricultural purposes (Ben Ismail et al., 1987a; Ben Rachid et al., 1992; Fichet-Calvet et al., 2003). *Psammonys* infection rate may reach 100 %. The hygrophilic nocturnal rodents, *Meriones shawi* and *Meriones libycus* act as secondary reservoirs and are responsible of the spread of the disease because of their migratory habits. *Meriones* are granivorous and build their burrows in jujube trees (*Zizyphus*) surrounding cereal fields and often cause important agricultural damage (Ben Ismail & Ben Rachid, 1989; Ben Rachid et al., 1992). All *Leishmania* strains isolated so far from humans, rodents and phlebotomine vectors are of the zymodeme MON 25 (Aoun et al., 2008; Ben Abda et al., 2009; Ben Ismail et al., 1986; Haouas et al., 2007).
ZCL was first described in 1882 in and around Gafsa oases, and termed "clou de Gafsa" (Deperet & Bobinet, 1884). From this date and up to the beginning of the 20th century, many additional outbreaks were reported in the same area. Then, the disease continued to occur on a very sporadic mode, and nearly disappeared (Chadli et al., 1968; Vermeil, 1956).
In 1982, a large outbreak arose in Nasrallah delegation (Kairouan governorate), near to the recently completed Sidi-Saad dam (Ben Ammar et al., 1984). From there, the disease rapidly spread to cover large rural and sub-urban parts of the central and south-western neighbouring governorates, so that, by 1986, 10 governorates were involved (Figure 6). In season 1991-1992, ZCL extended further south-east to Medenine and Tataouine governorates. All along the outbreak, Sidi Bouzid, Gafsa and Kairouan governorates have remained the leading areas in terms of incidence (Anonymous). However, from the early 1990s and up to date, Tataouine, Tozeur, Médenine, Kébili, Gabès, Sfax and Kasserine have emerged as active and stable foci (Figure 5). In the last few years, some level of ZCL spread towards the north (Siliana, Béja, Le Kef, Tunis and Zaghouan governorates) was registered, which is somewhat surprising (Ben Abda et al., 2009).
The number of annual recorded cases rapidly grew from 182 cases in 1982 to > 18000 cases by 1987, > 65000 cases by 1999. Up to date, > 120000 cases were reported and the epidemic is still going on. It should be mentioned however, that the actual number of cases is supposed to be underestimated and would exceed 150000 cases (Chahed et al., 2002). The number of annual recorded cases greatly varied and ranged from 1129 cases in 1995 to > 15000 in 2004,

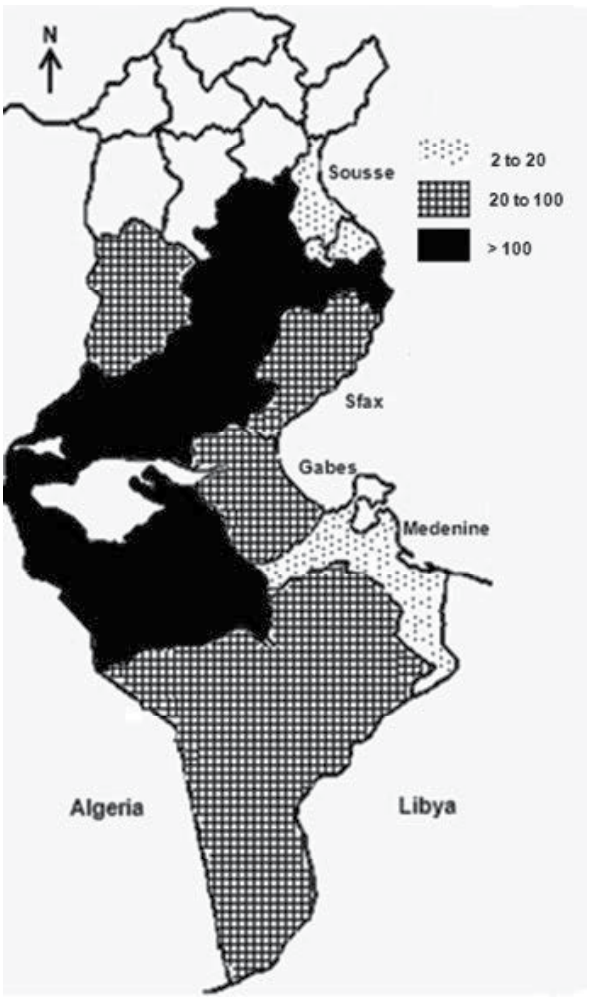


Fig. 5. Distribution of zoonotic cutaneous leishmaniasis according to incidence (expressed as $^{0}/_{00\,000}$).

with an average of 4700 cases/year (Figure 7). The annual incidence showed the same fluctuations. It ranged between 3 $^{0}/_{00\,000}$ in the less affected areas and > 500 in Kairouan, and > 1000 in Gafsa and Sidi Bouzid in 2004 (Anonymous). These fluctuations are at least in part related to the dynamics of rodents' populations. It was demonstrated that the distribution of *P. obesus* is of paramount importance in the epidemiology of the human disease (Ben Ismail et al., 1987a; Ben Ismail & Ben Rachid, 1989; Ben Salah et al., 2007; Fichet-Calvet et al., 2003). On the other hand, control programmes are undoubtedly very effective and beneficial in terms of incidence. Indeed, a rodent control project, consisting of ploughing lands with growing chenopods and reafforestation with acacia and other plants was launched in 1992 in Sidi Bouzid city and suburbs that resulted in a dramatic decrease in the incidence of the disease in the area; but the intensity of the epidemic grew again as soon as the control programme stopped (Figure 7). From the mid 2000s, similar control measures have been carried out again in Sidi Bouzid and then in Sidi El Heni

delegation which is the main transmission focus in Sousse governorate, and led to an obvious decrease of incidence in both foci.

The ongoing outbreak that started in 1982 near the Sidi-Saad dam may be explained by the interruption, as a result of the construction of the dam, in the flooding that frequently occurred in the area and used to decimate a high proportion of rodents every year. In addition, the enrichment of the area's ground water helped the chenopodiaceae to grow abundantly, thereby increasing the food source of *Psammomys*. On the other hand, *Atriplex*, a plant grown in large quantities as a sheep fodder is much appreciated by *Psammomys*. Furthemore, humidity created by the dam is highly suitable for the sandflies.

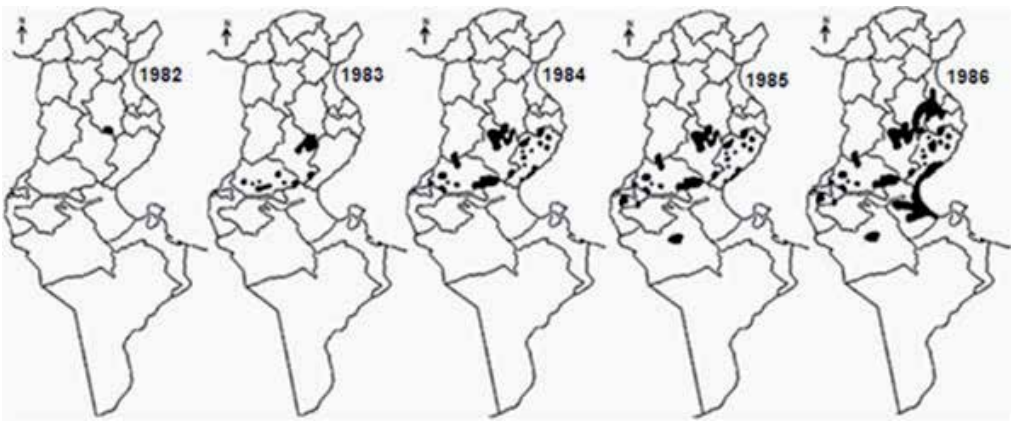


Fig. 6. Spread of zoonotic cutaneous leishmaniasis during the period 1982- 1986 (Ben Ismail R., unpublished).

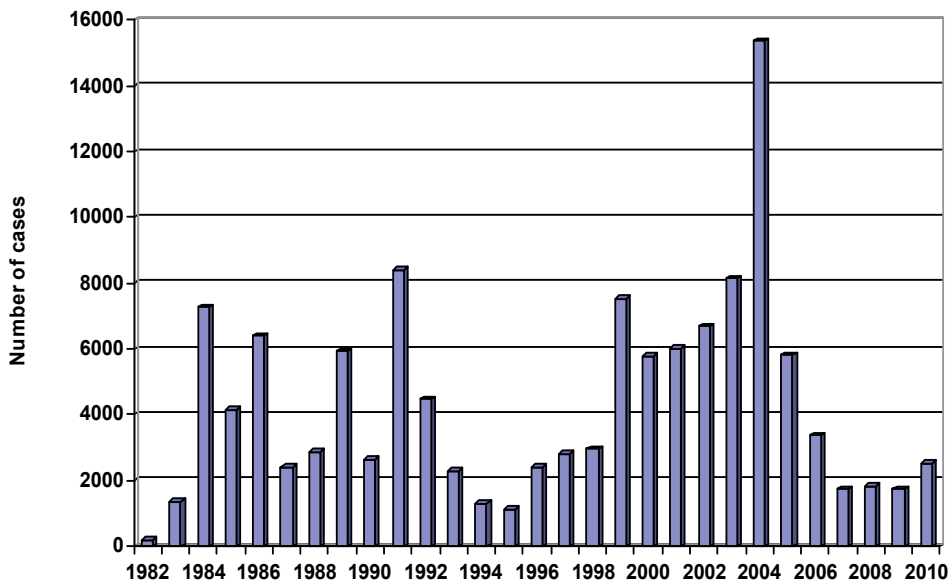


Fig. 7. Annual number of reported zoonotic cutaneous leishmaniasis cases at the national level from 1982 up to 2010.

One of the characteristics of ZCL is its marked seasonal occurence as most cases are observed between october and january (Ben Ismail & Ben Rachid 1989; Ben Rachid et al., 1992).
Clinically, ZCL most often presents as multiple, inflammatory ulcers on the face and the limbs that usually scar in less than 6-8 months, and affects all ages with a median of 24 years (Ben Abda et al., 2009; Ben Ismail & Ben Rachid, 1989; Chaffai et al., 1988; Masmoudi et al., 2007; Zakraoui et al., 1995).

2.4 Visceral leishmanasis (VL)

VL has been known to sporadically occur in Tunisia, since 1903 where the first case of mediterranean VL was described in a child living in the suburbs of Tunis. The disease is caused by *Leishmania infantum*, mostly zymodeme MON 1 and to a lesser extent zymodemes MON 24 and MON 80 (Aoun et al., 2001, 2008; Bel Hadj et al., 1996, 2000, 2002; Ben Ismail et al., 1986; Haouas et al., 2005; Kallel et al., 2008b). It is transmitted by *Phlebotomus perniciosus* sandfly, and the dog is the exclusive reservoir, with an infection prevalence rate ranging from 5% to 26 % (Ben Ismail et al., 1986; Ben Rachid et al., 1992; Bouratbine et al., 1998).
Since 1903, 2449 VL cases were reported in Tunisia (Table 2).

Period	Nb cases	Median/year
1903-1956	151	2.8
1957-1967	142	12.9
1968-1981	178	12.7
1982-1989	578	72.3
1990-2000	720	65.5
2001-2010	680	68.0
Total	2449	22.7

Table 2. Number of visceral leishmaniasis cases reported in Tunisia (1903-2010)

Up to 1981, incidence was low to moderate and nearly all cases were reported from Zaghouan, North-West (Le Kef, Béja, Jendouba, Siliana), Tunis and Sousse governorates, located in the humid, sub-humid and semi-arid zones (Anderson et al., 1934, 1938; Ben Ismail et al., 1986; Ben Rachid et al., 1983; Chadli et al., 1968; Khaldi et al., 1991; Nicolle, 1912; Vermeil, 1956). From the early 1980s, the incidence markedly increased and the disease progressed towards the south, mainly to Kairouan governorate and to a lesser extent to Sfax, Sidi Bouzid, Kasserine and Tozeur governorates, together with an increase in canine leishmaniasis (Ayadi et al., 1991; Bel Hadj et al., 1996; Ben Salah et al., 2000; Besbès et al., 1994; Bouratbine et al., 1998; Chargui et al., 2007; Pousse et al., 1995). Indeed, from the 1980s, the region of Kairouan emerged as a highly active VL focus, and by 1991-1992 it was recognized as the most active one, with 30 to 55 % of reported cases (Anonymous).
The distribution of VL is given in figure 8. Highest number of cases was reported in 1922 (n = 130), 1993 and 2006 (n = 122), and 2005 (n = 120). It is worth mentioning that VL has nearly disappeared between 1974 and 1980, as a result of the anti-malaria campaign which included extensive insecticide spraying (Ben Rachid et al., 1983). The disease sporadically occurs in rural and to a lesser extent in suburban areas and mainly affects children under five years, cases in immunocompetent adults being less than 5 % (Ben Ismail & Ben Rachid, 1989; Ben Rachid et al., 1983; Besbès et al., 1994; Bouratbine et al, 1998; Hammoud et al.,

2004; Pousse et al. 1995). In HIV Tunisian patients, VL is infrequent; it is best diagnosed by PCR (Kallel et al., 2007).

Fig. 8. Actual distribution of visceral leishmaniasis in Tunisia.

3. Materials and methods

The laboratory of parasitology of Sousse, the third major Tunisian city which is located in the central, coastal part of Tunisia, was first created in 1986. It can be considered as a central laboratory ensuring the diagnosis of parasitic diseases' cases including leishmaniasis, sent by generalists, dermatologists, pediatricians or hematologists either from the same hospital or other hospitals mainly from governorates of central Tunisia like Kairouan, Sidi Bouzid and Mahdia. Results are usually sent back to the referring doctors so the patients can be managed as appropriate. Since its creation, the laboratory has been involved in the diagnosis of all forms of leishmaniasis.

For purposes of this retrospective study, VL and CL patients' data, including age, sex, geographical origin and the likely place of contamination, clinical presentation and evolution without or after treatment were collected.

3.1 Diagnosis of cutaneous leishmaniasis

The diagnosis of CL is usually achieved by the demonstration of amastigotes in Giemsa-stained smears from the fluid obtained by scraping the edges of the cutaneous lesion with a

sterile lancet. In addition, the size of the identified *Leishmania* is carefully evaluated, because small amastigotes (< 3μ) are evocative of *L. infantum* and *L. killicki* rather than *L. major* whose amastigotes are larger. Over the last decade, lesions evocative of CL but found negative in direct examination were further submitted to PCR technique, known to be more sensitive.

3.2 Diagnosis of visceral leishmaniasis

3.2.1 Parasitological diagnosis

Diagnosis of VL is usually based on the demonstration of amastigotes in Giemsa stained bone-marrow aspirates. However, in our laboratory, bone-marrow smears are mainly carried out and read in doubtful cases, because they usually are performed and interpreted by the haematologists and sometimes the pediatricians themselves and the results of the examination are later sent to us together with sera specimens.

For the purpose of parasitological diagnosis of VL, since 1996, we have been using the cytoconcentration of peripheral blood according to the technique of Petithory et al, 1997. Cytoconcentration is usually practiced first, and when positive the painful bone marrow aspiration is no more needed and the diagnosis of VL is established.

3.2.2 Serodiagnosis of leishmaniasis

All sera from suspected or confirmed VL cases are sent to our laboratory for serodiagnosis. Our reference technique is the fluorescent antibody test (FAT). It is carried out by using *Leishmania*-spot IF® slides (bioMerieux, France) on sera serially diluted starting from 1/100. Sera showing fluorescence at ≥ 1/200 dilution are considered positive.

In addition to FAT, we have been using, since 2003, the rK39 dipstick test (DST). The first one we used was from Inbios International, USA (kalazar detect®). Then, since 2007 and up to date, we have been using the Dia.Med IT Leish® (Dia Med, Switzerland), according to Saghrouni et al., 2009.

3.3 Culture of *Leishmania*

Our laboratory has mainly been involved in the culture of dermotropic *Leishmania*, even though isolates from VL bone-marrow aspirates were occasionally cultured.

Culture was mainly carried out for the purpose of typing strains by isoenzyme electrophoresis (IEE) technique. Media we have been using are the NNN (Nicolle Novy Mc Neal), the BHI (brain heart infusion agar) and the CRS (coagulated rabbit serum), the last being best for cutaneotropic *L. infantum* strains. *Leishmania* isolates were typed either in the IEE unit of the LEEP or in the laboratory of parasitology of the Faculty of Pharmacy, Monastir, where an IEE unit was set up in 2001.

3.4 Molecular biology

Up to 2008, our laboratory was not equipped for molecular biology techniques. In 2007, as part of a collaborative project on "molecular tools for accurate diagnosis and better assessment of leishmaniases", codirected by the LEEP and our own laboratory and financed by the IAEA, a molecular biology unit was set up in our department and has just started working. In the meanwhile we have been collaborating with the molecular biology unit of the LEEP, mainly for identifying *Leishmania* strains. The first study, conducted in 2005, aimed at identifying strains isolated from patients suspected for having SCL, by using K-DNA-PCR according to Smyth et al., 1992 and PCR-RFLP according to Guerbouj et al., 2001 (Ben Said et al., 2006).

Later, additional strains isolated from CL patients were typed by a novel Multiplex PCR (Saadi et al., in preparation) as part of the IAEA project that is still ongoing.
In addition, specimens from patients with lesions highly evocative of CL but found negative in parasitological examination were addressed for PCR to the laboratory of parasitology of the Faculty of Pharmacy, Monastir, where a molecular biology unit has just been set up. PCR was performed according to Chargui et al., 2005.

4. Results

4.1 Cutaneous leishmaniasis

Over the 25 year period study, 4329 patients were investigated for CL. Most of them were referred to our laboratory by the service of Dermatology of Farhat Hached hospital, Sousse. *Leishmania* parasites were demonstrated in 2087 cases (48.2%). In addition, out of 86 PCR performed on samples found negative in direct examination of dermal Giemsa stained smears, 17 were positive. So, the total number of CL cases diagnosed during the study period was 2104. Most of them were diagnosed during the last decade.
Out of the 2104 diagnosed cases 50 were confirmed or very likely SCL form. Fourteen came from areas known to be endemic for SCL (Le Kef, Jendouba, Siliana, Zaghouan and Bizerte governorates). The remaining 36 cases were from areas located in central Tunisian governorates where SCL has never been described: 13 were from Monastir, 12 from Sousse, 6 from Mahdia and 5 from Kairouan. Out of the 36 cases, 13 originated from areas known to be endemic for ZCL, in Kairouan, Mahdia and Sousse governorates. The age of patients ranged from to 5.5 to 63 years (median = 28.5 years). Twenty six were males and 24 were females (sex ratio M/F = 1.08).
In three patients, the isolate proved to be *L. killicki*. The first patient was a 5 year old child from Meknassi in Sidi Bouzid governorate where CCL was unknown. The second was a 30 year old woman from Ghomrassen, known to be endemic for CCL. The third patient was a 21 year old woman who came from Nasrallah, one of the most active ZCL foci in Kairouan governorate and from where no CCL cases were reported before.
All the remaining 2051 patients were suffering from ZCL. 1182 were females and 869 were males (sex ratio F/M = 1.36). Their age ranged from 1 month to 90 years (median = 28 years). The annual distribution of ZCL cases diagnosed over the 25 year period is shown in figure 9. The number of cases ranged from 5 in 1997 to 443 in 2004.
The place of contamination could be ascertained in 1873 out of the 2051 patients. In the 178 remaining cases, the geographical origin of contamination could not be determined with certainty because of multiple displacements of patients across two, three or more endemic areas. In addition, some patients were originating from Libya and Algeria and were not included in the analysis of the spatio-temporal distribution of ZCL cases.
The distribution of the 1873 ZCL cases according to the area where the contamination took place is given in table 3. Most of the patients came from Sidi Bouzid (610 = 32.6%), Mahdia (494 = 26.4%), Kairouan (369 = 19.7%), and Sousse (306 = 16.3%) governorates.
The distribution of cases according to delegations inside the four governorates mentioned above is shown in figure 10, and the annual distribution of diagnosed cases in figure 11. According to seasonal distribution 1745 (85.1 %) cases were diagnosed between October and February. All confirmed CL patients were treated with local or parenteral N-methylglucamine antimoniate (glucantime®) together with cryotherapy in those with few lesions. Most of treated patients responded well to antimonial treatment, and scarring of

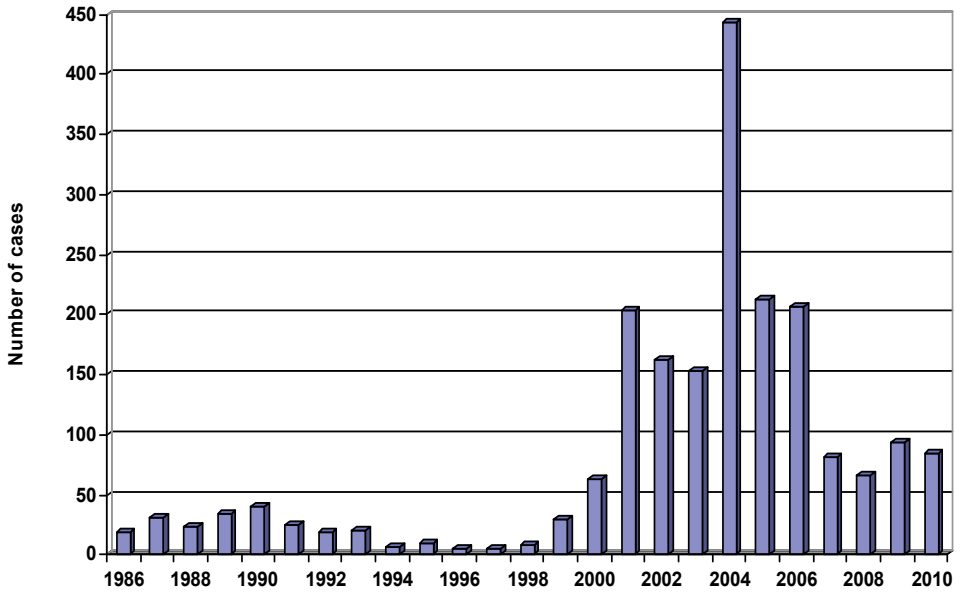


Fig. 9. Annual distribution of 2051 zoonotic cutaneous leishmaniasis cases (1986-2010).

Governorate	Delegation	Nb/delegation	Nb (% /governorate)
Sidi Bouzid	Ouled Haffouz	453	610 (32.6 %)
	Jelma	41	
	Sidi Bouzid city	33	
	Others	88	
Mahdia	Chorbène	321	494 (26.4 %)
	Souassi	54	
	Hbira	54	
	Ouled Chamekh	51	
	Others	14	
Kairouan	Nasrallah	127	369 (19.7 %)
	Bouhajla	56	
	Kairouan south	45	
	Hadjeb layoun	42	
	Others	99	
Sousse	Sidi El Heni	241	306 (16.3 %)
	Msaken	33	
	Others	32	
Others : Monastir, Gafsa, Kasserine, Sfax, Tataouine, Tozeur, Gabes, Kébili	-	-	94 (5%)
TOTAL	-	-	1873

Table 3. Distribution of 1873 zoonotic cutaneous leishmaniasis cases according to governorate and delegation.

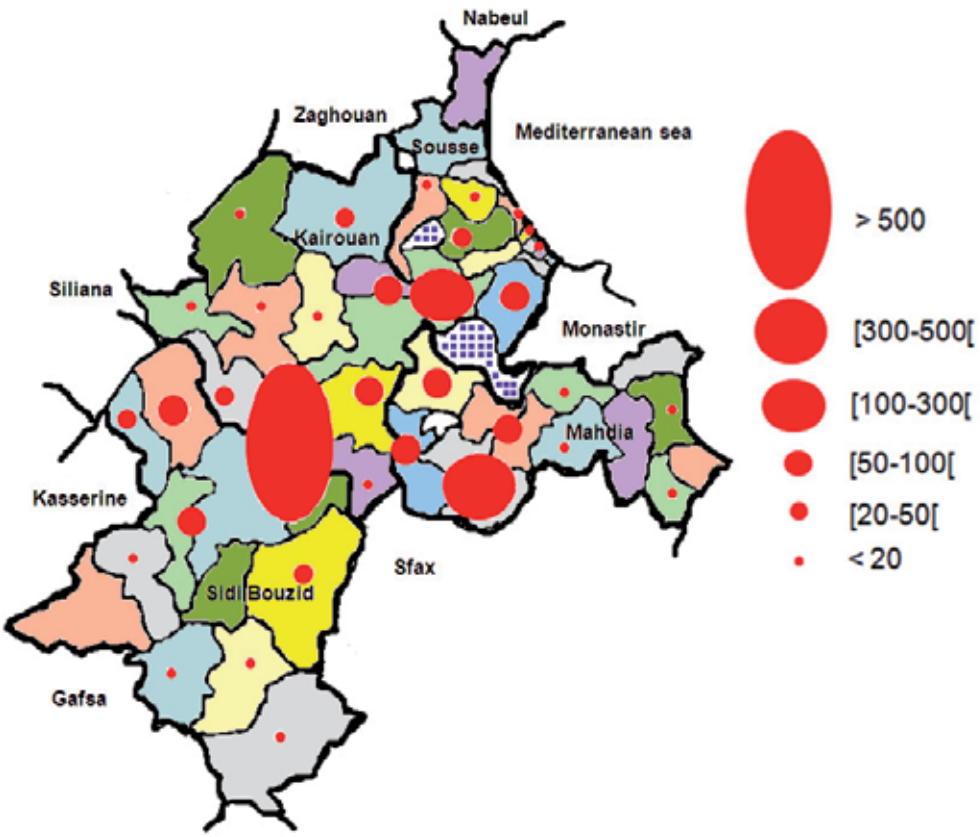


Fig. 10. Distribution of 1779 zoonotic cutaneous leishmaniasis cases diagnosed over the 25 year period originating from Sidi Bouzid, Mahdia, Kairouan and Sousse. The dots represent the number of cases. The limits of the governorates are shown in bold lines. The administrative district subdivisions are illustrated by different colours in each governorate.

lesions was obtained in a few weeks (one to three) after the treatment was initiated. However, in some patients, the outcome was unexpectedly atypical in that the lesions took much more delay to heal as demonstrated by the persistence of *Leishmania* in direct examination. In some adequately treated patients, the lesions persisted longer than one year and up to 4.5 years in one of them. In another patient, nearly 100 glucantime® injections were needed before the lesions resolved. In some additional cases, new lesions appeared while the patient was under specific treatment for previous ulcers. On the other hand, in many patients treated with *in situ* antimonial infiltrations, sporotrichoid nodules developed a few days or weeks later, next to the treated lesion.

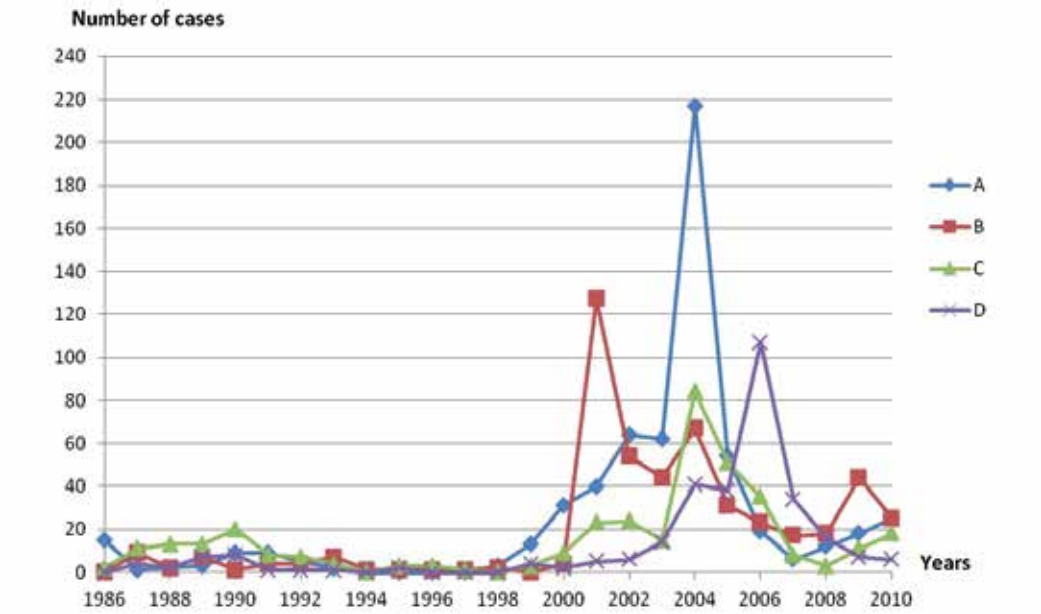


Fig. 11. Annual distribution of 1779 zoonotic cutaneous leishmaniasis cases in Sidi Bouzid (A), Mahdia (B), Kairouan (C) and Sousse (D) governorates (1986 to 2010).

4.2 Visceral leishmaniasis

Up to 2010, 2784 serum samples were addressed to our laboratory for suspected VL cases. Anti-*Leishmania* antibodies were demonstrated in 944 sera. FAT and rK39 DST were positive in 944 (99.2%) and 822 (87.1%) cases respectively. In all positive patients, VL was confirmed by the demonstration of amastigotes in bone marrow and/or cytocentrifuged blood and/or favourable outcome under antimonial treatment. In 8 confirmed VL cases, both serological tests were negative.

Thus, the total number of VL cases diagnosed over the 25 year period is 952.

Three hundred and eight blood samples were investigated by the cytoconcentration technique. Among them, 201 were from confirmed VL patients, and amastigotes were demonstrated in 88 (43.7%) of them. In six cases, bone marrow examination was also negative. Apart from the confirmed VL FAT negative cases, all the remaining negative sera were from patients suffering from diseases other than VL, mainly leukaemia and various haematological disorders. Nine hundred and thirty four of the VL patients were children, aged 2 months to 14 years (median: 26.8 months). Eighteen were adults aged 19 to 39 years (median: 25.9 years); one of them was a HIV+ woman. The number of annually diagnosed cases ranged from 14 cases in 2004 to 83 cases in 2005. The highest proportion of VL cases (60%) was from Kairouan governorate, known to include the most active foci of VL in Tunisia, followed by Sousse governorate and, to a lesser extent from other areas in central Tunisia (Mahdia, Monastir, Kasserine).

All confirmed VL patients were treated with glucantime®, usually a single 21 to 28 day course. The outcome was favourable in the majority of them. Death occurred in 18 (1.9%) patients. All were children. In 4 of them, no response to antimonials was observed despite an adequate treatment regimen. They were assumed to be resistant to antimonials even

though the resistance could not be assessed by *in vitro* testing of the strain. In an additional child, initially treated by three successive courses of glucantime®, healing was ultimately obtained with ambisome® (liposomal amphotericin B).

4.3 Typing of *Leishmania* strains

Over the 25 year study period 103 *Leishmania* strains were typed by either IEE or PCR or both. More than 150 isolates were *in vitro* cultured. Fifteen of them were from bone-marrow aspirates in VL cases. All were typed by IEE and found to be *L. infantum*. Most cultured isolates were from CL patients. Eighty one of them could be maintained in culture media, cryopreserved and typed by IEE. The remaining isolates were either lost after repeated subculture or contaminated during sampling or subculturing. It should be mentioned that strains addressed to the laboratory of Monastir, were typed and identified at the zymodeme level, whereas those typed in the LEEP were only identified at the species level.

Sixty four isolates were typed by molecular techniques: 4 were typed by K-DNA-PCR and PCR-RFLP, and 60 by multiplex PCR.

Overall, 88 CL strains were typed by either IEE or PCR technique: 7 were *L. infantum*, 3 were *L. killicki* and 78 were *L. major*. All *L. major* strains that were typed at the zymodeme level were found to be *L. major* MON 25.

5. Discussion

One of the main characteristics of the four leishmaniasis forms occurring in Tunisia is their changing epidemiological pattern, which has continuously been described over the last 30 years. These changes mainly consisted of the spread of all forms of the disease from their initial endemic foci towards neighbouring or farther areas.

The VL which has long been confined to the northern parts of Tunisia showed a noticeable spread towards the central and southern parts of the country, where the disease has never been previously reported. The Kairouan governorate where VL was unknown till 1980, has progressively emerged as a highly endemic area and by the early 1990s, it became the most active focus in terms of incidence (Anonymous; Bel Hadj et al., 1996; Besbès et al., 1994; Bouratbine et al., 1998). These findings contrast with earlier periods where VL was mainly reported from Zaghouan, North-western and Tunis governorates and to a lesser extent from the central coastal region (Sousse) (Anderson et al., 1938; Ben Ismail et al., 1986; Ben Rachid et al., 1983; Chadli et al., 1968). In the same time, VL progressively spread further south to southern and central western governorates (Sfax, Sidi Bouzid, Kasserine, Tozeur) (Ayadi et al., 1991; Chargui et al., 2007). While spreading, the VL incidence markedly increased from a median of 12 to 68 annual recorded cases, 50 % of them being from Kairouan foci.

Similar trends were described in SCL which, up to 1980, was very rarely reported south to the Tunisian ridge ("dorsale"). From the early 1980s, and up to date, SCL has continuously been reported from central and southern parts of the country (Aoun et al., 2000, 2008; Ben Saïd et al., 2006; Kallel et al., 2005, 2008a).

ZCL was first reported on the end of the 19th century causing large outbreaks in Gafsa and neighbouring areas. Later, it has nearly disappeared for half a centrury; but reappeared again in 1982 causing a widespread epidemic that is still going on and constitutes a major public health problem in extended areas of the country, with up to date more than 150000 cases (Anonymous; Ben Abda et al., 2009; Ben Ismail & Ben Rachid, 1989).

CCL, known to be confined to limited areas in the presaharian region of Tataouine, has itself shown spread towards areas far from its original endemic foci, up to Kairouan governorate (Aoun et al., 2008; Haouas et al., 2005).

The described changes are supposed to mainly originate in the agricultural development and the ecological transformations that occurred in the concerned areas and led to a marked increase in irrigated surfaces that in turn helped the reservoirs (mainly rodents) and vectors to abundantly proliferate, and created biotopes very suitable for the *Leishmania* cycles to establish and amplify (Aoun et al., 2008; Ben Abda et al., 2009; Ben Salah et al., 2007). In addition, urbanization of previously rural areas made the populations' access to medical facilities easier, and consequently more and more leishmaniasis cases could be detected and diagnosed. The impact of climate change and variability on leishmaniasis (due to el Niño) was shown in Colombia and is associated to shifts in insect and animal distributions (Cardenas et al., 2008). This phenomenon was also shown to impact on the incidence of VL in Brazil and models were established to predict high risk years for VL (Franke et al., 2002). In Tunisia, the relationship between climate variability and leishmaniasis is not well studied. However, incidence rate of VL was shown to be positively correlated with mean yearly rainfall and continentality index; a rainy year is followed 2 years later by an increase in VL cases likely modulated by the intensity of transmission to dogs and by the influence on sandfly abundance (Ben-Ahmed et al., 2009). Distribution of sandflies classically associated to VL was also shown to be dependent on bioclimate (Zhioua et al., 2007), likewise for the distribution of *Phlebotomus papatasi*, the vector of ZCL (Chelbi et al., 2009).

Knowledge on the epidemiological patterns and trends in leishmaniasis has much increased over the last 3 decades mainly because of a better identification of the *Leishmania* species involved in the natural cycles of the parasite. This knowledge greatly benefited of the availability of techniques used in typing isolates obtained from humans or from reservoirs and sandflies. In this respect, IEE or molecular techniques allowed a more precise identification of the *Leishmania* at the species, zymodeme and genomic (schizodeme) level (Ben Hammouda et al., 2000; Ben Ismail et al., 1992; Ben Said et al., 2006; Guerbouj et al., 2007; Guizani et al., 1993, 1994a, 1994b; Guizani et al., 2002; Hanafi et al., 2001; Kallel et al., 2008b).

As far as zymodemes are concerned, it was shown that *L. major* was much more homogeneous than *L. infantum*. Indeed, all *L. major* strains obtained from humans, rodents and sandflies proved to be identical to the *L. major* MON 25 reference strain (Aoun et al., 2008 ; Ben Ismail et al., 1986; Haouas et al., 2007 ; Kallel et al., 2005). In contrast, at least three *L. infantum* zymodemes occur in Tunisia : i) the MON 1, mainly causing VL and to a lesser extent SCL ; ii) the MON 24, the most common agent of SCL, that also causes VL ; iii) the MON 80, which is very rarely isolated (Aoun et al., 1999, 2008; Bel Hadj et al., 2000, 2002; Gramiccia et al., 1991; Haouas et al., 2007). These findings show that a single zymodeme may cause quite different diseases; and raise the as yet poorly documented question of pathogenesis and virulence of strains in cause. In addition, it was shown that the distribution of both MON 1 and MON 24 *L. infantum* zymodemes was different according to the geographical areas the patients originated from. Indeed, *L. infantum* MON 24 zymodeme was more frequently reported in VL cases from Kairouan governorate as compared to northern VL foci where MON 1 zymodeme is predominant. This was attributed to the emergent character of Kairouan foci which may favour and select atypical or rare variants (Aoun et al., 2008; Ben Abda et al., 2009). Involvement of different

sandfly species in transmission of different *L. infantum* parasites can not be excluded (Guerbouj et al. 2007). It is well established that different sandfly species belonging to the sub-genus *Larroussius* are involved in the transmission of *L. infantum*; in Tunisia clear association between sandfly species and bioclimate was demonstrated and presence of species of this subgenus in the different bioclimatic zones allowed explaining the extension of VL (Zhioua et al., 2007).

The reservoir of *L. killicki* is still debated. Some findings argue for its zoonotic origin as the disease is hypoendemic and frequently reported in rural populations. In addition, *L. killicki* in Kenya was isolated from hyracoides (Sang et al., 1994). However, CCL has also been reported from urban areas and from regions where *L. major* ZCL is highly prevalent and additional CCL cases were probably misdiagnosed. So, the CCL may be more frequent than previously thought. On the other hand, it was hypothesized that competition between *L. major* and *L. killicki* may lead to some degree of pressure exerted by the first species with a subsequent reduction in the incidence of the second one (Aoun et al., 2008). However, parasite identification still remains circumstantial; parasite isolation from cutaneous lesions is often contaminated by overgrowing microorganisms. In this situation, isolating and typing of much more additional strains is highly needed, in order to get further insight in the knowledge of the concerned *Leishmania* cycles.

Even though IEE has been demonstrated to be quite efficient in identifying isolates and still constitutes a reference tool, molecular techniques would be more relevant in assessing variability of strains, as even minor genomic variations may have important consequences on the epidemiological and the clinical levels. An additional advantage of the molecular techniques is their rapidity in that culture of isolates is no more needed. On the other hand, they are much adapted for both diagnostic (PCR, Multiplex PCR, Real time- PCR) and epidemiological (PCR-RFLP, Multiplex PCR, RAPD) purposes (Aoun et al., 2008; Bel Hadj et al., 2002; Ben Ismail et al., 1992; Ben Said et al., 2006; Chargui et al., 2005; Guerbouj et al., 2007; Guizani et al., 1994a, 1994b; Guizani et al., 2002; Hanafi et al., 2001; Saadi et al., in preparation). In addition, molecular techniques are specific and highly sensitive, allowing identification of parasite species hard to isolate or to maintain *in vitro* in culture or too scarce to be detected or cultured. It has been supposed that the dog is the reservoir of *L. infantum* MON 24 zymodeme and *P. perfiliewi* the phlebotomine vector. However, isolation of this zymodeme from either the dog or the sandfly has been unsuccessful, making the hypothesis questionable (Aoun et al., 2008). In this situation, molecular techniques would be much more suitable for this purpose. Using DNA tools, *P. langeroni* was found infected with *L. infantum* in an active transmission focus of SCL (Guerbouj et al., 2007). It may be concluded from all these findings that identification and typing of isolates are much needed in order to best assess the epidemiology and to investigate the diversity and changing patterns of leishmaniasis.

The incidence of leishmaniasis as reflected by the annually reported cases at the national level together with our own findings was shown to highly vary and fluctuate over the 25 year period study. These fluctuations were attributed first to the dynamics of the reservoirs' populations and sandflies, as a consequence of climatic variations, environmental and ecological changes occurring in the endemic areas (see section 2); and second, to the immunization, through the repeated outbreaks, of the infected human populations, that progressively makes previously infected people in the endemic area less receptive to the infection. This was best demonstrated in the ZCL form (Ben Salah et al., 2007).

In Tunisia, human activities, agricultural development and the subsequent ecological changes mainly benefited to rodents which dramatically proliferated in areas where their populations were previously too low for the *Leishmania* cycle to be completed. This can explain that ZCL is by far the predominant and the most widespread form of leishmaniasis in Tunisia, in terms of both incidence and geographical distribution. In addition, no sustained control programme for ZCL was carried out in most endemic areas, because of the difficulties that arose in the organization and the feasibility of such programmes. This made the ZCL epidemics still going on. It is worth mentioning however that control projects may be successful, as demonstrated by the results of those launched in 1992 in Sidi Bouzid area and again in the mid 2000s in Sidi Bouzid and Sidi El Heni, that led to an obvious decrease in the ZCL incidence over the mid 1990s in Sidi Bouzid and the last few years in both regions.

As compared to SCL, the spread of VL has been more important and obvious. This finding was related, among other factors, to the ecology of the phlebotomine vectors as it was shown that *P. perniciosus* (sub-genus *Larroussius*), the vector of MON 1 *L. infantum* zymodeme is more resistant to dry climate as compared to *P. perfiliewi*, the presumed vector of MON 24 zymodeme (Aoun et al., 2008 ; Ben Abda et al., 2009). Given the fact that presence and distribution of the different sandfly species generally associated to *L. infantum* transmission explained extension of VL (Zhioua et al., 2007) and that the extensions seem to concern in majority MON 24 parasites (Aoun et al., 2008; Ben Abda et al., 2009; Kallel et al., 2008a), explanations may well relate to parasite features not accounted for by the mere attribution to zymodemes and/or to differential distribution of reservoirs yet to identify.

Even though molecular techniques namely PCR and its variants have been increasingly used in the diagnosis of all forms of leishmaniasis, it is to be pointed out that conventional techniques should not be neglected. In VL, bone-marrow aspirate examination was shown to be sensitive enough, even though not optimal. Examination of cytoconcentrated peripheral blood was demonstrated to be positive in nearly 50 % of VL cases and was proposed to be carried out at first in order to avoid the painful bone-marrow puncture (Ben Said et al., 1998, Chemli et al., 2006). In serodiagnosis, FAT was found to be very efficient and suitable as its sensitivity exceeded 95 % and rK39 DST, even though less sensitive, very useful because it is highly specific, easy to perform and adapted to epidemiological investigations (Saghrouni et al., 2009). PCR is much more adapted to the diagnosis of the asymptomatic and subclinical forms of the disease because of its sensitivity and the scarcity of parasites in such situation. In CL, parasitological diagnosis by demonstrating amastigotes in dermal specimens is again very adequate, even though PCR was demonstrated to be more sensitive (Chargui et al., 2005). In contrast, serodiagnosis is much less useful in CL diagnosis as antibodies' amounts are often very low and results difficult to interpret.

One of the main objectives of this study was to assess whether the activity of our laboratory reflects the actual situation of the leishmaniases at the country level, and whether it could constitute an indicator of public health relevance. As far as VL is considered, most patients originating from Kairouan and Sousse governorates, where are located the most active foci, were investigated for serodiagnosis in our laboratory. So, we may conclude that our findings reflect the actual incidence of VL in the region. This is further confirmed by the comparison of our results to the statistics on the annually recorded cases of VL and edited by the primary health care direction of the Tunisian Ministry of Health (Anonymous). Obviously, our findings match these reports as highest number of registered cases at the national level was in 1992 (n = 130), 1993 (n = 122) and 2005 (n = 122); and highest number of

cases diagnosed in our laboratory was as follows: 74 cases in 1992, 72 cases in 1993 and 83 cases in 2005. This is not surprising as nearly 50 % or more of VL cases originate from Kairouan governorate.

Concerning CL, we have to point out that first SCL cases from central Tunisia were diagnosed in our laboratory in patients with clinical presentation found to be evocative of this form, and amastigotes, very suggestive of *L. infantum* as compared to *L. major* because of their small size (Ben Saïd et al., 2006). As mentioned in section 2, additional cases were further reported and the spread of SCL towards central and southern Tunisia confirmed. Similarly, the first CCL case originating from outside the original foci of Tataouine area was again diagnosed in our laboratory (Haouas et al., 2005); and CCL spread was further confirmed by additional reports leading to the revised geographical distribution of the disease (Bouratbine et al., 2005; Aoun et al., 2008). In our laboratory, we used to diagnose ZCL since 1986. Up to the late 1990s, the number of diagnosed cases was too moderate as compared to that of ZCL reported cases in the endemic regions (Figures 7 & 9). This, for at least, the two following reasons: **i)** at the beginning of the outbreak, many patients suffering from ZCL were only clinically diagnosed, so that only a few of them were addressed to the laboratory for parasitological confirmation, **ii)** from the 1990s, a great number of families originating from ZCL endemic areas migrated and settled in Sousse city and suburbs, where they progressively constituted a large community. Most of them used to return back to their home of origin for summer holidays where, because continuously exposed to phlebotomine bites, many of them contract leishmaniasis which is later addressed to us for diagnosis. It is worth mentioning that a high proportion of this community originates from Sidi Khelif in Ouled Haffouz delegation, Sidi Bouzid; and and many of them were contaminated there and were later diagnosed in our laboratory. From 2000 and onwards, the number of ZCL patients who attended our laboratory for diagnosis dramatically increased. We consider that since this date the activity of our laboratory indirectly but adequately reflects the actual status of ZCL in central Tunisia, namely in Sidi Bouzid, Mahdia, Kairouan and Sousse governorates. This is best illustrated by our findings in 2004 where > 400 ZCL cases were diagnosed in our laboratory. In the same year, the highest incidence of ZCL (> 15000 cases) was registered at the national level, because of the three epidemic peaks that occurred in Sidi Bouzid (Sidi Bouzid city, Regueb, Menzel Bouzayène), Gafsa (Gafsa city, Sned, Mdhilla) and Kairouan (Nasrallah, Hajeb Layoun, Chrarda) governorates (Anonymous). The peak we registered in 2001 was due to an outbreak that arose in Chahda, a small locality in Chorbène delegation (Mahdia governorate). Indeed, more than 50 % of cases we diagnosed in 2001 were from this locality. A similar peak in Sidi El Heni delegation (Sousse governorate) was registered in 2006, with 107 cases. This peak was followed by a noticeable decrease as soon as control activities were carried out in the area. Similarly the decrease in ZCL incidence shown in our study over the last 5 years can be attributed to an important decrease in the transmission in Sidi Bouzid region where control activities were launched in the mid 2000s (Figure 9). This decrease was reflected on the national level over the same period (Figure 7). Last, our results confirm the seasonal occurrence of ZCL.

Our findings and results show that the activity of our laboratory reflects the situation of leishmaniasis at the regional level and at least in part at the national level, and may be an indicator of public health relevance. It may thus contribute to early alert of health authorities on changing epidemiological trends or emergence of leishmaniasis in the region. However, our activities may not reflect the actual situation and epidemiological changes occurring in farther endemic areas like Gafsa, Tozeur, Kébili, Médenine and Gabès known to include

highly active foci because very few patients from these regions seek medical facilities in Sousse hospital. Work is in progress to identify and characterize the *Leishmania* parasites actually circulating in central Tunisia using molecular DNA techniques.

6. Acknowledgement

This work received financial support from IAEA (Project ref. TUN/6/012), and from the Ministry of Higher Education and Scientific Research (LR00SP04). We thank the staff of the LEEP, the laboratory of Parasitology of the Faculty of Pharmacy, Monastir and of the laboratory of Parasitology of Farhat Hached hospital, Sousse for their contribution.

7. References

Anderson, C. Chronique du kala-azar en Tunisie. *Arch. Inst.Pasteur Tunis* (1934), 23 : 455-464.

Anderson, C. Chronique du kala-azar.*Arch. Inst. Pasteur Tunis* (1938), 27 : 96-104.

Anonymous. Epidemiological Bulletins of the Direction of Primary Health care. Ministry of Health, Tunisia, 1980-2010.

Aoun, K., Bouratbine, A., Harrat, Z., Maherzi, A., Belkaid, M., Bousnina S. & Ben Ismail R. Confirmation of the presence of *Leishmania infantum* MON-80 in Tunisia. *Bul Soc Pathol Exot*. (1999), 92 : 29-30.

Aoun, K., Bouratbine, A., Harrat, Z., Guizani, I., Mokni, M., Bel Hadj Ali, S., Ben Osman, A., Belkaïd, M., Dellagi, K. & Ben Ismail, R. Epidemiologic and parasitologic data concerning sporadic cutaneous leishmaniasis in northern Tunisia. *Bull Soc Pathol Exot*. (2000), 93: 101-103.

Aoun, K., Bouratbine, A., Harrat, Z., Belkaïd, M. & Bel Hadj Ali S. Particular profile of the zymodemes of *Leishmania infantum* causing visceral in Tunisia. *Bull Soc Pathol Exot*. (2001), 94 : 375-377.

Aoun, K., Chahed, M.K., Mokni, M., Harrat, Z. & Bouratbine, A. Importance of amastigote forms morphology to differentiate *Leishmania infantum* and *Leishmania major* species. *Arch Inst Pasteur Tunis* (2003), 80 : 53-56.

Aoun. K., Amri. F., Chouihi. E., Haouas, N., Bedoui, K., Benikhelef, R. & Bouratbine, A. Epidemiology of *Leishmania (L) infantum*, *L. major* and *L. killicki* in Tunisia : results and analysis of the identification of 226 human and canine isolates. *Bull Soc Pathol Exot*. (2008), 101: 323-328.

Ayadi, A., Ben Ismail, R. & Ben Rachid, M.S. Spread of *Leishmania infantum* Kala-azar transmission area towards central and southern Tunisia. *Arch Ins Pasteur Tunis* (1991), 68 : 269-273.

Bel Hadj, S., Djaiet – Baraket, Z., Jemli, B., Ben Osman, A. & Chaker, E. Visceral and cutaneous leishmaniasis of the North. *Bull Soc Pathol exot*, (1996), 89: 269-273.

Bel Hadj, S., Pratlong, F., Mahjoub, H., Toumi, N.H., Azaiez, R., Dedet, J.P. & Chaker, E. Infantile visceral leishmaniasis from *Leishmania infantum* Mon-24 : a reality in Tunisia. *Bull Soc Pathol Exot*. (2000), 93: 12-13.

Bel Hadj, S., Pratlong, F., Toumi, N.H., Kallel, K., Mahjoub, H., Babba, H., Azaiez, R., Dedet, JP. & Chaker, E. Visceral leishmaniasis in Tunisia: result of the isoenzymatic characterization of 65 *Leishmania infantum* strains. *Trans R Soc Trop Med Hyg*. (2002), 96: 627-630.

Bel Hadj, S., Pratlong, F., Hammami, M., Kallel, K., Dedet, JP. & Chaker, E. Human cutaneous leishmaniasis due to *Leishmania infantum* in the Sidi Bourouis focus (Northern Tunisia): epidemiological study and isoenzymatic characterization of the parasites. *Acta Trop*. (2003), 85: 83-86.

Ben Abda, I., Aoun, K., Ben Alaya, N., Bousslimi, N., Mokni, M. & Bouratbine, A. Curent epidemiological, clinical and parasitological data concerning cutaneous leishmaniasis in Tunisia. *Revue Tunisiennne d'Infectiologie* (2009), 2: 31-36.

Ben-Ahmed, K., Aoun, K., Jeddi, F., Ghrab, J., El-Aroui, M.A. & Bouratbine, A. Visceral leishmaniasis in Tunisia: spatial distribution and association with climatic factors. *Am J Trop Med Hyg*. (2009), 81: 40-5.

Ben Ammar, R., Ben Ismail, R., Helal, H., Bacha-Hamba, D., Chaouch, A., Bouden, I. & Ben Rachid, M.S. A new focus of rural cutaneous leishmaniasis in Sidi Saad area (Tunisia). *Bull Soc Fr Parasitol*. (1984), 2: 9-12.

Ben Hammouda, A., Guizani, I., Jedidi, S., Dellagi, K. & Ben Ismail, R. *Leishmania infantum* species-specific kDNA probes: isolation an evaluation. *Arch Inst Pasteur Tunis*. (2000), 77: 37-43.

Ben Ismail, R., Gradoni, L., Gramiccia, M., Bettini, S., Ben Rachid, M.S. & Garraoui, A. Epidemic cutaneous leishmaniasis in Tunisia: biochemical characterization of parasites. *Trans R Soc Trop Med Hyg,* (1986), 80: 669-70.

Ben Ismail, R., Ben Rachid, M.S., Gradoni, L., Gramiccia, M., Helal, H. & Bach-Hamba, D. Zoonotic cutaneous leishmaniasis in Tunisia: study of the disease reservoir in the Douara area. *Ann Soc Belg Med Trop.* (1987a), 67: 335-343.

Ben Ismail, R., Gramiccia, M., Gradoni, L., Helal, H. & Ben Rachid, M.S. Isolation of *Leishmania major* from *Phlebotomus papatasi* in Tunisia. *Trans R Soc Trop Med Hyg*. (1987b), 81: 749.

Ben Ismail, R., Helal, H., Bach-Hamba, D. & Ben Rachid, M.S. Natural infestation of *Phlebotomus papatasi* in a focus of zoonotic cutaneous leismaniasis in Tunisia. *Bull Soc Pathol Exot*. (1987 c), 80: 613-614.

Ben Ismail, R. & Ben Rachid, M.S. Epidémiologie des leishmanioses en Tunisie, in : Maladies tropicales transmissibles. *AUPELF-UREF*, Paris, 1989, 73-80.

Ben Ismail, R., Smith, D.F., Ready, P.D., Ayadi, A., Gramiccia, M., Ben Osman, A. & Ben Rachid, M.S. Sporadic cutaneous leishmaniasis in north Tunisia: identification of the causative agent as *Leishmania infantum* by the use of a diagnostic deoxyribonucleic acid probe. *Trans R Soc Trop Med Hyg.* (1992), 86: 508-510.

Ben Rachid, M.S., Hamza, B., Tabbane, C., Gharbi, R., Jeridi, H. & Ben Saïd, M. Current status of leishmaniasis in Tunisia. *Ann Soc Belg Med Trop.* (1983), 63: 29-40.

Ben Rachid, M.S., Ben Ismail, R. & Ben Saïd, M. The ecology of visceral and cutaneous leishmaniasis in Tunisia. In: "Proceedings of the international worshop on leishmaniasis control strategies, 25-29 nov. 1991, Merida, Mexico, *IDRC and UPCH editors*, (1992, pp. 131-154.

Ben Saïd, M., Amri, F., Mili, A., Ardoin, F., Petithory, J.C. The cytoconcentration in the diagnosis of visceral leishmaniasis in immunocompetent children. *Bull Soc Fr Parasitol.* (1998), 16: 86-94.

Ben Saïd, M., Guerbouj, S., Saghrouni, F., Fathallah-Mili, A. & Guizani, I. Occurrence of *Leishmania infantum* cutaneous leishmaniasis in central Tunisia. *Trans R Soc Trop Med Hyg*. (2006), 100: 521-526.

Ben Salah, A., Ben Ismail, R., Amri, F., Chlif, S., Ben Rzig, F., Kharrat, H., Hadhri, H., Hassouna, M., & Dellagi, K. Investigation of the spread of human visceral leishmaniasis in central Tunisia. *Trans R Soc Trop Med Hyg.* (2000), 94: 382-386.

Ben Salah, A., Kamarianakis, Y., Chelif, S., Ben Alaya, N. & Prastacos, P. Zoonotic cutaneous leishmaniasis in central Tunisia : spatio temporal dynamics *Int J. Epidemiol.* (2007), 36: 991-1000.

Besbès, A., Pousse, H., Ben Saïd, M., Kharrat, H. & Ghenimi, L. Infantile visceral leishmaniasis in central Tunisia (report of 221 cases). *Med Mal Infect.* (1994), 24 : 628-634.

Bouratbine, A., Aoun, K., Chahed, M.K. & Ben Ismail, R. Epidemiological data on infantile visceral leishmaniasis in Tunisia. *Med Mal Infect*. (1998), 28 : 446-447.

Bouratbine, A., Aoun, K., Ghrab, J., Harrat, Z., Ezzedini, M.S. & Etlijani S. Spread of *Leishmania killick*i to Central and South-West Tunisia. *Parasite* (2005) 12: 59-63.

Cardenas, R., Sandoval, C.M., Rodríguez-Morales, A.J., Franco-Paredes, C. Impact of climate variability in the occurrence of leishmaniasis in northeastern Colombia. *Am J Trop Med Hyg*. (2006), 75: 273-7.

Chadli, A., Ben Rachid, M.S. & Fhaiel, A. Chroniques des leishmanioses en Tunisie. *Arch Inst Pasteur Tunis*. (1968), 45: 1-14.

Chaffai, M., Ben Rachid, M.S., Ben Ismail, R., Ben Osman, A. & Makni, N. Clinico-epidemiological forms of cutaneous leishmaniasis in Tunisia. *Ann Dermatol Vénéréol*. (1998), 115: 1255-1260.

Chahed, M.K., Hsairi, M., Bousnina, A., Achour, N., Bouratbine, A. & Hili, K. Assessment of the cutaneous leishmaniasis surveillance system in Tunisia. *Revue Tunisienne de la Santé Militaire*. (2002), 4: 40-47.

Chargui, N., Bastien, P., Kallel, K., Haouas, N., Akrout Messaadi, F, Masmoudi, A., Zili, J., Chaker, E., Othman, A.D., Azaiez, R., Crobu, L., Mezhoud, H. & Baba, H. Usefulness of PCR in the diagnosis of cutaneous leishmaniasis in Tunisia. *Trans R Soc Trop Med Hyg*. (2005), 99: 762-768.

Chargui, N., Haouas, N., Gorcii, M., Akrout Messaadi, F., Zribi, M. & Baba, H. Increase of canine leishmaniasis in a previously low-endemicity area in Tunisia. *Parasite*. (2007), 14: 247-251.

Chelbi I, Kaabi B, Béjaoui M, Derbali M, Zhioua E. Spatial correlation between *Phlebotomus papatasi* Scopoli (Diptera: Psychodidae) and incidence of zoonotic cutaneous leishmaniasis in Tunisia. *J Med Entomol*. (2009), 46: 400-2.

Chemli, J., Abroug, M., Fathallah, A., Abroug, S., Ben Said, M. & Harbi, A. Contribution of leucoconcentration in the diagnosis of kala-azar in Tunisia. *Med Mal Infect*. (2006), 36: 390-392.

Deperet, C. & Bobinet, E. Du bouton de Gafsa au camp de Sathonay. *Archs Med Pharm Milit.* (1884), 3: 296-302.

Fichet-Calvet, E., Jommaa, I., Ben Ismail, R. & Ashford, R.W. *Leishmania major* infection in the fat sand rat *Psammomys obesus* in Tunisia: interaction of host and parasite populations. *Ann Trop Med Parasitol*. (2003), 97: 593-603.

Franke CR, Ziller M, Staubach C, Latif M. Impact of the El Niño/Southern Oscillation on visceral leishmaniasis, Brazil. *Emerg Infect Dis.* (2002), 8: 914-7.

Ghrab, J., Rhim, A., Bach-Hamba, D., Chahed, M.K., Aoun, K., Nouira, S. & Bouratbine, A. Phlebotominae (Diptera: Psychodidae) of human leishmaniasis sites in Tunisia. *Parasite*. (2006), 13: 23-33.

Gramiccia, M., Ben Ismail, R., Gradoni, L., Ben Rachid, M.S. & Ben Said, M. A *Leishmania infantum* enzymatic variant, causative agent of cutaneous leishmaniasis in north Tunisia. *Trans R Soc Trop Med Hyg*. (1991), 85: 370-371.

Guerbouj, S., Victoir, K., Guizani, I., Seridi, N., Nuwayri-Salti, N., Belkaid, M., Ben Ismail, R., Le Ray, D. & Dujardin, JC. Gp63 gene polymorphism and population structure of *Leishmania donovani* complex: influence of the host selection pressure? *Parasitology*. (2001), 122: 25-35.

Guerbouj, S., Chemkhi, J., Kâabi, B., Rahali, A., Ben Ismail, R. & Guizani, I. Natural infection of the species *Phlebotomus Larroussius langeroni* (Diptera: Psychodidae) with *Leishmania infantum* in Tunisia. *Trans R Soc Trop Med Hyg.* (2007) 101: 372-377.

Guizani, I., Ben Hamouda, A., van Eys, G.J., Jedidi, S., Dellagi, K. & Ben Ismail, R. DNA probes development in Tunisia for the identification, the taxonomy and the eco-epidemiology of Old World *Leishmania* species. *Arch Inst Pasteur Tunis*. (1993), 70: 363-371.

Guizani, I., Ben Hamouda, A., Jedidi, S., Dellagi, K. & Ben Ismail, R. Molecular identification of *Leishmania*: development and application of DNA probes in Tunisia. *Arch Inst Pasteur Tunis*. (1994a), 71: 439-446.

Guizani, I., van Eys, G.J., Ben Ismail, R. & Dellagi, K. Use of recombinant DNA probes for species identification of Old World *Leishmania* isolates. *Am J Trop Med Hyg*. (1994b), 50: 632-640.

Guizani, I., Dellagi, K. & Ben Ismail, R. Random amplified polymorphic DNA technique for identification and differentiation of Old World *Leishmania* species. *Am J Trop Med Hyg*. (2002) 66: 152-156.

Haouas, N., Chargui, N., Chaker, E., Ben Said, M., Babba, H., Belhadj, S., Kallel, K., Pratlong, F., Dedet, JP., Mezhoud, H. & Azaiez, R. Anthroponotic cutaneous leishmaniasis in Tunisia: presence of *Leishmania killicki* outside its original focus of Tataouine. *Trans R Soc Trop Med Hyg*. (2005), 99: 499-501.

Haouas, N., Gorcii, M., Chargui, N., Aoun, K., Bouratbine, A., Messaadi Akrout F, Masmoudi, A., Zili, J., Ben Said, M., Pratlong, F., Dedet, JP., Mezhoud, H., Azaiez, R. & Babba, H. Leishmaniasis in central and southern Tunisia: current geographical distribution of zymodemes. *Parasite*. (2007), 14: 239-246.

Hammoud, M., Ben Hassine, L., Azzabi, S., Ben Abdelghani, K., Kooli, C., Kaouach, Z. & Khalfallah, N. Adult visceral leishmaniasis: about six cases. *Revue Tunisienne de la Santé Militaire*. (2004), 6: 54-57.

Hanafi, R., Barhoumi, M., Bel Hadj Ali, S., Guizani, I.. Molecular analysis of Old World *Leishmania* RAPD markers and development of a PCR assay selective for parasites of the *L. donovani* species complex. *Exp Parasitol*. (2001) 98: 90-99.

Harrat, Z., Boubidi, S.C., Pratlong, F., Benikhlef, R., Selt, B., Dedet, JP., Ravel, C. & Belkaid, M. Description of a dermatropic *Leishmania* close to *L. killicki* (Rioux, Lanotte & Pratlong 1986) in Algeria. *Trans R Soc Trop Med Hyg*. (2009), 103: 716-720.

Helal, H., Ben Ismail, R., Bach-Hamba, D., Sidhom, M., Bettini, S. & Ben Rachid, M.S. An epidemiological survey in the focus of zoonotic cutaneous leishmaniaisis

(*Leishmania major*) of Sidi Bouzid (Tunisia) in 1985. *Bull Soc Pathol Exot*. (1987), 80: 349-356.

Kallel, K., Pratlong, F., Belhadj, S., Cherif, F., Hammami, M., Dedet, JP. & Chaker, E. Cutaneous leishmaniasis in Tunisia: results of the iso-enzymatic characterization of 71 strains. *Ann Trop Med Parasitol*. (2005), 99: 11-19.

Kallel, K., Ammari, L., Kaouech, E., BelHadj, S., Anane, S., Kilani, B. & Chaker, E. Asymptomatic beading of *Leishmania infantum* among Tunisian HIV infected. *Pathol Biol*. (2007), 55: 521-524.

Kallel, K., Haouas, N., Pratlong, F., Kaouech, E., Bel Hadj, S., Anane, S., Dedet, JP., Babba, H. & Chaker, E. Cutaneous leishmaniasis caused by *Leishmania infantum* MON 24 in Tunisia : extension of the focus to the center of the country. *Bull Soc Pathol Exot*. (2008a), 101: 30-31.

Kallel, K., Pratlong, F., Belhadj, S., Cherif, F., Hammami, H. & Chaker, E. Isoenzyme variability of *Leishmania infantum* in Tunisia concerning 245 human strains. *Act Trop*. (2008b), 106: 132-136.

Khaldi, F., Achouri, E., Gharbi, A., Debabi, A. & Ben Naceur, B. Infantile visceral leishmaniasis. Data on hospitalized children cases from 1974 to 1988 in INSEE, Tunis. *Médecine Tropicale*. (1991), 51: 143-147.

Ladjimi, R. & Lakhoua, M. Premier cas de bouton d'orient dans la banlieue de Tunis. *Arch Inst Pasteur Tunis*. (1955), 32: 331-336.

Masmoudi, A., Ayadi, N., Boudaya, S., Mziou, T.J., Mseddi, M., Marrakchi, S. & Ayadi, A. Clinical polymorphism in cutaneous leishmaniasis of center of south Tunisia. *Bull Soc Pathol Exot*. (2007), 100: 36-40.

Nicolle C. Statistique des trente premières observations tunisiennes de kala-azar. *Arch Inst Pasteur Tunis*. (1912), 2: 65-67.

Nicolle, C. & Blanc, G. Extension de la région à bouton d'orient tunisienne. *Bull Soc Pathol Exot*. (1917), 10: 378-379.

Petithory, J.C., Ardoin, F., Ash, L.R., Vandemeulebroucke, E., Galeazzui, G., Dufour, M., Paugam, A. Microscopic diagnosis of blood parasites following a cytoconcentration technique. *Am J Trop Med Hyg*. (1997), 57 : 637-642.

Pousse, H., Besbès, A., Ben Said, M., Ghenimi, L. & Kharrat, H. Epidemiology of human visceral leishmaniasis in Tunisia. *J Trop Pediatr*. (1995), 41: 191-192.

Rioux, JA., Lanotte, G. & Pratlong, F. *Leishmania killicki* n.sp. (Kinetoplastida, Trypanosomatidae), in: *Leishmania, taxonomie et phylogenèse, applications éco-épidémiologiques*. IMEEE, Montpellier, 1986, 139-142.

Saghrouni, F., Gaied-Meksi, S., Fathallah, A., Amri, F., Ach, H., Guizani, I. & Ben Said, M. Immuno chromatographic rK39 strip test in the serodiagnosis of visceral leishmaniasis in Tunisia. *Trans R Soc Trop Med Hyg*. (2009), 103: 1273-1278.

Sang, D.K., Njeru, W.K. & Ashford, R.W. A zoonotic focus of cutaneous leishmaniasis due to *Leishmania tropica* at Utut, Rift Valley Province, Kenya. *Trans R Soc Trop Med Hyg*. (1994), 88: 35-37.

Smyth, A.J., Ghosh, A., Hassan, M.Q., Basu, D., De Bruijn, M.H.L., Adhya, S, Mallik, K.K. & Barker, D.C. Rapid and sensitive detection of *Leishmania* kinetoplast DNA from spleen and blood samples of kala-azar patients. *Parasitology*. (1992), 105: 183-192.

Vermeil, C. Chronique des leishmanioses en Tunisie. *Arch Inst Pasteur Tunis*. (1956), 33 : 195-201.

Zakraoui, H., Ben Salah, A., Ftaiti, A., Marrakchi, H., Zaatour, A., Zaafouri, B., Ahmadi, Z., Garraoui, A., Ben Osman, A. & Dellagi, K. Spontaneous course of lesions of *Leishmania major* cutaneous leishmaniasis in Tunisia. *Ann Dermatol Venereol*. (1995), 122: 405-407.

Zhioua, E., Kaabi, B. & Chelbi, I. Entomological investigations following the spread of visceral leishmaniasis in Tunisia. *J Vector Ecol*. (2007), 32: 371-374.

16

Current Advances in Computational Strategies for Drug Discovery in Leishmaniasis

Andrés F. Flórez[1,2], Stanley Watowich[3] and Carlos Muskus[2]
[1]*German Cancer Research Center (DKFZ)/ Division Theoretical Systems Biology, Heidelberg*
[2]*Universidad de Antioquia/Programa de Estudio y Control de Enfermedades Tropicales – PECET, Medellín*
[3]*University of Texas Medical Branch/Department of Biochemistry and Molecular Biology and the Sealy Center for Structural Biology and Molecular Biophysics, Galveston*
[1]*Germany*
[2]*Colombia*
[3]*USA*

1. Introduction

Leishmaniasis is a complex disease caused by several species of the *Leishmania* genus ranging in severity from cutaneous and mucocutaneous lesions to the chronic visceral form that if untreated adequately can cause death. It has a worldwide distribution in 98 countries and 85 out of 98 are developing or poor countries. One of the main problems in leishmaniasis is the limited number of drug options along with the adverse effects they can cause including death (Ahasan., et al. 1996; Sundar & Chakravarty 2010; Oliveira., et al. 2011). In addition, there are reports of treatment failures due to increased parasite resistance to the first drug of choice, the antimonials (Faraut-Gambarelli., et al. 1997; Goyeneche-Patino., et al. 2008). Second-choice drugs, such as amphotericin B, pentamidine, paromomycin, and more recently, miltefosine, have also toxic effects that require hospital management (Maltezou 2008; Oliveira., et al. 2011). Miltefosine, the only oral administered drug for leishmaniasis, has not been tested in many *Leishmania* species. Recently, a central nervous system toxicity was reported for liposomal amphotericin B therapy used to treat cutaneous leishmaniasis (Glasser & Murray 2011).

In the search for new drug targets in *Leishmania*, a group of proteins have been proposed based mainly on their known function, the expression level, and localization, or because they are involved in important metabolic processes in the parasite. Topoisomerases (Das., et al. 2008), kinases (de Azevedo & Soares 2009), proteins localized or targeted to lysosomes (Carrero-Lerida., et al. 2009) are some potential *Leishmania* drug targets. However, none of these protein targets have been used to successfully develop new drugs that can substitute the existing therapies.

Currently, the massive genome sequencing of many medically important microorganisms together with protein structure and drug databases and the development of new computational tools, will allow molecular targets and new drugs to be searched in a more rigorous manner. Three *Leishmania* genomes, *L. major*, *L. infantum* and *L. braziliensis*

(Peacock., et al. 2007) have been sequenced and annotated and a fourth species, *L. mexicana* and some *L. major* strains are in the process of being sequenced (GeneDB, http://www.genedb.org; University of Washington Genome Sequencing Center, http://genome.wustl.edu/gsc/gschmpg.html). The availability of these genomes and the annotated proteins can be used in a rational manner to predict novel drug targets and provide a basis to develop new drugs.

The computational prediction of drugs, in addition to the evaluation of drugs already synthesized and used in other diseases, must be coupled with automated in vitro assessment methodologies of these compounds. In this sense and in the case of *Leishmania*, the use of GFP (Varela., et al. 2009) or luciferase transgenic parasites (Lang., et al. 2005) coupled with techniques such as flow cytometry or fluorometry can be used to rapidly evaluate potential anti-leishmanial drugs. The WHO program for training in tropical diseases research has created a network based on reporter gene technology to foster the process of drug search not only against leishmaniasis but also against other diseases with limited therapeutic options.

2. Selection of drug targets

An initial step in the drug discovery process involves the search and selection of the drug target. This target is frequently a protein that is essential for the organism survival or critical for regulating a particular signaling pathway. In the specific case of parasites, the protein target when inhibited should impair or delay parasite viability. The classical approach of finding a new essential protein that can act as a potential target is the experimental characterization by using gene knockout or knock-down strategies in the target organism. Besides essentiality, some targets are selected for being specific for the pathogen; for example, the ergosterol pathway is present in fungi and *Leishmania spp*, but humans only contain the required enzymes for the synthesis of cholesterol. This is the reason why this pathway has been exploited for searching drugs against mycotic pathogens and also *Leishmania*. However, the experimental approach employing interference RNA (RNAi) is not feasible given *Leishmania* species do not carry the machinery for RNAi (Peacock., et al. 2007), with the exception of *Leishmania braziliensis* where some RNAi-associated genes have been found. In addition, depending on the parasite stage the essentiality of a particular protein could change dramatically. With all these constraints, a rational alternative for choosing effective targets is a more systematic study of the biology of the parasite, with the aim of uncovering important mechanisms that are not evident by studying descriptively isolated proteins. A starting point for this "systems view" of the parasite biology in the case of *Leishmania*, was the sequencing of its genome in 2005 (Ivens., et al. 2005). Since then, more high-throughput data have been generated, not at the same rate as other organisms but with important applications for drug discovery in tropical diseases. This leads to an important issue of data analysis, where computational tools can have a role in reducing the ocean of possibilities of finding a drug for this disease, making more efficient and less costly the experimental setup. In the following sections, we will describe the current computational methods that can be applied to find new drug targets, with special application to the *Leishmania* parasite.

2.1 Selection of targets by homology searching

The simplest approach for finding a drug target is the homology search of essential proteins. There are several organisms with available essential data at genome-wide level (Forsyth., et al. 2002; Kamath., et al. 2003; Hu., et al. 2007). In model organisms such as yeast, the

phenotypic effects of deletion of particular genes have been shown (Giaever., et al. 2002) and more recently the study of genetic interactions on a large scale (Costanzo., et al. 2010). This has been used to elucidate redundancy and possibly some synergistic effects among genes. Therefore, it is possible to find orthologs in the organism of interest that could be essential by comparing its sequences against the list of essential genes in model organisms. The Database of Essential Genes (http://tubic.tju.edu.cn/deg/) (Zhang & Lin 2009) provides information of essential genes in prokaryotes and eukaryotes, and it is also possible to do a BLAST search with the protein of interest. This resource is useful for an exploratory search of essentiality of a particular protein. Another important resource, for drug target data, is the DrugBank database (http://www.drugbank.ca/) (Knox., et al. 2011), which can be used to extract drug-target interactions along with additional pharmacological data. The same strategy can be employed in this case; with the advantage that the homology search will also return possible drug candidates that can be tested on the protein found to have homology to the target in DrugBank.

This methodology has been applied in *Pseudomonas aeruginosa* (Sakharkar., et al. 2004) with the aim of detecting new drug targets, given this bacterium is an important problem in nosocomial settings due to the rapid generation of resistance. In *Leishmania,* drug targets can be also identified by this approach. Tools like BLAST or PSI-BLAST can be employed, with PSI-BLAST being more sensitive for detecting distant relationships among proteins (Altschul., et al. 1997). However, some false positives still can occur due to alignments that are optimal according to the algorithm but not biologically meaningful. The E value helps to detect those alignments that are significant. As an example, running a PSI-BLAST search with the *Leishmania major* proteome against the DrugBank database, one can find among the potential *Leishmania* orthologs to known targets, the protein *LmjF36.2430,* which is similar to the sterol 14- alpha demethylase in fungi. Drugs such as miconazole are known inhibitors of this enzyme. Interestingly, the protein *LmjF19.0450* belongs to the group of protein kinases conserved in other *Leishmania* species; it is constitutively expressed and has significant similarity to other kinase targets in cancer. These are simple cases of how a homology search can generate a list of potential drug targets using existing genomic data. The main advantage of this methodology is that it offers a quick overview of potential targets and second use of drugs. In addition, the STITCH 2 database (http://stitch.embl.de/) (Kuhn., et al. 2010) compiles known and predicted drug-target relationships jointly with biological information about targets in a network-based view.

Despite its simplicity, the homology search strategy has some caveats. Proteins inside the cell perform specific functions depending on their interactions, and these interactions can vary between species. Even if sequences are highly related, pathway conservation is not necessarily present. In addition, temporal regulation is important, as not all the interactions are active at the same time, which can further complicate the analysis. These problems highlight the importance of detecting targets by incorporating more detailed information about the molecular interactions.

2.2 Selection of targets by topological analysis of protein networks

In order to better understand complex pathogens such as *Leishmania* and to improve the efficiency of the drug discovery process, it is crucial to gain deeper knowledge about how protein interactions are established and how these interactions are regulated. This is a central issue for a more accurate definition of essentiality and biological robustness. These interactions can be described as a *network*, a representation commonly used to describe

complex systems. The protein interaction network (interactome) describes all possible molecular interactions among proteins. The interactome is composed of *nodes* that represent the molecular components, in this case proteins, and *edges,* that are the interactions between components (Fig. 1). Depending on the biological function of the node, other types of networks can also be constructed; for example, gene networks involving transcription factors as nodes that regulate other genes by binding (edges) and metabolic networks where the nodes are the enzymes connected by the production of some metabolites. The study of networks comes from a mathematical discipline called *graph theory*, and the analysis of the interaction patterns in the network is defined as *network topology*.(Barabasi & Oltvai 2004)

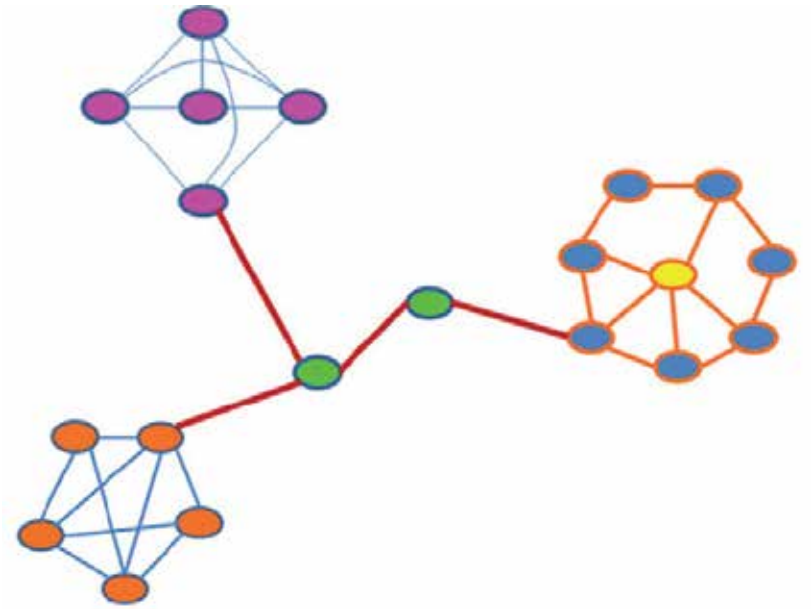


Fig. 1. Schematic representation of a protein network. Yellow circle corresponds to a hub protein, green circles correspond to bottleneck proteins connecting several sub-networks. Lines connecting circles represent the edges of the network.

To detect protein interactions in biological systems, large-scale methods have been developed that can map all possible pairwise interactions. Yeast two-hybrid is a popular technique of this kind, which was used to construct the first interactome (Uetz., et al. 2000). The technique involves the fusion of a protein with a transcription factor DNA-binding domain subunit. This protein is called the *bait*. The second protein is fused onto an activator domain subunit and it is called the *prey*. If the interaction between the bait and the prey is present, the two transcription factor subunits will come closer and the expression of the reporter gene is activated (Osman 2004). The most important limitation of this method is the presence of high number of false positives. However recent evidence has shown that a combination of experimental methods will reduce the number of false interactions (Dreze., et al. 2010).

The initial studies of the yeast interactome revealed that the network structure was not organized randomly, and in fact the organization pattern was similar to other experimentally-observed networks. This particular network structure was called *scale-free*

and it was elucidated by analyzing the number of interactions (or degree distribution) of proteins in the yeast interactome, showing that some nodes were more highly connected than others, and those nodes were in relatively low frequency in the network. This scale-free structure followed a *power law* distribution for the node degree and it described the probability of a node having a certain degree. An interesting consequence of having a scale-free structure is that the network was robust against random deletion of nodes, but susceptible to the deletion of highly connected nodes or *hubs* (Jeong., et al. 2001). The hubs can be detected by measuring the connectivity or degree of the network. In addition, the scale-free network was also susceptible to deletion of other types of nodes that were not highly connected but control the flux of the network; these nodes were called *bottlenecks* (Yu., et al. 2007). A classical example of bottleneck nodes is the scaffold proteins (Good., et al. 2011); these proteins facilitate the communication between signalling pathways very efficiently, although sometimes they are not highly connected. Deleting a bottleneck node will disrupt cellular homeostasis by destroying communication between processes in the cell. This network biology approach becomes an important step in a systems level understanding of the biology of parasites like *Leishmania*, and it becomes very useful for detecting essential nodes that may constitute potential new drug targets.

2.2.1 Construction of the *Leishmania* protein interaction network

The analysis of the *Leishmania* protein network could lead to the discovery of new and effective drug targets. However, current protein interaction data in *Leishmania* have only focused on a few specific proteins, and at this time, no yeast two-hybrid data is available for this organism. Despite this limitation, the use of a computationally-predicted protein network from orthology-based methods is a good first step for the exploration of drug targets that may be more informative than a traditional homology search. The results described in the next section will focus on the current status of the predicted *Leishmania major* interactome and will give some directions for future experimental studies for network and target validation.

Even when protein domain sequences are conserved, multiple combinations of these domains enable an organism to rewire the interactome in different ways. This can overcome the problem of the context of the targets that influence essentiality and enable new hubs or protein targets to be detected. A common disadvantage is the bias towards detection of conserved interactions, which could be a caveat in the case of organism-specific interactions that may also be important for survival. These specific interactions will be only detected when more data becomes available, which will also allow existent predictions to be validated.

In our recent study (Florez., et al. 2010), the protein interaction network in *Leishmania major* was predicted using only the parasite protein sequences and several protein interaction databases, in particular iPfam (Finn., et al. 2005), PSIMAP (Park., et al. 2005) and PEIMAP. These databases included protein-protein interactions defined by analysis of structures of protein complexes and experimental data extracted from literature, including high-throughput experiments. From the structures, the analysis of interacting structural domains was mapped to the sequence, using the domain definition by Pfam (Finn., et al. 2006) and SCOP (Hubbard., et al. 1997). These two databases contained information of domains with a systematic classification for protein families. In this particular case the physical distance between adjacent domains within a complex was used as the criteria for the definition of interaction and it was stored in iPfam and PSIMAP databases. This strategy has been used

in other organisms such as fungi and bacteria (He., et al. 2008; Kim., et al. 2008). The domain interaction analysis generated more diversity in the detection of possible interactions because modular exchange of protein domains allowed rewiring the network even if the isolated sequence of the domain was conserved. However, despite the high accuracy of this method, the prediction of protein interactions was limited as there was not an abundance of crystallized protein complexes. The PEIMAP database was also used, and it included sequences of protein interaction pairs detected by several methods, including co-immunoprecipitation (co-IP) and yeast two-hybrid.

To construct the *Leishmania major* network, protein sequences were extracted from the GeneDB database. This database included genomic and proteomic information of pathogens, including protozoan parasites. The protein sequences were aligned to the interacting domain pairs using PSI-BLAST against the SCOP 1.71 database with an E-value cutoff of 0.0001, as described previously (Kim., et al. 2008). The PSI-BLAST tool was used for the alignments because it had the advantage of detecting small conserved sequences, such as small domains that would be otherwise missed by using the standard BLASTP. The same strategy was applied for the alignments concerning the iPfam database. In this case, the domain assignment for the *Leishmania* proteins was carried out using the Pfam database (release 18.0) with the hmmpfam tool employed for the alignments. The final set of predicted interactions was carried out by homology search over the PEIMAP database using BLASTP, with a minimal cutoff of 40% sequence identity and 70% length coverage. The PEIMAP database included protein-protein interaction (PPI) information from six source databases: DIP (Xenarios., et al. 2000), BIND (Bader., et al. 2001), IntAct (Hermjakob., et al. 2004), MINT (Zanzoni., et al. 2002), HPRD(Peri., et al. 2004), and BioGrid (Stark., et al. 2006).

2.2.2 Filtering interactions by using a combined confidence score

As discussed earlier, the reliability of this analysis and its bias to certain types of protein interactions was dependent on the experimental method employed. Therefore, it was necessary to combine results from different databases to increase the coverage and the confidence of the predicted interactions. In the *Leishmania major* interactome, we used a simple scoring system to identify high confidence interactions. A previous study classified the experimental methods according to their reliability (Chua., et al. 2006), and we used this data in addition to the significance of the sequence alignments to calculate the confidence of the interactions. This scoring system was called the 'combined score' method, and it was applied for the confidence calculations in the STRING database (von Mering., et al. 2005). This database is useful for searching predicted protein interactions detected by other methods, although the definitions are beyond the scope of this chapter.

The score was calculated according to the formula (1):

$$score = 1 - \prod_{i \in E} (1 - R_i)^n \quad (1)$$

where *score* was the confidence value ranging from 0-1 with 1 equals to 100% accuracy, E was the set of methods under analysis (PEIMAP, PSIMAP, iPfam); R_i was the reliability of method i, and n was the number of interactions predicted by method i. The results of these calculations represented pairs of interactions with their respective confidence. With this information, it was possible to select those interactions that fulfilled a particular confidence threshold. In this case, a confidence score of 0.7 was chosen to select the core *Leishmania major* network. The threshold

selection can vary depending on how strongly supported the interactions were required. For us, a 0.70 confidence value gave a smooth fit to the power law distribution and this was an important condition for reliable detection of hubs and bottlenecks.

2.2.3 Topological analysis of the network

Topological metrics such as clustering coefficient and mean shortest path help to describe global characteristics of the network. They measure the density of the connections within the network. Highly dense connected networks are characterized by modular components which also maintain the robustness of the network against failures. Biological networks tend to have a modular structure (Jeong., et al. 2001) and one additional way to test for reliability of the predicted network is by comparing the values of the clustering coefficient and mean shortest path to randomly generated networks with the same number of nodes and edges. These metrics should be statistically different between predicted and random networks. In the case of *Leishmania* network, 1,000 random networks were generated and the metrics calculated and compared to the original network.

The power law fitting for the definition of scale-free structure can be calculated using the plug-in Network Analyzer v.2.6.1(Assenov., et al. 2008) available in the platform Cytoscape (Shannon., et al. 2003). This platform includes a very advanced environment for network visualization and analysis. Network topology metrics, such as betweenness centrality, and connectivity were calculated using the Hubba server (http://hub.iis.sinica.edu.tw/ Hubbawebcite). (Lin., et al. 2008) A plug-in version of this tool in Cytoscape was recently made available. For the calculation of the metrics, the confidence scores of the interactions were used so the detection could be focused on the nodes most likely to be essential in the group of highly supported interactions. From this analysis, a potential list of targets was selected. However, it was possible that some proteins detected could also be conserved in terms of sequence and function among several organisms including humans. This becomes a problem if drugs targeting some of these proteins interfere with important biological process in humans, generating unwanted toxic effects. To avoid this, an additional filter was used for the list of predicted targets and it consisted of aligning the *Leishmania* proteins to the human proteins and excluding proteins that were conserved between these two species.

2.2.4 Prediction of protein function from network clusters

An important feature of network analysis was the prediction of protein function. The normal procedure for inferring function involved a homology search of the unknown protein versus a curated protein database such as UniProt (http://www.uniprot.org/). In some occasions, the detection of protein function was not feasible as significant similarity could not be found. When this approach failed, protein interaction network analysis helped to uncover potential functions. The prediction of protein function based on network analysis involved the assumption suggested by experimental data that interacting proteins tended to have related functions. This implied that it was possible to predict the function of neighboring nodes by clustering network modules and knowing the function of some of the nodes inside of the module. This analysis was carried out over the *Leishmania* network using the Markov Clustering (MCL) algorithm (Enright., et al. 2002) which has been demonstrated to be a robust and fast algorithm for detecting clusters or modules in protein networks (Brohee & van Helden 2006). The algorithm was implemented in the NeAT tool (Brohee., et

al. 2008). For proteins of unknown function in the GeneDB database, we predicted their possible biological roles by evaluating the results of Gene Ontology terms for biological processes using the BinGO plug-in available in Cytoscape.

2.2.5 Selection of candidate drug targets from the network analysis

We constructed a protein-protein interaction (PPI) map, combining the results generated by PEIMAP, iPfam and PSIMAP (Fig. 2). The number of interactions detected for each database is described in (Table 1). By merging the data from the different approaches, bias to a specific class of interactions was avoided. The predicted network also contained isolated sub-networks which were difficult to analyze. These sub-networks appeared as a consequence of the inability to assign domains or from the lack of homology of those proteins to the known pairs of protein interactions. These sub-networks could be investigated by further experimental validation of the network. The total number of high confidence predicted interactions were 33,861 for 1,366 nodes

Number of Proteins	PEIMAP		PSIMAP		IPFAM	
8,335	Nodes	Edges	Nodes	Edges	Nodes	Edges
	718	14,839	3,184	158,984	2,336	50,398

Table 1. Number of nodes and predicted interactions for each database.

By using the topological metrics of connectivity and betweenness centrality we identified 384 potential targets. From these targets, those that had homology to human proteins were eliminated. This substantially reduced the number of potential targets, although higher specificity of drug effects was expected. As explained earlier, toxicity becomes a very important issue when designing or searching for a drug, since many clinical trials failed because of undesired and severe side effects. After this filter, the final number of targets was reduced to 142. Further filters can be applied to this list to select those targets that were most attractive for drug design (Table 2).

From the group of targets, 91 kinases were predicted as essential proteins in the network with no homology to the human kinome. Kinases are very important regulators of signaling in the cell, and in the case of *Leishmania,* kinases are crucial to enable the different metabolic changes needed to adapt to a human host. Perhaps by intensive pharmacological investigation, drugs that are very successful in treating cancer (e.g., Gleevec) could be used against *Leishmania* parasites. One particular example from the group of predicted kinases detected on the network is the protein LMPK (*LmjF36.6470*). This protein has been shown to be essential in *Leishmania mexicana* (Wiese 1998) and it has conserved orthologs in other species such as *L. amazonensis*, *L. major*, *L. tropica*, *L. aethiopica*, *L. donovani, L. infantum*, and *L. braziliensis* (Wiese & Gorcke 2001). Therefore, this kinase was an interesting candidate for experimental validation and possibly its upstream and down-stream interacting partners could also be inhibited by a combination of drugs. In addition, one of the challenges in this disease is to find a broad-spectrum drug that can have therapeutic effects on several *Leishmania* species that cause different forms of leishmaniasis. Further analysis of this target can help to elucidate drugs or combination of drugs that are active against amastigotes, the stage responsible for the disease in mammals. Three ABC transporters that were *Leishmania* specific -

LmjF34.0670, LmjF27.0470, LmjF32.2060 - were also predicted as essential. They confer resistance to antimonials and pentamidine by extruding the drug outside of the cell (Perez-Victoria., et al. 2002). Based upon our analysis, these proteins could be also interesting drug targets due to their role in the homeostasis of the intracellular parasite environment.

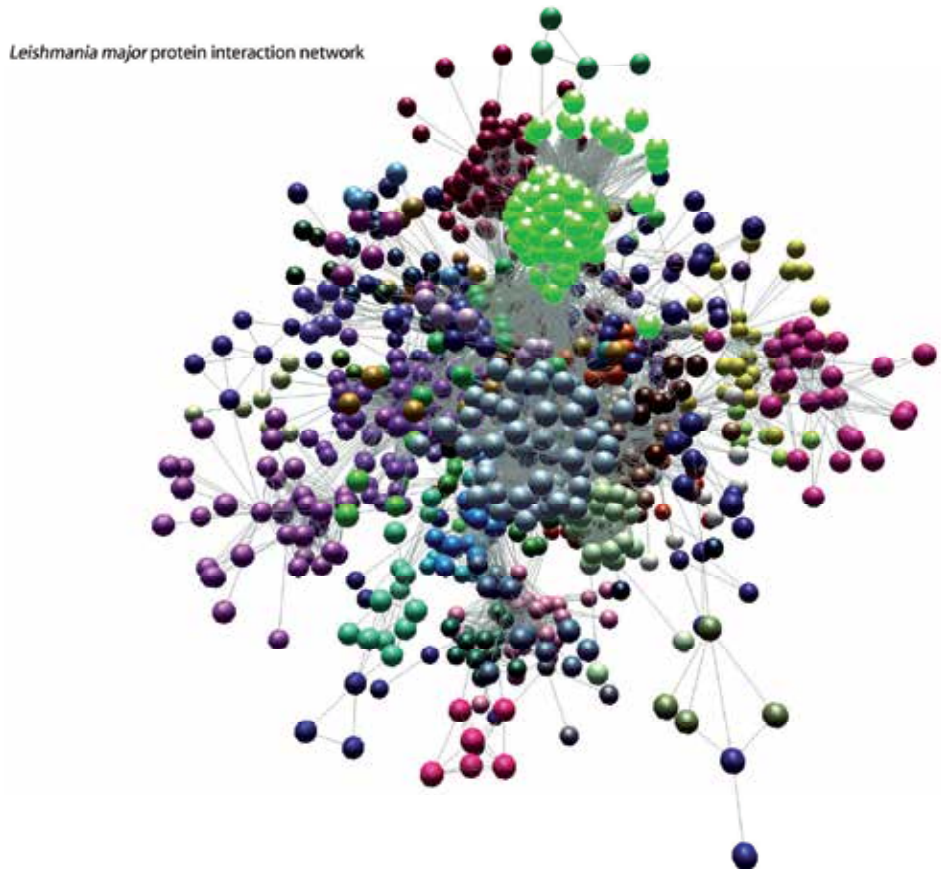


Fig. 2. Visualization of the predicted *Leishmania major* interactome

GeneDB ID	Uniprot ID	Description
LmjF15.0770	Q4QFA8	Protein kinase.
LmjF07.0250	Q4QIR9	Protein kinase
LmjF11.0330	Q4QH47	PIF1 helicase-like protein
LmjF35.2450	Q4FWM4	Hypothetical protein conserved
LmjF25.1990	Q4Q9P0	Protein kinase
LmjF21.0853	Q4QCC1	Hypothetical protein conserved
LmjF27.1800	Q4FYE1	Protein kinase-like
LmjF35.1000	Q4FX16	Casein kinase I
LmjF26.0660	Q4Q9C8	Protein disulfide isomerase
LmjF25.2050	Q4Q9N4	Helicase-like protein

Table 2. Top 10 list of predicted targets from the *L. major* interactome.

It has been shown that modular organization is a prevalent feature in biology, and this modular organization of pathways can be used to infer protein function (Rives & Galitski 2003). We detected 63 clusters or modules in the network, and assigned potential biological processes to 263 proteins with no prior functional description. By examining the proportion of predicted targets by biological process, 64% of the proteins in the network were predicted to participate in the protein phosphorylation (GO:0006468). In addition, 8% of proteins were predicted to be involved in nucleosome assembly (GO:0006334), 4% in nucleic acid metabolic process (GO:0006139), 4% in electron transport (GO:0006118), 4% in transport processes (GO:0006810), and 2% in protein amino acid alkylation (GO:0006139). The remaining 14% of target proteins were distributed across processes with one protein per process. This result highlighted the importance of protein kinases as the main protein class to characterize and explore as drug targets in *Leishmania* parasites.

3. Selection of drug targets by metabolic flux balance analysis and *in silico* deletions

Proteins involved in metabolism constitute another important source of drug targets. The energetic balance in the cell is controlled by enzymes that regulate the transformation of substrates in a coordinated and efficient manner. These enzymes are needed specifically for producing energy or as building blocks for other molecules being essential for the viability of the organism. However, a different approach needs to be used for modeling metabolism because the interactions between enzymes depend upon the rate of turnover of molecules or *fluxes*, not specifically through physical interactions as described for the case of the interactome.

The reconstruction of metabolic networks is more established compared to interactome generation. Since glycolysis was elucidated in 1930, several metabolic pathways have been discovered in many organisms. Metabolic networks reconstructed from this source of data started with *E. coli* (Reed & Palsson 2003) and was followed later on by reconstructions in eukaryotic organisms such as *Saccharomyces cerevisiae* (Duarte., et al. 2004) and *Aspergillus niger* (David., et al. 2003). More recently, metabolic networks of *Plasmodium falciparum* (Plata., et al. 2010) and *Leishmania major* (Chavali., et al. 2008) were reconstructed with the aim of detecting drug targets, and some details about the network generation and analysis will be discussed.

In order to build a metabolic network it is necessary to list all the substances with their concentrations and the reactions between substances. In living systems these reactions are catalyzed by enzymes and the transport processes are carried out by transporters or channels. The reactions are influenced by the *stoichiometric coefficients* which denote the proportion of substrate and product molecules involved in a reaction. The reaction:

$$S1 + S1 \rightarrow 2P$$

describes the generation of product P from S1. Therefore, the stoichiometric coefficients for this particular reaction are -1,-1 and 2 respectively. For a metabolic network consisting of m substances and r reactions, the systems dynamics are described by *systems equations* (2) (or balance equations, since the balance of substrate production and degradation is considered):

$$\frac{dSi}{dt} = \sum_{j=1}^{r} n_{ij} v_j \tag{2}$$

The term n_{ij} are the stoichiometric coefficients of metabolite i in reaction j. In this case, diffusion is not considered in the system. These equations can be applied to compartments, where the flux between compartments has to be considered as a different reaction. The stoichiometric coefficients n_{ij} can be combined into the so-called *stoichiometric matrix,* where each column belongs to a reaction and each row to a substance (Klipp et al., 2005).
According to this, a mathematical model for a metabolic network can be described as a system with a vector S = ($S1$, $S2$, …, Sn) of concentration values for the different species, a vector v = ($v1$, $v2$, …, vr) of reaction rates and the stoichiometric matrix N. With these definitions, the balance equation (3) can be rewritten as follows:

$$\frac{dS}{dt} = Nv \tag{3}$$

3.1 Predicted drug targets in the *Leishmania major* metabolic network

The total number of genes included in the network was 560, with 1,112 reactions and 1,101 metabolites. The process for the reconstruction involved different data sources, in particular literature and biological databases. Reaction stoichiometry and subcellular localization were also extracted by examining the existing literature. Some reactions were assigned as non-gene-associated to account for spontaneously generated metabolites. The gene-associated reactions were further adjusted according to the specified constraints.
Flux balance analysis is a method that has been used extensively to analyze metabolic networks. The important advantage of this method is that does not require detailed knowledge of the enzyme kinetics. The principle of the method relies on investigation of the fluxes that have the greatest influence in the growth (or production of biomass) by preserving a set of constraints such as physicochemical, thermodynamic, topological and environmental. In the case of the *Leishmania* model, constraints included reaction reversibility rules, promastigote/amastigote protein expression data, various medium conditions and prevalence of transport reactions across cellular compartments were used. The model was simulated under *steady state* conditions, which means that the net change of any metabolite in the network during time should be zero.
Virtual knockouts were carried out over the network with the aim to detect potential drug targets. The knockout genes were classified as being lethal (essential for survival), growth reducing, or with no effect. From this analysis, only 12% of single knockouts were predicted as lethal and 10% as growth reducing. Approximately 83% of all lethal genes belonged to three metabolic processes: lipid, carbohydrate or amino-acid metabolism, highlighting how critical these are to general function.
From the group of lethal genes, the authors selected those that were exclusive or without human orthologs as potential candidates (a strategy that was also employed for the interactome analysis). The gene *LmjF05.0350*, which encodes for trypanothione reductase, was lethal *in silico*. This enzyme participates in the reduction of oxidative agents by using trypanothione and this molecule is only present in kinetoplastids. This enzyme has been studied extensively as drug target, confirming the predictions of the mathematical model (Eberle., et al. 2011). *LmjF31.2940* and *LmjF21.0845*, encoding for squalene synthase and hypoxanthine-guanine phosphoribosyltransferase respectively, were also predicted as lethal in the network. The squalene synthase inhibitors affect the sterol biosynthesis pathway, taking advantage of the trypanosomatid requirement for specific endogenous sterols (e.g.

ergosterol). Interestingly, proteins belonging to this process in *Leishmania* were also detected by homology and interactome analysis showing consistency between methods.
Double knockouts were also simulated to identify lethal combination of genes. Out of 152,520 double deletions, 19,341 were lethal. From this group, 19,285 double deletions were trivial lethal, which means that at least one of the genes involved was lethal in a single deletion. There were 56 non-trivial lethal double deletions that could be interesting to test experimentally. The main participation of these double knockouts was in the lipid and carbohydrate metabolism with 57.6% of the genes in these groups. One explanation for the large number of double deletions that were not essential is the high degree of redundancy in the network. These results show the utility of a different methodology that uses mathematical modeling for the detection of essential genes in metabolism.

4. Discovery of new drugs using virtual screening

Once the protein target is identified, the next step is finding an effective and non-toxic inhibitor that can be administrated in human patients. However, the process for identifying compounds, as in the case for drug targets, requires expensive equipment for testing millions of chemicals with a final result of few *hits* that can advance in further drug development stages. This process is unpractical and time consuming. A computational strategy has been used to improve the drug search by using computational chemistry to select those compounds that are likely to work in the experimental setting, this computational technique is called *virtual screening*. The aim of this technique is to identify novel small molecules that could bind the target of interest. This is carried out by *docking* compound libraries over the protein structure of the target, using optimization algorithms that search for the best conformation of the target and the ligand, which by definition has the lowest conformational energy (McInnes 2007). The libraries are usually compounds available from chemical vendors or predicted in some cases.
The procedure for virtual screening involves the selection of the target as a first step. The target can be chosen by the results from RNAi screenings or from computational approaches as the ones previously described. The target must have a 3D structure with an acceptable resolution, which is critical for the accuracy of the predictions. The structures determined by experimental methods can be easily accessed through the Protein Data Bank (PDB; http://www.pdb.org/). This database contains protein structures from different organisms determined by X-ray crystallography or NMR methods. In addition, predicted structures by homology-modeling methods can be found in the ModBase database (http://modbase.compbio.ucsf.edu/) (Pieper., et al. 2009). In contrast, the resources for compounds or *ligands* are restricted due to patent protection. However an interesting resource that contains millions of ligands ready to use for virtual screening experiments is the ZINC database (http://zinc.docking.org/) (Irwin & Shoichet 2005). This database includes commercial ligands and it is also free for academic use. This combination of resources of protein structures and ligand databases facilitates enormously the development of virtual screening projects which can be very productive for finding new drugs, especially for neglected diseases such as leishmaniasis.
The tools employed for virtual screening are also very diverse. One very popular tool is AutoDock (Goodsell., et al. 1996), which was developed by the Scripps Institute and used in several virtual screening projects, demonstrating good performance and accuracy. However, a recent study (Chang., et al. 2010) compared the accuracy and reproducibility of AutoDock

against a recently developed tool, also freely available, called AutoDock Vina (Trott & Olson 2010). The experiments consisted of screening ligands with known activity against the HIV protease and *decoys* or non-binders. Autodock Vina performed very well in terms of speed, being ~10 times faster, and more accurate in ranking larger molecules compared to AutoDock. We are currently using AutoDock Vina in a virtual screening project called "Drug Search for Leishmaniasis" in association with IBM-World Community Grid (http://www.worldcommunitygrid.org/research/dsfl/overview.do) to speed up the process of finding new active compounds.

As an example of the application of this strategy in *Leishmania,* a recent study demonstrated the utility of virtual screening to identify potential MAPK inhibitors. The target, MAPK was first modeled by using *homology modeling* techniques. Essentially, the technique predicts the 3D structure of a particular protein by finding sequence homology to a model protein with experimentally determined structure. This model was refined by molecular dynamics. Structural features, such as ATP binding pocket, phosphorylation lip, and common docking site were identified. Virtual screening was carried out using this target with several compounds from the class of ATP inhibitors. Interestingly, the docking analysis suggested that the indirubin class of molecules could act as putative inhibitors of *Leishmania* MAPK. By testing this result experimentally, the authors found reasonably good correlation between *in vitro* activity and calculated binding energy for indirubin class of inhibitors obtained in the virtual screening study. These molecules make strong hydrogen bonding interactions with Lys43, Arg57, Asp155, Glu94, and Ile96 amino acid residues of the *Leishmania* MAPK model. These residues belong to the catalytic domain and inhibition of the catalytic domain leads to impaired kinase activity (Awale., et al. 2010). This is a clear example of the synergy between computational and experimental methods to accelerate drug discovery.

5. Selection of new drugs using machine learning techniques

The *Leishmania* proteome is estimated to contain ~8,150 proteins based on the annotated genome of the sequenced species (Peacock., et al. 2007). However, fewer than 150 proteins have 3D structures in the PDB. This limits the use of docking-based strategies to search for anti-Leishmania compounds. An alternative strategy to associate active compounds with *Leishmania* targets is by using machine learning techniques. This approach is intended to find patterns on protein targets such as domains, post-translational modifications etc, that can be linked to a specific class of compounds. This system will "learn" these patterns and when challenged by proteins from the organism of interest it will predict the potential association for a particular compound. Two studies have applied this strategy to a particular set of protein targets (Bulashevska., et al. 2009; Thangudu., et al. 2010), employing the different techniques such as support vector machines (SVM) and Bayesian classifiers (BC). As a perspective, these methods could also be applied for drug search in *Leishmania;* however, the definition of protein patterns would be critical for establishing robust drug-target relationships.

6. Experimental approaches for drug testing in Leishmania

Several *in vitro* assays to test *Leishmania* susceptibility to new potential inhibitors have been developed for the two stages of *Leishmania*, namely promastigotes and amastigotes. These two stages are morphologically and biochemically different and these differences are likely responsible for their differing susceptibility to proven anti-leishmanial compounds. Assays developed with intracellular amastigotes have the advantage of being more "disease

appropriate" since this is the stage responsible for mammalian disease. In addition, an intracellular *in vitro* model resembles the natural event when the parasite is in the mammalian host. Axenic amastigotes are also employed, although a more efficient alternative is the use of intracellular fluorescent or bioluminescent amastigotes.

The effectiveness of compounds to kill the parasites has been evaluated using different methodological approaches. Several years ago, direct parasite counting was the most used method (Gaspar., et al. 1992; Chan-Bacab., et al. 2003; Khan., et al. 2003). However, this method lacked accuracy and precision, likely due to human errors. This made necessary to develop new automated methods based on colorimetric, radioactive, fluorescent and luminometer detection (Fumarola., et al. 2004). Colorimetric methods like MTT [3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide] have been used frequently. Recently, these methods are being replaced by transgenic parasites with reporter genes that do not interfere with cellular mitochondria. Parasites genetically engineered to express green fluorescent protein (GFP) or luciferase, have been developed and are currently used in automatized protocols involving flow cytometer or luminometer.

On the other hand, it is important to measure cytotoxicity to evaluate the possibility that a compound might produce side effects in humans. Mammalian or mouse cell lines are usually employed in these assays. The most used cells are U-937 human histiocytes, TPH-1 human peripheral blood monocytes, and hamster peritoneal macrophages (Robledo., et al. 1999; Weniger., et al. 2001; Varela., et al. 2009; Taylor., et al. 2010). To increase the selectivity of promising drugs, liposomal formulations of the compounds may be evaluated in order to reduce the toxicity as was observed for amphotericin B (Mehta., et al. 1985; Lopez-Berestein 1987).

The leishmanicidal activity of compounds that show high anti-leishmanial activity and low toxicity for mammalian cells *in vitro*, is next evaluated *in vivo*. This is normally performed in mouse or the golden hamster (*Mesocricetus auratus*) models depending on the *Leishmania* subgenus (Travi., et al. 2002). Monkey models can also used but these studies are limited to only a few laboratories worldwide (Grimaldi., et al. 2010).

7. Conclusion

Through the current chapter, the more relevant techniques for finding drugs and targets employing computational approaches were described. Special considerations should be made when using the homology searching approach for finding drug targets as the context of protein interactions is important for the definition of essentiality. Protein interaction network analysis together with metabolic flux balance analysis are becoming useful alternatives to understand protein function and to systematically select drug targets. The selection of new inhibitory compounds can be done by using virtual screening. Predicted structures can also be used in virtual screening when experimentally derived structures are absent. Finally, machine learning techniques are a promising option to search for anti-leishmanial drugs, especially when experimental or predicted structures are not available, as may occur with many *Leishmania* proteins. It is important to state that any computational analysis is considered exploratory, and experimental validation is necessary to guide final decisions about potential compounds that can advance to the next stage of drug discovery. However, by using computational tools the search space for drugs and targets is reduced, allowing more focused experiments that could reduce the cost and time of drug development.

8. Acknowledgment

This book chapter was supported by Colciencias, through the projects with the following contracts and codes: Contract 538 with code 111549326124 and contract 197 with code 111551929015.

9. References

Ahasan, H. A., M. A. Chowdhury, M. A. Azhar, A. K. Rafiqueuddin & K. A. Azad (1996). "Deaths in visceral leishmaniasis (Kala-azar) during treatment." *Med J Malaysia* 51(1): 29-32.

Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller & D. J. Lipman (1997). "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs." *Nucleic Acids Res* 25(17): 3389-3402.

Assenov, Y., F. Ramirez, S. E. Schelhorn, T. Lengauer & M. Albrecht (2008). "Computing topological parameters of biological networks." *Bioinformatics* 24(2): 282-284.

Awale, M., V. Kumar, P. Saravanan & C. G. Mohan (2010). "Homology modeling and atomic level binding study of Leishmania MAPK with inhibitors." *J Mol Model* 16(3): 475-488.

Bader, G. D., I. Donaldson, C. Wolting, B. F. Ouellette, T. Pawson & C. W. Hogue (2001). "BIND--The Biomolecular Interaction Network Database." *Nucleic Acids Res* 29(1): 242-245.

Barabasi, A. L. & Z. N. Oltvai (2004). "Network biology: understanding the cell's functional organization." *Nat Rev Genet* 5(2): 101-113.

Brohee, S., K. Faust, G. Lima-Mendez, G. Vanderstocken & J. van Helden (2008). "Network Analysis Tools: from biological networks to clusters and pathways." *Nat Protoc* 3(10): 1616-1629.

Brohee, S. & J. van Helden (2006). "Evaluation of clustering algorithms for protein-protein interaction networks." *BMC Bioinformatics* 7: 488.

Bulashevska, A., M. Stein, D. Jackson & R. Eils (2009). "Prediction of small molecule binding property of protein domains with Bayesian classifiers based on Markov chains." *Comput Biol Chem* 33(6): 457-460.

Carrero-Lerida, J., G. Perez-Moreno, V. M. Castillo-Acosta, L. M. Ruiz-Perez & D. Gonzalez-Pacanowska (2009). "Intracellular location of the early steps of the isoprenoid biosynthetic pathway in the trypanosomatids Leishmania major and Trypanosoma brucei." *Int J Parasitol* 39(3): 307-314.

Costanzo, M., A. Baryshnikova, J. Bellay, Y. Kim, E. D. Spear, C. S. Sevier, H. Ding, J. L. Koh, K. Toufighi, S. Mostafavi, J. Prinz, R. P. St Onge, B. VanderSluis, T. Makhnevych, F. J. Vizeacoumar, S. Alizadeh, S. Bahr, R. L. Brost, Y. Chen, M. Cokol, R. Deshpande, Z. Li, Z. Y. Lin, W. Liang, M. Marback, J. Paw, B. J. San Luis, E. Shuteriqi, A. H. Tong, N. van Dyk, I. M. Wallace, J. A. Whitney, M. T. Weirauch, G. Zhong, H. Zhu, W. A. Houry, M. Brudno, S. Ragibizadeh, B. Papp, C. Pal, F. P. Roth, G. Giaever, C. Nislow, O. G. Troyanskaya, H. Bussey, G. D. Bader, A. C. Gingras, Q. D. Morris, P. M. Kim, C. A. Kaiser, C. L. Myers, B. J. Andrews & C. Boone (2010). "The genetic landscape of a cell." *Science* 327(5964): 425-431.

Chan-Bacab, M. J., E. Balanza, E. Deharo, V. Munoz, R. D. Garcia & L. M. Pena-Rodriguez (2003). "Variation of leishmanicidal activity in four populations of Urechites andrieuxii." *J Ethnopharmacol* 86(2-3): 243-247.

Chang, M. W., C. Ayeni, S. Breuer & B. E. Torbett (2010). "Virtual screening for HIV protease inhibitors: a comparison of AutoDock 4 and Vina." *PLoS One* 5(8): e11955.

Chavali, A. K., J. D. Whittemore, J. A. Eddy, K. T. Williams & J. A. Papin (2008). "Systems analysis of metabolism in the pathogenic trypanosomatid Leishmania major." *Mol Syst Biol* 4: 177.

Chua, H. N., W. K. Sung & L. Wong (2006). "Exploiting indirect neighbours and topological weight to predict protein function from protein-protein interactions." *Bioinformatics* 22(13): 1623-1630.

Das, B. B., A. Ganguly & H. K. Majumder (2008). "DNA topoisomerases of Leishmania: the potential targets for anti-leishmanial therapy." *Adv Exp Med Biol* 625: 103-115.

David, H., M. Akesson & J. Nielsen (2003). "Reconstruction of the central carbon metabolism of Aspergillus niger." *Eur J Biochem* 270(21): 4243-4253.

de Azevedo, W. F., Jr. & M. B. Soares (2009). "Selection of targets for drug development against protozoan parasites." *Curr Drug Targets* 10(3): 193-201.

Dreze, M., D. Monachello, C. Lurin, M. E. Cusick, D. E. Hill, M. Vidal & P. Braun (2010). "High-quality binary interactome mapping." *Methods Enzymol* 470: 281-315.

Duarte, N. C., M. J. Herrgard & B. O. Palsson (2004). "Reconstruction and validation of Saccharomyces cerevisiae iND750, a fully compartmentalized genome-scale metabolic model." *Genome Res* 14(7): 1298-1309.

Eberle, C., B. S. Lauber, D. Fankhauser, M. Kaiser, R. Brun, R. L. Krauth-Siegel & F. Diederich (2011). "Improved inhibitors of trypanothione reductase by combination of motifs: synthesis, inhibitory potency, binding mode, and antiprotozoal activities." *ChemMedChem* 6(2): 292-301.

Enright, A. J., S. Van Dongen & C. A. Ouzounis (2002). "An efficient algorithm for large-scale detection of protein families." *Nucleic Acids Res* 30(7): 1575-1584.

Faraut-Gambarelli, F., R. Piarroux, M. Deniau, B. Giusiano, P. Marty, G. Michel, B. Faugere & H. Dumon (1997). "In vitro and in vivo resistance of Leishmania infantum to meglumine antimoniate: a study of 37 strains collected from patients with visceral leishmaniasis." *Antimicrob Agents Chemother* 41(4): 827-830.

Finn, R. D., M. Marshall & A. Bateman (2005). "iPfam: visualization of protein-protein interactions in PDB at domain and amino acid resolutions." *Bioinformatics* 21(3): 410-412.

Finn, R. D., J. Mistry, B. Schuster-Bockler, S. Griffiths-Jones, V. Hollich, T. Lassmann, S. Moxon, M. Marshall, A. Khanna, R. Durbin, S. R. Eddy, E. L. Sonnhammer & A. Bateman (2006). "Pfam: clans, web tools and services." *Nucleic Acids Res* 34(Database issue): D247-251.

Florez, A. F., D. Park, J. Bhak, B. C. Kim, A. Kuchinsky, J. H. Morris, J. Espinosa & C. Muskus (2010). "Protein network prediction and topological analysis in Leishmania major as a tool for drug target selection." *BMC Bioinformatics* 11: 484.

Forsyth, R. A., R. J. Haselbeck, K. L. Ohlsen, R. T. Yamamoto, H. Xu, J. D. Trawick, D. Wall, L. Wang, V. Brown-Driver, J. M. Froelich, K. G. C, P. King, M. McCarthy, C. Malone, B. Misiner, D. Robbins, Z. Tan, Z. Y. Zhu Zy, G. Carr, D. A. Mosca, C. Zamudio, J. G. Foulkes & J. W. Zyskind (2002). "A genome-wide strategy for the identification of essential genes in Staphylococcus aureus." *Mol Microbiol* 43(6): 1387-1400.

Fumarola, L., R. Spinelli & O. Brandonisio (2004). "In vitro assays for evaluation of drug activity against Leishmania spp." *Res Microbiol* 155(4): 224-230.

Gaspar, R., F. R. Opperdoes, V. Preat & M. Roland (1992). "Drug targeting with polyalkylcyanoacrylate nanoparticles: in vitro activity of primaquine-loaded

nanoparticles against intracellular Leishmania donovani." *Ann Trop Med Parasitol* 86(1): 41-49.

Giaever, G., A. M. Chu, L. Ni, C. Connelly, L. Riles, S. Veronneau, S. Dow, A. Lucau-Danila, K. Anderson, B. Andre, A. P. Arkin, A. Astromoff, M. El-Bakkoury, R. Bangham, R. Benito, S. Brachat, S. Campanaro, M. Curtiss, K. Davis, A. Deutschbauer, K. D. Entian, P. Flaherty, F. Foury, D. J. Garfinkel, M. Gerstein, D. Gotte, U. Guldener, J. H. Hegemann, S. Hempel, Z. Herman, D. F. Jaramillo, D. E. Kelly, S. L. Kelly, P. Kotter, D. LaBonte, D. C. Lamb, N. Lan, H. Liang, H. Liao, L. Liu, C. Luo, M. Lussier, R. Mao, P. Menard, S. L. Ooi, J. L. Revuelta, C. J. Roberts, M. Rose, P. Ross-Macdonald, B. Scherens, G. Schimmack, B. Shafer, D. D. Shoemaker, S. Sookhai-Mahadeo, R. K. Storms, J. N. Strathern, G. Valle, M. Voet, G. Volckaert, C. Y. Wang, T. R. Ward, J. Wilhelmy, E. A. Winzeler, Y. Yang, G. Yen, E. Youngman, K. Yu, H. Bussey, J. D. Boeke, M. Snyder, P. Philippsen, R. W. Davis & M. Johnston (2002). "Functional profiling of the Saccharomyces cerevisiae genome." *Nature* 418(6896): 387-391.

Glasser, J. S. & C. K. Murray (2011). "Central nervous system toxicity associated with liposomal amphotericin B therapy for cutaneous leishmaniasis." *Am J Trop Med Hyg* 84(4): 566-568.

Good, M. C., J. G. Zalatan & W. A. Lim (2011). "Scaffold proteins: hubs for controlling the flow of cellular information." *Science* 332(6030): 680-686.

Goodsell, D. S., G. M. Morris & A. J. Olson (1996). "Automated docking of flexible ligands: applications of AutoDock." *J Mol Recognit* 9(1): 1-5.

Goyeneche-Patino, D. A., L. Valderrama, J. Walker & N. G. Saravia (2008). "Antimony resistance and trypanothione in experimentally selected and clinical strains of Leishmania panamensis." *Antimicrob Agents Chemother* 52(12): 4503-4506.

Grimaldi, G., Jr., R. Porrozzi, K. Friedrich, A. Teva, R. S. Marchevsky, F. Vieira, N. Miekeley & F. J. Paumgartten (2010). "Comparative efficacies of two antimony regimens to treat Leishmania braziliensis-induced cutaneous Leishmaniasis in rhesus macaques (Macaca mulatta)." *Antimicrob Agents Chemother* 54(1): 502-505.

He, F., Y. Zhang, H. Chen, Z. Zhang & Y. L. Peng (2008). "The prediction of protein-protein interaction networks in rice blast fungus." *BMC Genomics* 9: 519.

Hermjakob, H., L. Montecchi-Palazzi, C. Lewington, S. Mudali, S. Kerrien, S. Orchard, M. Vingron, B. Roechert, P. Roepstorff, A. Valencia, H. Margalit, J. Armstrong, A. Bairoch, G. Cesareni, D. Sherman & R. Apweiler (2004). "IntAct: an open source molecular interaction database." *Nucleic Acids Res* 32(Database issue): D452-455.

Hu, W., S. Sillaots, S. Lemieux, J. Davison, S. Kauffman, A. Breton, A. Linteau, C. Xin, J. Bowman, J. Becker, B. Jiang & T. Roemer (2007). "Essential gene identification and drug target prioritization in Aspergillus fumigatus." *PLoS Pathog* 3(3): e24.

Hubbard, T. J., A. G. Murzin, S. E. Brenner & C. Chothia (1997). "SCOP: a structural classification of proteins database." *Nucleic Acids Res* 25(1): 236-239.

Irwin, J. J. & B. K. Shoichet (2005). "ZINC--a free database of commercially available compounds for virtual screening." *J Chem Inf Model* 45(1): 177-182.

Ivens, A. C., C. S. Peacock, E. A. Worthey, L. Murphy, G. Aggarwal, M. Berriman, E. Sisk, M. A. Rajandream, E. Adlem, R. Aert, A. Anupama, Z. Apostolou, P. Attipoe, N. Bason, C. Bauser, A. Beck, S. M. Beverley, G. Bianchettin, K. Borzym, G. Bothe, C. V. Bruschi, M. Collins, E. Cadag, L. Ciarloni, C. Clayton, R. M. Coulson, A. Cronin, A. K. Cruz, R. M. Davies, J. De Gaudenzi, D. E. Dobson, A. Duesterhoeft, G. Fazelina, N. Fosker, A. C. Frasch, A. Fraser, M. Fuchs, C. Gabel, A. Goble, A. Goffeau, D. Harris, C. Hertz-Fowler, H. Hilbert, D. Horn, Y. Huang, S. Klages, A. Knights, M. Kube, N. Larke, L. Litvin, A.

Lord, T. Louie, M. Marra, D. Masuy, K. Matthews, S. Michaeli, J. C. Mottram, S. Muller-Auer, H. Munden, S. Nelson, H. Norbertczak, K. Oliver, S. O'Neil, M. Pentony, T. M. Pohl, C. Price, B. Purnelle, M. A. Quail, E. Rabbinowitsch, R. Reinhardt, M. Rieger, J. Rinta, J. Robben, L. Robertson, J. C. Ruiz, S. Rutter, D. Saunders, M. Schafer, J. Schein, D. C. Schwartz, K. Seeger, A. Seyler, S. Sharp, H. Shin, D. Sivam, R. Squares, S. Squares, V. Tosato, C. Vogt, G. Volckaert, R. Wambutt, T. Warren, H. Wedler, J. Woodward, S. Zhou, W. Zimmermann, D. F. Smith, J. M. Blackwell, K. D. Stuart, B. Barrell & P. J. Myler (2005). "The genome of the kinetoplastid parasite, Leishmania major." *Science* 309(5733): 436-442.

Jeong, H., S. P. Mason, A. L. Barabasi & Z. N. Oltvai (2001). "Lethality and centrality in protein networks." *Nature* 411(6833): 41-42.

Kamath, R. S., A. G. Fraser, Y. Dong, G. Poulin, R. Durbin, M. Gotta, A. Kanapin, N. Le Bot, S. Moreno, M. Sohrmann, D. P. Welchman, P. Zipperlen & J. Ahringer (2003). "Systematic functional analysis of the Caenorhabditis elegans genome using RNAi." *Nature* 421(6920): 231-237.

Khan, K. M., M. Rasheed, U. Zia, S. Hayat, F. Kaukab, M. I. Choudhary, R. Atta ur & S. Perveen (2003). "Synthesis and in vitro leishmanicidal activity of some hydrazides and their analogues." *Bioorg Med Chem* 11(7): 1381-1387.

Kim, J. G., D. Park, B. C. Kim, S. W. Cho, Y. T. Kim, Y. J. Park, H. J. Cho, H. Park, K. B. Kim, K. O. Yoon, S. J. Park, B. M. Lee & J. Bhak (2008). "Predicting the interactome of Xanthomonas oryzae pathovar oryzae for target selection and DB service." *BMC Bioinformatics* 9: 41.

Klipp, E., Herwig, R., Kowald, A.,Wierling, C. & Lehrach, H (2005). *Systems Biology in Practice: Concepts, Implementation and Application.* Wiley-VCH Verlag GmbH & Co. KGaA, ISBN: 9783527310784, Weinheim, Germany.

Knox, C., V. Law, T. Jewison, P. Liu, S. Ly, A. Frolkis, A. Pon, K. Banco, C. Mak, V. Neveu, Y. Djoumbou, R. Eisner, A. C. Guo & D. S. Wishart (2011). "DrugBank 3.0: a comprehensive resource for 'omics' research on drugs." *Nucleic Acids Res* 39(Database issue): D1035-1041.

Kuhn, M., D. Szklarczyk, A. Franceschini, M. Campillos, C. von Mering, L. J. Jensen, A. Beyer & P. Bork (2010). "STITCH 2: an interaction network database for small molecules and proteins." *Nucleic Acids Res* 38(Database issue): D552-556.

Lang, T., S. Goyard, M. Lebastard & G. Milon (2005). "Bioluminescent Leishmania expressing luciferase for rapid and high throughput screening of drugs acting on amastigote-harbouring macrophages and for quantitative real-time monitoring of parasitism features in living mice." *Cell Microbiol* 7(3): 383-392.

Lin, C. Y., C. H. Chin, H. H. Wu, S. H. Chen, C. W. Ho & M. T. Ko (2008). "Hubba: hub objects analyzer--a framework of interactome hubs identification for network biology." *Nucleic Acids Res* 36(Web Server issue): W438-443.

Lopez-Berestein, G. (1987). "Liposomes as carriers of antimicrobial agents." *Antimicrob Agents Chemother* 31(5): 675-678.

Maltezou, H. C. (2008). "Visceral leishmaniasis: advances in treatment." *Recent Pat Antiinfect Drug Discov* 3(3): 192-198.

McInnes, C. (2007). "Virtual screening strategies in drug discovery." *Curr Opin Chem Biol* 11(5): 494-502.

Mehta, R. T., G. Lopez-Berestein, R. L. Hopfer, K. Mehta, R. A. White & R. L. Juliano (1985). "Prophylaxis of murine candidiasis via application of liposome-encapsulated

amphotericin B and a muramyl dipeptide analog, alone and in combination." *Antimicrob Agents Chemother* 28(4): 511-513.

Oliveira, L. F., A. O. Schubach, M. M. Martins, S. L. Passos, R. V. Oliveira, M. C. Marzochi & C. A. Andrade (2011). "Systematic review of the adverse effects of cutaneous leishmaniasis treatment in the New World." *Acta Trop* 118(2): 87-96.

Osman, A. (2004). "Yeast two-hybrid assay for studying protein-protein interactions." *Methods Mol Biol* 270: 403-422.

Park, D., S. Lee, D. Bolser, M. Schroeder, M. Lappe, D. Oh & J. Bhak (2005). "Comparative interactomics analysis of protein family interaction networks using PSIMAP (protein structural interactome map)." *Bioinformatics* 21(15): 3234-3240.

Peacock, C. S., K. Seeger, D. Harris, L. Murphy, J. C. Ruiz, M. A. Quail, N. Peters, E. Adlem, A. Tivey, M. Aslett, A. Kerhornou, A. Ivens, A. Fraser, M. A. Rajandream, T. Carver, H. Norbertczak, T. Chillingworth, Z. Hance, K. Jagels, S. Moule, D. Ormond, S. Rutter, R. Squares, S. Whitehead, E. Rabbinowitsch, C. Arrowsmith, B. White, S. Thurston, F. Bringaud, S. L. Baldauf, A. Faulconbridge, D. Jeffares, D. P. Depledge, S. O. Oyola, J. D. Hilley, L. O. Brito, L. R. Tosi, B. Barrell, A. K. Cruz, J. C. Mottram, D. F. Smith & M. Berriman (2007). "Comparative genomic analysis of three Leishmania species that cause diverse human disease." *Nat Genet* 39(7): 839-847.

Perez-Victoria, J. M., A. Di Pietro, D. Barron, A. G. Ravelo, S. Castanys & F. Gamarro (2002). "Multidrug resistance phenotype mediated by the P-glycoprotein-like transporter in Leishmania: a search for reversal agents." *Curr Drug Targets* 3(4): 311-333.

Peri, S., J. D. Navarro, T. Z. Kristiansen, R. Amanchy, V. Surendranath, B. Muthusamy, T. K. Gandhi, K. N. Chandrika, N. Deshpande, S. Suresh, B. P. Rashmi, K. Shanker, N. Padma, V. Niranjan, H. C. Harsha, N. Talreja, B. M. Vrushabendra, M. A. Ramya, A. J. Yatish, M. Joy, H. N. Shivashankar, M. P. Kavitha, M. Menezes, D. R. Choudhury, N. Ghosh, R. Saravana, S. Chandran, S. Mohan, C. K. Jonnalagadda, C. K. Prasad, C. Kumar-Sinha, K. S. Deshpande & A. Pandey (2004). "Human protein reference database as a discovery resource for proteomics." *Nucleic Acids Res* 32(Database issue): D497-501.

Pieper, U., N. Eswar, B. M. Webb, D. Eramian, L. Kelly, D. T. Barkan, H. Carter, P. Mankoo, R. Karchin, M. A. Marti-Renom, F. P. Davis & A. Sali (2009). "MODBASE, a database of annotated comparative protein structure models and associated resources." *Nucleic Acids Res* 37(Database issue): D347-354.

Plata, G., T. L. Hsiao, K. L. Olszewski, M. Llinas & D. Vitkup (2010). "Reconstruction and flux-balance analysis of the Plasmodium falciparum metabolic network." *Mol Syst Biol* 6: 408.

Reed, J. L. & B. O. Palsson (2003). "Thirteen years of building constraint-based in silico models of Escherichia coli." *J Bacteriol* 185(9): 2692-2699.

Rives, A. W. & T. Galitski (2003). "Modular organization of cellular networks." *Proc Natl Acad Sci U S A* 100(3): 1128-1133.

Robledo, S. M., A. Z. Valencia & N. G. Saravia (1999). "Sensitivity to Glucantime of Leishmania viannia isolated from patients prior to treatment." *J Parasitol* 85(2): 360-366.

Sakharkar, K. R., M. K. Sakharkar & V. T. Chow (2004). "A novel genomics approach for the identification of drug targets in pathogens, with special reference to Pseudomonas aeruginosa." *In Silico Biol* 4(3): 355-360.

Shannon, P., A. Markiel, O. Ozier, N. S. Baliga, J. T. Wang, D. Ramage, N. Amin, B. Schwikowski & T. Ideker (2003). "Cytoscape: a software environment for integrated models of biomolecular interaction networks." *Genome Res* 13(11): 2498-2504.

Stark, C., B. J. Breitkreutz, T. Reguly, L. Boucher, A. Breitkreutz & M. Tyers (2006). "BioGRID: a general repository for interaction datasets." *Nucleic Acids Res* 34(Database issue): D535-539.

Sundar, S. & J. Chakravarty (2010). "Antimony toxicity." *Int J Environ Res Public Health* 7(12): 4267-4277.

Taylor, V. M., D. L. Munoz, D. L. Cedeno, I. D. Velez, M. A. Jones & S. M. Robledo (2010). "Leishmania tarentolae: utility as an in vitro model for screening of antileishmanial agents." *Exp Parasitol* 126(4): 471-475.

Thangudu, R. R., M. Tyagi, B. A. Shoemaker, S. H. Bryant, A. R. Panchenko & T. Madej (2010). "Knowledge-based annotation of small molecule binding sites in proteins." *BMC Bioinformatics* 11: 365.

Travi, B. L., Y. Osorio, P. C. Melby, B. Chandrasekar, L. Arteaga & N. G. Saravia (2002). "Gender is a major determinant of the clinical evolution and immune response in hamsters infected with Leishmania spp." *Infect Immun* 70(5): 2288-2296.

Trott, O. & A. J. Olson (2010). "AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading." *J Comput Chem* 31(2): 455-461.

Uetz, P., L. Giot, G. Cagney, T. A. Mansfield, R. S. Judson, J. R. Knight, D. Lockshon, V. Narayan, M. Srinivasan, P. Pochart, A. Qureshi-Emili, Y. Li, B. Godwin, D. Conover, T. Kalbfleisch, G. Vijayadamodar, M. Yang, M. Johnston, S. Fields & J. M. Rothberg (2000). "A comprehensive analysis of protein-protein interactions in Saccharomyces cerevisiae." *Nature* 403(6770): 623-627.

Varela, M. R., D. L. Munoz, S. M. Robledo, B. K. Kolli, S. Dutta, K. P. Chang & C. Muskus (2009). "Leishmania (Viannia) panamensis: an in vitro assay using the expression of GFP for screening of antileishmanial drug." *Exp Parasitol* 122(2): 134-139.

von Mering, C., L. J. Jensen, B. Snel, S. D. Hooper, M. Krupp, M. Foglierini, N. Jouffre, M. A. Huynen & P. Bork (2005). "STRING: known and predicted protein-protein associations, integrated and transferred across organisms." *Nucleic Acids Res* 33(Database issue): D433-437.

Weniger, B., S. Robledo, G. J. Arango, E. Deharo, R. Aragon, V. Munoz, J. Callapa, A. Lobstein & R. Anton (2001). "Antiprotozoal activities of Colombian plants." *J Ethnopharmacol* 78(2-3): 193-200.

Wiese, M. (1998). "A mitogen-activated protein (MAP) kinase homologue of Leishmania mexicana is essential for parasite survival in the infected host." *EMBO J* 17(9): 2619-2628.

Wiese, M. & I. Gorcke (2001). "Homologues of LMPK, a mitogen-activated protein kinase from Leishmania mexicana, in different Leishmania species." *Med Microbiol Immunol* 190(1-2): 19-22.

Xenarios, I., D. W. Rice, L. Salwinski, M. K. Baron, E. M. Marcotte & D. Eisenberg (2000). "DIP: the database of interacting proteins." *Nucleic Acids Res* 28(1): 289-291.

Yu, H., P. M. Kim, E. Sprecher, V. Trifonov & M. Gerstein (2007). "The importance of bottlenecks in protein networks: correlation with gene essentiality and expression dynamics." *PLoS Comput Biol* 3(4): e59.

Zanzoni, A., L. Montecchi-Palazzi, M. Quondam, G. Ausiello, M. Helmer-Citterich & G. Cesareni (2002). "MINT: a Molecular INTeraction database." *FEBS Lett* 513(1): 135-140.

Zhang, R. & Y. Lin (2009). "DEG 5.0, a database of essential genes in both prokaryotes and eukaryotes." *Nucleic Acids Res* 37(Database issue): D455-458.

17

Advances in Serological Diagnosis of Chagas' Disease by Using Recombinant Proteins

Iván S. Marcipar[1] and Claudia M. Lagier[2]
[1]*Laboratorio de Tecnología Inmunológica (LTI), Fac. Bioquímica y Cs. Biológicas, Universidad Nacional del Litoral*
[2]*Instituto de Química Rosario (IQUIR-CONICET), División Química Analítica, Fac. Cs. Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario*
Argentina

1. Introduction

Chagas' disease is an infection caused by the parasite *Trypanosoma cruzi*, mainly occurring in American countries where the parasite vector bug, *Triatoma infestans*, is widespread. One hundred million individuals are currently under threaten of infection, as well as 16 million people are considered affected by the illness in Latin America alone.(Editorial, 2009) Considering indicators such as the disability-adjusted life years, DALY, and from a social point of view, Chagas' disease accounts for the third most important tropical illness of the World, following malaria and schistosomiasis.(Bitran *et al.*, 2009) Moreover, Chagas disease epidemiology nowadays impacts in non-endemic regions due to globalization, being the infection disseminated all over the world (Gascon *et al.* 2009). Certainly, the non-vectorial disease transmission (mother to child, transfusional and by organ transplantation) is the way the illness spreads in Europe, North America, Japan, and Australia, because many infected people have migrated from endemic regions to distant cities. (Bowling & Walter, 2009; Lescure *et al.*, 2009; Yadon & Schmunis, 2009)

The parasite distribution and living habits of rural Latin-American people have determined that the main transmission route is the vectorial one (via *Triatoma infestans*), leading to up 80% of human infection.(WHO, 2003) The main contagion way in urban areas arise from blood-transfusion, being responsible for 5-20% of the reported cases, while vertical transmission (mother-to-child) accounts for 2-10% of the infections.(Carlier & Torrico, 2003)

The infection transmission by oral route because of consumption of contaminated food and drinks is lower than that reported for the previously-mentioned routes.(Dias *et al.*, 2011) However, it has to be taken into account that the success of the non-oral infection prevention has increased the importance of the oral route of transmission.(Dias *et al.*, 2011; Nóbrega *et al.*, 2009) It is noteworthy that the acute outcome of the oral infection is particularly severe.(Bastos et al 2010).

From the clinical point of view, the illness presents variable unspecific symptoms, depending on its stage: the acute one, shortly after primary infection, and the chronic stage, which may last many years if the individual is not treated. Human infection typically

appears with an incubation period of 4-10 days, which is generally asymptomatic. Afterwards, the infection may advance to the short acute phase, followed by the long-lasting chronic stage, which may occur in a syntomatic or asymptomatic way.

The acute stage of the infection usually lasts 2-4 months. When symptoms appear, these are often light and atypical. The infection proceeds generally unnoticed, and this prevents its diagnosis. Most of the patients recover within 3-5 months. However, global mortality during this phase reaches 5-10%,(WHO, 2002) and may be higher in children.(Pinto *et al.*, 2004) Deaths can be caused by complications related to myocarditis and/or meningoencephalitis,(Pittella, 2009) which has been proved by the presence of the amastigote form of parasites in cardiac, skeletal, glial and soft tissue cells.(da Silveira *et al.*, 2007)

The undetermined, chronic phase generally begins 8-10 weeks after the acute infection, and may last several years or even the whole life of the individual. The illness overtakes asymptomatically, and generally, infected individuals keep their full working capacity without being aware of the infection. However, during this stage, patients display positive serology for specific IgG antibodies and low parasitemia.(Coura, 2007) Over 50% of the infected individuals show themselves healthy and do not develop serious outcomes. In the other cases, the infection is often detected once its sequels appear, after 10-20 years of the first parasite entry. The illness features that mainly develop are cardiac, digestive and/or neurological damages. Chagas' pathologic alterations can be summarized as chronic myocarditis, 27%, esophageal or colonic expansion, 5%, and abnormalities of central nervous system, ca. 3%.(Teixeira *et al.*, 2006) The most severe impact of the illness occurs during chronic phase, and after many years that the infection has been established, leading to high mortality rate among people who have develop cardiac pathology.(Rassi, Jr. *et al.*, 2007)

Undoubtedly, the most effective alternative to prevent Chagas' disease spreading is to control vectorial and transfusional ways of transmissions. Nevertheless, once the individual has been infected by *T. cruzi*, he/she may be treated. Two main aspects are covered by therapy, aiming to eliminate the parasite with trypanocidal medication, and/or medicating to relief symptoms of the several clinical outlines of the illness. Nowadays, two chemicals are used as trypanocidal agents namely, nifurtimox (Lampit®, Bayer; 5-nitrofuran 3-methyl-4-(5'-nitrofurfurylideneamine) tetrahydro-4H-1,4-tiazine-1,1-dioxide) and benznidazole (Rochagan® and Radanil®, Roche; N-benzyl-2-nitroimidazole acetamide). When infection is treated during the acute stage, it has been reported that parasitemia disappeared in 60% of the cases and serology turned into negative. Nevertheless, the outcome after therapy is finished shows variable effectiveness percentages, depending mainly on the age of the individual and the geographical region.(Perez-Molina *et al.*, 2009)

Currently, studies trying to expand the indicators on treatment effectiveness are in progress and will be accomplished by 2012.(Marin-Neto *et al.*, 2008) Though these studies have not yet finished, there is a consensus on treating Chagas' disease, taking into account the clear-cut results when using trypanocidal medication with children, during the indeterminate phase of the infection.(Lescure *et al.*, 2010;Perez-Molina *et al.*, 2009;Sosa-Estani & Segura, 2006) It therefore follows that it is highly advisable to count with reliable methods to early diagnose the disease, since this may accelerate the patient treatment, helping to reduce the serious consequences that the long term infection may cause.

1.1 *T. cruzi* infection diagnostic during the different stages of the illness

The infection diagnosis is not a simple task because the features of the illness development as well as the immunological response of the host must be taken into account when performing the laboratory diagnosis. Different diagnostic methods are used, depending on the illness stage and the particular clinical entity of the patient.

The alternative ways to get acute Chagas' infection are mainly vectorial and vertical transmission, and the reactivation of the chronic disease in immunosuppressed individuals. Therefore, diagnosis at this stage limits to: *i)* the uncommon cases of symptomatic patients, *ii)* newborns delivered from chagasic mothers, and *iii)* immunosuppressed patients that have been previously diagnosed as infected.

In the above mentioned cases, parasites are usually, easily found in blood, therefore being their direct microscopic observation the chosen method for a safe diagnosis.(WHO, 2002;Rosenblatt, 2009) In contrast, indirect serological techniques present low sensitivity in recently infected individuals because humoral immune response is delayed.(Zuniga *et al.*, 2000) Moreover, even specific IgM immunoglobulin has a window period that requires up to several weeks to be produced at detectable concentrations. Other pitfalls such as the lack of IgM anti-*T. cruzi* commercial kits prevents from choosing serology as the infection diagnostic method. This methodology is only used when the parasite is not found in fresh blood smears, by Struot and/or microhematocrit methods.(Luquetti & Schmunis, 2010)

During the chronic phase, the *T. cruzi* infection diagnosis is required, *i)* as pregnancy or occupational routine control analysis -in countries where according legislation is in force-, *ii)* when in presence of a Chagas' compatible cardiopathy, *iii)* during transfusion and transplantation screening, and *iv)* to achieve a reliable illness prognosis of patients who were already diagnosed. One particular case among the latter ones is monitoring the antiparasitic treatment effectiveness.

Conversely to what happens in the acute phase, during the chronic infection, a significant humoral response is found in immunocompetent individuals, along with low parasite concentration in blood. Under these circumstances, indirect techniques are highly sensitive, while the direct ones fail. Hence, Chagas' disease serologic diagnoses intend to verify the occurrence of several specific antibodies against *T. cruzi*. However, conventional serology presents different sensitivity and selectivity, depending on the immunological technique used to perform the determination, and mainly in the nature of the antigen used to capture the specific antibodies.(Belluzo *et al.*, 2011)

In this Chapter, we will describe the most recent contributions of our and other groups to improve the analytical tools available to properly and safely diagnose Chagas' disease using recombinant proteins, in each one of the clinical entities mentioned above.

2. Conventional diagnosis of *T. cruzi* infection

Direct parasite detection in whole blood is the simplest, regular procedure used to diagnose acute infection whereas, indirect serological tests are the chosen ones to diagnose the undetermined, chronic state.(Rosenblatt, 2009;WHO, 2002)

2.1 Parasitological and serological diagnosis of acute entities

The direct microscopic observation of parasites from patients peripheral blood is the elected methodology to confirm acute infection. The Strout concentration method is the routinely

performed parasitologic analysis in adults since more than 50 years,(Strout, 1962) and it has been reported about 95% sensitivity.(Freilij & Storino, 1994)

The other commonly used concentration method is the pediatric, more recent version, named the direct micromethod or microhematocrit, which requires a lower blood volume than Strout method, and is mainly used to diagnose congenital Chagas' disease and acute infection in children.(Freilij *et al.*, 1983;Freilij & Altcheh, 1995) However, newborn babies usually present low parasitemia, therefore making difficult to perform a proper conventional parasitologic analysis. It is then recommended to perform serologic tests to diagnose the congenital infection. The evaluation of specific anti-*T. cruzi* IgA and IgM is not recommended due to the high rate of false-negative results in neonates.(Moya *et al.*, 2005) Considering that maternal specific anti-*T. cruzi* IgG antibodies are commonly present in newborn circulating blood, even up to the ninth month, it is not advisable to perform serologic IgG determinations as routine, in newborns younger than 9 months old. In this line, if the micromethod is negative or if it has not been performed during the first months of life of the newborn, then congenital infection should be serologically diagnosed using peripheral blood not before the child is 9 months old, once maternal antibodies have disappeared.(Gomes *et al.*, 2009) Following, when specific IgG presence is negative after the ninth month of life, then vertical transmission is ruled out. Alternatively, during the first months of life of babies, it is possible to forego results using other non-standard, more expensive techniques such as the polymerase chain reaction, PCR.(Diez *et al.*, 2008) This technology is particularly preferred when the health center counts with the supplies to carry out the methodology.

Indirect parasitological methods are also used, mainly when the parasite is not easily found in samples. These methods are the hemoculture and xenodiagnosis, and consist of enriching the parasites present in the patient's blood sample, through allowing their replication.(Chiari *et al.*, 1989) Both of these latter techniques are also used when diagnosing chronic infection. These methods demand long periods of time to arise to the results (weeks or months), together with other drawbacks. For example, xenodiagnostic method has the disadvantage of producing rather variable sensitivity results, 20-50%, alongside the requirement of a suitable building infrastructure and trained personnel to deal with insect breeding. Thus, this method is not commonly performed in basic health centers.(Luquetti & Schmunis, 2010)

When searching for reappearance of acute infection in immunosuppressed individuals under risk, negative serological results are not always associated with absence of the infection. This is a consequence of the immunological status of the patient that shows difficulties to produce detectable amounts of specific IgG. As mentioned previously, in the particular, difficult cases, expensive PCR techniques are the recommended diagnostic method.

2.2 Serological diagnosis of chronic entities

The widely used serological assays to diagnose *T. cruzi* infection in present clinical practice are indirect haemagglutination (IHA), indirect immunofluorescence (IIF), and enzyme-linked immunosorbent assay (ELISA).(WHO, 2002;Yadon & Schmunis, 2009) The analyst's choice of the particular technique depends on sanitary-authority recommendations, market impositions, and the lab-technician preference. This latter one is generally related to the

methodology simplicity, and the personal confidence he/she has in a particular technique after having performed it for a long while.

IHA is an inexpensive technique, which is easy to be performed and interpreted, and it has been used for more than 50 years, therefore being appropriately settled among lab technicians. Similarly, IIF was developed in the sixties and presents equivalent features to IHA though, more skillful technicians are required to perform the analysis and produce accurate readings, as well as it needs a fluorescence microscope. IIF is a very sensitive, specific and cheap alternative for those who have the equipment and the trained personnel. However, regular health centers do not count with both of them.

ELISA is a more recent technique which was firstly described during 1975 to diagnose Chagas' disease,(Voller *et al.*, 1975) and its usage was settled just at the ends of the eighties. This technique has the advantage of being widely extended as a diagnostic tool of many infections. Therefore, most of regular laboratories have the required equipment and trained personnel to appropriately perform the analysis. Contrarily to IHA and IIF, ELISA may be performed with automatic equipment at large health institutions. Moreover, even though ELISA is more expensive than the other two techniques, its notable performance in terms of sensitivity and specificity, has made of this the preferred methodology to diagnose *T. cruzi* infection.(Saez-Alquezar *et al.*, 1997)

Recently, one very fast technique with a different format has been developed namely, lateral chromatography.(Ponce *et al.*, 2005;Barfield *et al.*, 2011) This methodology uses small volume samples such as one serum drop, and allows acquiring results in 15 min, therefore being useful to perform the test in the field, without the need of refrigerator to preserve the reagents. Several multicenter studies have demonstrated that a commercial lateral chromatography kit show more than 92% sensitivity, whereas specificity is ca. 96%.(Ponce *et al.*, 2005;Brutus *et al.*, 2008;Roddy *et al.*, 2008;Sosa-Estani *et al.*, 2008)

The fundamental problems of *T. cruzi* infection serological diagnostic methods are the lack of reproducibility that sometimes occur, deficient immunological reaction specificity, what produces false-positive results, and the occasional insufficient sensitivity translated into false-negative outcomes.

Chagasic infection serology tests may produce cross-reactions with antibodies produced during the course of other illnesses. In this line, unspecific reactivity has been described for infections caused by *T. cruzi* phylogenetically related microorganisms, such as *T. rangeli* and *Leishmania sp.*(Soto *et al.*, 1996;Araujo, 1986;Saez-Alquezar *et al.*, 2000) Moreover, other false-positive results due to cross-reactions have been described when testing samples from patients with autoimmune diseases,(Reiche *et al.*, 1996) or from individuals suffering from other acute infections or pregnant women who display an important, polyclonal unspecific response.(Konishi, 1993)

The clinical practice often finds an important number of inconsistent results regarding reproducibility and confidence when diagnosing chagasic infection. The lack of reproducibility and confident results has also been reported in a multicenter study.(Saez-Alquezar *et al.*, 1997) In this work, it was proved the deficiency of reagents standardization, what produced incongruent results when testing the same serum panel. Along the same direction, since the early nineties, several works accounted for the huge losses caused by disposal of whole blood reservoirs typified as undetermined for *T. cruzi* infection.(Carvalho *et al.*, 1993;Salles *et al.*, 1996;Saez-Alquezar *et al.*, 2000) Taking into account tests discrepancies, one of WHO

recommendations states that *T. cruzi* infection must be diagnosed when the sample produces positive results by two different serological methods, whereas the undetermined condition is established for samples rendering dissimilar outcomes.
Traditionally, whole parasites, or extracts from laboratory strains of *T. cruzi* epimastigotes cultures, have been the source of antigens used for the serological infection diagnosis. However, this yields to complex protein mixtures of unknown composition, which display severe difficulties to be standardized, and additionally lead to false-positive results.
The diagnostic problems arising from serology deficient specificity, as well as the deprived reagents standardization, can be resolved through the use of defined antigens, such as the proteins obtained by molecular biology technology procedures.(Saez-Alquezar *et al.*, 2000;Umezawa *et al.*, 2003;Umezawa *et al.*, 2004;Aguirre *et al.*, 2006)
The following sections will be focused in this issue and the most important contributions that several research groups have recently made.

3. Use and prospects of recombinant DNA technology

Since the emergence of recombinant DNA technology, many protein molecules have been designed and prepared to eventually be assessed for serological diagnosis. The proteins obtained through this technology may be used as antigens to capture antibodies, to evaluate exclusively defined molecules, avoiding potential interferences from other components that usually occur when the antigens have been obtained by purifying native source proteins. (da Silveira *et al.*, 2001) It follows that the usage of recombinant proteins as antigens to detect or quantify specific antibodies markers of a disease permits enhancing the specificity of the immunological reaction involved, therefore leading to more accurate diagnosis.(Aguirre *et al.*, 2006;Camussone *et al.*, 2009)
In this methodology the proteins are usually prepared by heterologous expression, mainly in *Escherichia coli* cells.(da Silveira *et al.*, 2001) Sequences of *T. cruzi*-protein codifying-DNA are inserted in a bacterial plasmid, which is transformed in competent bacteria. The proteins encoded by the plasmid are expressed in the bacterial culture, and are afterward purified into a highly pure product. The advantage of the proteins thus obtained is that they are an entirely characterized antigen, which may be evaluated individually for antibody determination in different clinical conditions. The prepared antigens can therefore be characterized considering the clinical information they provide, and may then be used to prepare specific diagnostic reagents. These proteins count with one desired feature of diagnostic reagents, as it is that their production and evaluation can be highly standardized. From another point of view, recombinant antigens do not require manipulation of the infective agent as do the antigens obtained by purification procedures from rough cultures. This has been a significant progress when considering the characteristics of viral infective agents, for which reagents production has substantially switched to that derived from recombinant DNA technology. Not less important is the major saving financial benefit of these reagents. Indeed, once bacteria are transformed into competent, protein producing strain, they can be used to prepare substantial amounts of antigen with low cost of production.
Using this technology, many gene expression clones have been create, a fact that has made available the obtainment of massive amounts of highly pure, standardized *T. cruzi* proteins.(da Silveira *et al.*, 2001)

During the latest three decades, many parasite antigens have been cloned and characterized. The cloned antigens correspond to different parasite stages namely, the trypomastigote sanguineous, the amastigote intracellular and the epimastigote, which is the form found inside the vector bowel and that can be cultured. Several of these antigens were obtained by immunological tracing through expression of cDNA libraries from chagasic patient sera, as well as from immunized animals.(Lafaille *et al.*, 1989;Affranchino *et al.*, 1989;Levin *et al.*, 1989;Cotrim *et al.*, 1990;Gruber & Zingales, 1993) The antigen codifying genes have been identified from cDNA present in the libraries accomplished from epimastigote or trypomastigote forms.(Affranchino *et al.*, 1989;Levin *et al.*, 1989;Gruber & Zingales, 1993;Godsel *et al.*, 1995) Lately, Da Rocha et al. have proposed using amastigote proteins since this is the intracellular parasite form, being these antigens more significant for serodiagnosis.(DaRocha *et al.*, 2002)

The usage of DNA technology brought into light the existence of many parasite antigens with repetitive sequences, a fact that had been previously described when cloning proteins of other parasites.(Hoft *et al.*, 1989) Generally, these are the most immunogenic antigens, and are the mainly selected when performing immunological tracing in cDNA libraries cloned in phages. Therefore, it was initially stated that these were the most valuable antigens for diagnosis.(Frasch & Reyes, 1990) However, it was afterward proved that some non-repetitive antigens have equivalent diagnostic value than repetitive ones. Certainly, a multicenter study evaluated in parallel 4 repetitive recombinants antigens (H49, JL7, B13, JL8) together with 2 non-repetitive ones (A13 y 1F8).(Umezawa *et al.*, 1999) The results demonstrated that both type of antigens were similarly useful for *T. cruzi* infection diagnosis, and the authors suggested that if they were to be used together in a mixture, they could supplemented each other enhancing the sensitivity of the assay. This was afterwards proved by the same group, see Tables 1 A,B and C.(Umezawa *et al.*, 2003)

Once the complete genome sequence of *Trypanosoma cruzi* was annotated, (El Sayed *et al.*, 2005) alternative antigenic candidates have been searched in the parasite genome. The studies have been supported by bioinformatic prediction of putative proteins and antigenicity predictors.(Goto *et al.*, 2008;Cooley *et al.*, 2008;Hernandez *et al.*, 2010) Using these tools, it has been possible to choose antigens which display the lowest homology level with proteins of organisms related to *T. cruzi*.(Hernandez *et al.*, 2010) Moreover, the bioinformatic analysis has allowed describing for the first time a specific antigen to type discrete typing units (DTUs). (Di Noia *et al.*, 2002)

The results published by many different laboratories point towards considering recombinant proteins as the chosen molecules to be used in immunoassays to diagnose *T. cruzi* infection. Moreover, the lack of specificity leading to false-positive results can be overcome by deleting sequence regions encoding for proteins which cross-react when analyzing negative sera,(Aguirre *et al.*, 2006), or using recombinant proteins that are specific for anti-*T. cruzi* antibodies, yet keeping a high sensitivity.(Belluzo *et al.*, 2011;Camussone *et al.*, 2009) Indeed, the largest studies on the diagnosis reveal the convenience of using these antigens, regarding not only specificity but also the possibility of standardizing both, the methodology and the protein production.(Umezawa *et al.*, 1999;Saez-Alquezar *et al.*, 2000;Umezawa *et al.*, 2003)

The following table display the key recombinant antigens discussed in the present chapter, which were evaluated by several authors for *T. cruzi* infection diagnosis. Notice,

that many of these antigens particularly named by one author have amino acid sequences, which may be very similar to those obtained by other authors who have named them differently (e.g. FRA, Ag1, JL7, H49). Identical or highly similar antigens were grouped in the same row.

Antigen (grouped by high identity)	Characteristics	Diagnostic use	Described by
CRA Ag30 JL8 TCR27 RP4	Cytoplasmic antigen	Chronic infection	Lafaille *et al*., 1989 Ibáñez *et al*., 1988 Levin et al., 1989 Hoft *et al*., 1989 Camussone *et al*., 2009
FRA Ag1 JL7 H49 RP1	Cytoskeleton associated protein	Chronic infection	Lafaille *et al*., 1989 Ibañez *et al*., 1988 Levin *et al*., 1989 Cotrim *et al*., 1995 Camussone *et al*., 2009
B13 Ag2 TCR39 PEP-2 RP5	Trypomastigote surface protein	Chronic infection	Gruber *et al*., 1993 Ibañez *et al*., 1988 Hoft *et al*., 1989 Peralta *et al*., 1994 Camussone *et al*., 2009
Ag36 JL9 MAP-like RP3	Microtubule associated protein	Chronic and acute infection. Antibodies against this protein render cross-reactions with mammal cell cytoskeleton.	Ibañez *et al*., 1988 Levin *et al*., 1989 Kerner *et al*., 1991 Camussone *et al*., 2009

Table 1A. Relevant repetitive recombinant antigens proposed for diagnostic uses. Abbreviations used: CRA, cytoplasmic repetitive antigen; FRA, flagellar repetitive antigen; MAP, microtubule associated protein. RP1, RP3, RP4 and RP5, repetitive peptide 1, 3, 4 and 5, respectively.

Antigen name	Characteristics	Diagnostic use	Described by
SAPA RP2	Trans-sialidase family	Acute and congenital infections. Chronic infection in leishmaniasis endemic regions	Frasch & Reyes, 1990 Russomando *et al.*, 2010 Breniere et al., 1997 Gil *et al.*, 2011 Camussone *et al.*, 2009
TcLo1.2	Trans-sialidase family	Chronic infection	Houghton *et al.*, 1999
TcD	Trans-sialidase family	Chronic and acute infection	Burns, Jr. *et al.*, 1992
Trans-sialidase catalytic region	Trans-sialidase family	Confirmation of chronic infection	Buchovsky *et al.*, 2001
FL-160 CEA CRP160	Complement regulatory protein from TS-like family	Chronic infection and cure monitoring Chronic infection and cure monitoring Cure monitoring	Cetron *et al.*, 1992 Jazin *et al.*, 1995 Meira *et al.*, 2004
TSSAI	Trypomastigote muscin of TS-like family	*T. cruzi* typing (named lineage Tc I, in the previous nomenclature)	Di Noia *et al.*, 2002
TSSAII	Trypomastigote muscin of TS-like family	*T. cruzi* typing (named lineage Tc II, in the previous nomenclature) DTUII, V and VI in the current nomenclature Confirmatory diagnostic in Chagas and leishmaniasis co-endemic regions	Di Noia *et al.*, 2002 Bhattacharyya *et al.*, 2010 Cimino *et al.*, 2011

Table 1B. Relevant recombinant antigens which belong to tran-sialidase (TS) and TS-like family, proposed for diagnostic uses. Abbreviations used: CEA, chronic exoantigen (160 KDa); CRP, complement regulatory protein; FL-160, surface flagellar protein (160 KDa); RP2, repetitive peptide 2; SAPA, shed-acute phase antigen.

Antigen name	Characteristics	Diagnostic use	Described by
R13	Last 13 amino acids from ribosomal protein.	Specific of cardiac disease Stages of cardiac disease	Aznar *et al.*, 1995 Diez *et al.*, 2006
P2β	Full length ribosomal P2β protein	Stages of cardiac disease All stages	Fabbro *et al.*, 2011 Breniere *et al.*, 2002
TcE	Ribosomal protein	Chronic infection	Houghton et al., 1999
FcaBP 1F8 Tc-24 F29 Tc-29 Calflagin	Flagellar calcium binding protein	Chronic and acute infection Chronic infection Cure monitoring patients Cure monitoring patients Chronic infection Chronic infection	Engman *et al.*, 1989 Gonzalez *et al.*, 1985 Krautz *et al.*, 1995 Fabbro *et al.*, 2007 Abate *et al.*, 1993 Marcipar *et al.*, 2005
cy-hsp70 mt-hsp70 grp-hsp78	Heat Shock Proteins	Chronic infection and cure monitoring	Krautz *et al.*, 1998
TcAg29	Alginate regulatory protein	Chronic infection	DaRocha *et al.*, 2002
TcAg48	RNA binding protein	Chronic infection	DaRocha *et al.*, 2002
Tc1	Repetitive proteins obtained by bioinformatic analysis of the genome	Chronic infection	Goto *et al.*, 2008
Tc3	Idem	Chronic infection	Goto *et al.*, 2008
Tc4	Idem	Chronic infection	Goto *et al.*, 2008
Tc9	Idem	Chronic infection	Goto *et al.*, 2008
Tc10	Idem	Chronic infection	Goto *et al.*, 2008
Tc12	Idem	Chronic infection	Goto *et al.*, 2008
Tc15	Idem	Chronic infection	Goto *et al.*, 2008

Table 1C. Other relevant recombinant antigens proposed for diagnostic uses. Abbreviations used: cy-hsp70, cytoplasmic thermal-shock protein; FCaBP, flagellar calcium-binding protein; grp.hsp 78, endoplasmic reticule thermal-shock protein (78 KDa); mt-hsp 70, thermal-shock mitochondrial protein (70 KDA).

4. Recombinant proteins use: Mixtures vs. fusion proteins

The first works dealing with a single recombinant protein for diagnostic purposes reported lack of sensitivity when using only one of those antigens.(Levin *et al.*, 1991;Moncayo & Luquetti, 1990;Peralta *et al.*, 1994) Consequently, most of these proteins have been evaluated not only alone and independently from others, but also together as part of mixtures or as fusion proteins, carrying several recombinant epitopes.(Umezawa *et al.*, 1999;Umezawa *et al.*, 2004;Camussone *et al.*, 2009;Foti *et al.*, 2009) Accordingly, a multicenter study evaluating 6 recombinant proteins separately with a serum panel composed by sera from patients of several countries, described that using the set of results of the 6 proteins together had yield a sensitivity and specificity compatible with the reference assays.(Umezawa *et al.*, 1999) Later, the same group evaluated the mixture of the 6 proteins, supporting the use of the mixture to reach the same sensitivity and specificity.(Umezawa *et al.*, 2003) Soon after, the reactivity of individual antigens vs. antigen mixtures was systematically assessed by ELISA.(Umezawa *et al.*, 2004) This study confirmed that the results obtained with recombinant protein mixtures led to higher media values of optical densities, ODs, than the results produced when using the individual recombinant proteins. Moreover, sera rendering low ODs when examined with individual recombinant proteins produced higher ODs outcomes when using the protein mixtures. Along with this, several commercial ELISA kits with recombinant protein mixtures display equivalent or even higher sensitivities and specificities than those produced by kits with total parasite homogenate.(Gadelha *et al.*, 2003;Pirard *et al.*, 2005;Remesar *et al.*, 2009;Caballero *et al.*, 2007) These works have studied kits using Ag1, Ag2, Ag30, Ag13 together with Ag36 recombinant antigens (Chagatest Rec from Wiener lab, Argentina), and FRA and CRA recombinant antigens (Biomanguinhos, Friocruz, Brazil). However, another study reported that Chagatest Rec v3.0 (Wiener) displayed a rather low 95% sensitivity.(Ramirez *et al.*, 2009)

One of the strategies proposed to enhance reagents production standardization is to obtain multiepitope molecules, designed as a unique construction by fusing several relevant diagnostic antigens.(Houghton *et al.*, 1999;Aguirre *et al.*, 2006;Camussone *et al.*, 2009) It has recently been proved that when using these constructions, the ODs of sera with low reactivity increases, as well as it had been reported for mixtures.(Camussone *et al.*, 2009) Moreover, by this approach the attachment of the antigen turned out to be homogenous and reproducible when using different surfaces such as ELISA plaques, latex particles or bioelectrodes.(Camussone *et al.*, 2009;Gonzalez *et al.*, 2010;Belluzo *et al.*, 2011) It has been proposed that when there is only one molecule exposed to the surface, competition for the active sites is prevented, therefore resulting in a uniform attachment. Furthermore, sensitivity may be increased because a higher number of freely accessible epitopes are available to capture the antibodies present in samples, as depicted in Fig. 1.(Camussone *et al.*, 2009)

A few articles report on the use of this strategy to produce commercial ELISA kits which have demonstrated to be highly satisfying. One of these works, analyzes the performance of the TcF antigen, previously described by Houghton et al in 1999, with which the Biolab Merieux reagent was prepared.(Ferreira *et al.*, 2001) In this case, the recombinant protein used bears the PEP2, TcD, TcE and TcLo1.2 peptides. Recently, Abbot Laboratories have presented a new kit which uses a 4-antigen multiepitope protein containing TcF, FP3 -built up with TcR27 and FcaBP-, FP6 –with TcR39 and FRA- and FP10 -with SAPA and MAP-.(Praast *et al.*, 2011) According to the authors, this kit performed even better than the Biolab Merieux one.

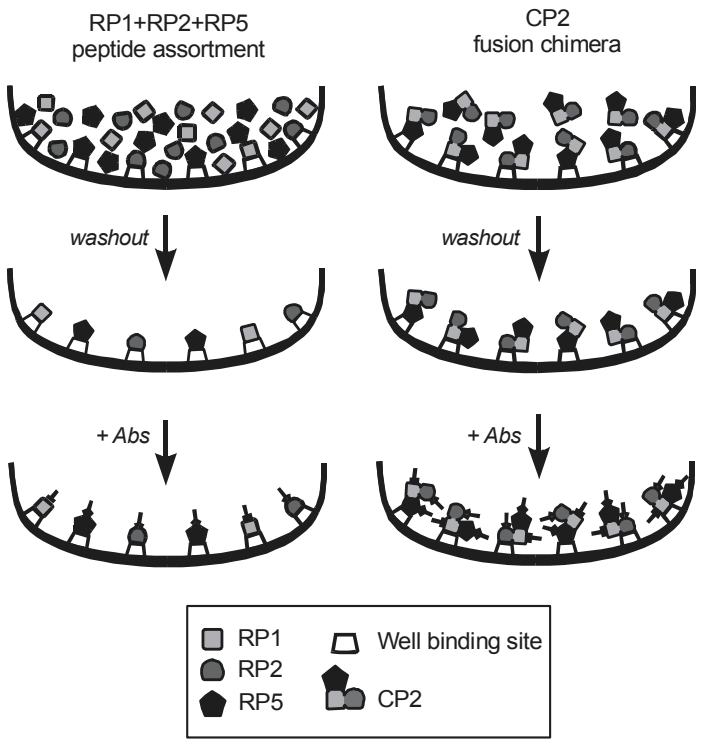


Fig. 1. Illustration of the ELISA plaque sensitizing process and the exposure to the sample: left-hand side, when using a protein mixture of three recombinant peptides RP1+RP2+RP5; right-hand side, when using a multiepitope chimeric protein bearing the same peptides fused in a single protein, CP2. RP: recombinant peptide, CP: chimeric protein obtained by fusion of peptides RP1, RP2 and RP5 together in only one molecule. Reproduction from Camussone et al 2009.

5. Recombinant antigens use for the diagnosis of the different clinical entities

5.1 Acute phase diagnosis

As previously mentioned, during acute infection, serological methods are poorly sensitive. In the case of immunosuppressed individuals, as they do not develop an appropriate humoral immunological response, the direct method or molecular techniques are advisable. Serology is pertinent for in the neonatal congenital diagnostic when microhematocrit repeatedly shows negative results, or when diagnosis has not been made during the first month of life of the newborn. In these cases, it is necessary to serologically monitor congenital infection between 6 to 9 months, no matter if conventional reagents or recombinant ones are used.

Nevertheless, the search for antibodies which are usually triggered during acute phase could enhance results. In the early nineties a shed acute phase antigen (SAPA) was proposed to discriminate between acute and chronic infection.(Reyes *et al.*, 1990) This antigen was described when a panel of recombinant proteins obtained from a cDNA library was used to analyze the reactivity of IgG antibodies occurring in sera of chronic chagasic

mothers and their newborns. The recognized IgGs against different *T. cruzi* antigens produced the same signal in sera from newborns and their respective mothers but SAPA antigen was recognized most frequently by antibodies from the infected newborns than it was by antibodies occurring in their mothers serum. Accordingly, the authors proposed it to be used to detect specific anti-*T. cruzi* IgG antibodies in neonates. Other works report that antibodies anti-SAPA allows the discrimination between acute and chronic *T. cruzi* infection because they were not present in the later stage of the infection.(Lorca *et al.*, 1993) Nevertheless, later works described SAPA as reactive when assessed with sera from chronic infected individuals.(Breniere *et al.*, 1997;Camussone *et al.*, 2009) This apparent contradiction could be explained considering the significant differential reactivity of anti-SAPA antibodies generated during the different stages of the infection. Indeed, anti-SAPA antibodies are detected in almost all infected individuals but its reactivity is higher in the acute infection. It has recently been performed a study by following up of 2283 chagasic mothers, from which 209 transmitted the infection to the newborns.(Russomando *et al.*, 2010) This work provides evidence on SAPA utility to serologically diagnose congenital infection before the third month of life, thus turning the protein into a promising inexpensive reagent to reduce the required time to detect the neonatal infection, and proceed to its early treatment.

Although different reactivity patterns have been described in Western Blot assays which use native *T. cruzi* excretion antigens, to discriminate between acute and chronic infection (Umezawa *et al.*, 1996) no other useful recombinant antigen different from SAPA has been described to diagnose the acute phase.

5.2 Chronic infection diagnosis

It has been already mentioned above that, when *T. cruzi* homogenate is used to perform ELISA tests, the assay sensitivity is high leading to a quite reliable result, therefore some authors have suggested that a single assay could be enough to test sera in blood banks. (Sosa Stani *et al.*, 2008; Otani *et al.*, 2009)

Several multicenter studies carried out on samples from blood-banks, report that ELISA tests which use parasite homogenates perform similarly than those which used recombinant proteins.(Remesar *et al.*, 2009; Otani *et al.*, 2009) However, cross-reactivity of antibodies towards antigens from *T. cruzi* and *Leishmania sp* has been frequently informed, and can be explained considering the phylogenic proximity between both parasite species.(Chiller *et al.*, 1990;Vexenat *et al.*, 1996;Chiaramonte *et al.*, 1996;Desquesnes *et al.*, 2007;Aguirre *et al.*, 2006) When sera from patients infected with *Leishmania ssp* parasites are included in the evaluations, specificity of recombinant proteins are higher (Umezawa *et al.*, 1999; Ferreira *et al.*, 2001; Aguirre *et al.*, 2006; Caballero *et al.*, 2007; Camussone *et al.*, 2009)

It has been recently reported that the antigen TSSA2 displays 87.8% sensitivity and 100% specificity to discriminate between chagasic and leishmaniasic patients.(Cimino *et al.*, 2011) TSSA2 is the only reported recombinant antigen, which has displayed specificity to type *T. cruzi* genotypes DTUII, DTUV or DTUVI by specific antibodies from infected patients. (di Noia *et al.*, 2002; Bhattacharyya *et al.*, 2010) As these DTUs are those predominant in South America, the authors proposed using this antigen in confirmatory *T. cruzi* infection diagnostic tests, in regions which are co-endemic for both infections. It was also described that SAPA antigen could be specific and sensitive enough to be used when trying to distinguish between chronic *T. cruzi* and leishmaniasic infections, in regions where both illnesses are co-endemic.(Gil *et al.*, 2011)

It should be considered that it is difficult to discard *T. cruzi* infection in patients suffering from leishmaniasis, because these are co-endemic diseases. That is why antigens cross-reactivity is normally assayed testing sera from patients who live in Chagas' disease non-endemic regions, because this allows ruling out *T. cruzi* infection from an epidemiological point of view. (Hernandez *et al*., 2010;Caballero *et al*., 2007;Aguirre *et al*., 2006) It therefore follows the need to discuss and define new criteria to study the performance of immunochemical tests at those regions. In this regard, the enhanced sensitivity displayed by PCR techniques should allow overcoming the mentioned drawback.

The lack of cross-reactions of *T. cruzi* recombinant proteins towards samples from *T. rangeli* infected individuals was also described. (Caballero *et al*., 2007) However, these are only preliminary results, and large evaluations have not been still performed since *T. cruzi* and *T. rangelli* are co-endemic and mixed infections are difficult to exclude with conventional or epidemiologic analysis. The first studies reporting discrimination of both infections using molecular approaches have been published in the last year and will allow to compose a serum panel with samples from patients suffering from either only one or both infections.(Botero *et al*., 2010)

In spite of the advantages yielded when using recombinant antigens in ELISA, it has also been described that sensitivity varies according to the antigen used to sensitize plaques and the geographical region. Thus, sensitivity obtained when performing tests in Colombia using recombinant antigens related to the predominant strain in South America is different from that obtained when assays are carried out in regions at the South of the continent.(Ramirez *et al*., 2009) The same holds true when examining samples from Panamá patients, where *T. cruzi* strains are very similar to those from Colombia. (Caballero *et al*., 2007) These works point out that serum level of antibodies in Panamanian patients were significantly lower than those from Brazilian individuals, from where the recombinant proteins were obtained using parasite genotypes isolated in Brazil. These data are in agreement with the serological differential reactivity produced by experimental infections in a mouse model, when strains representatives of different DTUs are inoculated.(dos Santos *et al*., 2009).

5.3 Laboratory treatment monitoring

The evaluation of treatment effectiveness is normally carried out through serological analysis. Direct parasitological techniques miss reliability because of the extremely low parasitemia, which after treatment diminishes more, even when total parasite elimination could have not been reached.

Conventional serology turns into negative for more than 80% of the patients treated during the acute phase, once passed about 2 years after treatment. However, this percentage drops to less than 10% for patients who have been treated during the chronic phase, this taking several years.(Cancado, 1999)

The patient status is classified, according to the laboratory tests results. Thus, patients are considered cured when parasitological tests and conventional serology are negative. When parasitological tests are negative and 2 of 3 conventional serological tests are positive, the patient is classified as dissociated. Patients are considered not to be cured when the 3 tests are positive.

The serological test that demonstrated to be especially useful to monitor treatment effectiveness is the assessment of lytic antibodies. This test showed 100% correlation with parasitological cure, when lytic antibodies were evaluated in sera from patients who were

confirmed to be cured.(Krettli *et al.*, 1979) The drawback of this test is the need to count with in vivo trypomastigotes culture, thus not being available in clinical diagnostic laboratories. Consequently, several recombinant antigens to evaluate patient's treatment response have been proposed and assessed by ELISA as follows.

The target antigen of lytic antibodies was identified as a 160 KDa molecule, a complement regulatory protein, usually named CRP.(Krettli, 2009) The assessment of antibodies against this protein displayed 100% correlation with that of lytic antibodies, when using both the native protein and the recombinant one.(Meira *et al.*, 2004)

Cruzipain and Tc24 are other recombinant proteins which were also evaluated to monitor patient's treatment, and displayed 70% and 80% correlation with the lytic antibodies method, respectively.(Gazzinelli *et al.*, 1993;Krautz *et al.*, 1995) F29, which is Tc24 homologous, was used to follow up the treatment in children younger than 12 years old, after 48 months of initiated the medication.(Sosa *et al.*, 1998) This work reports that 67% of sera from treated children showed lack of anti-F29 antibodies whereas 100% of untreated children showed positive results for the specific antibodies. Therefore, the authors proposed to confirm the lack of anti-F29 antibodies as a serological marker of children cure. More recently, F29 was again evaluated as antigen of treatment monitoring in adults.(Fabbro *et al.*, 2007) Results showed lack of the specific antibodies in 82.4% of sera from treated patients who still showed positive conventional serology. The same group has recently evaluated the levels of specific antibodies against the ribosomal protein TcP2β, as a cure marker. (Fabbro *et al.*, 2011) Their results showed a significant decrease of specific anti-TcP2β in sera from treated patients, although no negative results occurred, which is a similar behavior than that displayed when performing conventional serology. Therefore, anti-TcP2β does not resemble to be an apparently good candidate to be used as an early marker of the infection cure.

Another interesting study is the one where CRA and FRA antigens were evaluated, and displayed a 67% correlation with the reference technique. This makes CRA and FRA as quite promising antigens to be used for cure monitoring. The results are interesting considering that the Bio-manguinhos, Fiocruz commercial kit, commercialized in Brazil is manufactured with a single mixture of these two recombinant proteins.

5.4 Chronic infection monitoring

In the context of Chagas disease autoimmune hypothesis, during the nineties, it was proposed that different antigens contributed to the generation of autoantibodies, which could be used as illness evolution markers.(Leon & Engman, 2001) Among these antigens we should mention cruzipain, (Giordanengo *et al.*, 2000;Goin *et al.*, 1999;Duschak *et al.*, 2001) sulfo-cerebrosides (Avila *et al.*, 1993) and the ribosomal protein TcP2β.(Levitus *et al.*, 1991) Precursor works had described that this ribosomal antigen that shares the C terminal region with its homologous from humans, generated autoimmune antibodies, whose concentration was increased in patients who had developed chagasic cardiopathy.(Levin *et al.*, 1991;Aznar *et al.*, 1995). Our group evaluated the concentration of antibodies against cruzipain, sulfo-cerebrosides and ribosomal TcP2β in three different groups of patients: those classified as asymptomatic, those who only displayed electrocardiographic alterations and those who had evident cardiopathy.(Diez *et al.*, 2006) In our experience, only those samples from patients with evident cardiopathy had increased specific anti-TcP2β concentration. However, these results have not yet been confirmed analyzing a larger number of patients. In another study, anti-TcP2β concentration was higher in sera from patients at indeterminate

stage than in sera from symptomatic individuals.(Breniere *et al.*, 2002) In a longitudinal evaluation of asymptomatic and cardiac groups of patients, we described that only the individuals who evolved to a more severe clinical status increased specific anti-TcP2β concentration in late stages of the infection.(Fabbro *et al.*, 2011) However, the transversal comparison of the sera from patients with and without cardiopathy revealed that anti-TcP2β concentration between both groups was not significantly different. The discrepant results mentioned above show that it is not still clear if anti-TcP2β can be used as a serological marker of myocardic damage.

Also, muscarinic acetylcholine receptor subtype II, in this case a host antigen, has shown to be quite auspicious to monitor the chronic infection.(Goin *et al.*, 1999) Nevertheless, recent studies suggest that it is not apparent that this protein is useful to discriminate between different stages of the illness.(Tovar *et al.*, 2009;Talvani *et al.*, 2006)

The difficulties to establish clear illness evolution markers lead to the present state, where we do not count yet with useful tools to evaluate Chagas' disease prognosis.

6. Future prospects

The number of recombinant proteins assessed and proposed as candidates to be used as tools for *T. cruzi* infection diagnosis is quite high. However, there is no serum panel to be used as international reference. Therefore, the reports on results produced by using these proteins can hardly be compared. Usually, serum panels, previously typified with other validated methods are used to appraise new serology reagents. These already standardized serological reagents have their own sensitivity and specificity, which may lead to a bias when typifying the panel.

Currently, several diagnostic tests have been proposed as the reference one, such as immunofluorescence or different versions of Western blot.(Otani *et al.*, 2009;Caballero *et al.*, 2007) However, no consensus exists among researchers and regulatory agencies on which tests are preferable.

Another inconvenience is that there is evidence on the absence of humoral response in some patients of endemic regions, whose infection was proved by parasitological techniques or by tests evaluating the cellular immune response.(Salomone *et al.*, 2003;Olivera *et al.*, 2010) These reports alert us on a potential overestimation of the sensitivity and specificity underestimation of the immunochemical assays when they are assessed with serum panels typified by conventional serology.

Conventional serology is still a crucial tool to diagnose the different entities studied during the chronic phase of the infection. The main multicenter studies carried out in regions where leishmaniasis is not endemic have shown that ELISAs using both parasite extractive antigens, as well as the recombinant ones, display optimal sensitivity and specificity. Although both kind of antigens perform similarly in those leishmaniasis-free places, when determinations are carried out where Chagas' disease and leishmaniasis are co-endemic, ELISAs using recombinant proteins have demonstrated to be the most useful.

In the same line, presently, several authors who have evaluated ELISA commercial kits with plaques sensitized with parasite homogenate or with recombinant antigens, have shown that it is not mandatory to carry out 2 different tests. Certainly, when performing only one ELISA, it is feasible to diagnose the infection, if the result is positive. This is acknowledged because results obtained with both kinds of ELISA correlate appropriately. (Remesar *et al.*, 2009;Otani *et al.*, 2009) However, considering the poor sensitivity of IHA, this latter one would not be recommended as a second test. (Remesar *et al.*, 2009)

Even though some recombinant proteins have been used to monitor Chagas´ disease treatment, it could not be still demonstrated that these proteins give diagnostic information to evaluate cardiopathy diagnosis and prognosis. During recent years, the description of the whole genome of *T. cruzi* has prompt to systematically analyze new antigens, some of which have been described as putative antigens, but has not yet confirmed. This is being evaluated nowadays by different research groups which, it is expected will suggest new interesting markers that are useful for cure monitoring and cardiopathy prognosis.

Lately, several research works on infection diagnostic tools have reported on the development of latex particle agglutination and amperometric biosensors to diagnose *T. cruzi* infection.(Gonzalez *et al.*, 2010;Belluzo *et al.*, 2011;Ribone *et al.*, 2006) Latex particle vs. conventional agglutination has the advantage of allowing particle sensitization with recombinant proteins, what leads to a more reproducible, standardized reagents production.(Gonzalez *et al.*, 2010) Biosensors technology admits reutilization of the device, potentially yielding to automation, thus facilitating laboratory operation. Moreover, the simplicity of the equipment required, permits the analysis to be performed in the field, which is an important attribute because infected people generally live in the countryside and do not attend health centers.(Belluzo *et al.*, 2011) The electrochemical biosensor technology developed follows the same ELISA format, exchanging the colorimetric signal readout by the amperometric one.(Belluzo *et al.*, 2011) Although no commercial device is yet available, the results of our studies are quite promising. This methodology could allow reducing costs and time of analysis in the near future, keeping the same or even higher standards of sensitivity and specificity than ELISA.(Belluzo *et al.*, 2011)

7. References

Affranchino,J.L.; Ibanez,C.F.; Luquetti,A.O.; Rassi,A.; Reyes,M.B.; Macina,R.A.; Aslund,L.; Pettersson,U. & Frasch,A.C. (1989) Identification of a Trypanosoma cruzi antigen that is shed during the acute phase of Chagas' disease. *Mol. Biochem. Parasitol.* 34(3), 221-228.

Aguirre,S.; Silver,A.M.; Brito,M.E.F.; Ribone,M.E.; Lagier,C.M. & Marcipar,I.S. (2006) Design, Construction and Evaluation of a Specific Chimeric Antigen to Diagnose Chagasic Infection. *J. Clin. Microbiol.* 44, 1043-1046.

Araujo,F.G. (1986) Analysis of *Trypanosoma cruzi* antigens bound by specific antibodies and by antibodies to related trypanosomatids. *Infect. Immun.* 53(1), 179-185.

Avila,J.L.; Rojas,M. & Carrasco,H. (1993) Elevated levels of antibodies against sulphatide are present in all chronic chagasic and dilated cardiomyopathy sera. *Clin. Exp. Immunol.* 92(3), 460-465.

Aznar,C.; Lopez-Bergami,P.; Brandariz,S.; Mariette,C.; Liegeard,P.; Alves,M.D.; Barreiro,E.L.; Carrasco,R.; Lafon,S.; & Kaplan,D. (1995) Prevalence of anti-R-13 antibodies in human *Trypanosoma cruzi* infection. *FEMS Immunol. Med. Microbiol.* 12(3-4), 231-238.

Barfield,C.A.; Barney,R.S.; Crudder,C.H.; Wilmoth,J.L.; Stevens,D.S.; Mora-Garcia,S.; Yanovsky,M.J.; Weigl,B.H. & Yanovsky,J. (2011) A highly sensitive rapid diagnostic test for Chagas disease that utilizes a recombinant Trypanosoma cruzi antigen. *IEEE Trans. Biomed. Eng.* 58(3), 814-817.

Belluzo,M.S.; Ribone,M.E.; Camussone,C.; Marcipar,I.S. & Lagier,C.M. (2011) Favorably orienting recombinant proteins to develop amperometric biosensors to diagnose Chagas' disease. *Anal. Biochem.* 408(1), 86-94.

Bhattacharyya,T.; Brooks,J.; Yeo,M.; Carrasco,H.J.; Lewis,M.D.; Llewellyn,M.S. & Miles,M.A. (2010) Analysis of molecular diversity of the Trypanosoma cruzi trypomastigote small surface antigen reveals novel epitopes, evidence of positive selection and potential implications for lineage-specific serology. *Int. J. Parasitol.* 40(8), 921-928.

Bitran,R.; Martorell,B.; Escobar,L.; Munoz,R. & Glassman,A. (2009) Controlling and eliminating neglected diseases in Latin America and the Caribbean. *Health Aff. (Millwood.)*. 28(6), 1707-1719.

Botero,A.; Ortiz,S.; Munoz,S.; Triana,O. & Solari,A. (2010) Differentiation of Trypanosoma cruzi and Trypanosoma rangeli of Colombia using minicircle hybridization tests. *Diagn. Microbiol. Infect. Dis.* 68(3), 265-270.

Bowling,J.; Walter,E.A. (2009) Recognizing and meeting the challenge of Chagas disease in the USA, *Expert Rev. Anti. Infect. Ther.* 7, 1223-1234.

Breniere,S.F.; Yaksic,N.; Telleria,J.; Bosseno,M.F.; Noireau,F.; Wincker,P. & Sanchez,D. (1997) Immune response to Trypanosoma cruzi shed acute phase antigen in children from an endemic area for Chagas' disease in Bolivia. *Mem. Inst. Oswaldo Cruz.* 92(4), 503-507.

Breniere,S.F.; Bosseno,M.F.; Noireau,F.; Yacsik,N.; Liegeard,P.; Aznar,C. & Hontebeyrie,M. (2002) Integrate study of a Bolivian population infected by Trypanosoma cruzi, the agent of Chagas disease. *Mem. Inst. Oswaldo Cruz.* 97(3), 289-295.

Brutus,L.; Schneider,D.; Postigo,J.; Romero,M.; Santalla,J. & Chippaux,J.P. (2008) Congenital Chagas disease: diagnostic and clinical aspects in an area without vectorial transmission, Bermejo, Bolivia. *Acta Trop.* 106(3), 195-199.

Buchovsky,A.S.; Campetella,O.; Russomando,G.; Franco,L.; Oddone,R.; Candia,N.; Luquetti,A.; Gonzalez Cappa,S.M. & Leguizamon,M.S. (2001) trans-sialidase inhibition assay, a highly sensitive and specific diagnostic test for Chagas' disease. *Clin. Diagn. Lab. Immunol.* 8(1), 187-189.

Burns,J.M.; Jr. Shreffler,W.G.; Rosman,D.E.; Sleath,P.R.; March,C.J. & Reed,S.G. (1992) Identification and synthesis of a major conserved antigenic epitope of *Trypanosoma cruzi*. *Proc. Natl. Acad. Sci. U. S. A.* 89(4), 1239-1243.

Caballero,Z.C.; Sousa,O.E.; Marques,W.P.; Saez-Alquezar,A. & Umezawa,E.S. (2007) Evaluation of serological tests to identify Trypanosoma cruzi infection in humans and determine cross-reactivity with Trypanosoma rangeli and Leishmania spp. *Clin. Vaccine Immunol.* 14(8), 1045-1049.

Camussone,C.; Gonzalez,V.; Belluzo,M.S.; Pujato,N.; Ribone,M.E.; Lagier,C.M. & Marcipar,I.S. (2009) Comparison of recombinant Trypanosoma cruzi peptide mixtures versus multiepitope chimeric proteins as sensitizing antigens for immunodiagnosis. *Clin. Vaccine Immunol.* 16(6), 899-905.

Cancado,J.R. (1999) Criteria of Chagas disease cure. *Mem. Inst. Oswaldo Cruz.* 94(1), 331-335.

Carlier,Y. & Torrico,F. (2003) Congenital Infection with Trypanosoma cruzi: From mechanisms of transmission to strategies for diagnosis and control. *Rev. Bras. Med. Trop.* 36(6), 767-771.

Carvalho,M.R.; Krieger,M.A.; Almeida,E.; Oelemann,W.; Shikanai-Yassuda,M.A.; Ferreira,A.; Pereira,J.B.; Saez-Alquezar,A.; Dorlhiac-Llacer,P.E.; Chamone,D.F. &

Goldenberg,S. (1993) Chagas' disease diagnosis: evaluation of several tests in blood bank screening. *Transfusion*. 33, 830-834.

Cetron,M.S.; Hoff,R.; Kahn,S.; Eisen,H. and Van Voorhis,W.C. (1992) Evaluation of recombinant trypomastigote surface antigens of Trypanosoma cruzi in screening sera from a population in rural northeastern Brazil endemic for Chagas' disease. *Acta Trop.* 50(3), 259-266.

Chiaramonte,M.G.; Zwirner,N.W.; Caropresi,S.L.; Taranto,N.J. & Malchiodi,E.L. (1996) Trypanosoma cruzi and Leishmania spp. human mixed infection. *Am. J. Trop. Med. Hyg.* 54(3), 271-273.

Chiari,E.; Dias,J.C.; Lana,M. & Chiari,C.A. (1989) Hemocultures for the parasitological diagnosis of human chronic Chagas' disease. *Rev. Soc. Bras. Med. Trop.* 22(1), 19-23.

Chiller,T.M.; Samudio,M.A. & Zoulek,G. (1990) IgG antibody reactivity with Trypanosoma cruzi and Leishmania antigens in sera of patients with Chagas' disease and leishmaniasis. *Am. J. Trop. Med. Hyg.* 43(6), 650-656.

Cimino,R.O.; Rumi,M.M.; Ragone,P.; Lauthier,J.; Alberti,D.'A.; Quiroga,I.R.; Gil,J.F.; Cajal,S.P.; Acosta,N.; Juarez,M.; Krolewiecki,A.; Orellana,V.; Zacca,R.; Marcipar,I.; Diosque,P. & Nasser,J.R. (2011) Immuno-enzymatic evaluation of the recombinant TSSA-II protein of Trypanosoma cruzi in dogs and human sera: a tool for epidemiological studies. *Parasitology.*; 1-8.

Cooley,G.; Etheridge,R.D.; Boehlke,C.; Bundy,B.; Weatherly,D.B.; Minning,T.; Haney,M.; Postan,M.; Laucella,S. & Tarleton,R.L. (2008) High throughput selection of effective serodiagnostics for Trypanosoma cruzi infection. *PLoS. Negl. Trop. Dis.* 2(10), e316.

Cotrim,P.C.; Paranhos,G.S.; Mortara,R.A.; Wanderley,J.; Rassi,A.; Camargo,M.E. & da Silveira,J.F. (1990) Expression in *Escherichia coli* of a dominant immunogen of *Trypanosoma cruzi* recognized by human chagasic sera. *J. Clin. Microbiol.* 28(3), 519-524.

Cotrim,P.C.; Paranhos-Baccala,G.; Santos,M.R.; Mortensen,C.; Cano,M.I.; Jolivet,M.; Camargo,M.E.; Mortara,R.A. & da Silveira,J.F. (1995) Organization and expression of the gene encoding an immunodominant repetitive antigen associated to the cytoskeleton of Trypanosoma cruzi. *Mol. Biochem. Parasitol.* 71(1), 89-98.

Coura,J.R. (2007) Chagas disease: what is known and what is needed--a background article. *Mem. Inst. Oswaldo Cruz*. 102 Suppl 1, 113-122.

da Silveira,J.F.; Umezawa,E.S. & Luquetti,A.O. (2001) Chagas disease: recombinant *Trypanosoma cruzi* antigens for serological diagnosis. *Trends Parasitol.* 17(6), 286-291.

da Silveira,A.B.; Lemos,E.M.; Adad,S.J.; Correa-Oliveira,R.; Furness,J.B. & D'Avila,R.D. (2007) Megacolon in Chagas disease: a study of inflammatory cells, enteric nerves, and glial cells. *Hum. Pathol.* 38(8), 1256-1264.

DaRocha,W.D.; Bartholomeu,D.C.; Macedo,C.D.; Horta,M.F.; Cunha-Neto,E.; Donelson,J.E. & Teixeira,S.M. (2002) Characterization of cDNA clones encoding ribonucleoprotein antigens expressed in *Trypanosoma cruzi* amastigotes. *Parasitol. Res.* 88(4), 292-300.

de Moraes,M.H.; Guarneri,A.A.; Girardi,F.P.; Rodrigues,J.B.; Eger,I.; Tyler,K.M.; Steindel,M. & Grisard,E.C. (2008) Different serological cross-reactivity of Trypanosoma rangeli forms in Trypanosoma cruzi-infected patients sera. *Parasit. Vectors.* 1(1), 20.

Desquesnes,M.; Bosseno,M.F. & Breniere,S.F. (2007) Detection of Chagas infections using Trypanosoma evansi crude antigen demonstrates high cross-reactions with Trypanosoma cruzi. *Infect. Genet. Evol.* 7(4), 457-462.

Di Noia,J.M.; Buscaglia,C.A.; De Marchi,C.R.; Almeida,I.C. & Frasch,A.C. (2002) A Trypanosoma cruzi small surface molecule provides the first immunological evidence that Chagas' disease is due to a single parasite lineage. *J. Exp. Med.* 195(4), 401-413.

Dias,J.C.; Amato Neto, V.; Luna, E.J. (2011) Alternative transmission mechanisms of *Trypanosoma cruzi* in Brazil and proposals for their prevention. *Rev. Soc. Bras. Med. Trop.* 44(3) 375-9.

Diez,C.N.; Gea,S.; Marcipar,I.S.; Pezzotto,S.M.; Beloscar,J.; Pellizzon,O.; Marcipar,A. & Bottasso,O. (2006) Cardiovascular risk factors in chronic Chagas' disease are associated with a different profile of putative heart-pathogenic antibodies. *FEMS Immunol. Med. Microbiol.* 48(1), 26-33.

Diez,C.N.; Manattini,S.; Zanuttini,J.C.; Bottasso,O. & Marcipar,I.S. (2008) The value of molecular studies for the diagnosis of congenital Chagas disease in northeastern Argentina. *Am. J. Trop. Med. Hyg.* 78(4), 624-627.

dos Santos,D.M.; Talvani,A.; Guedes,P.M.; hado-Coelho,G.L.; de,L.M. & Bahia,M.T. (2009) Trypanosoma cruzi: Genetic diversity influences the profile of immunoglobulins during experimental infection. *Exp. Parasitol.* 121(1), 8-14.

Duschak,V.G.; Riarte,A.; Segura,E.L. & Laucella,S.A. (2001) Humoral immune response to cruzipain and cardiac dysfunction in chronic Chagas disease. *Immunol. Lett.* 78(3), 135-142.

Editorial (2009) Chagas disease: a neglected emergency. *Lancet.* 373(9678), 1820.

El Sayed,N.M.; Myler,P.J.; Bartholomeu,D.C.; Nilsson,D.; Aggarwal,G.; Tran,A.N.; Ghedin,E.; Worthey,E.A.; Delcher,A.L.; Blandin,G.; Westenberger,S.J.; Caler,E.; Cerqueira,G.C.; Branche,C.; Haas,B.; Anupama,A.; Arner,E.; Aslund,L.; Attipoe,P.; Bontempi,E.; Bringaud,F.; Burton,P.; Cadag,E.; Campbell,D.A.; Carrington,M.; Crabtree,J.; Darban,H.; da Silveira,J.F.; de Jong,P.; Edwards,K.; Englund,P.T.; Fazelina,G.; Feldblyum,T.; Ferella,M.; Frasch,A.C.; Gull,K.; Horn,D.; Hou,L.; Huang,Y.; Kindlund,E.; Klingbeil,M.; Kluge,S.; Koo,H.; Lacerda,D.; Levin,M.J.; Lorenzi,H.; Louie,T.; Machado,C.R.; McCulloch,R.; McKenna,A.; Mizuno,Y.; Mottram,J.C.; Nelson,S.; Ochaya,S.; Osoegawa,K.; Pai,G.; Parsons,M.; Pentony,M.; Pettersson,U.; Pop,M.; Ramirez,J.L.; Rinta,J.; Robertson,L.; Salzberg,S.L.; Sanchez,D.O.; Seyler,A.; Sharma,R.; Shetty,J.; Simpson,A.J.; Sisk,E.; Tammi,M.T.; Tarleton,R.; Teixeira,S.; Van Aken,S.; Vogt,C.; Ward,P.N.; Wickstead,B.; Wortman,J.; White,O.; Fraser,C.M.; Stuart,K.D. & Andersson,B. (2005) The genome sequence of *Trypanosoma cruzi*, etiologic agent of Chagas disease. *Science.* 309(5733), 409-415.

Engman,D.M.; Krause,K.H.; Blumin,J.H.; Kim,K.S.; Kirchhoff,L.V. & Donelson,J.E. (1989) A novel flagellar Ca2+-binding protein in trypanosomes. *J. Biol. Chem.* 264(31), 18627-18631.

Fabbro,D.L.; Streiger,M.L.; Arias,E.D.; Bizai,M.L.; del,B.M. & Amicone,N.A. (2007) Trypanocide treatment among adults with chronic Chagas disease living in Santa Fe city (Argentina), over a mean follow-up of 21 years: parasitological, serological and clinical evolution. *Rev. Soc. Bras. Med. Trop.* 40(1), 1-10.

Fabbro,D.L.; Olivera,V.; Bizai,M.L.; Denner,S.; Diez,C.; Marcipar,I.; Streiger,M.; Arias,E.; del,B.M.; Mendicino,D. & Bottasso,O. (2011) Humoral immune response against

P2beta from Trypanosoma cruzi in persons with chronic Chagas disease: its relationship with treatment against parasites and myocardial damage. *Am. J. Trop. Med. Hyg.* 84(4), 575-580.

Ferreira,A.W.; Belem,Z.R.; Lemos,E.A.; Reed,S.G. & Campos-Neto,A. (2001) Enzyme-linked immunosorbent assay for serological diagnosis of Chagas' disease employing a Trypanosoma cruzi recombinant antigen that consists of four different peptides. *J. Clin. Microbiol.* 39(12), 4390-4395.

Foti,L.; Fonseca,B.P.; Nascimento,L.D.; Marques,C.F.; da Silva,E.D.; Duarte,C.A.; Probst,C.M.; Goldenberg,S.; Pinto,A.G. & Krieger,M.A. (2009) Viability study of a multiplex diagnostic platform for Chagas disease. *Mem. Inst. Oswaldo Cruz*. 104 Suppl 1, 136-141.

Frasch,A.C. & Reyes,M.B. (1990) Diagnosis of Chagas disease using recombinant DNA technology. *Parasitol. Today*. 6(4), 137-139.

Freilij,H.; Muller,L. & Gonzalez Cappa,S.M. (1983) Direct micromethod for diagnosis of acute and congenital Chagas' disease. *J. Clin. Microbiol.* 18(2), 327-330.

Freilij,H. & Storino,R. (1994) Diagnóstico de laboratorio. In: *Enfermedad de Chagas,* Storino,R. & Milei,J. (eds.), Mosby-Doyma, Buenos Aires.

Freilij,H. & Altcheh,J. (1995) Congenital Chagas' disease: diagnostic and clinical aspects. *Clin. Infect. Dis.* 21(3), 551-555.

Gadelha,A.A.; Vercosa,A.F.; Lorena,V.M.; Nakazawa,M.; Carvalho,A.B.; Souza,W.V.; Ferreira,A.G.; Silva,E.D.; Krieger,M.A.; Goldenberg,S. & Gomes,Y.M. (2003) Chagas' disease diagnosis: comparative analysis of recombinant ELISA with conventional ELISA and the haemagglutination test. *Vox Sang.* 85(3), 165-170.

Gascon,J.A.; Bern,C.B.; Pinazo,M.J. (2009) Chagas disease in Spain, the United States and other non-endemic countries. *Acta Tropica.* 115 (1-2) 22-27.

Gazzinelli,R.T.; Galvao,L.M.; Krautz,G.; Lima,P.C.; Cancado,J.R.; Scharfstein,J. & Krettli,A.U. (1993) Use of Trypanosoma cruzi purified glycoprotein (GP57/51) or trypomastigote-shed antigens to assess cure for human Chagas' disease. *Am. J. Trop. Med. Hyg.* 49(5), 625-635.

Gil,J.; Cimino,R.; Lopez,Q.; I, Cajal,S.; Acosta,N.; Juarez,M.; Zacca,R.; Orellana,V.; Krolewiecki,A.; Diosque,P. & Nasser,J. (2011) Reactivity of GST-SAPA antigen of Trypanosoma cruzi against sera from patients with Chagas disease and leishmaniasis. *Medicina (B Aires)*. 71(2), 113-119.

Giordanengo,L.; Fretes,R.; Diaz,H.; Cano,R.; Bacile,A.; Vottero-Cima,E. & Gea,S. (2000) Cruzipain induces autoimmune response against skeletal muscle and tissue damage in mice. *Muscle Nerve*. 23(9), 1407-1413.

Godsel,L.M.; Tibbetts,R.S.; Olson,C.L.; Chaudoir,B.M. & Engman,D.M. (1995) Utility of recombinant flagellar calcium-binding protein for serodiagnosis of *Trypanosoma cruzi* infection. *J. Clin. Microbiol.* 33(8), 2082-2085.

Goin,J.C.; Sterin-Borda,L.; Bilder,C.R.; Varrica,L.M.; Iantorno,G.; Rios,M.C. & Borda,E. (1999) Functional implications of circulating muscarinic cholinergic receptor autoantibodies in chagasic patients with achalasia. *Gastroenterology*. 117(4), 798-805.

Gomes,Y.M.; Lorena,V.M. & Luquetti,A.O. (2009) Diagnosis of Chagas disease: what has been achieved? What remains to be done with regard to diagnosis and follow up studies? *Mem. Inst. Oswaldo Cruz*. 104 Suppl 1, 115-121.

Gonzalez,A.; Lerner,T.J.; Huecas,M.; Sosa-Pineda,B.; Nogueira,N. & Lizardi,P.M. (1985) Apparent generation of a segmented mRNA from two separate tandem gene families in Trypanosoma cruzi. *Nucleic Acids Res.* 13(16), 5789-5804.

Gonzalez,V.D.; Garcia,V.S.; Vega,J.R.; Marcipar,I.S.; Meira,G.R. & Gugliotta,L.M. (2010) Immunodiagnosis of Chagas disease: Synthesis of three latex-protein complexes containing different antigens of Trypanosoma cruzi. *Colloids Surf. B Biointerfaces.* 77(1), 12-17.

Goto,Y.; Carter,D. & Reed,S.G. (2008) Immunological dominance of *Trypanosoma cruzi* tandem repeat proteins. *Infect. Immun.* 76(9), 3967-3974.

Gruber,A. & Zingales,B. (1993) *Trypanosoma cruzi*: characterization of two recombinant antigens with potential application in the diagnosis of Chagas' disease. *Exp. Parasitol.* 76(1), 1-12.

Hernandez,P.; Heimann,M.; Riera,C.; Solano,M.; Santalla,J.; Luquetti,A.O. & Beck,E. (2010) Highly effective serodiagnosis for Chagas' disease. *Clin. Vaccine Immunol.* 17(10), 1598-1604.

Hoft,D.F.; Kim,K.S.; Otsu,K.; Moser,D.R.; Yost,W.J.; Blumin,J.H.; Donelson,J.E. and Kirchhoff,L.V. (1989) *Trypanosoma cruzi* expresses diverse repetitive protein antigens. *Infect. Immun.* 57(7), 1959-1967.

Houghton,R.L.; Benson,D.R.; Reynolds,L.D.; McNeill,P.D.; Sleath,P.R.; Lodes,M.J.; Skeiky,Y.A.; Leiby,D.A.; Badaro,R. & Reed,S.G. (1999) A multi-epitope synthetic peptide and recombinant protein for the detection of antibodies to *Trypanosoma cruzi* in radioimmunoprecipitation-confirmed and consensus-positive sera. *J. Infect. Dis.* 179(5), 1226-1234.

Ibáñez,C.F.; Affranchino,J.L.; Macina,R.A.; Reys,M.B.; Aslund,U.; Petterson,U. & Frasch,A.A.C. (1988) Multiple *Trypanosoma cruzi* antigens containing tandemly amino acid sequence motif. *Mol. Biochem. Parasitol.* 30, 27-34.

Jazin,E.E.; Bontempi,E.J.; Sanchez,D.O.; Aslund,L.; Henriksson,J.; Frasch,A.C. & Pettersson,U. (1995) Trypanosoma cruzi exoantigen is a member of a 160 kDa gene family. *Parasitology.* 110 (Pt 1), 61-69.

Kerner,N.; Liegeard,P.; Levin,M.J. & Hontebeyrie-Joskowicz,M. (1991) *Trypanosoma cruzi:* antibodies to a MAP-like protein in chronic Chagas' disease cross-react with mammalian cytoskeleton. *Exp. Parasitol.* 73(4), 451-459.

Konishi,E. (1993) Naturally occurring antibodies that react with protozoan parasites. *Parasitol. Today.* 9(10), 361-364.

Krautz,G.M.; Galvao,L.M.; Cancado,J.R.; Guevara-Espinoza,A.; Ouaissi,A. & Krettli,A.U. (1995) Use of a 24-kilodalton *Trypanosoma cruzi* recombinant protein to monitor cure of human Chagas' disease. *J. Clin. Microbiol.* 33(8), 2086-2090.

Krautz,G.M.; Peterson,J.D.; Godsel,L.M.; Krettli,A.U. & Engman,D.M. (1998) Human antibody responses to Trypanosoma cruzi 70-kD heat-shock proteins. *Am. J. Trop. Med. Hyg.* 58(2), 137-143.

Krettli,A.U.; Weisz-Carrington,P. & Nussenzweig,R.S. (1979) Membrane-bound antibodies to bloodstream Trypanosoma cruzi in mice: strain differences in susceptibility to complement-mediated lysis. *Clin. Exp. Immunol.* 37(3), 416-423.

Krettli,A.U. (2009) The utility of anti-trypomastigote lytic antibodies for determining cure of Trypanosoma cruzi infections in treated patients: an overview and perspectives. *Mem. Inst. Oswaldo Cruz*. 104 Suppl 1, 142-151.

Lafaille,J.J.; Linss,J.; Krieger,M.A.; Souto-Padron,T.; de Souza,W. & Goldenberg,S. (1989) Structure and expression of two *Trypanosoma cruzi* genes encoding antigenic proteins bearing repetitive epitopes. *Mol. Biochem. Parasitol.* 35(2), 127-136.

Leon,J.S. & Engman,D.M. (2001) Autoimmunity in Chagas heart disease. *Int. J. Parasitol.* 31(5-6), 555-561.

Lescure,F.X.; Paris,L.: Elghouzzi,M.H.; Le,L.G.; Develoux,M.; Touafek,F.; Mazier,D.; Pialoux, G. (2009) Experience of targeted screening of Chagas disease in Ile-de-France, *Bull. Soc. Pathol. Exot.* 102, 295-299.

Lescure,F.X.; Le,L.G.; Freilij,H.; Develoux,M.; Paris,L.; Brutus,L. & Pialoux,G. (2010) Chagas disease: changes in knowledge and management. *Lancet Infect. Dis.* 10(8), 556-570.

Levin,M.J.; Mesri,E.; Benarous,R.; Levitus,G.; Schijman,A.; Levy-Yeyati,P.; Chiale,P.A.; Ruiz,A.M.; Kahn,A.; and Rosenbaum,M.B. (1989) Identification of major *Trypanosoma cruzi* antigenic determinants in chronic Chagas' heart disease. *Am. J. Trop. Med. Hyg.* 41(5), 530-538.

Levin,M.J.; da Silveira,J.F.; Frasch,A.C.; Camargo,M.E.; Lafon,S.; Degrave,W.M. & Rangel-Aldao,R. (1991) Recombinant *Trypanosoma cruzi* antigens and Chagas' disease diagnosis: analysis of a workshop. *FEMS Microbiol. Immunol.* 4(1), 11-19.

Levitus,G.; Hontebeyrie-Joskowicz,M.; Van Regenmortel,M.H. & Levin,M.J. (1991) Humoral autoimmune response to ribosomal P proteins in chronic Chagas heart disease. *Clin. Exp. Immunol.* 85(3), 413-417.

Lorca,M.; Gonzalez,A.; Reyes,V.; Veloso,C.; Vergara,U. & Frasch,C. (1993) [The diagnosis of chronic Chagas disease using recombinant antigens of Trypanosoma cruzi]. *Rev. Med. Chil.* 121(4), 363-368.

Luquetti,A.O. & Schmunis,G.A. (2010) Diagnosis of T. cruzi infection. In: *American Trypanosomiasis. Chagas Disease One Hundred Years Of Research,* Telleria,J. & Tibayrenc,M. (eds.), pp. 743-792. Elsevier, Madrid, Spain.

Marin-Neto,J.A.; Rassi,A.; Jr.; Morillo,C.A.; Avezum,A.; Connolly,S.J.; Sosa-Estani,S.; Rosas,F. & Yusuf,S. (2008) Rationale and design of a randomized placebo-controlled trial assessing the effects of etiologic treatment in Chagas' cardiomyopathy: the BENznidazole Evaluation For Interrupting Trypanosomiasis (BENEFIT). *Am. Heart J.* 156(1), 37-43.

Meira,W.S.; Galvao,L.M.; Gontijo,E.D.; hado-Coelho,G.L.; Norris,K.A. & Chiari,E. (2004) Use of the Trypanosoma cruzi recombinant complement regulatory protein to evaluate therapeutic efficacy following treatment of chronic chagasic patients. *J. Clin. Microbiol.* 42(2), 707-712.

Moncayo,A. & Luquetti,A.O. (1990) Multicentre double blind study for evaluation of Trypanosoma cruzi defined antigens as diagnostic reagents. *Mem. Inst. Oswaldo Cruz*. 85(4), 489-495.

Moya,P.; Basso,B. & Moretti,E. (2005) [Congenital Chagas disease in Cordoba, Argentina: epidemiological, clinical, diagnostic, and therapeutic aspects. Experience of 30 years of follow up]. *Rev. Soc. Bras. Med. Trop.* 38 (Suppl 2), 33-40.

Nóbrega,A.A.; Garcia,M.H.; Tatto,E. Obara,M.T.; Costa,E.; Sobel,J.; Araujo,W.N. (2009) Oral Transmission of Chagas Disease by Consumption of Açaí Palm Fruit, Brazil. *Emer. Infect. Dis.* 15 (4) 653-655.

Olivera,G.C.; Albareda,M.C.; Alvarez,M.G.; De Rissio,A.M.; Fichera,L.E.; Cooley,G.; Yachelini,P.; Hrellac,H.A.; Riboldi,H.; Laucella,S.A.; Tarleton,R.L. & Postan,M. (2010) Trypanosoma cruzi-specific immune responses in subjects from endemic areas of Chagas disease of Argentina. *Microbes. Infect.* 12(5), 359-363.

Otani,M.M.; Vinelli,E.; Kirchhoff,L.V.; del,P.A.; Sands,A.; Vercauteren,G. & Sabino,E.C. (2009) WHO comparative evaluation of serologic assays for Chagas disease. *Transfusion*. 49(6), 1076-1082.

Peralta,J.M.; Teixeira,M.G.; Shreffler,W.G.; Pereira,J.B.; Burns Jr,J.M.; Sleath,P.R. & Reed,S.G. (1994) Serodiagnosis of Chagas' disease by enzyme-linked immunosorbent assay using two synthetic peptides as antigens. *J. Clin. Microbiol.* 32(4), 971-974.

Perez-Molina,J.A.; Perez-Ayala,A.; Moreno,S.; Fernandez-Gonzalez,M.C.; Zamora,J. & Lopez-Velez,R. (2009) Use of benznidazole to treat chronic Chagas' disease: a systematic review with a meta-analysis. *J. Antimicrob. Chemother.* 64(6), 1139-1147.

Pinto,A.Y.; Valente,S.A. & Valente,V.C. (2004) Emerging acute Chagas disease in Amazonian Brazil: case reports with serious cardiac involvement. *Braz. J. Infect. Dis.* 8(6), 454-460.

Pirard,M.; Iihoshi,N.; Boelaert,M.; Basanta,P.; Lopez,F. & Van der,S.P. (2005) The validity of serologic tests for Trypanosoma cruzi and the effectiveness of transfusional screening strategies in a hyperendemic region. *Transfusion*. 45(4), 554-561.

Pittella,J.E. (2009) Central nervous system involvement in Chagas disease: a hundred-year-old history. *Trans. R. Soc. Trop. Med. Hyg.* 103(10), 973-978.

Ponce,C.; Ponce,E.; Vinelli,E.; Montoya,A.; de,A.; V, Gonzalez,A.; Zingales,B.; Rangel-Aldao,R.; Levin,M.J.; Esfandiari,J.; Umezawa,E.S.; Luquetti,A.O. & da Silveira,J.F. (2005) Validation of a rapid and reliable test for diagnosis of chagas' disease by detection of Trypanosoma cruzi-specific antibodies in blood of donors and patients in Central America. *J. Clin. Microbiol.* 43(10), 5065-5068.

Praast,G.; Herzogenrath,J.; Bernhardt,S.; Christ,H. & Sickinger,E. (2011) Evaluation of the Abbott ARCHITECT Chagas prototype assay. *Diagn. Microbiol. Infect. Dis.* 69(1), 74-81.

Ramirez,J.D.; Guhl,F.; Umezawa,E.S.; Morillo,C.A.; Rosas,F.; Marin-Neto,J.A. & Restrepo,S. (2009) Evaluation of adult chronic Chagas' heart disease diagnosis by molecular and serological methods. *J. Clin. Microbiol.* 47(12), 3945-3951.

Rassi,A.; Jr.; Rassi,A. & Rassi,S.G. (2007) Predictors of mortality in chronic Chagas disease: a systematic review of observational studies. *Circulation*. 115(9), 1101-1108.

Reiche,E.M.; Inouye,M.M.; Pontello,R.; Morimoto,H.K.; Itow,J.S.; Matsuo,T. & Jankevicius,J.V. (1996) Seropositivity for anti-trypanosoma cruzi antibodies among blood donors of the "Hospital Universitario Regional do Norte do Parana", Londrina, Brazil. *Rev. Inst. Med. Trop. Sao Paulo*. 38(3), 233-240.

Remesar,M.C.; Gamba,C.; Colaianni,I.F.; Puppo,M.; Sartor,P.A.; Murphy,E.L.; Neilands,T.B.; Ridolfi,M.A.; Leguizamon,M.S.; Kuperman,S. & Del Pozo,A.E. (2009) Estimation of sensitivity and specificity of several Trypanosoma cruzi antibody assays in blood donors in Argentina. *Transfusion*. 49(11), 2352-2358.

Reyes,M.B.; Lorca,M.; Munoz,P. & Frasch,A.C. (1990) Fetal IgG specificities against *Trypanosoma cruzi* antigens in infected newborns. *Proc. Natl. Acad. Sci. U. S. A.* 87(7), 2846-2850.

Ribone,M.E.; Belluzo,M.S.; Pagani,D.; Marcipar,I.S. & Lagier,C.M. (2006) Amperometric bioelectrode for specific human immunoglobulin G determination: optimization of the method to diagnose American trypanosomiasis. *Anal. Biochem.* 350(1), 61-70.

Roddy,P.; Goiri,J.; Flevaud,L.; Palma,P.P.; Morote,S.; Lima,N.; Villa,L.; Torrico,F. and bajar-Vinas,P. (2008) Field evaluation of a rapid immunochromatographic assay for detection of Trypanosoma cruzi infection by use of whole blood. *J. Clin. Microbiol.* 46(6), 2022-2027.

Rosenblatt,J.E. (2009) Laboratory diagnosis of infections due to blood and tissue parasites. *Clin. Infect. Dis.* 49(7), 1103-1108.

Russomando,G.; Sanchez,Z.; Meza,G. & de,G.Y. (2010) Shed acute-phase antigen protein in an ELISA system for unequivocal diagnosis of congenital Chagas disease. *Expert. Rev. Mol. Diagn.* 10(6), 705-707.

Saez-Alquezar,A.; Luquetti,A.; Borges-Pereira,J.; Moreira,M.; Gadhaela,M.T.; García Zapata,M.T. & Strugo Arruda,A.H. (1997) Estudo multicéntrico: validação do desempenho de conjuntos diagnósticos de hemaglutinação indirecta disponíveis no Brasil para o diagnóstico serológico da infeção pelo *Trypanosoma cruzi*. *Rev. Patol. Trop.* 26, 343-374.

Saez-Alquezar,A.; Sabino,E.C.; Salles,N.; Chamone,D.F.; Hulstaert,F.; Pottel,H.; Stoops,E. & Zrein,M. (2000) Serological confirmation of Chagas' disease by a recombinant and peptide antigen line immunoassay: INNO-LIA chagas. *J. Clin. Microbiol.* 38(2), 851-854.

Salles,N.A.; Sabino,E.C.; Cliquet,M.G.; Eluf-Neto,J.; Mayer,A.; Almeida-Neto,C.; Mendonca,M.C.; Dorliach-Llacer,P.; Chamone,D.F. & Saez-Alquezar,A. (1996) Risk of exposure to Chagas' disease among seroreactive Brazilian blood donors. *Transfusion*. 36(11-12), 969-973.

Salomone,O.A.; Basquiera,A.L.; Sembaj,A.; Aguerri,A.M.; Reyes,M.E.; Omelianuk,M.; Fernandez,R.A.; Enders,J.; Palma,A.; Barral,J.M. & Madoery,R.J. (2003) Trypanosoma cruzi in persons without serologic evidence of disease, Argentina. *Emerg. Infect. Dis.* 9(12), 1558-1562.

Silva,E.D.; Pereira,V.R.; Gomes,J.A.; Lorena,V.M.; Cancado,J.R.; Ferreira,A.G.; Krieger,M.A.; Goldenberg,S.; Correa-Oliveira,R. & Gomes,Y.M. (2002) Use of the EIE-recombinant-Chagas-biomanguinhos kit to monitor cure of human Chagas' disease. *J. Clin. Lab Anal.* 16(3), 132-136.

Sosa,E.S.; Segura,E.L.; Ruiz,A.M.; Velazquez,E.; Porcel,B.M. & Yampotis,C. (1998) Efficacy of chemotherapy with benznidazole in children in the indeterminate phase of Chagas' disease. *Am. J. Trop. Med. Hyg.* 59(4), 526-529.

Sosa-Estani,S.; Gamboa-Leon,M.R.; Del Cid-Lemus,J.; Althabe,F.; Alger,J.; Almendares,O.; Cafferata,M.L.; Chippaux,J.P.; Dumonteil,E.; Gibbons,L.; Padilla-Raygoza,N.; Schneider,D.; Belizan,J.M. & Buekens,P. (2008) Use of a rapid test on umbilical cord blood to screen for Trypanosoma cruzi infection in pregnant women in Argentina, Bolivia, Honduras, and Mexico. *Am. J. Trop. Med. Hyg.* 79(5), 755-759.

Sosa-Estani,S. & Segura,E.L. (2006) Etiological treatment in patients infected by Trypanosoma cruzi: experiences in Argentina. *Curr. Opin. Infect. Dis.* 19(6), 583-587.

Soto,M.; Requena,J.M.; Quijada,L. & Alonso,C. (1996) Specific serodiagnosis of human leishmaniasis with recombinant *Leishmania* P2 acidic ribosomal proteins. *Clin. Diagn. Lab. Immunol.* 3(4), 387-391.

Strout,R. (1962) A method for concentrating hemoflagellates. *J. Parasitol.* 48, 100.

Talvani,A.; Rocha,M.O.; Ribeiro,A.L.; Borda,E.; Sterin-Borda,L. & Teixeira,M.M. (2006) Levels of anti-M2 and anti-beta1 autoantibodies do not correlate with the degree of heart dysfunction in Chagas' heart disease. *Microbes. Infect.* 8(9-10), 2459-2464.

Teixeira,A.R.; Nitz,N.; Guimaro,M.C.; Gomes,C. & Santos-Buch,C.A. (2006) Chagas disease. *Postgrad. Med. J.* 82(974), 788-798.

Tovar,N.C.; Echeverry,M.C. & Mora,G. (2009) [Presence of antibodies to cardiac neuroreceptors in patients with Chagas disease]. *Biomedica.* 29(3), 476-494.

Umezawa,E.S.; Nascimento,M.S.; Kesper,N.; Jr.; Coura,J.R.; Borges-Pereira,J.; Junqueira,A.C. & Camargo,M.E. (1996) Immunoblot assay using excreted-secreted antigens of *Trypanosoma cruzi* in serodiagnosis of congenital, acute, and chronic Chagas' disease. *J. Clin. Microbiol.* 34(9), 2143-2147.

Umezawa,E.S.; Bastos,S.F.; Camargo,M.E.; Yamauchi,L.M.; Santos,M.R.; Gonzalez,A.; Zingales,B.; Levin,M.J.; Sousa,O.; Rangel-Aldao,R. & da Silveira,J.F. (1999) Evaluation of recombinant antigens for serodiagnosis of Chagas' disease in South and Central America. *J. Clin. Microbiol.* 37(5), 1554-1560.

Umezawa,E.S.; Bastos,S.F.; Coura,J.R.; Levin,M.J.; Gonzalez,A.; Rangel-Aldao,R.; Zingales,B.; Luquetti,A.O. & da Silveira,J.F. (2003) An improved serodiagnostic test for Chagas' disease employing a mixture of *Trypanosoma cruzi* recombinant antigens. *Transfusion*. 43(1), 91-97.

Umezawa,E.S.; Luquetti,A.O.; Levitus,G.; Ponce,C.; Ponce,E.; Henriquez,D.; Revollo,S.; Espinoza,B.; Sousa,O.; Khan,B. & da Silveira,J.F. (2004) Serodiagnosis of chronic and acute Chagas' disease with *Trypanosoma cruzi* recombinant proteins: results of a collaborative study in six Latin American countries. *J. Clin. Microbiol.* 42(1), 449-452.

Vexenat,A.C.; Santana,J.M. & Teixeira,A.R. (1996) Cross-reactivity of antibodies in human infections by the kinetoplastid protozoa Trypanosoma cruzi, Leishmania chagasi and Leishmania (viannia) braziliensis. *Rev. Inst. Med. Trop. Sao Paulo*. 38(3), 177-185.

Voller,A.; Draper,C.; Bidwell,D.E. & Bartlett,A. (1975) Microplate enzyme-linked immunosorbent assay for chagas' disease. *Lancet*. 1(7904), 426-428.

WHO (2002) Control of Chagas disease. Second report of the WHO Expert Committee. *World Health Organization*. http://whqlibdoc.who.int/trs/WHO_TRS_905.pdf(.

WHO (2003) Chagas disease. Burdens and trends. (2003). *World Health Organization*. http://www.who.int/ctd/chagas/burdens.htm(.

Yadon,Z.E. & Schmunis,G.A. (2009) Congenital Chagas disease: estimating the potential risk in the United States. *Am. J. Trop. Med. Hyg.* 81(6), 927-933.

Zuniga,E.; Motran,C.; Montes,C.L.; Diaz,F.L.; Bocco,J.L. & Gruppi,A. (2000) Trypanosoma cruzi-induced immunosuppression: B cells undergo spontaneous apoptosis and lipopolysaccharide (LPS) arrests their proliferation during acute infection. *Clin. Exp. Immunol.* 119(3), 507-515.

18

Echinococcosis/Hydatidosis

Antoni Soriano Arandes and Frederic Gómez Bertomeu
University Hospital Joan XXIII of Tarragona
Spain

1. Introduction

Echinococcosis is a zoonotic infection caused by adult or larval (metacestode) stages of cestodes belonging to the genus *Echinococcus* and the family *Taeniidae*. Life cycles imply two mammal hosts. Definitive hosts are carnivores containing adult forms in the gut. The infection is acquired by the intermediate hosts and humans after the ingestion of eggs from the feces of carnivorous definitive hosts, which harbour the adult egg-producing stage in the intestine. Eggs ingested by infected human develop into the infective metacestode stage causing various forms of Echinococcosis. The disease in humans and intermediate hosts is called Hydatidosis and is characterized according to the morphologic features of the larval stages: cystic echinococcosis (CE) caused by *Echinococcus granulosus* and related organisms, alveolar echinococcosis (AE) caused by *Echinococcus multilocularis*, and polycystic echinococcosis (PE) caused by *Echinococcus vogeli* or *Echinococcus oligarthrus*.
This disease is becoming an important public health problem in many parts of the world where dogs are used for cattle breeding. Control measures are unable to be implemented everywhere, and where control programs were initiated the success of them have been incomplete generating a re-emergence of the disease. This has also lead to the interruption of control measures excluding Echinococcosis from the list of notifiable diseases. As a consequence of this the incidence and prevalence of CE in Mediterranean countries in humans and animals are not known (Dakkak, 2010). Also, there are a number of factors that contribute to the increase of prevalence and to the spreading of CE in the Mediterranean Region. Cyprus is the only country where an eradication programme has been successfully implemented. However, CE has obtained important developments in the last decade, in the epidemiology, in the diagnosis of canine infection, in strain characterisation and in immune strategies against CE in animals. This scientific progress, together with effective health education programmes, will likely improve control programmes and reduce the time required to achieve significant decreases in prevalence or eradication. Thus, European authorities recognized, through the directive 2003/99/CE, Echinococcosis as a disease to be reported to the European Food Safety Authority (EFSA).
The "WHO/OIE Manual on Echinococcosis in Humans and Animals: a Public Health Problem of Global Concern", published in 2001, has been used as a fundamental issue for the redaction of this chapter.

2. Echinococcosis

The first part of this chapter is dedicated to explain the etiology to know the taxonomy and the life transmission cycles which perpetuate the agent in nature. The biology of the

causative agents of various forms of Echinococcosis is faced because it can help to understand the maintaining of these parasites in different geographic areas. Following to this part the epidemiology and clinical presentation forms are assessed. Finally, diagnosis, treatment and prevention are developed and emphasis is given to the identification of species and strains within the genus as an essential prerequisite to the establishment of local control programmes.

2.1 Etiology and life cycles

Echinococcus presents certain unique characteristics that set it apart from the other major genus in the family, *Taenia.* The adult form is only few millimetres long, has no gut and all metabolic interchange takes place across the syncytial outer covering, the tegument. Scolex is the anterior part of the *Echinococcus* becoming an attachment organ with four muscular suckers and two rows of hooks. The body is the strobila and is segmented in a different number of reproductive units called proglottids. The adult worm is hermaphrodite with reproductive ducts opening at a common, lateral, genital pore, the position of which may vary depending on species and strain. The uterus dilates after fertilisation, eventually occupying most of the terminal segment when the eggs are fully developed. The eggs are ovoid, consisting of a hexacanth embryo surrounded by several envelopes and are morphologically indistinguishable to those of other tapeworms of the genus *Taenia*. The metacestode is the second larval stage and consists of a bladder with an outer acellular laminated layer and an inner nucleated germinal layer. Protoscoleces arise from the inner wall of the brood capsules (figure 1). The structure and development of the metacestode differs between the four species of *Echinococcus*.

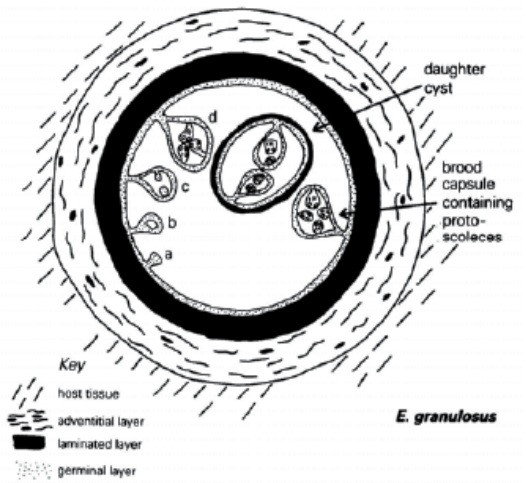


Fig. 1. Representation of the metacestode of *Echinococcus granulosus* (WHO/OIE publication: *Manual on echinococcosis in humans and animals*)

The life cycle of *Echinococcus spp*. requires two mammalian hosts for its completion. Gravid proglottids containing eggs or free eggs are passed in the faeces of the definitive host, a carnivore. These eggs are ingested by an intermediate host, in which the metacestode stage and protoscoleces develop. The cycle is completed if such an intermediate host is eaten by a suitable carnivore. Eggs are highly resistant to the environmental factors being infective for

months at lower ranges of temperatures (from +4ºC to +15ºC). However, they are very sensitive to desiccation and to high temperatures as 60-80ºC.
Intermediate hosts are represented by a wide range of mammals which acquire the infection by the ingestion of eggs. The oncosphere is released from the keratinised embryophore in the stomach and small intestine. Bile activates the oncosphere which penetrates the wall due to the hook movements and secretions and arrives to the liver where some of them are retained (figure 2). All mammals (including man) in which metacestodes of *Echinococcus* species develop after infection with eggs may be referred to as 'intermediate hosts'. However, man is an aberrant host because metacestode stages do not become fertile in this host or because does not interact in the transmission cycle. Once the oncosphere has reached its final location, it develops into the metacestode stage. Time of development is variable and it may take several months before protoscoleces are produced (fertile metacestode). There may be several thousand protoscoleces within a single cyst of *E. granulosus* or an aggregation of vesicles of *E. multilocularis.* Each single protoscolex is capable of developing into a sexually mature adult worm. Not all metacestodes produce protoscoleces (sterile metacestode).

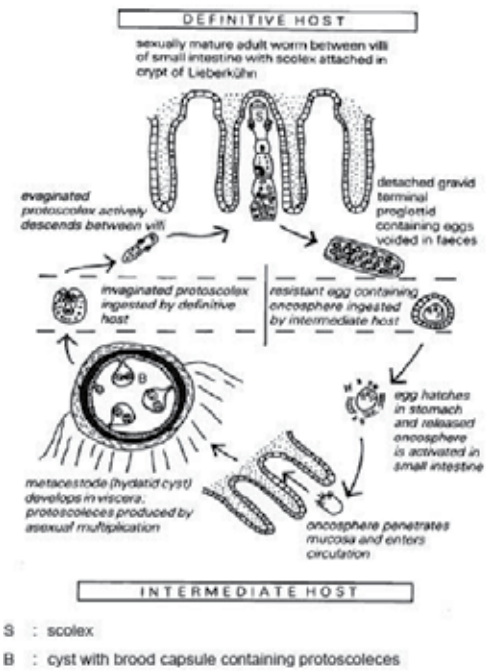


Fig. 2. Life-cycle of Echinococcus (WHO/OIE publication: Manual on echinococcosis in humans and animals)

Strain identification is possible for all four species of *Echinococcus* using morphological and biological features and/or molecular techniques, such as sequence comparison of a 366 bp-fragment of the mitochondrial cytochrome oxidase subunit 1 DNA (CO1) and a 471 bp-region in the mitochondrial NADH dehydrogenase gene 1 (ND1), by analysis of a ribosomal (r) DNA fragment (1ST2) or by the random amplified polymorphic DNA-PCR (RAPD-PCR). Recent genetic studies have principally confirmed the concept of strain diversity within the

species *E. granulosus*, previously based on morphological and biological features. Several molecular techniques are now available which would quite easily allow the identification of certain *E. granulosus* strains using genetic markers. To prepare the identification of the strain using molecular techniques protoscoleces are collected from *E. granulosus* cysts being washed several times in physiological saline solution and preserved in 70% ethanol. The material needs to be examined by an experienced laboratory. The rDNA ITS1 (internal transcribed spacer) region has been shown to be a potentially very useful genetic marker for distinguishing strains and species of *Echinococcus* and small quantities of *Echinococcus* material can be characterised using a PCR-RFLP 'fingerprinting' technique (Bowles & McManus, 1993). Other method is the single strand conformation polymorphism (SSCP) which is technically simple and has high resolution capacity under optimised conditions. The utility of SSCP has been demonstrated for the categorisation of different *Echinococcus* genotypes (Gasser et al., 1998). The different strains of *Echinococcus* have an epidemiological significance for the prognosis in infected patients. Epidemiological studies have evidenced that the sylvatic strain of *E. granulosus* in northern North America is causing a benign infection with low pathogenicity, and affecting the lungs. Also, these epidemiological observations have been demonstrated in the People's Republic of China. In contrast, in parts of Kenya and Libya, it has been suggested that there are local virulent strains of *E. granulosus* (Thompson, 1995). Isoenzyme and molecular studies have confirmed that sheep strain is infective to humans (Bowles & McManus, 1993). Developmental differences between species and strains of *Echinococcus* are likely to be a limiting factor in control programmes which employ regular adult cestocidal treatment of definitive hosts for breaking the cycle of transmission (Thompson, 1995).

2.2 Clinical forms of Echinococcosis

The metacestodes of all four recognised *Echinococcus* species can infect humans and cause various forms of echinococcosis (Table 1). Among these forms cystic and alveolar echinococcosis are of special medical importance.

Forms of Echinococcosis	Causative agent	Disease synonyms
Cystic Echinococcosis	*Echinococcus granulosus*	Hydatid disease, Hydatidosis, *E. granulosus* echinococcosis
Alveolaar Echinococcosis	*Echinococcus multilocularis*	Alveolar hydatid disease, *E. multilocularis* echinococcosis
Polycystic Echinoccosis	*Echinococcus vogeli*	*E.vogeli* echinococcosis
Polycystic Echinococcosis	*Echinococcus oligarthrus*	*E. oligarthrus* echinococcosis

Table 1. Forms of Echinococcosis

Primary echinococcosis is established when metacestodes develop in various sites of the human body from oncospheres liberated from ingested eggs of *Echinococcus* spp. In CE liver and lung are the most frequently affected organs.

Secondary echinococcosis occurs when metacestode material spreads from primary site to adjacent or distant organs and proliferates. Regarding CE this form occurs after release of viable parasite material during invasive treatment procedures.

2.2.1 Cystic echinococcosis (CE)

The causative agent of CE is the metacestode of *Echinococcus granulosus*, becoming a cystic structure filled with a clear fluid. Most of the cysts grow slowly in size and become surrounded by host tissue (pericyst) encompassing the endocyst of metacestode origin. The endocyst consists of the outer laminated layer and the inner cellular germinal layer, which may form brood capsules and protoscoleces. The minimum time required for the development of protoscoleces in cysts in humans is not exactly known, but it is expected to be 10 months or longer after infection (Pawlowski, 1997). Fertile (with protoscoleces) and sterile (without protoscoleces) cysts may coexist in the same patient. Frequently, smaller daughter cysts are formed within a larger mother cyst. If these smaller cysts are growing in close proximity to each other forming clusters the appearance of "polycystic" needs to be distinguished from AE or PE.

The initial phase of primary infection is always asymptomatic remaining as this for many years or permanently. However, the infection may become symptomatic when cysts press adjacent tissues or induce other pathological events. When symptoms appear suddenly a spontaneous or traumatic cyst rupture has to be suspected. Spontaneous cure is possible but improbable, and is due to the collapse and resolution of cysts or due to the cyst rupture into the bile duct or the bronchial tree. The fatality rate is highly dependent on the severity of the infection and on facilities for treatment.

The age of the symptomatic infected patients can vary from below 1 year of age to over 75 years old. In a study from Madrid (Spain), over 1,473 patients admitted to a children's hospital, 2%were <1 year old, 21% between 1 and 4 years and 77% between 5 and 14 years (Utrilla et al., 1991). Many patients (about 40% up to 80%) with CE have a single organ involved and harbour a solitary cyst.

2.2.1.1 Clinical presentation of CE

Clinical symptoms of CE are variable and depend on the organ involved, the size of the cysts, the interaction between the expanding cysts and the adjacent organ structures, and the complications related to the cyst rupture and bacterial infection. CE involving the liver can remain asymptomatic for more than ten years (Frider et al., 1999). Liver and lungs are the two more frequent organ sites involved. Complications affecting the biliary tract are the most common and include the cystic rupture into bile ducts. Other complications are bacterial infection of the cyst, intraperitoneal rupture, and lung involvement.

2.2.1.2 Diagnosis of CE

Diagnosis of CE is done through different steps as follows:

a. Clinical suspect or screening
b. Confirmation by imaging and identification of suspicious cyst structures
c. Confirmation by detection of specific antibodies with immunodiagnostic tests
d. If doubt diagnostic puncture may be considered
e. Material obtained by biopsy puncture or surgery is examined.

Ultrasonography (US) is used for the diagnosis of the cystic structure and portable units are suitable to take into account in field situations. Immunodiagnostic tests for detecting specific antibodies are commonly used for the aetiological confirmation of the findings of imaging examinations.

Protoscoleces or hooks of *E. granulosus* are found in aspirated fluid samples. This technique is not performed frequently because the material can only be available after a surgical intervention, therapeutic puncture (PAIR) or diagnostic puncture. Direct diagnosis can also

be made by macroscopic identification of the *E. granulosus* obtained by surgery or biopsy. Other methods include the identification of specific *E. granulosus* antigen (antigen 5) in the fluid from sterile cysts or DNA markers in the cysts fluid or parasite tissue (e.g. by PCR).
Imaging techniques for diagnosis:

- Standard radiology: chest radiography detects uncomplicated cyst structures displaying a homogeneous shadow that indicates a fluid-filled space. Calcification in lung imaging is rare and cysts may be located anywhere as solitary or multiple. For differential diagnosis, cysts filled with clear fluid, with an air shadow or with water-lily sign are pathognomonic. If a rounded parenchymatous opacity is seen, it is necessary to consider tuberculoma, a tumour or pulmonary sequestration. A fluid and air shadow will lead to consideration of a bacterial, fungal or amoebic abscess.
- Ultrasonography (US): an expert committee of the WHO Working Group on echinococcosis presented an internationally agreed classification of US images in hepatic CE in 2001 (WHO, 2001), according to the use of PAIR (Puncture, Aspiration, Injection, Re-aspiration). This technique was proposed in 1986 by the Tunisian team that first used it in a prospective study. PAIR is a minimally invasive therapeutic option for percutaneous drainage of echinococcal cysts located in the abdomen, complementing or replacing surgery in most of the settings. PAIR also helps the use of benzimidazoles (albendazole and mebendazole) for the treatment of CE. The drainage is performed with a fine needle or a catheter, followed by the killing of the protoscolices remaining in the cyst cavity by a protoscolicide agent. If numerous and large daughter cysts are present, an alternative percutaneous technique "Percutaneous Puncture with Drainage and Curettage" (PPDC) may be used.

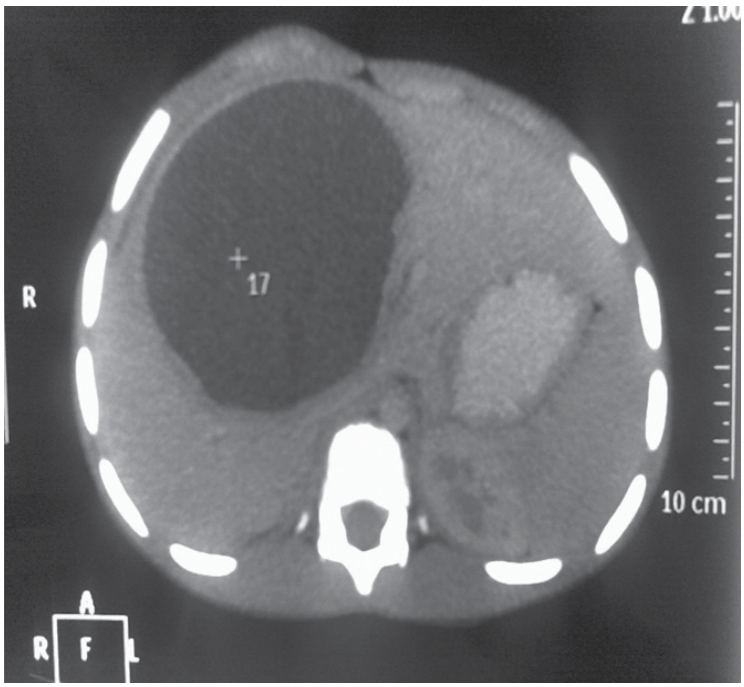
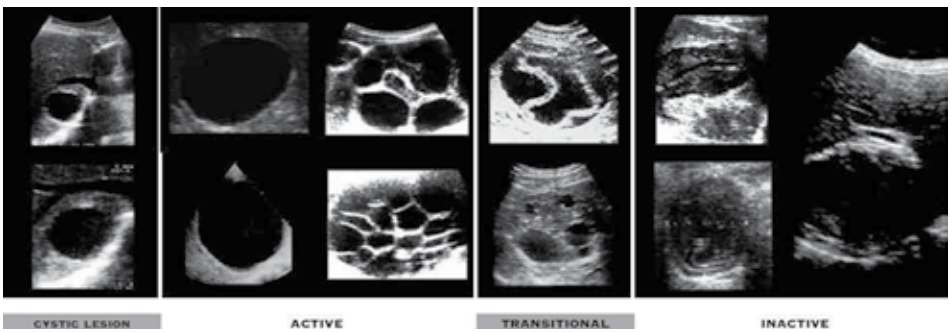


Fig. 3. Image from a computed tomography study of the abdomen of a patient (Soriano Arandes et al., 2010).

- Computed tomography (CT) can detect small cysts, and it also facilitates differential diagnosis of lesions caused by *Echinococcus* metacestodes from non-parasitic lesions (figure 3) (Soriano Arandes et al., 2010). CT is only indicated when US diagnosis is uncertain, mainly in cysts CE4 or CE5. However, CT is the principal method for diagnosis of cerebral cysts showing a spherical cyst with a thin wall, not enhanced after injection of contrast medium, without perilesional oedema the adjacent structures.

2.2.1.3 WHO classification of CE

The WHO classification of CE cysts (WHO, 2001) is done according to the US images:



Cystic lesion (CL): Unilocular, cystic lesion (s) (CL) with uniform anechoic content, not clearly delimited by a hyperechoic rim (= cyst wall not visible).

- Normally round but may be oval.
- Size: variable but usually small. CLs (< 5.0 cm), CLm (5 – 10 cm), CLl (> 10cm).
- Status: If CE - active.If these cystic lesions are caused by CE at an early stage of development then usually these cysts are not fertile.
- Ultrasound does not detect any pathognomonic signs.
- Differential diagnosis of these cystic lesions requires further diagnostic techniques.

CE1: Unilocular, simple cyst with uniform anechoic content. Cyst may exhibit fine echoes due to shifting of brood capsules which is often called hydatid sand ("snow flake sign") (figure 4).

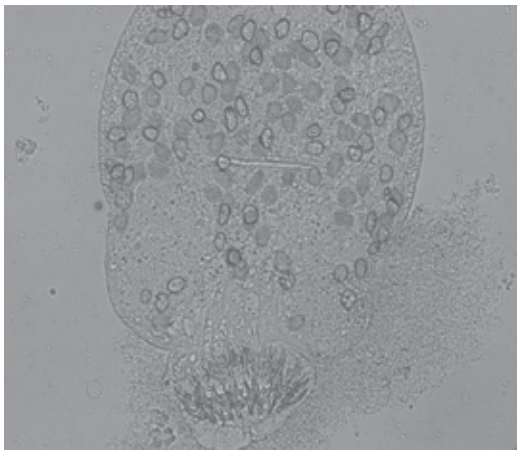


Fig. 4. Hydatid sand containing a protoscolex of *Echinococcus granulosus* seen by light microcopy (Soriano Arandes et al., 2010).

- Cyst wall is visible.
- Normally round or oval.
- Size variable: CE1s (< 5.0 cm), CE1m (5 – 10 cm), CE1l (> 10cm)
- Status: active.
- Usually fertile.
- Pathognomonic signs include visible cyst wall and snow flake sign.

CE2: Multivesicular, multiseptated cysts; cysts septations produce "wheel-like" structures, and presence of daughter cysts is indicated by "rosette-like" or "honeycomb-like" structures. Daughter cysts may partly or completely fill the unilocular mother cyst.

- Cyst wall normally visible.
- Normally round or oval.
- Size variable: CE2s (< 5.0 cm), CE2m (5 – 10 cm), CE2l (> 10cm).
- Status: active.
- Usually fertile.
- Ultrasound features are pathognomonic.

CE3: Unilocular cyst which may contain daughter cysts. Anechoic content with detachment of laminated membrane from the cyst wall is visible as floating membrane or as "water-lily sign" which is indicative of wavy membranes floating on top of remaining cyst fluid.

- Cyst form may be less rounded because of decrease of intra-cystic fluid pressure.
- Size variable: CE3s (< 5.0 cm), CE3m (5 – 10 cm), CE3l (> 10cm).
- Status: transitional.
- Transitional stage. Cyst which may degenerate further or may give rise to daughter cysts.
- Ultrasound features are pathognomonic.

CE4: Heterogenous hypoechoic or hyperechoic degenerative contents. No daughter cysts.

- May show a "ball of wool" sign which is indicative of degenerating membranes.
- Size variable: CE4s (< 5.0 cm), CE4m (5 – 10 cm), CE4l (> 10cm).
- Status: inactive.
- Most cysts of this type are not fertile.
- US features are not pathognomonic and further diagnostic tests are required to ascertain a diagnosis.

CE5: Cysts characterized by thick calcified wall that is arch shaped, producing a cone shaped shadow.

- Degree of calcification varies from partial to complete.
- Size variable: CE5s (< 5.0 cm), CE5m (5 – 10 cm), CE5l (> 10cm).
- Status: inactive.
- Cyst not fertile in most cases.
- Diagnosis is uncertain. Features are not pathognomonic but highly suggestive for *E. granulosus*.

2.2.1.4 Laboratory findings of CE

Routine laboratory tests show non-specific results. Patients with rupture of a cyst into the biliary tree can show transient elevations of GGT or alkaline phosphatase concentrations, often associated with hyperamylasaemia and eosinophilia (>500/µl). When cyst is ruptured eosinophilia achieves higher concentrations.

2.2.1.5 Immunodiagnosis of CE

Immunodiagnosis procedures for serum antibody detection are used for the aetiological confirmation of imaging structures suggestive for CE. Certain proportion of patients with echinococcosis is unable to be diagnosed with highly sensitive diagnostic tests such as IgG-ELISA and false-negative results are obtained. Cysts in the brain or eye and calcified cysts often induce low or no antibody titres. Antibody response may also be low in certain human population groups and in young children. False positive results may also occur, especially in patients with other helminthic diseases. Approaches to the diagnosis of CE using immune methods are specified in table 2.

Fisrt step: Primary antibody test Test for serum antibody detection: IgG-ELISA or IgE-ELISA with E. Granulosus antigen A combination of two or more primary tests may increase sensitivity		
	Subsequent steps	
Seronegative samples **+** **No suggestive images for CE**	**Seronegative samples** **+** **Suggestive images for CE**	**Seropositive samples** **+** **With or without suggestive images for CE**
No further serological follow-up or further steps for differential diagnosis	**Asymptomatic cases** Extended and/or advanced imaging and repeated serological examinations, including differential diagnosis for AE* 'Wait and observe' approach with repeated serological examinations **Symptomatic cases** Consideration of cyst puncture Consideration of surgical intervention and/or chemotherapy without further serological examinations	**Asymptomatic and symptomatic cases** Secondary antibody test: Arc 5 test IgG4-ELISA Immunoblot for antibodies reactive with subunits of *E. granulosus* antigens Serological differential diagnosis for AE (ELISA-Em2plus, immunoblot)

Table 2. Approaches for immunodiagnosis of CE

IgG-ELISA is the preferable test used as a primary test for detecting anti-*Echinococcus* serum antibodies. Most of the routine laboratory test systems or commercialized test kits are based on crude or semi-purified preparations of *E. granulosus* antigens. The use of the two major hydatid cyst fluid antigens, antigen 5 (thermolabile) and antigen B (thermostable), is predominantly restricted to scientific applications, and these antigens are not generally

available. Secondary tests for antibody detection are used to increase specificity and these are: arc 5, identification of IgG subclasses, and immunoblotting which demonstrates the reactivity of serum antibodies with subunits of *E. granulosus* antigens (Craig, 1997; Di Felice, 1986; Ioppolo, 1996; Leggatt & McManus, 1994; Leggatt, 1992; Ligthowlers & Gottstein, 1995; Profumo, 1994; Sheperd & McManus, 1987; Siracusano & Vuitton, 1997; Wen & Craig, 1994). Generally, these tests are less sensitive, but more specific than primary test systems. Putative hydatid cyst fluid samples obtained by puncture or after surgical intervention can be tested for the presence or absence of *Echinococcus* antigen through binding of enzyme-labelled anti-*Echinococcus* (hydatid cyst fluid) antibodies in an ELISA with a monoclonal antibody against antigen 5 (Ag5) that may be useful in confirmation of the *Echinococcus* nature of the fluid (Paul & Stefaniak, 1997).

2.2.1.6 Treatment of CE

Surgery is still the treatment that has the potential to remove *E. granulosus* cysts and lead to complete cure (WHO, 1996). Up to 90% of the patients can be treated surgically if a cyst does not have a risky localisation or if the disease is not too far advanced. However, surgery may be impractical in patients with multiple cysts localised in several organs and if surgical facilities are inadequate. Chemotherapy and PAIR offer an attractive option for treatment, especially in inoperable patients and for cases with a high surgical risk.

2.2.1.6.1 Surgery

Surgery is indicated for large liver cysts with multiple daughter cysts; single liver cysts, situated superficially that may rupture spontaneously or as a result of trauma; cysts that are infected; cysts communicating with biliary tree and/or exerting pressure on adjacent vital organs; cysts in the lung, brain and kidney, bones and other organs.
Surgery of CE is contraindicated as defined for surgical procedures in general, i.e. patients refusing surgery, patients at the extremes of age, pregnant women, and patients with concomitant severe diseases (i.e. cardiac, renal or hepatic diseases, diabetes and hypertension). Also, surgery is contraindicated in patients with multiple cysts or cysts difficult to access, dead cysts either partly or totally calcified, and in patients with very small cysts.
The protoscolicides appareantly effective are: 70-95% ethanol, 15-20% hypertonic saline solution, and 0.5% cetrimide solution. These substances should be left in the cyst cavity for at least 15 minutes to obtain an optimal efficacy.
The risks of surgical intervention include secondary echinococcosis owing to spillage of viable parasite material during the intervention. Recurrence can be due to incomplete cyst removal or to previously undetected cysts. Anaphylactic reactions represent a further risk on rare occasions. Postoperative fatality is about 2% or less, but may be higher in the second or further operations or if medical facilities are inadequate.

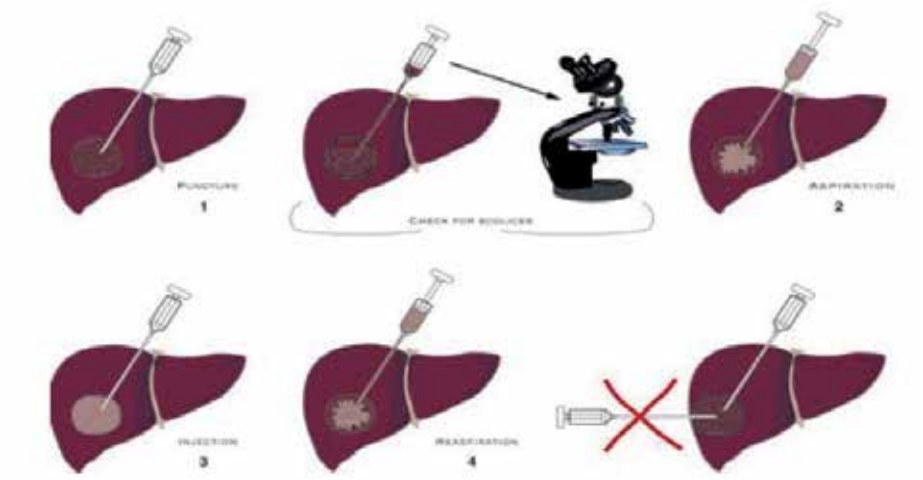
2.2.1.6.2 Puncture, aspiration, injection, re-aspiration (PAIR)

This technique includes the following steps (WHO, 2001):

- percutaneus puncture of cysts under ultrasonic guidance
- aspiration of a substantial amount of cyst fluid
- injection of protoscolicidal substance (preferably 95% ethanol)
- re-aspiration of the fluid cyst content after 15 min to 20 min.

PAIR should be accompanied by a chemotherapeutic coverage to minimise risks of secondary echinococcosis and should be reserved for use by skilled and well experienced physicians with a surgical and intensive care back-up team well prepared to deal

immediately with complications. Aspirates of liver cysts must be analysed immediately for traces of bilirubin and protoscoleces or hooks. PAIR should only be performed under chemotherapeutic coverage, except in early pregnant patients (Filice & Brunetti, 1997).
The PAIR sequence is (WHO, 2001):



There are some critical points to take into account when proceeding with the PAIR protocol:

- Prophylaxis pre- and post-procedure: albendazole is administered 24 to 4 hours before intervention and 15 days to 30 days after intervention according to the cystic size. No treatment for pregnant women.
- Communication with biliary tree: the minimum requirements are to search for bile in the fluid with fast test.
- Scolicidal agent to be used: hypertonic saline (at least 15% final concentration in cyst) or 95% alcohol.
- Quantity of scolicide injected: at least 1/3 of the aspirated quantity.
- Evaluation of viability: microscopic examination.
- Needle vs. catheter: needle for cysts <5 cm. or in multiloculated cysts. Catheter for cysts >5 cm. (PAIRD)
- Follow-up: every week for the 1st month, then every month for the 1st year, the every year for 10 years.

Indications for PAIR: we use this technique for patients with:

- Non-echoic lesion ≥ 5 cm in diameter (CE1m and l)
- Cysts with daughter cysts (CE2), and/or with detachment of membranes (CE3)
- Multiple cysts if accessible to puncture
- Infected cysts
- Also for pregnant women, children >3 years old, patients who fail to respond to chemotherapy alone, patients in whom surgery is contraindicated, patient who refuse surgery, and patients who relapse after surgery.

Contraindications for PAIR:

- Non-cooperative patients and inaccessible or risky location of the cyst in the liver
- Cyst in spine, brain and/or heart
- Inactive or calcified lesion

- Cysts communicating with the biliary tree
- Cysts open into the abdominal cavity, bronchi and urinary tract.

2.2.1.6.3 Chemotherapy

The documentation of the experience about chemotherapy with benzimidazoles in CE is now extensive. Third part of the patients treated with benzimidazoles is achieving the cure with a complete and definitive disappearing of the cysts, and higher proportion (30-50%) has obtained a considerable reduction of the cyst size and also relieve of their symptoms. However, 20-40% of the cases don't respond as expected. Smaller and isolated cysts (less than 7mm.), surrounded by a minimal adventitial membrane respond better than complicated with multiple separations or with son cysts, or surrounded by a thick or calcified adventitial membrane which are refractory to the treatment. There are two main drugs for chemotherapy: albendazole (10-15 mg/kg/day twice per day; 3- to more than 6 monthly courses with free intervals of 14 days) for treating patients with single or multiple cysts (Gemmell & Roberts, 1995), and mebendazole (40-50 mg/kg/day; everyday in three doses per day during 3-6 months). Albendazole continuous courses have shown equal or improved efficacy for 3 to 6 months or longer without an increase of adverse effects (Franchi et al., 1999). When comparing both drugs, albendazole and mebendazole, some researchers concluded that albendazole was better regarding complete cure rates and relieve of the symptoms (Franchi et al., 1999). Albendazole has a better pharmacokinetic profile than mebendazole facilitating higher intestinal absorption and penetration into the cysts. There are described some adverse reactions (neutropenia, hepatic toxicity, alopecia, and others) in a few number of patients which are reversible when treatment is interrupted. Doses, duration, and follow-up of treatment must be taken individually for each patient. However, it seems that minimum duration has to be for three months. It is difficult to predict the long-term prognosis for every patient; therefore it's necessary to do a long-term follow-up, with US or other imaging methods to be able to evaluate the result of the treatment.

Chemotherapy is also useful as a surgical complement and albendazole has been used as a pre-surgical treatment to facilitate the surgical manipulation of the cysts inactivating the protoscoleces previously, modifying the integrity of the cyst membranes and reducing the consistency of the cysts. Treatment with benzimidazoles is recommended to prevent the relapse of the disease, which is the secondary echinococcosis when the content of the cysts is spread after its spontaneous or accidental rupture. When it occur the best option is to treat with three cycles of albendazole or the continuous administration of mebendazole during 1-3 months.

Praziquantel at doses of 40 mg/kg is a potent protoscolicide and could be used as a preventive drug after the cyst content spillage when the rupture of the cyst or as a protoscolicide when PAIR is applied.

Other drugs as nitazoxamide (at doses of 500mg/12h for 3-24 months) has been evaluated in the effectiveness in disseminated cystic echinococcosis (DCE) that failed to respond to surgical and antiparasitic therapy. Three patients improved: one with muscle involvement (clinico-radiological response), one with lung involvement (radiological response), and another with soft tissue and bony involvement (clinico-radiological response of soft tissue cysts) (Pérez-Molina et al., 2011).

Benzimidazoles are contraindicated in pregnancy because they are teratogenic.

Monitoring of the patients is needed and medical and laboratory examinations for adverse reactions are necessary initially every 2 weeks then monthly (WHO, 1996). Leukocyte counts

should be checked at 2-week intervals during the first 3 months because in rare instances severe and not always reversible leukopaenia has been observed in early phases of chemotherapy. Serum drug concentrations (ABZ-sulfoxide or MBZ parent compound) should be monitored after 2 and 4 weeks of chemotherapy, respectively, in order to identify levels too high (possibly toxic) or too low (ineffective). For MBZ, it has been recommended to determine serum or plasma levels 4 h after the morning dose. Oral drug doses can be adapted to individual patients in order to achieve adequate serum levels, but such attempts are not always effective. Unfortunately, only few laboratories have the capability to measure ABZ-sulfoxide or MBZ serum drug levels (see also section on AE). Follow-up examinations, including imaging if needed, should be carried out at intervals of about 3 to 6 months for 1 to 3 years after termination of chemotherapy because of the relatively high rate of relapses.

2.2.1.6.4 Vaccines

A new vaccine against echinococcosis would be highly desirable in order to provide long-term prevention of the disease and to complement control programs. Vaccines against ovine hydatidosis have demonstrated its efficacy when targeting the larval stage of the parasite (Lightowlers, 2001). However, if used in the field we would need to vaccinate all the animals in a herd to achieve good results and this would be very costly to control programs. Otherwise, a vaccine protecting dogs against the adult worm would have to be given to only a few animals to protect the environment, because dogs are less numerous than other animals in the herd. Also, domestic dogs are the key in the transmission to livestock and humans. Therefore, some authors have proposed a recombinant oral vaccine given to the small number of dogs keeping the herd would decrease the number of *E. granulosus* adult worms and, consequently, the number of infective eggs. This measure would help reduce the contamination risk factors for humans and livestock, would be cost-effective for the owners of the dogs, and could help increase the overall efficacy of control programs in endemic countries (Petavy, 2008).

Some candidates have been used to induce immune response with vaccination. One of those is Eg-95 encoding gene that is expressed in the oncosphere, protoscoleces, and immature and mature adult worms of *E. granulosus*. EG95 vaccine antigen is a secreted glycosylphosphatydilinositol (GPI)-anchored protein containing a fibronectin type III domain, which is ubiquitous in modular proteins involved in cell adhesion. EG95 protein represents one of the targets of immunity induced by the vaccine because there is a high degree of sequence gene conservation between different isolates (Zhang, 2003). A mixture of different EG95 isoforms increases the ability of *E. granulosus* to invade different hosts and could possibly maximize vaccine efficacy (Haag, 2009). Other candidates for vaccine are the homologous sequences of two of the S3Pvac peptides, GK1 and KETc1, identified and further characterized in *Taenia crassiceps* WFU, *Taenia solium*, *Taenia saginata*, *E. granulosus* and *E. multilocularis*. Comparisons of the nucleotide and amino acid sequences coding for KETc1 and GK1 revealed significant homologies in these species and the results of a study indicate that GK1 and KETc1 may be considered candidates to be included in the formulation of a multivalent and multistage vaccine against these cestodiases because of their enhancing effects on other available vaccine candidates (Rassy, 2010).

2.2.2 Alveolar echinococcosis (AE)

Alveolar echinococcosis (AE) is an infection caused by the metacestode stage of *E. multilocularis*, which is characterised by a tumour-like, infiltrative and destructive growth

with the potential to induce serious disease with a high fatality rate. Metacestodes develop primarily almost exclusively in the liver varying from small foci of a few millimetres in size to large areas of infiltration (15-20 cm.). From the liver, the metacestode tends to spread to both the adjacent and distant organs by infiltration or metastasis formation (Eckert, 1998). Cases of AE are characterized by an initial asymptomatic incubation period of 5-15 years duration and a subsequent chronic course. Fatality rate in untreated or inadequately treated persons is high.

High burden of AE is known to be common in certain rural communities in China whilst it is generally rare and sporadic elsewhere. Recently, a study was carried out to estimate the global incidence of this disease by country (Torgerson, 2010). They undertook a detailed review of published literature and data from other sources suggesting that there are approximately 18,235 (CI 11,900–28,200) new cases of AE per annum globally with 16,629 (91%) occurring in China and 1,606 outside China. Most of these cases are in regions where there is little treatment available and therefore will be fatal cases. They were able to calculate that AE results in a median of 666,434 DALYs per annum (CI 331,000-1.3 million).

2.2.2.1 Clinical presentation of AE

Age at the time of diagnosis of AE is significantly higher than for CE. The primary site of metacestode development is almost exclusively in the liver. The right lobe is predominantly infected, but the liver hilus together with one or two lobes may also be involved. Extra hepatic primarily locations are rare. During the infection, secondary echinococcosis may occur in variety of adjacent or distant organs. Symptoms of AE are primarily cholestatic jaundice (1/3 of cases) and/or epigastric pain (1/3 of the cases). In the remaining third of patients, AE is detected incidentally during medical examination for symptoms such as fatigue, weight loss, hepatomegaly, or abnormal routine laboratory findings.

2.2.2.2 Classification and staging of AE

PNM system for classification of human alveolar echinococcosis
Classification of findings
P: Hepatic localisation of the parasite
PX: Primary lesion cannot be assessed
P0: No detectable lesion in the liver
P1: Peripheral lesions without proximal vascular and/or biliar involvement
P2: Central lesions with proximal vascular and/or biliar involvement of one lobe (a)
P3: Central lesions with hilar vascular and biliar involvement of both lobes and/or with involvement of two hepatic veins
P4: Any liver lesion with extension along the vessels (b) and the biliary tree
N: Extrahepatic involvement of neighbouring organs
Diaphragm, lung, pleura, pericardium, heart, gastric and duodenal wall, adrenal glands, peritoneum, retroperitoneum, parietal wall (muscles, skin, bone), pancreas, regional lymph nodes, liver ligaments, kidney
NX: Not evaluable
N0: No regional involvement (see above)
N1: Regional involvement of contiguous organs or tissues
M: Absence or presence of distant metastases
Lung, distant lymph nodes, spleen, CNS, orbital, bone, skin, muscle, distant peritoneum and retroperitoneum]

MX: Not completely evaluated
M0: No metastasis(c)
M1: Metastasis

a. For classification, the plane projecting between the bed of the gallbladder and the inferior vena cava divides the liver in two lobes
b. Vessels means inferior vena cava, portal vein and arteries
c. Chest X-ray and cerebral CT negative

Source: European Network for Concerted Surveillance of AE: PNM system for the classification of human cases of AE.

Staging of alveolar echinococcosis cases based on PNM classification

Stage of alveolar echinococcosis	**PNM classification**
Stage I	P1 N0 M0
Stage II	P2 N0 M0
Stage IIIa	P3 N0 M0
Stage IIIb	P1-3 N1 M0
	P4 N0 M0
Stage IV	P4 N1 M0
	Any P Any N M1

Source: European Network for Concerted Surveillance of Alveolar Echinococcosis: PNM system for the classification of human cases of alveolar echinococcosis.

2.2.2.3 Diagnosis of AE

Diagnosis of AE is based on similar findings and criteria as in CE.
Hepatic lesions are characterised in US and CT by heterogenous hypodense masses, often associated with necrotic cavities. The lesion contours are irregular and there is lack of a welldefined wall. Calcifications are often found and exhibit a typical pattern in regard to shape and distribution: clusters of microcalcifications or irregular plaque-like calcified foci are located in the central or peripheral parts of the lesions. Discrepancies between US and CT patterns can be found. Hyperechoic haemangioma-like nodules could represent early forms of AE lesions. Quite frequently an extension of the lesions beyond the liver is found toward diaphragm, lungs, pericardium, retroperitoneum, hepatoduodenal ligament and pancreas.
Magnetic resonance imaging is used to observe compression or obstruction of inferior vena cava, the hepatic veins or the portal branches. Pathognomonic aspects are represented by multicystic honeycomb-like images.

2.2.2.4 Laboratory findings of AE

The routine laboratory tests do not yield specific findings. The blood sedimentation rate is elevated in most of the cases. The numbers of leucocytes and platelets may be depressed in patients with splenomegaly. Lymphopaenia is frequent in advanced cases, and eosinophilia is usually absent. Cholestasis with or without jaundice is observed in patients with intrahepatic bile duct compression or obstruction. Cholangitis and/or liver abscesses, which usually result from bile duct obstruction, are associated with typical alterations of the laboratory parameters. Hypergammaglobulinaemia is present in most of the patients and reflects the specific and polyclonal antibody response. In about one-half of the patients, the presence of specific anti-*E. multilocularis* – IgE can be demonstrated.

2.2.2.5 Immunodiagnosis of AE (table 3)

Fisrt step: Primary antibody test Tests with high sensitivity and less specific value **Subsequent steps**		
Seronegative samples **+** **No suggestive images for AE**	**Seronegative samples** **+** **Suggestive images for AE**	**Seropositive samples** **+** **With or without suggestive images for AE**
No further serological follow-up. Persons with suspected infection risk may require repeated serological examinations after 3-6 months, and US imaging.	**Asymptomatic cases** Extended and/or advanced imaging and repeated serological examinations. Fine needle biopsy for PCR or immunohistology may be considered in rare cases. If lesions are fully calcified, serological and imaging follow-up after 6 months to confirm parasite abortion. **Symptomatic cases** Consideration of surgical intervention and/or chemotherapy without further serological examinations	**Asymptomatic and symptomatic cases** Secondary antibody test: Em2Plus-ELISA (Gottstein et al., 1993) Em alkaline phosphatise-antigen-ELISA (Sarciron et al., 1997) Immunoblot for specific bands or similar test Serological differential diagnosis for CE

Table 3. Approaches for immunodiagnosis of AE

2.2.2.6 Pathological and histological examination of AE

Metacestode of *E. multilocularis* typically exhibits an alveolar structure composed of numerous irregular cysts with diameters between less than 1 mm and 30 mm. when is examined in a macroscopic section of the liver. Due to necrosis of the lesion, cavities filled with liquid and necrotic material may be formed in the central parts of the parasite (Eckert, 1998). Microscopically, the cysts consist of a relatively thin PAS-positive laminated layer and a delicate germinal layer often with only a few nuclei. Brood capsules and protoscoleces are rarely formed in the human host (Eckert, 1998). The cysts are surrounded by an inner zone of necrotic tissue and outer layers of histiocytes and lymphocytes. In later phases, tissue reactions of chronic inflammation, often with giant cell foreign body reaction, fibrous tissue and calcifications are seen around cysts.

2.2.2.7 Treatment of AE

There are a variety of options to select the adequate treatment for each individual patient. Clinical experience is crucial for AE; therefore patients should be referred to a recognised national or regional reference centre. Early diagnosis of AE is of special importance for successful treatment because the lesion is acting as a malignant tumour. Screening programmes in Japan and Europe have shown that early diagnosis reduces mortality and morbidity due to AE. Some considerations for treatment of AE are generally accepted: the first choice treatment in all operable cases is radical surgical resection of the entire parasitic lesion from the liver and other affected organs, chemotherapy is indicated after radical surgery for a limited period of time, and long-term chemotherapy is mandatory after incomplete resection of the lesions, in inoperable patients and in AE patients after liver transplantation.

Chemotherapy has several indications, as follows:

- Applicable for limited period of time after radical surgery. Since residual parasite tissue may remain undetected at radical surgery, post-operative chemotherapy for at least 2 years should be carried out and patients should be monitored for a minimum of 10 years for possible recurrence
- Long-term chemotherapy for several years is mandatory in inoperable AE patients, in cases following incomplete surgical resection of the parasite lesions and after liver transplantation
- Pre-surgical chemotherapy is not indicated in cases of AE. However, in rare cases that surgery was contraindicated at the time of diagnosis of AE, surgery can be carried out after a prolonged course of chemotherapy.

Benzimidazoles are preferentially used for AE:

Mebendazole (MBZ) is given as 500-mg tablets in daily doses of 40-50 mg/kg bw in three divided doses postprandially. After an initial continuous treatment of 4 weeks, it is advisable to adjust the oral doses in order to obtain plasma drug levels of >250 nmol/l (= 74 ng/ml). The duration of treatment is at least 2 years after radical surgery or continuously for many years in inoperable cases, as well as for patients who have undergone incomplete resection or liver transplantation.

Albendazole (ABZ) is given as 400-mg tablet or as a 4% suspension at daily doses of 10 -15 mg/kg bw (in two divided doses). In practice, a daily dose of 800 mg is given to adults, divided into two doses of 400 mg. Repeated cycles of 28 days treatment should be followed by a 'wash out' phase without chemotherapy of 14 days. However, data from the People's Republic of China (Liu, 1997) and Italy indicate that a continuous ABZ treatment of AE is at least equally or more effective and well tolerated. The duration of necessary chemotherapy has not yet been determined but might well be life-long for most of the patients without complete resection of the AE lesions.

Adverse effects of the chemotherapy with benzimidazoles are neutropaenia, alopecia and liver dysfunction. They are contraindicated in pregnancy due to its potential embryotoxicity and teratogenicity. Monitoring of the AE patients is similar to that in CE patients. A long-term follow-up of more than 10 years is recommended.

Interventional procedures are indicated for AE patients for whom surgery is contraindicated. Some of them are dilation and stent implantation in vessels or bile ducts, and endoscopic sclerosing of oesophageal varices.

Liver transplantation should only be considered in patients with very severe hilar extension, leading to uncontrolled biliary infections, symptomatic secondary biliary cirrhosis with ascites or severe variceal bleeding owing to portal hypertension (Bresson-Hadni, 1997). It requires long-term and continuous postoperative chemotherapy.

2.2.3 Polycystic echinococcosis (PE)

Forms of human polycystic echinococcosis (PE) are caused by *E. vogeli* and *E. oligarthrus*, which are confined in their distribution to Latin American countries.

Metacestode of *E. vogeli* is characterized by its polycystic form filled with liquid with a tendency to form conglomerates with multiples small spaces inside. The most affected organ in the intermediate host is the liver. Metacestode of *E. oligarthrus* is similar to *E. vogeli* but the division in secondary spaces is less frequent and the laminar membrane is significantly thinner.

Wild and domestic dogs as definitive hosts and paca (*Cuniculus paca*) as intermediate host participate in the life-cycle of *E. vogeli*. Polycystic echinococcosis due to *E. vogeli* has been communicated in the majority of the countries belonging to the neotropical region of America; including Panama, Colombia, Argentina, Ecuador, Brasil, Bolivia and Venezuela.

E. oligarthrus is the unique *Echinococcus* specie that uses felids as definitive hosts. Infections naturally acquired have been demonstrated affecting pumas, jaguars, and other wild felids.

2.2.3.1 Polycystic echinococcosis due to *E. vogeli*

Clinical and radiological presentation is very similar to infection with multiple cysts of *E. granulosus*, and differential diagnosis depends on isolation of protoscoleces and morphological hook characteristics (D'Alessandro, 1997). Immunodiagnosis using a purified antigen of *E. vogeli* allowed discrimination between cases of PE and CE, but differentiation between PE and AE was not always possible (Gottstein, 1995). Albendazole with doses of 10 mg/kg/day has been used for chemotherapy in six cases with success of treatment in four and improvement in two (D'Alessandro, 1997).

2.2.3.2 Polycystic echinococcosis due to *E. oligarthrus*

Only three human cases have been reported to date, two orbital in Venezuela and Surinam and one cardiac in Brazil with 2 cysts (1.5 cm diameter) (D'Alessandro, 1997). The diagnosis was based on morphology of protoscolex hooks.

3. Conclusion

Human echinococcosis is a zoonotic larval cestode disease usually caused by *Echinococcus granulosus* or *E. multilocularis*. Infection is chronic taking years for symptoms to develop. The medical impact of the late stages of human cystic or alveolar echinococcosis may be significant though morbidity and mortality are usually grossly under-reported in endemic areas. Because of diagnosis and treatment are difficult and reservoirs of infection are maintained in domestic livestock, dogs or wildlife, the disease is difficult to assess in terms of public health and requires long-term control interventions. Globally, 3.6 million disability-adjusted life-years (DALYs) could be lost due to echinoccocosis, and this disease is included in an important group of neglected non vector-borne zoonotic infections that are currently not sufficiently prioritised (WHO/DFID-AHP, 2006).

Echinococcosis is therefore a neglected disease which is under-reported and requires urgent attention in common with a number of other zoonoses in order to reduce morbidity and to help alleviate poverty in poor pastoral areas of the sub-tropics and temperate zones. It's also difficult to formulate interventions and to apply cost-effective control programmes in this disease.

Human behaviour is crucial in facilitating transmission of this infection between domestic animal hosts as a result of traditional pastoral and husbandry practices (Mcpherson, 2005; Craig, 2007). Dogs are also susceptible to infection with *E. multilocularis* and *E. vogeli* (whose intermediate hosts are principally rodents) and therefore dogs may constitute a greater zoonotic reservoir of infection compared to natural wild canid hosts. Peri-domestic transmission may occur and could for example sustain a level of transmission of *E. multilocularis* in highly endemic communities (Li, 2005), but is probably not responsible for long-term maintenance of these Echinococcus species adapted to small mammals. Therefore, echinococcosis is a disease where humans may acquire infection from wild or domestic animal hosts but the parasite cannot be directly transmitted between humans (Wolfe, 2007), and due to all of these concepts treatment of human echinococcosis cases will have no effect on pathogen transmission. We will need to apply interventions to reduce human exposure or break transmission cycles in order to control the disease. This places echinococcosis in a 'difficult-to-deal-with' category; firstly, unlike the other neglected parasitic diseases humans can not act as a definitive host, and secondly, echinococcosis in livestock (or dogs) is not perceived as an animal health problem.

Clinical symptoms and subsequent diagnosis occur in adults (20-60 years) but infections in children may also become symptomatic (Soriano Arandes et al., 2010), and imaging techniques are the basis for diagnosis preferably accompanied by a specific serological test (Craig et al., 2003). Surgical removal of cysts/cystic masses, cyst drainage or organ resection, are the main form of treatment, often supported by high dose albendazole cover; the latter also has a benefit in medically-only treated cases (WHO, 2001).

The key factors of echinococcosis as a neglected disease are best described in a recent paper (Craig et al., 2007):

- Human echinococcosis is a zoonosis, non vector-borne zoonosis, that is not transmitted between humans. Therefore, it's a disease that is not amenable to vector-based control nor to direct human-treatment-approaches for case prevention.
- Human CE and AE are chronic diseases with very long asymptomatic periods so that endemic communities and health authorities fail to properly recognise the negative health impacts. Prevalence values represent infection events some years previously. CE remains an important health problem in many regions of the world, both where no control measures have been implemented, and where control programs have been incompletely successful with ensuing re-emergence of the disease. In Spain, official data on CE show an increase in the proportion of intermediate hosts with CE during the last few years, and autochthonous pediatric patients have been reported, a sign of active local transmission of disease. However, several crucial aspects related to CE that would help better understand and control the disease have not been tackled appropriately, in particular the emergence of infection in specific geographical areas. The introduction of national registries for CE with online data entry, following the example set by the European Registry for Alveolar Echinococcosis, would help streamline data collection on CE by eliminating the need for evaluating and

integrating data from multiple regions, by avoiding duplication of data from patients who access several different health facilities over time, and by providing much needed clinical and epidemiological data that are currently accessible only to clinicians (Rojo-Vazquez et al., 2011).

- Echinococcosis is difficult to detect or diagnose in humans without access to imaging tools (eg. ultrasound, CT scan), furthermore the basis for confirmatory laboratory diagnosis is usually expensive serological tests.
- Treatment is very difficult and not always very effective, relying largely on costly major surgical or percutaneous hospital-based interventions to remove or sterilise cystic lesions. Furthermore, anthelmintic therapy is not ashighly effective as for other helminthic diseases (eg. gastro-intestinal helminthiases, schistosomiasis or onchocerciasis), and is based on long-term high dose albendazole usually re- quiring a minimum 6 months daily course, for which follow-up is very difficult especially in poor-resource areas. In this regard echinococcosis treatment more closely resembles tumour treatments or TB therapy, than that for a parasitic disease.
- Medical records are usually not very explicit/specific, may involve complex follow-up notes, and may be dispersed across several specialities within/between hospitals/clinics and therefore data is not usually properly collated.
- In under-developed regions, human echinococcosis generally occurs in poor, often remote marginalised pastoral societies that may be ethnically/socio-culturally isolated from the general population. Consequently they are not usually very well prioritised by the predominant agricultural-based community district health authorities, and so access to affordable health care is also poor and/or difficult.
- The burden of echinococcosis disease is therefore difficult to quantify, and official hospital or district records often inaccurate, and in any case represent gross under-estimates of the real burden in an endemic area.
- CE is a chronic, asymptomatic infection in domestic animals and is therefore also not recognised by livestock owners as an animal health or economic problem.
- Dogs are the main carrier and spreader of the parasite, but are asymptomatic. In contrast to livestock numbers, official accurate estimates of dog population sizes (owned and stray) are almost never kept/known by municipal, veterinary or agricultural authorities. Canine echinococcosis is treatable with the anthelmintic praziquantel but requires frequent dosing. Unlike for rabies, there is currently no dog vaccine against canine echinococcosis.
- Control of echinococcosis is difficult and exacerbated by the requirement of cooperation between agricultural/veterinary services and medical authorities.

4. Acknowledgment

To my family, and especially to my wife Marta and my sons Marc and Toni for their support everywhere and every moment of my life.

5. References

Bowles, J. & McManus, D.P. (1993). Molecular variation in *Echinococcus. Acta trop.*, 53, 291-305.

Bowles, J. & McManus, D.P. (1993). Rapid discrimination of *Echinococcus* species and strains using a polymerase chain reaction-based RFLP method. *Molec. biochem. Parasitol.*, 57, 231-239.

Bresson-Hadni, S., Koch, S., Beutron, L., Vuitton, DA., Bartholomot, B., Hrusovsky, S., Heyd, B., Lenys, D., Minello, A., Becker, MC., Vanlemmens, C., Mantion, GA. & Miguet, JP. (1997). Primary disease recurrence after liver transplantation for alveolar echinococcosis: long-term evaluation in 15 patients. *Hepatology*, 30, 857-863.

Craig, PS. (1997). Immunodiagnosis of *Echinococcus granulosus* and a comparison of techniques for diagnosis of canine echinococcosis, *In:* Compendium on cystic echinococcosis in Africa and Middle Eastern Countries with special reference to Morocco (F.L. Andersen, H. Ouhelli & M. Kachani, eds). Brigham Young University, Print Services, Provo, 85-118.

Craig, PS., Budke, CM., Schantz, PM., Li, T., Qiu, J., Yang, Y., Zeyhle, E., Rogan, MT., and Ito, A. (2007). Human echinococcosis: a neglected disease?, *Trop. Med. Int. Health,* 35(4), 283-292.

Craig, PS., McManus, DP., Lightowlers, MW., Chabalgoity, JA., Garcia, HH., Gavidia, CM., Gilman, RH., Gonzalez, AE., Lorca, M., Naquira, C., Schantz, PM. (2007). Prevention and control of cystic echinococcosis. *Lancet Inf Dis*., 7, 385-394.

Craig, PS., Rogan, MT., Campos-Ponce, M. (2003). Echinococcosis: disease, detection and transmission. *Parasitology*, 127, S5-S20.

Dakkak, A. (2010). Echinococcosis/hydatidosis: a severe threat in Mediterranean countries. *Vet Parasitol.* 174(1-2), 2-11.

D'Alessandro, A. (1997). Polycystic echinococcosis in tropical America: *Echinococcus vogeli* and *E. oligarthrus. Acta trop*., 67 (1-2), 43-65.

Di Felice, G., Pini, C., Afferni, C. & Vicari, G. (1986). Purification and partial characterization of the major antigen of *E. granulosus* (antigen 5) with monoclonal antibodies. *Molec. biochem. Parasitol*., 20, 133-142.

Eckert, J. (1998). Alveolar echinococcosis (*Echinococcus multilocularis*) and other forms of echinococcosis (*Echinococcus oligarthrus* and *Echinococcus vogeli*), *In:* Zoonoses (S.R. Palmer, E.J.L. Soulsby & D.I.H. Simpson, eds). Oxford University Press, Oxford, 689-716.

Filice, C. & Brunetti, E. (1997). Use of PAIR in human cystic echinococcosis. *Acta trop*., 64, 95-107.

Franchi, C., Di Vico, B. & Teggi, A. (1999). Long-term evaluation of patients with hydatidosis treated with benzimidazole carbamates. *Clin. infect Dis.,* 29, 304-309.

Frider, B., Larrieu, E. & Odriozola, M. (1999). Long-term outcome of asymptomatic liver hydatidosis. *J. Hepatol.,* 30, 228-231.

Gasser, RB., Zhu, XQ. & McManus, DP. (1998). Display of sequence variation in PCR-amplified mitochondrial DNA regions of *Echinococcus* by single-strand conformation polymorphism. *Acta trop*., 71, 107-115.

Gemmell, M.A. & Roberts, M.G. (1995). Modelling *Echinococcus* life cycles, *In: Echinococcus* and hydatid disease (R.C.A. Thompson & A.L. Lymbery, eds). CAB International, Wallingford, Oxon, 333-354.

Gottstein, B., D'Alessandro, A. & Rausch, R.L. (1995). Immunodiagnosis of polycystic hydatid disease/polycystic echinococcosis due to *Echinococcus vogeli. Am. J. trop. Med. Hyg.*, 53, 558-563.

Gottstein, B., Jacquier, P., Bresson-Hadni, S. & Eckert, J. (1993). Improved primary immunodiagnosis of alveolar echinococcosis in humans by an enzyme-linked immunosorbent assay using the Em2plus-antigen. *J. clin. Microbiol.*, 31, 373-376.

Haag, KL., Gottstein, B., Ayala, FJ. (2009). The EG95 Antigen of Echinococcus spp. Contains Positively Selected Amino Acids, which May Influence Host Specificity and Vaccine Efficacy. *PLoS ONE* 4(4): e5362. doi:10.1371/journal.pone.0005362.

Ioppolo, S., Notargiacomo, S., Profumo, E., Franchi, C., Ortona, E., Rigano, R. & Siracusano, A. (1996). Immunological responses to antigen B from *Echinococcus granulosus* cyst fluid in hydatid patients. *Parasite Immunol.*, 18, 571-578.

Leggatt, GR. & McManus, DP. (1994). Identification and diagnostic value of a major antibody epitope on the 12 kDa antigen from *Echinococcus granulosus* (hydatid disease) cyst fluid. *Parasite Immunol.*, 16, 87-96.

Leggatt, GR., Yang, W. & McManus, DP. (1992). Serological evaluation of the 12 kDa subunit of antigen B in *Echinococcus granulosus* cyst fluid by immunoblot analysis. *Trans. roy. Soc. trop. Med. Hyg.*, 86, 189-192.

Li, T., Qiu, J., Yang, W., Craig, PS., Chen, X., Xiao, N., Ito, A., Giraudoux, P., Wulamu, M., Yu, W., Schantz, PM. (2005). Echinococcosis in Tibetan populations, western Sichuan Province, China. *Emerg Inf Dis.*, 11, 1866-1873.

Lightowlers MW & Gauci CG (2001). Vaccines against cysticercosis and hydatidosis. *Vet Parasitol* 101: 337–352.

Ligthowlers, MW. & Gottstein, B. (1995). Echinococcosis/hydatidosis: antigens, immunological and molecular diagnosis, *In: Echinococcus* and hydatid disease (R.C.A. Thompson & A.J. Lymbery, eds). CAB International, Oxon, 355-410.

Liu, YH. (1997). Continuous or intermittent treatment with albendazole? *Arch. int. Hidatid*, 32, 171-173.

Macpherson, CNL. (2005). Human behaviour and the epidemiology of parasitic zoonoses. *Int J Parasito.l*, 35, 1319-1331.

Paul, M. & Stefaniak, J. (1997). Detection of specific *Echinococcus granulosus* antigen 5 in liver cyst bioptate from human patients. *Acta trop.*, 64, 65-77.

Pawłowski, ZS. (1997). Critical points in the clinical management of cystic echinococcosis: a revised review, *In*: Compendium on cystic echinococcosis in Africa and in Middle Eastern Countries with special reference to Morocco (F.L. Andersen, H. Ouhelli & M. Kachani, eds). Brigham Young University, Print Services, Provo, Utah, 119-135.

Pérez-Molina, JA., Díaz-Menéndez, M, Gallego, JI, et al. (2011). Evaluation of nitazoxanide for the treatment of disseminated cystic echinococcosis: report of five cases and literature review. *Am. J. Trop. Med. Hyg.*, 84(2), 351-6.

Petavy A-F, Hormaeche C, Lahmar S, Ouhelli H, Chabalgoity A, et al. (2008). An Oral Recombinant Vaccine in Dogs against Echinococcus granulosus, the Causative Agent of Human Hydatid Disease: A Pilot Study. *PLoS Negl Trop Dis* 2(1): e125.

Profumo, E., Ortona, E., Rigano, R., Gioia, I., Notargiacomo, S., Ioppolo, S. & Siracusano, A. (1994). Cellular and humoral responses to antigenic subunits of *Echinococcus granulosus* cyst fluid in hydatid patients. *Parasite Immunol*., 16, 393-398.

Rassy, D., Bobes, RJ., Rosas, G., Anaya, VH., Brehm, K., et al. (2010). Characterization of S3Pvac Anti-Cysticercosis Vaccine Components: Implications for the Development of an Anti-Cestodiasis Vaccine. *PLoS ONE* 5(6): e11287.

Rojo-Vazquez, FA., Pardo-Lledias, J., Francos-Von Hunefeld, M., et al. (2011). Cystic echinococcosis in Spain: current situation and relevance for other endemic areas in Europe. *PLoS neglected tropical diseases*., 5 (1): e893.

Sarciron, EM., Bresson-Hadni, S., Mercier, M., Lawton, P., Duranton, C., Lenys, D., Petavy, AF. & Vuitton, DA. (1997). Antibodies against *Echinococcus multilocularis* alkaline phosphatase as markers for the specific diagnosis and the serological monitoring of alveolar echinococcosis. *Parasite Immunol*., 19, 61-68.

Sheperd, A. & McManus, D.P. (1987). Specific and cross reactive antigens of *Echinococcus granulosus* cyst fluid. *Molec. biochem. Parasitol*., 25, 143-154.

Siracusano, A. & Vuitton, D. (1997). Immunology and immunopathology of *Echinococcus granulosus* and *Echinococcus multilocularis* infections. *Arch. int. Hidatid.*, 32, 132-135.

Soriano Arandes, A., Gómez Bertomeu, F., and Maldonado Artero, J. (2010). Microscopic Image of the Protoscolex of *Echinococcus granulosus* on the "Hydatid Sand". *Am. J. Trop. Med. Hyg.*, 82(6), 980.

Thompson, RCA. (1995). Biology and systematics of *Echinococcus*, *In: Echinococcus* and hydatid disease (R.C.A. Thompson & A.J. Lymbery, eds). CAB International, Wallingford, 1-50.

Torgerson, PR., Keller, K., Magnotta, M., Ragland, N. (2010). The Global Burden of Alveolar Echinococcosis. *PLoS Negl Trop Dis* 4(6): e722.

Utrilla, JG., Eyre, FP., Muguerza, R., Alami, H. & Bueno, J. (1991). Hidatidosis en la infancia. *Arch. Hidatid*., 30, 721-730.

Wen, H. & Craig, PS. (1994). Immunoglobulin G subclass responses in human cystic and alveolar echinococcosis. *Am. J. trop. Med. Hyg*., 51, 741-748.

Wolfe, ND., Dunavan, CP., Diamond, J. (2007). Origins of major human infectious diseases. *Nature.*, 447, 279-283.

World Health Organization (WHO) (1996). Guidelines for treatment of cystic and alveolar echinococcosis. WHO Informal Working Group on Echinococcosis. *Bull. WHO*, 74, 231-242.

World Health Organization (WHO) (2001). International classification of ultrasound images in cystic echinoccosis for application in clinical and field epidemiological settings., *In:* PAIR, an option for the treatment of cystic echinococcosis. WHO, Geneva.

World Health Organization (WHO) (2001). WHO/OIE Manual on Echinococcosis in Humans and Animals: a Public Health Problem of Global Concern. WHO, Geneva.

WHO/DFID-AHP (2006). The Control of Neglected Zoonotic Diseases-A Route to Poverty Alleviation.World Health Organization, Dept of Food Safety, Zoonoses, and Food-borne Diseases, Geneva.

Zhang, W., Li, J., You, H., Zhang, Z., Turson, G., Loukas, A. & McManus, DP. (2003). Short report: *Echinococcus granulosus* from Xinjiang, PR China: CDNAs encoding the EG95 vaccine antigen are expressed in different cycle stages and are conserved in the oncosphere. *Am. J. Trop. Med. Hyg*., 68, 40–43.

19

A Programme to Control Taeniosis-Cysticercolsis (*Taenia solium*) in Mexico

Aline S. de Aluja[1], Julio Morales Soto[2] and Edda Sciutto[3]
[1]*Departamento de Patología, Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México, Ciudad Universitaria, D. F.,*
[2]*Lab. MVZ. Aline S. de Aluja, Facultad de Medicina Veterinaria y Zootecnia, Universidad Nacional Autónoma de México, Ciudad Universitaria, D. F.*
[3]*Lab. de Inmunología, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Ciudad Universitaria, D. F.*
Mexico

1. Introduction

Taenia solium cysticercosis is a zoonosis which affects animals, mainly pigs, and human beings.

In pigs the cysticerci are found both in muscles and in the brain, in human beings predominantly in the form of neurocysticercosis (Escobar,1983; Fleury et al., 2003, 2006, 2010) but muscular, subcutaneous and ocular forms have also been reported. (Larralde & Aluja, 2006)

The disease is found mainly in countries where poverty prevails, hygiene is lacking and people live in close contact with pigs.

In Mexico the disease is present in marginated rural areas of the southern states, (Guerrero, Oaxaca, Puebla, Veracruz, Tabasco, Yucatan, parts of Morelos and others) where the above mentioned conditions exist and the parasite encounters a favorable environment for its survival. Many of the inhabitants of these areas are extremely poor, their dwellings are made of reed, wood or other cheap materials available in their region. Their number is calculated to be 47,190,000 million (INEGI 2009; CEPAL 2010). Few hamlets or villages have piped water and get their supply from wells that may dry up during the hot season or from ponds where rain water accumulates. Donkeys frequently bring water to isolated dwellings in 20 liter containers of which they carry four. There are villages where water is rationed during the very dry months and people get one bucket per day.

Roads are rarely paved, and many become impassable during the rainy season. Children often have to walk long distances to get to school and depending on the weather and road conditions do not go, some barely learn how to read and write. In many of these remote areas the inhabitants speak their indigenous languages, have difficulties understanding Spanish, and an interpreter is needed to communicate with them.

People usually own a few animals and some land, where they grow corn, the harvest being barely enough to feed their family. Their animals, a few cattle, pigs and chicken receive very little care, and in particular the pigs roam about freely searching for food (Copado et al, 2004).

Some peasants keep their pigs confined, tying them to a tree or in primitive enclosures during the time when the corn grows.
In some of the bigger villages, health centers may be found, where people get rudimentary medical care but in the smaller ones where none is available, they mostly rely on their ancestral remedies, among them herbs and medicinal plants.
Hygiene is understandably one of the big problems for people who live under these conditions of extreme poverty and scarcity of water. Toilets do not exist in many villages and hamlets and people defecate in the open. The health authorities have made efforts to improve hygiene and have introduced latrines, but this has not been accompanied by the necessary control and follow up interventions, with the result that they are not used and are transformed into store rooms, or are built on inadequate grounds and spill over with the torrential rains.
The pigs, being copropaghes, roaming about freely in villages and fields to find their food, and receiving very little attention from their owners, ingest the human feces, which constitute an important addition to their diet (Aluja et al. 1987; Acevedo, 1989; Aluja & Villalobos, 2000; Copado et al., 2004). If the owner keeps his pigs confined in yards next to his dwellings, the animals may have direct access to the outlet of a latrine and consume the feces. If the feces come from a *Taenia solium* carrier, the pig becomes infected and develops cysticerci in its tissues, predominantly skeletal muscles and brain. Occasionally people also ingest eggs which may come from contaminated food or by way of autoinfection if they themselves are the *T. solium* carrier, in which case they develop cysticercosis, mostly in the form of neurocysticercosis. (Escobar 1983; Villagran & Olvera, 1988; Larralde &Aluja, 2006, Aluja 2008)
If human beings eat insufficiently cooked meat of a pig with cysticerci they develop *Taenia solium* in their intestine. (Quiroz 2002; Larralde & Aluja 2006)
Another problem and the reason for the continuation of the Taeniosis-cysticercosis cycle in these remote areas, is that slaughter houses where meat inspection is carried out do not exist. There may be places where animals for meat are being killed, but the methods and the hygiene are unacceptable and meat is not inspected. Animals are mostly slaughtered by their owners for family or other festivities and are consumed without any inspection.
The prerequisites for the continuation of the *Taenia solium* cycle thus are:

- People who live in conditions of poverty,
- Lack of toilets or latrines,
- Scarcity of water,
- Lack of education,
- Rambling pigs.
- Taenia solium carriers.
- Absence of meat inspection.

These conditions still can be found in many of the developing countries, among them Mexico. (Molinari et al, 1983; Sarti et al. 1988; 1992; Larralde et al 1992; Martinez et al. 1997; Aluja et al 1998; Morales et al. 2006; 2008)
The Ministries of Health and Agriculture in Mexico issue monthly reports with information on the diseases in human beings and animals that are diagnosed in the country. Cysticercosis in pigs and human beings and taeniosis in people are nowadays rarely reported, which has led to the believe that they are practically extinct in the country (Flisser et al. 2010). The explanation for this is that, as has been described, because of the absence of medical and veterinary personal in the areas where it occurs, it is not notified. Pigs are not

slaughtered in official establishments, and health care for the inhabitants does either not exist or is rudimentary with the result that neither enters the official surveillance system.
Nine million four hundred thousand of the total pig population (15´107,785 (SIAP-SAGARPA, 2009; Rodriguez Licea & del Moral Barrera, 2010) in México belong to highly technified and controlled farms where *Taenia solium* carriers and cysticercosis are not found. The meat of these animals proceeds from strictly controlled federal slaughterhouses and cases of pig cysticercosis are almost never seen. These are the animals that enter the official statistics. The rest, 5´600,000 pigs belong to semitechnified farms and to the free roaming group, the latter being estimated around 3`000,000 pigs. (Rodriguez Licea & del Moral Barrera 2010).
By doing serological tests (ELISA) both in humans and in pigs, it was found that the prevalence of positive reactors is rather high in those states of Mexico where the above conditions are found, both in human beings (Larralde et al. 1992) and in pigs (Sosa, 2010; Sciutto [in process]).
Tongue inspection of rural pigs confirm the presence of cysticercosis in marginated areas. In a remote region of the state of Morelos, the prevalence of porcine cysticercosis ranges between 4 and 33%.(Morales et al. 2002). In the state of Guerrero it has also been shown in some villages that the frequency is high (Molinari et al.1983; Keilbach et al., 1989; Martinez et al. 1997; 2000) and in recent unpublished data it was found to range between 0 and 13,5%.
Official data on the frequencies of neurocysticercosis and teniosis in human beings in the country have not been published. As has been pointed out, methods to diagnose cysticercosis in the population are not available in isolated regions. During conversations with people in villages, one may hear of a relative who gets epileptic fits or another one who suffers from intense headaches, both among the symptoms of neurocisticercosis (Ortiz et al. 2006). However, in the absence of diagnostic tools and in view of the fact that the financial means to travel to the city are lacking, these cases remain undiagnosed. In a recent study Fleury, using computerized tomography found that in a rural community of the state of Morelos the frequency of neurocysticercosis was 9,6% (Fleury et al. 2006) and the same author reports that in hospitalized patients in a neurological institution it has remained unchanged during the last 10 years (Fleury et al. 2010).
Evaluating all these factors, a group of Veterinarians, Immunologists and Neurologists of the National Autonomous University of Mexico (UNAM) and of the Institute of Neurology of the Ministry of Health considered that, *T. solium* teniosis-cysticercosis, still being an important disease in parts of Mexico, a programme to control it is needed. One of the reasons that justifies this is that neurocysticercosis is being reported again in the United States of America (USA) and that most cases are traced back to Mexican or other Latin American immigrants (Sorvillo et al. 2007; 2011).
Isolated interventions to control the zoonosis have been carried out in Mexico and elsewhere, but to our knowledge none of them considered follow up activities (Sanchez et al. 1999; Keilbach et al. 1989; Sarti et al. 1997; Boa et al. 2003; Eddi et al. 2003; Engels et al. 2003; Martinez et al. 2003; Pawlowski et al. 2005).
Among the methods to control teniosis-cysticercosis, several strategies have been proposed, like improving infrastructure for sanitation, confinement of pigs, the obligatory installation of latrines, regular antihelmintic treatment of the population, treatment with albendazol of infected pigs, obligatory meat inspection, health education and quite sophisticated measures like meat irradiation (Larralde & Aluja, 2006; Flores et al. 2006).

Most of these proposals cannot be introduced on a short term basis under the conditions that still prevail in those areas where the disease exists. The health authorities offer antihelminthic treatment to adults and children, however the doses they prescribe may not suffice to eliminate taeniae. The suggestion to treat infected pigs is not practical, as it is not possible to inspect all free roaming animals to find the ones with cysticerci and besides, owners would have to wait at least 3 to 4 months before they can sell them for slaughter, in order to eliminate the remnants of the larvae from the muscles.

Integrated into our control programme we decided to use vaccination of pigs.

Vaccines against cysticercosis, both pig, cattle and sheep have been employed in other countries and in México (Johnson et al. 1989; Plancarte et al. 1999; Huerta et al. 2001; Flisser et al. 2004; Sciutto et al. 2007; Harrison et al. 2005; Gonzalez et al. 2005; Assana et al. 2010).

The vaccine S3Pvac that we use in our control programme is produced in the Institute for Biomedical Research of UNAM. It contains 3 protective peptides: KETc12 of 8 aminoacids, KETc1 of 12 and KETc7 of 110. (Manoutcharian et al. 1996; Manoutcharian reference 2004; Rosas et al. 1998; Toledo et al. 1999; 2001) In the peptide KETc7 2 protective epitopes GK1 and PT1 of 18 and 10 aminoacids respectively were identified (Manoutcharian et al. 1999). These peptides belong to different developmental stages of *Taenia crassiceps* and *Taenia solium* (Toledo et al. 1999; 2001; Rosas et al. 1998, 2002; Sciutto et al. 1995, 2007, 2008) and are found in different anatomical structures of the cysticerci, in the eggs and in the adult taeniae. Their sequences are detected in both cestodes with minimal differences in certain aminoacids which do not modify their tridimensional structure nor their protective capacity (Rassy et al. 2010).

The first version of the vaccine, S3Pvac, was evaluated in an endemic area in the state of Puebla (Huerta et al. 2001).It reduced the number of infected animals 50% and 98% the number of vesicular and colloidal cysticerci that are capable of developing into the adult worm.

The second version was the recombinant vaccine S3Pvac-phage, which consists of the SP3vac peptides which are expressed in filamentous phages. It was evaluated in communities in the state of Morelos and reduced 54% the prevalence of porcine cysticercosis and 89% the number of established cysticerci. (Morales et al. 2008)

In order to plan a programme with possibilities of success one has to consider all the factors that contribute to the persistence of the zoonosis and which have been enumerated above.

We selected communities in the state of Guerrero, where we had detected a high prevalence in the marginated areas, to start our activities.

The first task was to inform and convince federal and local authorities of the need to include the control of this zoonosis in their official health programmes. This was achieved by underscoring that the disease continues to be a health issue in areas where poverty prevails and that due to migrant workers it has spread to other countries.

The project was well received and the authorities collaborated by facilitating funds to purchase a vehicle to transport the members of the teams and to pay for their travels and salaries. They also authorized funds to purchase ultrasonographs for the diagnosis of porcine cysticercosis. This method has proven to be reliable and easier for both veterinarians and pigs, because it eliminates the often cruel handling of pigs and the strenuous efforts of veterinarians and assistants to immobilize them. (Herrera G. S.C. et al 2007)

Before we started with the activities the village authorities, teachers and peasants had to be informed of the programme, by explaining the cycle of the parasitosis, the consequences of getting neurocysticercosis, and the loss of income if their pigs acquire cysticercosis.

Teams are formed of at least one veterinarian who is responsible for the activitities of the group, last year veterinary students who vaccinate and volunteers, usually veterinary students who want to help and learn. People of the village are employed on a daily basis to help find, catch and hold the animals.
Other teams are trained to educate people. They give talks, whenever possible with audiovisual aids, to inform children, teachers, parents and the population in general on the disease, its consequences and how to avoid becoming infected, how to keep their pigs and the importance of using latrines and how.
The strategy we follow in our programme to control the disease is twofold:
1. Vaccination of pigs
2. Education of the population

2. Vaccination of pigs

After having agreed upon the day of vaccination, the owners keep their pigs confined and their tongues are examined by members of the team and each animal is registered in a data base, appointing the name of the owner, his or her address, age and sex of the animal, and where it was borne. All pigs are then vaccinated. If cysticerci were detected in the tongue, the pig is carefully examined with ultrasonography and if found positive, we try to purchase it from the owner. An attempt has been made to exchange it for a better bred animal, but this has not been very successful as these newly introduced piglets do not resist the hardship of their new lives and often die. The vaccine is applied subcutaneously, and repeated 3 times, with 3 months interval between each.

3. Education

The teams make appointments with the village authorities, with teachers, schools, parents, medical staff and nurses in order to explain how to improve their hygiene, how to keep their pigs, the importance of using latrines and other topics that may arise during the discussions. The importance of detecting a *T. solium* carrier is of course stressed and people are advised to get treatment for intestinal parasites, which the government offers free of charge in the health centers, whenever possible.
In order to determine how much people know about the disease and also what their living conditions are, before the programme gets under way, a questionnaire is distributed and people who know how to read and write are asked to answer it. The team members help those who cannot to fill it out. The same questionnaire will be distributed at the end of the project and compared with the initial one, which will show whether the educational campaign was successful. At present (August 2011) we have finished with vaccination and 3 months after the last application we shall start to examine all pigs in the communities and compare the frequency of infected animals with the one registered at the beginning.
During our work we have found the attitude of the people very positive. They are grateful for the dedication of the teams, and for the time they spend with them to show how they can improve their pig breeding methods and their own hygienic habits. The children are thrilled with the audiovisual presentations offered in their schools and go home to explain to their family what they have learnt. It is our conviction that to invest time to teach children is most rewarding, as they are more open to new knowledge than the older

generations, who often resist change arguing that "their ancestors have done it this way and why should they do it differently".

Problems may arise identifying pigs. We ask the owners not to introduce new ones into their group during the vaccination period and not to sell or kill animals without letting us know, but this has proven to be almost impossible as some get lost or die according to the owners and others that have been added to the group. We try to identify each animal by their special colors or markings, but inevitably there are failures with this system and to mark all pigs with microchips would be too costly. The possibility thus exists that not all pigs get the 3 planned vaccinations but we hope to be able to cover the majority.

By vaccinating as many pigs as possible, we hope to interrupt the cycle of the zoonosis and by educating the population we are confident that people will acquire the habit of using correct latrines, of washing their hands whenever possible before preparing food and of abstaining from ingesting meat with cysticerci, also of building correct stalls for their pigs.

4. Acknowledgement

We thank SENASICA-SAGARPA for their interest in the programme and for their financial support. Ms Isabel Aguilar for her competent secretarial help and all the authorities, peasants and their families and all other inhabitants of the communities for their collaboration. We are grateful to all members of the working teams, most particularly to Juanita Pérez, Antonio Celis, Arturo López, Alfredo Figueroa and Raul Suárez for their dedication.

5. References

Acevedo H. (1989). Epidemiología de la cisticercosis porcina. En: Cisticercosis Humana y Porcina, pp 251-253. (Editor. Flisser A, Malagón F) Limusa, Noriega. México, D.F

Aluja A. S. de, et al. (1987). *Cisticercosis: una recopilación actualizada de los conocimientos básicos para el manejo y control de Taenia solium*. Biblioteca de la Salud. ISP and Fondo de Cultura Económica, México, D. F.

Aluja A.S. de. (2008). Cysticercosis in pigs. Curr Top Med Chem. 8(5):368-74.

Aluja A. S. de, Villalobos N. (2000). Cisticercosis por *Taenia solium* en cerdos de México. *Veterinaria México*. 31(3):239-244

Aluja, A. S. de,M. J.Martínez y A. N.Villalobos (1998). Cysticercosis in young pigs: Age at first Infection and histological characteristics", *Veterinary Parasitology* 76:71-79.

Assana E, Kyngdon CT, Gauci CG, Geerts S, Dorny P, De Deken R, Anderson GA, Zoli AP, Lightowlers MW. (2010). Elimination of *Taenia solium* transmission to pigs in a field trial of the HP6-TSOL18 vaccine in Cameroon. Int J Parasitol. 40(5):515-9.

Boa M, Mukaratirwa S, Willingham AL, Johansen MV. (2003).Regional action plan for combating *Taenia solium* cysticercosis/taeniosis in Eastern and Southern Africa. Acta Trop. 87(1):183-6.

Comisión Económica Para América Latina – CEPAL- 2010. América Latina: evolución de la pobreza y la indigencia, 1980-2010.

Copado, B. F., A. S. de Aluja, L.Mayagoitia y F. Galindo (2004). The behaviour of free ranging pigs in the Mexican tropics and its relationships with human faeces consumption. Applied Animal Behaviour Science 88:243-252.

Eddi C, Nari A, Amanfu W. (2003). *Taenia solium* cysticercosis/taeniosis: potential linkage with FAO activities; FAO support possibilities. Acta Trop. 87(1): 145-8.

Engels, D., Urbani, C., Beloto, A., Meslin, F., Savioli, L. (2003). The control of human (neuro) cysticercosis: which way forward? Acta Trop., 87:177-82.

Escobar A. (1983). The Pathology of Neuro-cysticercosis. In: E. Palacios, J. Rodríguez Carbajal, J. M. Traveras (eds). Cysticercosis of the Nervous System. Charles C. Thomas, Springfield, Cap. 4:27-54.

Fleury A., Gomez T., Alvarez I., Meza D., Huerta M., Chavarria A., Carrillo Mezo R. A., Lloyd C., Dessein A., Preux P. M., Dumas M., Larralde C., Sciutto E. (2003). High prevalence of calcified silent neurocysticercosis in a rural village of Mexico. Neuroepidemiology. 22(2):139-145.}

Fleury A., Morales J., Bobes R.J., Dumas M., Yánez O., Piña J., Carrillo-Mezo R., Martínez J.J., Fragoso G., Dessein A., Larralde C., Sciutto E. (2006). An epidemiological study of familial neurocysticercosis in an endemic Mexican community. Trans R Soc Trop Med Hyg. 100(6):551-8.

Fleury A., Moreno Garcia J., Valdez Aguerrebere P., de Sayve Duran M., Becerril Rodriguez P., Larralde C., Sciutto E. (2010). Neurocysticercosis, a persisting health problem in Mexico. PLoS Negl Trop Dis. 4(8): e805

Flisser A., Gauci C. G., Zoli A., Martinez-Ocana J., Garza-Rodriguez A., Dominguez-Alpizar J. L., Maravilla P., Rodriguez-Canul R., Avila G., Aguilar-Vega L., Kyngdon C., Geerts S., Lightowlers M. W. (2004). Induction of protection against porcine cysticercosis by vaccination with recombinant oncosphere antigens. Infect Immun., 72: 5292-97.

Flisser A., Correa D. (2010). Neurocysticercosis May No Longer Be a Public Health Problem in Mexico. Plos Negl Trop Dis, 4(12):e8831 doi:10.1371/journal.pntd.0000831

Flores Pérez I., Aluja A. S. de y Martínez J. J. (2006). Efectos en el desarrollo del metacestodo de *Taenia solium* inducidos por dosis bajas de radiación Gamma. Rev. Vet. Méx. , Vol. 37:3

González AE, Gauci CG, Barber D, Gilman RH, Tsang VC, Garcia HH, Verastegui M, Lightowlers MW. (2005). Vaccination of pigs to control human neurocysticercosis. Am J Trop Med Hyg., 72(6):837-9.

Harrison L.J., Garate T., Bryce D. M., Gonzalez L. M., Foster-Cuevas M., Wamae L. W., Onyango-Abuje J. A., Parkhouse R. M. (2005). Ag-ELISA and PCR for monitoring the vaccination of cattle against *Taenia saginata* cysticercosis using an oncospheral adhesion protein (HP6) with surface and secreted localization. Trop Anim Health Prod. 37:103-20.

Herrera G. C., Aluja A. S. de y Méndez A. R. (2007). El uso de la ultrasonografía para el diagnóstico de la cisticercosis porcina. Rev. Vet. México 38;1:125-133.

Huerta M, Aluja A. S. de, Fragoso G, Toledo A, Villalobos N, Hernandez M, Gevorkian G, Acero G, Diaz A, Alvarez I, Avila R, Beltran C, Garcia G, Martinez JJ, Larralde C, Sciutto E. (2001). Synthetic peptide vaccine against *Taenia solium* pig cysticercosis: successful vaccination in a controlled field trial in rural Mexico. Vaccine. 20(1-2):262-6.

Instituto Nacional de Estadística Geografía e Informática, Censo de Población y Vivienda 2010. INEGI, México.

Johnson K.S., Harrison G.B., Lightowlers M.W., O'Hoy K.L., Cougle W.G., Dempster R.P., Lawrence S.B., Vinton J.G., Heath D.D., Rickard M.D. (1989). Vaccination against ovine cysticercosis using a defined recombinant antigen. Nature, 338: 585-587.

Keilbach N, Aluja A, Sarti E. (1989). A programme to control teniosis and cyticercosis (*Taenia solium*), experiences in a Mexican village. Acta Leiden. 57(2):181-189.

Larralde C., Padilla A., Hernández M., Govezensky T., Sciutto E., Gutiérrez G., Tapia-Conyer R., Salvatierra B., Sepúlveda J. (1992). Seroepidemiology of cysticercosis in Mexico. Salud Pública Mex. 34(2): 197-210.

Larralde C., y Aluja A. S. de (2006). Cisticercosis guía para profesionales de la salud. México: FCE, Secretaría de Salud, Instituto Nacional de Salud Pública, Fundación Mexicana para la Salud.

Manoutcharian K., Díaz-Orea A., Gevorkian G., Fragoso G., Acero G., González E., Aluja A. S. de, Villalobos N., Gómez-Conde E., Sciutto E. (2004). Recombinant bacteriophage-based multiepitope vaccine against *Taenia solium* pig cysticercosis. Vet Immunol Immunopathol. May; 99(1-2):11-24.

Manoutcharian K, Terrazas L.I., Gevorkian G., Acero G., Petrossian P., Rodriguez M., Govezensky T. (1999). Phage-displayed T-cell epitope grafted into immunoglobulin heavy-chain complementarity-determining regions: an effective vaccine design tested in murine cysticercosis. Infect Immun. 67(9):4764-70.

Manoutcharian K. Rosas, G. Hernández, M. Fragoso, G. Aluja, A. Villalobos, N. Rodarte, L. F. Sciutto, E. (1996). Cysticercosis: identification and cloning of protective recombinant antigens. J Parasitol. 82(2): 250-4.

Martínez, M. J., Aluja A. S. de y Gemmell M. (2000), Failure to incriminate domestic flies *(Diptera: Muscidae)* as mechanical vectors of *Taenia* eggs *(Cyclophillidea: Taeniidae)* in rural Mexico. *Journal of Medical Entomology* 37(4):489-491.

Martínez, M. J., A. S. de Aluja, A. N. M. Villalobos, A. C. Jaramillo y M. Gemmell. (1997). "Epidemiología de la cisticercosis en cerdos de una comunidad rural del estado de Guerrero, México", Veterinaria México 28(4):281-286.

Martínez, M. J., Aluja A. S. de, R. G. Ávila, V. L. Aguilar, C. A. Plancarte y A. C. Jaramillo (2003). Teniosis y detección de anticuerpos anticisticercos en personas de una comunidad rural del estado de Guerrero. *Salud Pública de México* 45(2):84-89.

Molinari J. L.; Meza R.; Suarez B.; Palacios S.; Tato P.; Retana A. (1983). *Taenia solium*: immunity in hogs to the Cysticercus. Exp Parasitol., 55, 340-57.

Morales J., Martínez J.J., Rosseti M. Fleury A., Maza V., Hernández M, Villalobos N, Fragoso G, de Aluja AS, Larralde C, Sciutto E. (2008). Spatial Distribution of Taenia solium Porcine Cysticercosis within a Rural Area of Mexico. PloS. NeGL. Trop. Dis., 2(9):e284. Sep 3.

Morales J. T. Velasco, V. Tovar, G. Fragoso, A Fleury, C. Beltran, N. Villalobos, A. Aluja, L.F. Rodarte, E. Sciutto. and C. Larralde. (2002). Castration and pregnancy of rural pigs significantly increase the prevalence of naturally acquired *Taenia solium* cysticercosis. Veterinary Parasitol., June, 108:41-48.

Morales J., Martínez J.J. García-Castella. J., Peña N., Maza V., Villalobos N., Aluja AS., Fleury A., Fragoso G., Sarralde C. Sciutto E. (2006). *Taenia solium*: the complex interactions, of biological, social, geographical and commercial factors, involved in the transmission dynamics of pig cysticercosis in highly endemic areas. Ann Trop Med Parasitol, 100(2):123-35.

Ortiz-Trejo J.M., Correa-Chacón A.J., Sctelo-Ham E.I., Torres-Valenzuela A., Alvarado-Esquivel C. (2006). Risk factors associated with neurocysticercosis in a public hospital in México. Gac Med Mex. May-Jun;142(3):175-9. Spanish.

Pawlowski Z. S., Allan J., Sarti E. (2005). Control of *Taenia solium* taeniasis/cysticercosis: from research towards implementation. Int J Parasitol. 35, 1221-32.

Plancarte A., Flisser A., Gauci C.G., Lightowlers M.W. (1999). Vaccination against *Taenia solium* cysticercosis in pigs using native and recombinant oncosphere antigens. Int J Parasitol, 9:643-7.

Quiroz R.H. (2002). Parasitologia y enfermedades parasitarias de animales domésticos. Grupo Noriega Editores, Editorial Limusa.

Rassy D., Bobes R. J., Rosas G., Anaya V. H., Brehm K., Hernandez B., Cervantes J., Pedraza S., Morales J., Villalobos N., de Aluja A. S., Laclette, J. P. Nunes, C. M. Biondi, G. F. Fragoso, G. Hernández, M. Sciutto, E. (2010). Characterization of S3Pvac anti-cysticercosis vaccine components: implications for the development of an anti-cestodiasis vaccine. PLoS-One. 5(6):e11287

Rodríguez L. G. y Del Moral B.L.E. (2010). Perspectivas del sector porcícola mexicano para 2010: recuperación de los efectos de la crisis económica y de la influenza (A)H1/N1. Revista Trimestral de Análisis de Coyuntura Económica, Vol. III, No. 2, Abril-Junio.

Rosas G., Cruz-Revilla C., Fragoso G., López-Casillas F., Pérez A., Bonilla M. A., Rosales R., Sciutto E. (1998). *Taenia crassiceps* cysticercosis: humoral immune response and protection elicited by DNA immunization. J Parasitol. 84(3): 516-23

Rosas G., Fragoso G., Garate T., Hernandez B., Ferrero P., Foster-Cuevas M., Parkhouse R. M., Harrison L. J., Briones S. L., Gonzalez L. M., Sciutto E. (2002). Protective immunity against *Taenia crassiceps* murine cysticercosis induced by DNA vaccination with a *Taenia saginata* tegument antigen. Microbes Infect., 4, 1417-26.

Sánchez,A. L., J. Lindback, P. M. Schantz, M. Sone, H. Sakai, M. T. Medina y I. Ljungstrom (1999), "A population-based, case-control study of *Taenia solium* taeniasis and cysticercosis", *Annals of Tropical Medicine and Parasitology* 93(3A):247-258.

Sarti E., Flisser A., Schantz P., Gleizer M., Loya M., Plancarte A., Ávila G., Allan J., Craig P., Bronfman M., Wijeyaratne P. (1997). "Development and evaluation of a health education intervention against *Taenia solium* in a rural community in Mexico". Am J Tropl Med Hyg. 56(2):127-132.

Sarti E., P. M. Schantz, R. Lara-Aguilera, H. Gómez y A. Flisser (1988), "*Taenia solium* taeniasis and cysticercosis in a Mexican village", *Tropical Medicine and Parasitology* 39(3):194-198.

Sarti E., P. Schantz, A. Plancarte, M. Wilson, I. Gutiérrez, A. López, J. Roberts y A. Flisser (1992). Prevalence and risk factors for *Taenia solium* taeniasis and cysticercosis in humans and pigs in a village in Morelos, México. *American Journal of Tropical Medicine and Hygiene* 46:677-685.

Sciutto E., Aluja A., Fragoso G., Rodarte L.F., Hernández M., Villalobos M.N., Padilla A., Keilbach N., Baca M., Govezensky T. (1995). Immunization of pigs against *Taenia solium* cysticercosis factors related to effective protection. Vet Parasitol, 60(1-2):53-67.

Sciutto E., Fragoso G., de Aluja A.S., Hernández M., Rosas G., Larralde C. (2008). Vaccine against cysticercosis. Curr Top Med Chem. 8(5):415-23.

Sciutto E., Morales J., Martínez J.J., Toledo A., Villalobos M.N., Cruz-Revilla C., Meneses G., Hernández M., Díaz A., Rodarte L.F., Acero G., Gevorkian G., Manoutcharian K., Paniagua J., Fragoso G., Fleury A., Larralde R., De Aluja A.S., Larralde C. (2007). Further evaluation of the synthetic peptide vaccine S3Pvac against *Taenia solium* cysticercosis in pigs in an endemic town of Mexico. Parasitology, 134:129-133.

Sciutto E., Rosas G., Hernández M., Morales J., Cruz-Revilla C., Toledo A., Manoutcharian K. Gevorkian G., Blancas A., Acero G., Hernádez B., Cervantes J., Bobes R.J., Goldbaum F.A., Huerta M., Diaz-Orea A., Fleury A., de Aluja A.S., Cabrera-Ponce J.L., Herrera-Estrella L., Fragoso G., Larralde C. (2007). Improvement of the synthetic tri-peptide vaccine (S3Pvac) against porcine *Taenia solium* cysticercosis in search of a more effective, inexpensive and manageable vaccine. Vaccine, 25(8):1368-78

Sciutto E., Morales J., Camacho C., Nájera M., Fleury A., Fragoso G., Aluja A. S. de, Hernández M., Martínez J.J., Villalobos N. (In press). Meaning of the presence of anti-cysticercal antibodies in pigs: usefulness in sensing cysticercososis transmission.

SIAP-SAGARPA. 2010. Estadísticas sobre la producción porcina para los años 2008 y 2009. (Abril 2010). http://siap.gob.mx

Sorvillo F., Wilkins P., Shafir S., Eberhard M. (2011). Public health implications of cysticercosis acquired in the United States. Emerg Infect Dis. Jan;17(1):1-6.

Sorvillo F.J., DeGiorgio C. & Watermant S.H. (2007). Deaths from cysticercosis, United States. Emerg Infect Dis. 13: 230-235.

Sosa J. (2010). Determinación de la frecuencia de cisticercosis por *T. solium* mediante la prueba de ELISA y su impacto en algunos indicadores productivos en cerdos del traspatio de Morelos 2008-2009. Tesis de licenciatura, Facultad de Ciencias Agropecuarias, Universidad Autónoma del Estado de Morelos. Sept. *System,* Charles C. Thomas, Springfield Illinois, pp. 27-54.

Toledo A., Fragoso G., Rosas G., Hernández M., Gevorkian G., Lopez-Casillas F., Hernández B., Acero G., Huerta M., Larralde C., Sciutto E. (2001). Two epitopes shared by *Taenia crassiceps* and *Taenia solium* confer protection against murine *T. crassiceps* cysticercosis along with a prominent T1 response. Infect Immun. 69, 1766-73.

Toledo A., Larralde C., Fragoso G., Gevorkian G., Manoutcharian K., Hernández M., Acero G., Rosas G., Lopez-Casillas F., Garfias C. K., Vazquez R., Terrazas I., Sciutto E. (1999). Towards a *Taenia solium* cysticercosis vaccine: an epitope shared by *Taenia crassiceps* and *Taenia solium* protects mice against experimental cysticercosis. Infect Immun., 67, 2522-30.

Villagrán J. Olvera J. E. (1988). Cisticercosis humana: Estudio clínico patológico de 481 casos de autopsia, Patología 26:149–156.

20

Antischistosomal Natural Compounds: Present Challenges for New Drug Screens

Josué de Moraes
Supervisão de Vigilância em Saúde, SUVIS Casa Verde,
Secretaria de Saúde da Cidade de São Paulo,
Rua Ferreira de Almeida, São Paulo, SP
Brazil

1. Introduction

Schistosomiasis, or bilharzias, is a neglected disease that remains a considerable public health problem in tropical and subtropical regions. This parasitic disease is the most important human helminth infection in terms of morbidity and mortality and is a growing concern worldwide. It is estimated that more than 200 million people have been infected and that 779 million are at risk of infection, resulting in 280,000 deaths annually (van der Werf et al., 2003; Steinmann et al., 2006). Schistosomiasis is caused by blood-dwelling fluke worms of the genus *Schistosoma* and is endemic in African, Asian and South American countries. The main disease-causing species are *S. mansoni*, *S. haematobium*, and *S. japonicum. S. mansoni* is the most widely distributed, affecting people in Africa, the Middle East, South America, and the Caribbean, while *S. japonicum* is confined to China, Indonesia, and the Philippines. *S. haematobium* is found in Africa and the Middle East. The adult worms colonise the veins of either the portal system (*S. mansoni* and *S. japonicum*) or the urinary bladder plexus (*S. haematobium*) and can live for years or even decades in human hosts; thus, the disease runs a chronic and debilitating course. Egg production is responsible for both the transmission of the parasite and the aetiology of the disease. Schistosomal species are distinguished by differences in their morphology, both in their parasite stages and in their eggs; further species distinction is made by the species of intermediate host snails that support transmission of the parasite (Gryseels et al., 2006).

The global strategy for the control of schistosomiasis is by chemotherapy. Systematic searching for chemotherapeutic drugs began almost a century ago, and the development of praziquantel (PZQ) in 1970 was essential for a reduction in morbidity and mortality due to schistosomiasis. Currently, treatment is still based on the use of PZQ, but the long-term application of PZQ results in decreased efficiency and serious concerns regarding the onset of resistance. In addition, PZQ has no prophylactic properties and is ineffective against larval stages of parasites (schistosomula), meaning that for effective treatment and sustainable control, PZQ must be given on a regular basis. Thus, it is prudent to search for novel therapeutics, and recent discussions have focused on reawakening the need to search for alternatives to PZQ (Caffrey, 2007; Doenhoff et al., 2008; Fenwick et al., 2003; Hagan et al., 2004; Keiser & Utzinger, 2007).

Natural products, mainly plants, have been the source of medicines for thousands of years. The discovery of pure compounds as active principles in plants was first described at the beginning of the 19th century, and the art of exploiting natural products has become part of the molecular sciences (Kayser et al., 2003). Several extracts or bioactive constituents from living organisms have been used in many communities worldwide against parasitic diseases, including schistosomiasis, and in the past decades, natural products have attracted renewed interest (Kayser et al., 2003; Mølgaard et al., 2001; Ndamba et al., 1994; Sanderson et al., 2002; Tagboto & Townson, 2001).

In vitro screening systems are useful and affordable ways to discover potential anthelmintic candidates for *in vivo* tests (Keiser, 2010; Ramirez et al., 2007; Yousif et al., 2007). Because a molecular-target approach is still rarely employed in schistosomicidal drug discovery, a more common strategy has been the complementary approach of whole-organism phenotypic screening *in vitro* to measure compound efficacy (Keiser, 2010; Ramirez et al., 2007; Yousif et al., 2007). In this context, screening for natural products that are active against schistosome is important in the establishment of future strategies for new antischistosomal drug discovery to control schistosomiasis (Yousif et al., 2007).

Considerable efforts are ongoing to develop novel schistosomicidal agents. As a result, many natural compounds with promising antischistosomal properties have been identified (Braguine et al., 2009; de Moraes et al., 2011; Magalhães et al., 2009, 2010; Moraes et al., 2011; Mølgaard et al., 2001; Parreira et al., 2010; Sanderson et al., 2002). The efficacy of these new compounds against schistosome is defined using three strategies: a) curative, by killing the adult worm; b) prophylactic, by killing schistosomula; and c) suppressive, by inhibiting worm egg-laying. Thus, several parameters, such as motor activity, tegumental changes, and oviposition, are often evaluated as indicators of biological activity and toxicity in studies with schistosome species.

This chapter reviews the present state of *in vitro* drug screening strategies used to discover new compounds active against *S. mansoni*, the most important species infecting humans, with an emphasis on natural products. Also highlighted are the best practices and challenges for drug screenings. Furthermore, information is provided about toxicity, susceptible *Schistosoma* stages, and other interesting laboratory studies on potential antischistosomal compounds, both natural products and natural product-derived compounds.

2. Antischistosomal drugs

For the control of schistosomiasis, which at present is dependent on chemotherapy, it is not satisfactory to have only one single effective treatment (Caffrey, 2007; Doenhoff et al., 2008; Fenwick et al., 2003). Ideally, other antischistosomal drugs would be available so that the classical strategy of alternating treatments to avoid the development of resistance could be used. Unfortunately, the other drugs used before the advent of PZQ, oxamniquine and metrifonate, are restricted in their use. Metrifonate, a drug that exhibits activity against *S. haematobium*, has recently been withdrawn from the market because of medical, operational, and economic criteria (Reich & Fenwick, 2001; Utzinger et al., 2003). Oxamniquine is the only alternative antischistosomal drug, but it is effective only against *S. mansoni*. In the 1970s, oxamniquine was used for individual and mass treatment of schistosomiasis, with satisfactory results regarding efficacy and tolerance. However, its use is currently declining and is being replaced by praziquantel (Cioli, 2000; Reich & Fenwick, 2001; Utzinger et al.,

2001, 2003). As there is currently no available vaccine for this disease in people (Bergquist et al., 2008), chemotherapy may now be at a crucial point.

Chemotherapy against schistosomiasis was reviewed extensively by Cioli et al. (1995), with an emphasis on compounds that were used in the past. Additionally, Cioli (1998) summarised some interesting laboratory studies on potential antischistosomal compounds and the possible emergence of praziquantel-resistant schistosomes. More recently, Ribeiro-dos-Santos et al. (2006) reviewed results from a comprehensive search of the scientific literature for substances and compounds tested for schistosomiasis therapy over the past century. The authors gathered information on the therapeutic action in humans or animal models and the mechanisms of action of over 40 drugs.

Briefly, antimonial compounds were introduced in 1918, and this group of drugs has been the major point of schistosome chemotherapy for approximately 50 years. However, they cause numerous side effects, such as nausea, vomiting, diarrhoea, anorexia, and cardiovascular, hepatic, and dermatological disturbances. Lethality from cardiac syncope and anaphylactic shock was also reported. Emetine, a drug used to treat amoebiasis, was employed in the second decade of the past century, but the doses required against schistosomiasis were at the very limit of toxicity. The introduction of 2,3-dehydroemetine reduced the toxicity of the parent compound, but patients had to be hospitalised over a month for treatment. Thus, the use of 2,3-dehydroemetine as an antischistosomal agent was abandoned (Cioli et al., 1995). Only in the 1960s was there a breakthrough in the treatment of schistosomiasis, with the rise of metrifonate, nitrofurans, lucanthone, niridazole, hycanthone, and, finally, oxamniquine. In the 1970s, several schistosomicidal drugs emerged, such as tubercidin, amoscanate, PZQ and its benzodiazepine derivative Ro11-3128, and oltipraz. Nevertheless, the therapeutic doses of most of these drugs were found to cause major side effects. PZQ, an isoquinoline-pyrazine derivative, immediately proved to be superior to any other schistosomicidal drug and quickly became the drug of choice in most endemic areas (Cioli et al., 1995; Fenwick & Webster, 2006). Because of the reliance on a single drug for the treatment and control of schistosomiasis and the considerable concern regarding the development of PZQ resistance, it is timely to review potential alternatives, with an emphasis on natural products.

2.1 Antischistosomals: Natural product and natural product-derived compounds

The use of natural products for curative and therapeutic purposes has a long history, and compounds derived from natural products have made a big impact on the pharmaceutical industry (Newman, 2003; Newman & Cragg, 2007). In addition to microbes and plants, there has been growing interest in other living organisms, such as arthropods and amphibians, as important sources of biologically active compounds (Kayser et al., 2003). However, the potential for using living beings as sources of new antischistosomal drugs is still poorly explored. In recent decades, there has been a growing interest in the scientific community to search for extracts and pure compounds, especially those derived from plants, that exhibit potential schistosomicidal properties, as one alternative method to the conventional chemical control.

Plants have been traditionally used in the treatment of different diseases, including schistosomiasis, especially in Africa and Asia (Ndamba et al., 1994). In general, medicinal plants are prepared by traditional healers, who have empirical knowledge and

cultural communities throughout the world. For example, in Zimbabwe, Ndamba et al. (1994) investigated the herbal remedies used in the treatment of schistosomiasis. Based on interviews with 286 traditional healers, they composed a list of 47 plant species most widely used to treat urinary schistosomiasis. Based on this survey, the seven most commonly used plants, *Abrus precatorius* (Leguminosae) *Ozoroa insignis* (Anacardiaceae), *Dicoma anomala* (Cornpositae), *Ximenia caffra* (Oleaceae), *Lannea edulis* (Anacardiaceae), *Elephantorrhiza goetzei* (Leguminosae) and *Pterocarpus angolensis* (Leguminosae), were collected, prepared as described by the traditional healers, their efficacy was evaluated using laboratory animals previously exposed to *S. haematobium* cercariae, and the activity from the extract of *P. angolensis* bark was almost comparable to that of praziquantel. Later, Mølgaard et al. (2001) screened extracts of 23 plant species, popularly used against schistosomiasis in Zimbabwe, for their anthelmintic effect against schistosomula of *S. mansoni*, and the best results against larval forms were obtained with stem and root extracts from *Abrus precatorius* (Fabaceae) and stem bark from *Elephantorrhiza goetzei* (Mimosaceae). All families and names of the plants that are used by traditional healers to treat urinary schistosomiasis in Zimbabwe are described by Ndamba et al. (1994).

Some of the most interesting antischistosomal compounds are derivatives of artemisinin, such as artemether and artesunate (Utzinger et al., 2001; Xiao et al., 2002). They are highly effective in the treatment of malaria and have also been shown to exhibit antischistosomal properties. Artemisinin is a sesquiterpene lactone with an endoperoxide group, which was isolated from the leaves of *Artemisia annua* L. This plant has been used for centuries in Chinese traditional medicine as antidote to many different ailments (Lee, 2007; Utzinger et al., 2001). Artemisinin has been used as an antimalarial since the early 1970s, and its antischistosomal activity was discovered in 1980 by a group of Chinese scientists. In 1982, antischistosomal properties were confirmed for artemether, the methyl ether derivative of artemisinin. Interestingly, artemether has been shown to be active against immature schistosome in experimentally infected animals, but it is less effective against adult worms (Utzinger et al., 2001). Significant progress has been made with artemether and its potential for the control of schistosomiasis, which has been reviewed by Utzinger et al. (2001). The mechanism of action of artemisinin and its derivatives appears to involve an interaction with heme, which cleaves the endoperoxide bridge of the drug to produce carbon-centred free radicals that then alkylate parasite proteins (Golenser et al., 2006). In addition, scanning electron microscopy showed that artemether caused extensive and severe damage to the tegument in 21-day-old *S. mansoni* harboured in mice (Xiao et al., 2000). Considering that artemether and praziquantel exhibit the highest activity against schistosomula and adult worms, respectively, combined treatment has been proposed to enhance the reduction in worm burden (Utzinger et al., 2003). Currently, new trials to use artemisinin and its synthetic derivatives as lead molecules for drug discovery against schistosomiasis and various other diseases are rapidly growing, and the studies are ongoing (Lee, 2007; Utzinger et al., 2003, 2007; Xiao et al., 2002). Likewise, research on other natural products and natural product-derived compounds against schistosome has been performed by many groups. Accordingly, several plants with antischistosomal properties have been described in the literature (Braguine et al., 2009; de Moraes et al., 2011; Magalhães et al., 2009, 2010; Moraes et al., 2011; Mohamed et al., 2005; Mølgaard et al., 2001; Parreira et al., 2010; Sanderson et al., 2002).

Here, data on natural products and related natural product-derived compounds are reviewed, especially those from recent years or that have received considerable attention due to their antischistosomal properties (Table 1).

Date *	Extract/ Compound and Biological Source	Relevant notes
1980	Artemisinin, active principle from the plant *Artemisia annua* L. (Asteraceae)	Artemisinin derivatives: artemether (1982) and artenusate (1983); effective against immature schistosome in experimentally infected animals; morphological alteration on the tegument (Utzinger et al., 2001)
1989	Extracts from the plant *Pavetta owariensis* P. Beauv (Rubiaceae) contain proanthocyanins	Effective in mice infected with *S. mansoni* (Baldé et al., 1989)
1997	Goyazensolide isolated from the plant *Eremanthus goyazensis* (Gardner) Sch. Bip. (Compositae)	*In vitro* activity on *Schistosoma* adult worms; inhibitory effect on egg-laying; female more susceptible than male; not tested on schistosomula (Barth et al., 1997)
2000	Extract of leaf from the plant *Vernonia amygdalina* Del (Compositae)	Active against *S. mansoni* in mice (Ogboli, 2000)
2001	Mirazid myrrh, an oleo-gumresin from the stem of the plant *Commiphora molmol* (Burseraceae)	There is a great debate about the efficacy and effectiveness of myrrh in the treatment of schistosome infections, both in laboratory and clinical settings (Abdul-Ghani et al., 2009; Badria et al., 2001; Botros et al., 2004, 2005; Sheir et al., 2001)
2002	Oil from the plant *Nigella sativa* L. (Ranunculaceae)	Active on *S. mansoni*-infected mice; crushed seed also has *in vitro* effects against *S. mansoni* miracidia, cercariae, and adult worms, and an inhibitory effect on egg-laying; not tested on schistosomula (Mahmoud et al., 2002; Mohamed et al., 2005)
2002	Extract of the seeds and isoflavonoids from the plant *Millettia thonningii* (Schum. et Thonn.) Baker (Leguminosae)	*In vitro* against *S. mansoni* adult worms; no egg production was observed for experimental worms; not tested on schistosomula. Schistosomicidal activity against *S. mansoni* cercariae and miracidia has been previously described (Lyddiard et al., 2002)

* Dates of introduction or publication are only approximate.

Table 1. *In vitro* and *in vivo* antischistosomal characteristics of natural products.

Date *	Extract/ Compound and Biological Source	Relevant notes
2005	Extract of rhizomes from the plant *Zingiber officinale* Roscoe (Zingiberaceae)	*In vitro* male worms seemed more susceptible than female; reduction in egg output; activity against *S. mansoni* in mice was conflicting between Mostafa et al. (2011) and Sanderson et al. (2005); morphological alteration on the tegument; not tested on schistosomula
2007	Extract from plant *Curcuma longa* L. (Zingiberaceae)	Effective on *S. mansoni*-infected mice (El-Ansary et al., 2007; El-Banhawey et al., 2007); the *in vitro* schistosomicidal activity of curcumin, the major constituent in the rhizome, and reduction in egg production has been reported (Magalhães et al., 2009)
2007	Extract from garlic *Allium sativum* L. (Liliaceae)	Active against *S. mansoni* in mice (50 mg/kg) and not effective in high dose (100 mg/kg); affects the development and maturity of *S. mansoni* eggs in mice and seems to be an agent in protecting hepatic tissue against oxidative damage due to *S. mansoni* infection. *In vitro* allicin (2011), the main constituent of garlic, causes alterations on the tegument of male worm in high doses (10 to 20 µg/ml), but toxicity not assessed; not tested on schistosomula (El Shenawy et al., 2008; Lima et al., 2011; Riad et al., 2007)
2009	Extract from the plant *Clerodendrum umbellatum* Poir (Verbenaceae)	Effective in *S. mansoni* mice model (Jatsa et al., 2009)
2009	Extract from the plant *Zanthoxylum naranjillo* Griseb (Rutaceae) and its isolated compounds	*In vitro* against *S. mansoni* adult worms; reduction in egg-laying; not tested on schistosomula; not toxic in mammalian cells (Braguine et al., 2009)
2010	Phloroglucinol compounds from plants of the *Dryopteris* genus (Dryopteridaceae)	*In vitro* against *S. mansoni* adult worms; reduction in egg-laying; not tested on schistosomula; not toxic in mammalian cells (Magalhães et al., 2010)
2010	Essential oil from the plant of *Baccharis dracunculifolia* DC. (Asteraceae)	*In vitro* against *S. mansoni* adult worms; reduction in egg-laying; not tested on schistosomula; not toxic in mammalian cell (Parreira et al., 2010)
2011	Essential oil from plant *Ageratum conyzoides* L. (Asteraceae)	*In vitro* against *S. mansoni* adult worms; reduction in egg-laying; not tested on schistosomula; not toxic in mammalian cells (de Melo et al., 2011)

Table 1. Continued

Date *	Extract/ Compound and Biological Source	Relevant notes
2011	Sulfated polysaccharide α-D-glucan extracted from lichen *Ramalina celastri* (Spreng.) Krog. & Swinsc	Effective in *S. mansoni*-infected mice (Araújo et al., 2011)
2011	Piplartine, an amide isolated from plant *Piper tuberculatum* Jacq. (Piperaceae)	*In vitro* against *S. mansoni* adult worms; reduction in egg-laying; causes alterations on the tegument of worms; not tested on schistosomula; not toxic in mammalian cells (Moraes et al., 2011)
2011	Dermaseptin 01, an antimicrobial peptide found in the skin of frog of the genus *Phyllomedusa* (Hylidae)	*In vitro* against *S. mansoni* adult worms; reduction in egg-laying; causes alterations on the tegument of worms; not tested on schistosomula; not toxic in mammalian cells (de Moraes et al., 2011)
2011	Epiisopiloturin, an alkaloid isolated from plant *Pilocarpus microphyllus* Stapf ex Holm (Rutaceae)	*In vitro* against *S. mansoni* adult worms; causes alterations on the tegument of worm; not tested on schistosomula; not toxic in mammalian cells (Leite et al., 2011)
2011	Extract from plants of the *Artemisia* genus (Asteraceae)	*In vitro* against *S. mansoni* adult worms; not tested on schistosomula (Ferreira et al., 2011)

Table 1. Continued

As shown in Table 1, several *in vitro* studies have been conducted to search for new natural substances with schistosomicidal activity. These natural products and natural product-derived compounds mostly come from plants. Extensive phytochemical investigations of many species have revealed the presence of a large number of novel compounds belonging to different classes (Kayser et al., 2003; Kato & Furlan, 2007; Parmar et al., 1997; Prassad et al., 2005). For example, various secondary metabolites have been isolated from the family Piperaceae, and these plants have generated great interest as a result of their biologically active metabolites, such as pyrones, terpenes, lactones, chromenes, chalcones, lignoids, amides, and alkaloids (Kato & Furlan, 2007; Parmar et al., 1997). Regarding the variety of biological properties in particular, Moraes et al. (2011) demonstrated the *in vitro* schistosomicidal activity of piplartine, an amide found in several *Piper* species. The authors showed that at low concentrations (9.5 μM) this amide can kill *S. mansoni* adult worms (male and female coupled) and that the sub-lethal concentration of piplartine (6.3 μM) caused a 75% reduction in egg production. Additionally, piplartine was not cytotoxic against mammalian cells when given at concentrations up to three times higher than what is needed for a schistosomicidal effect (31.5 μM). Furthermore, *Piper* species are widely distributed in tropical and subtropical regions of the world, and they are among the most important medicinal plants used in various systems of medicine (Jaramillo & Manos, 2001; Parmar et

al., 1997). In addition to the wide geographical distribution and their use in folk medicine, the interest in these compounds and plant extracts is based on the fact that it is easy to isolate secondary metabolites and to propagate the plant, which has a short reproductive cycle. Thus, considering the *in vitro* schistosomicidal activity of the amide piplartine, the importance of more research on the biological activity of the natural compounds isolated from the family Piperaceae and other plants is apparent.

3. *Schistosoma mansoni* life cycle and maintenance in the laboratory

Schistosome species are dioecious (having male and female reproductive organs in separate individuals) platyhelminthes and have complex life cycles comprising multiple morphologically distinct phenotypes in definitive mammalian and intermediate snail hosts. *S. mansoni* is one of the most common etiological agents of human schistosomiasis and is the most widely used schistosome model for chemotherapeutic studies.Schistosome infection of humans (or another definitive host) occurs by direct contact with freshwater containing free-swimming larval forms of the parasite, known as cercariae. Cercariae penetrate the intact human skin and transform into schistosomula, which reside in the skin for up to 72 hours before entering a blood vessel. Within the vascular system, schistosomula migrate via complex routes to their final venous destination, where they mature into male and female adults. The mature flukes dwell in the human portal vasculature, depositing eggs in the intestinal wall that either pass to the gut lumen and are expelled in the faeces or travel to the liver and trigger immune-mediated granuloma formation and peri-portal fibrosis. Egg production commences 5 to 6 weeks after infection and continues for the life of the worm. The life cycle is completed when the eggs passed in the faeces hatch in the water, releasing the larval form miracidia, which then infect freshwater snails of the *Biomphalaria* spp. The infected snails, bearing schistosomal sporocysts, release cercariae into the water, which in turn penetrate the skin of their definitive host (Gryseels et al., 2006).

To complete a life cycle in the laboratory, *S. mansoni* is commonly maintained using rodents, ranging from hamsters to mice, as the definitive hosts and *Biomphalaria glabrata* as the intermediate host snail species (Figure 1). Infections of rodents of the same gender, 3 to 4 weeks of age and weighing 18 to 25 g, with *S. mansoni* are commonly initiated by subcutaneous injection of 100 to 150 cercariae (infective larvae). At 42, 49 or 56 days postinfection, animals are sacrificed with CO_2, dissected, and miracidia are hatched from *S. mansoni* eggs taken from animal livers. Each intermediate host snail is exposed to approximately 10 miracidia. All animals should be handled in strict accordance with good animal practice adhering to the institutional guidelines for animal husbandry. Thus, all studies should have a statement from their ethics committee or institutional review board indicating the approval of the research.

The use of *in vivo* animal models in drug discovery and the techniques used for these studies in the laboratory have recently been described in detail elsewhere by Keiser (2010) and Ramirez et al. (2007). This chapter will focus on the techniques used for *in vitro* studies. Many methods have been described that aim to determine the antischistosomal activity of drugs *in vitro*. The assessment of the viability of different stages of schistosome, tegumental changes, oviposition, toxicity in mammalian cells and other parameters are important in the search for antischistosomal substances. The techniques detailed here will be the key to better assess the methodology employed during screening tests.

4. Parasite culture system

In vitro studies with schistosomula, juvenile and adult worms of *S. mansoni* are frequently used in screening strategies for the discovery of new antischistosomal drugs (Abdulla et al., 2009; Keiser, 2010; Mølgaard et al., 2001; Peak et al., 2010; Ramirez et al., 2007; Smout et al., 2010; Yousif et al., 2007). Parasites at different stages might show differences with regard to drug sensitivity. The *in vitro* methods currently utilised have recently been reviewed, and following the establishment of the *S. mansoni* life cycle in the laboratory, *in vitro* parasite culture techniques were developed (Keiser, 2010; Ramirez et al., 2007). For *in vitro* trials, parasites of different ages are used, such as 3-h-old and 1-, 3-, 5- and 7-day-old schistosomula, 21 day-old juveniles, and 42- to 56-day-old adults. Figure 1 shows the life cycle of *S. mansoni* in the laboratory, illustrating the collection points for *in vitro* chemotherapeutic studies.

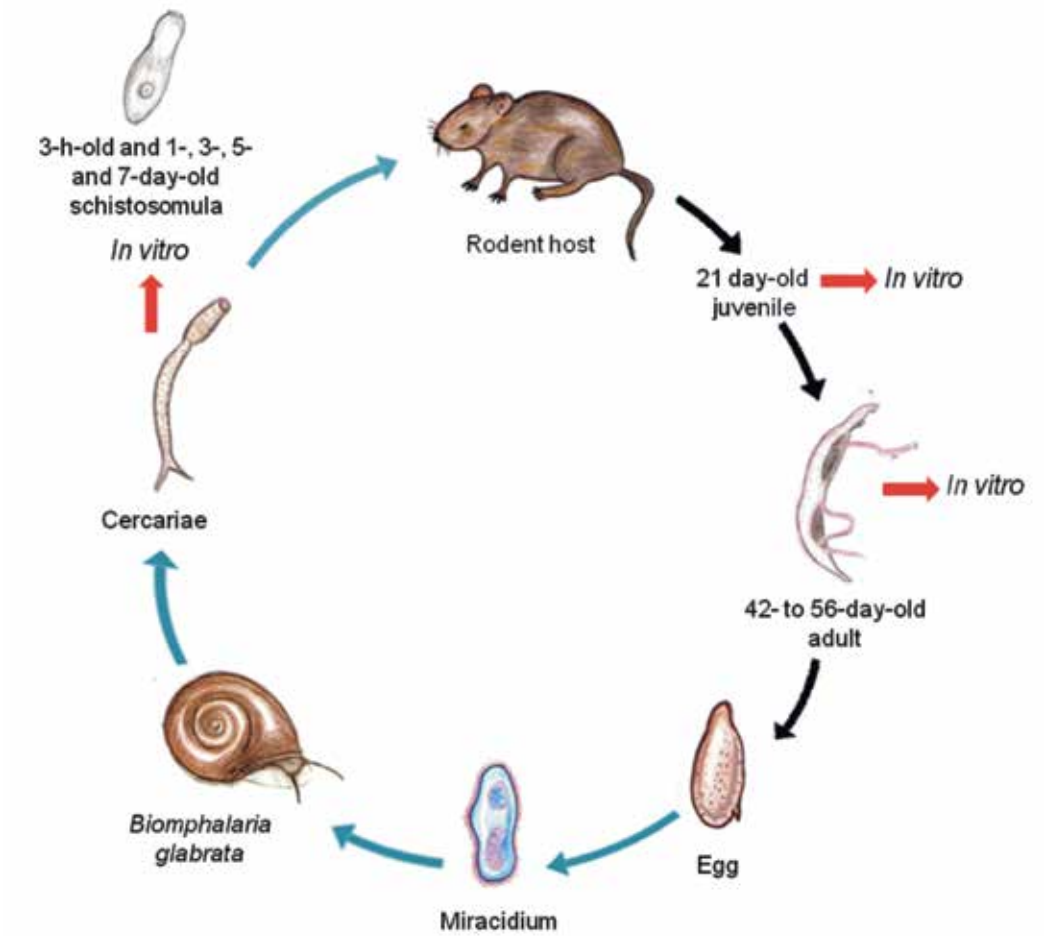


Fig. 1. Life cycle of *S. mansoni*, illustrating the collection points for *in vitro* chemotherapeutic studies. Black arrow: maturation of parasite within final host. Blue arrow: aquatic phase

4.1 Juvenile and adult schistosomes

Adult worms have been more commonly used for antischistosome drug discovery. Today, *in vitro* chemotherapeutic studies using juvenile worms are also highly recommended. For these assays, each rodent is commonly infected with either 100-150 or 400-500 cercariae. Rodents exposed to 400-500 cercariae are sacrificed 21 days after infection for juvenile recovery, while mice exposed to 100-150 cercariae are sacrificed 42 to 56 days after infection for adult recovery. Juveniles or adults are collected using a perfusion technique with Hanks' balanced salt solution (HBSS), Dulbecco's Modified Eagle's Medium (DMEM), or Roswell Memorial Park Institute (RPMI) 1640 medium, containing an anticoagulant such as heparin at a concentration range of 5-20 U/ml (Smithers & Terry, 1965). The worms are washed in RPMI 1640 medium, kept at pH 7.5 with 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) containing 200 U/ml penicillin, 200 µg/ml streptomycin, and 0.25 µg/ml amphotericin B. After washing, ten to fifteen juveniles or one pair of adult worms (male and female coupled) are transferred to each well of a 24-well culture plate containing 2 ml of the same medium supplemented with 10% bovine foetal serum and incubated at 37 °C in a humid atmosphere containing 5% CO_2. Only viable, contractile worms showing total tegument integrity as assessed by light microscopy should be included in the different investigations (Figure 2).

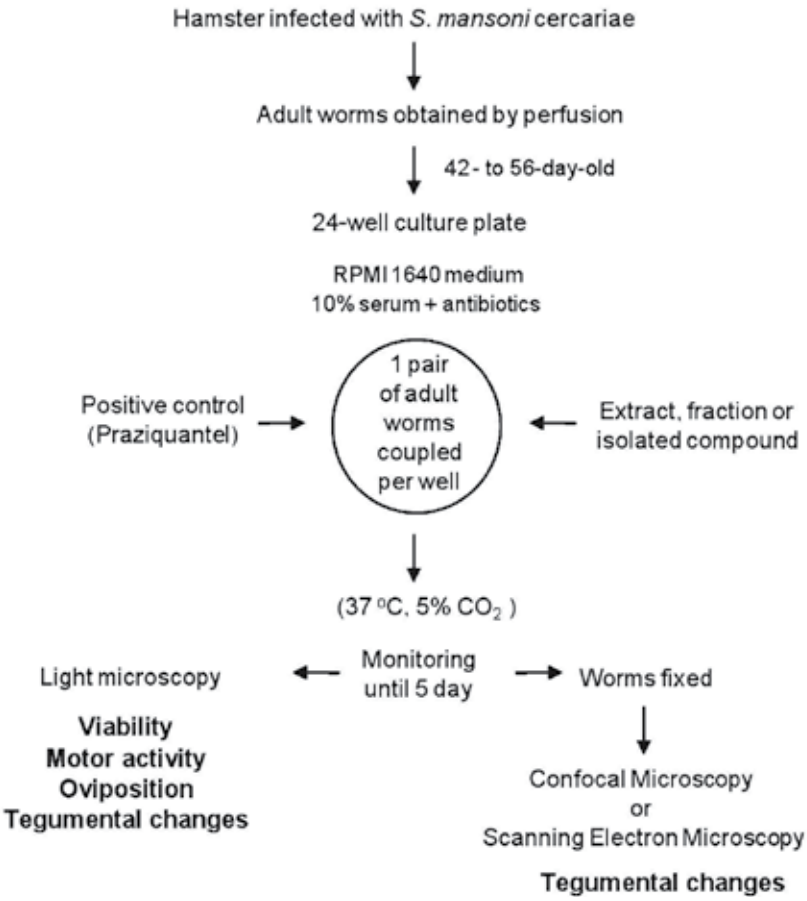


Fig. 2. *In vitro* assay models with *Schistosoma mansoni* adult worms.

4.2 Schistosomula

Obtaining sufficient quantities of schistosomula directly from skin or lung tissue for most research purposes is time consuming and involves working with mammalian hosts. These difficulties led to the development of techniques for transforming cercariae and maintaining schistosomula. In fact, schistosomula can be obtained by transforming cercariae using simple techniques, such as centrifugation, vortexing, repeated aspiration through a syringe needle, or chemical stimulation, and these schistosomula are easily maintained *in vitro* for several days (Basch, 1981). These *in vitro* strategies are advantageous because they confer uniformity in parasite maturation, which cannot be achieved *in vivo* due to the variation in the time required for individual parasites to penetrate the host skin and enter the vasculature. In addition, the use of mechanically obtained schistosomula is an alternative method that reduces, refines and replaces the use of animals in laboratory research in accordance with animal protection principles (Broadhead & Bottrill, 1997).

Among the techniques available for the production of schistosomula, the current methods most commonly used for the removal of tails from cercariae are the repeated aspiration through a syringe needle, based on Colley and Wikel (1974), and the use of a Vortex mixer, based on Ramalho-Pinto et al. (1974). In our laboratory, for example, schistosomula are mechanically transformed according to Ramalho-Pinto et al. (1974) and cultured *in vitro* in 169 medium, as described by Bash (1981). This method is recommended over the syringe-passage technique, which severely stresses and damages the parasites. In addition, the mechanical transformation procedure in a Vortex mixer is simpler than the passage of the parasites under pressure many times through a needle. Briefly, to obtain cercariae, *B. glabrata* snails infected with miracidia are exposed to incandescent light for 2 h, and then cercariae are collected, concentrated in glass conical centrifuge tubes and cooled in a ice bath for 10 minutes to reduce the motility of the worms. The ice-cold cercarial suspension is centrifuged, resuspended in RPMI 1640 medium with 25 mM HEPES, 200 UI/ml penicillin, 200 µg/ml streptomycin, and 0.25 µg/ml amphotericin B, and vortexed for 2 minutes to trigger tail loss. The resulting cercarial bodies are isolated from free tails by centrifugation through a 60% Percoll gradient (Lazdins et el., 1982) or decantation (Ramalho-Pinto et al., 1974). Microscope examination is used to assess the quantity and quality of purified schistosomula. Finally, schistosomula are cultivated in 169 medium containing antibiotics and supplemented with 10% bovine foetal serum at 37 ^{o}C in a 5% CO_2 atmosphere. Schistosomula are cultured until day 7, which corresponds to the lung-stage worm. For *in vitro* drug screening assays, schistosomula are transferred into 96-well culture microplates, with approximately 50 parasites per well, and maintained in 200 µl 169 medium under the conditions described above.

After penetrating the definitive host, significant morphological, physiological, and biochemical changes occur in the developing schistosomula (Gobert et al., 2007; Skelly & Alan Wilson, 2006). Although mechanically transformed schistosomula are different from the schistosomula that have penetrated the skin, these larval cultures have been maintained in *vitro* for different amounts of time to produce all the mammalian host stages, including skin- and lung-stage schistosomula and paired, mature adult males and females (Basch, 1981). Mechanically transformed schistosomula are now commonly used in studies of behaviour, development, metabolic activity, biochemistry, molecular biology, immunology,

and in vaccine development and drug screening protocols (Abdulla et al., 2009; Gobert et al., 2007; Harrop & Wilson, 1993; Peak et al., 2010). There is evidence that mechanically transformed schistosomula are structurally similar to their lung schistosomula counterparts (Chai et al., 2006), and after 7 days in culture, the larvae have the morphological features of lung worms and are capable of maturation when introduced into the portal vein of mice (Harrop & Wilson, 1993). Furthermore, mechanically transformed schistosomula are able to develop steadily until adult worm pairing (Basch, 1981). Because of these reasons, drug-screening assays in our laboratory are based on mechanically transformed schistosomula of different ages *in vitro* (3-h-, 1-, 3-, 5- and 7-day-olds). It takes roughly 3 h for the cercariae to secrete the contents of their acetabular glands; the 1- to 7-day-olds correspond to the skin- and lung-stage schistosomula.

5. Operating procedures for antischistosomal drug screening and the techniques employed

In recent years, the search for new anthelmintics has intensified, but little significant progress has been made in developing new techniques. The *in vitro* drug screening approaches must take into account some specific concerns, particularly to be simple and inexpensive. Important methodologies can objectively and rapidly distinguish helminth viability or phenotype. *In vitro* screening could identify novel anthelmintics and could eventually translate into practical applications. Herein, a general overview is given of the most common methodologies used for screening antischistosomal compounds and their effects on the whole organism.

5.1 Compound storage and handling

To test new compounds, including synthetic or natural products and crude extracts or fractions of a natural source, it is necessary to consider factors such as solubility and stability. The compounds are usually stored in hermetically sealed glass containers, covered with aluminium foil to protect the contents from light, and kept refrigerated at 8 ^{o}C or at ambient temperature until used. The compounds are commonly dissolved in dimethyl sulfoxide (DMSO), which should not exceed a final concentration of 2% in the culture medium containing parasites. Control schistosomes are incubated in the presence of the highest concentration of solvent used.

For *in vitro* screening assays, there is not a set maximum concentration to evaluate the activity of compounds on schistosomes, as long as no toxicity occurs on mammalian cells. However, in our laboratory, *in vitro* screenings are performed at final maximum concentrations of up to 500 μg/ml for crude extracts or fractions and 1,000 μM for isolated compounds. The reference drug praziquantel is used as a positive control at final concentrations ranging from 5 to 10 μM (Figure 2).

5.2 Assessment of parasite viability

The effects of compounds on *S. mansoni* are commonly assessed by phenotypic changes. The parasites are kept for 5 days as described above, and monitored at different time points (e.g., 24, 48, 72, 96, and 120 h) to evaluate their general condition, using parameters such as motor activity, morphological changes, and mortality rate (Figure 2).

Current methods utilised to assess schistosomal viability have recently been reviewed, and most of these methods involve microscopic techniques (Keiser, 2010; Ramirez et al., 2007). The phenotypic changes are scored by using a viability scale. For example, a scale of 0 – 4, where 4= normally active, 3= slowed activity, 2= minimal activity, 1= absence of motility apart from gut movements, and 0= total absence of mobility, is based on standard procedures for compound screening at the Special Programme for Research and Training in Tropical Diseases, World Health Organization, WHO-TDR (Ramirez et al., 2007). Alternatively, as described by Manneck et al. (2010, 2011), drug activity is defined as 3= totally vital, normally active, and no morphological changes; 2= slowed activity, primary morphological changes and visible granularity; 1= minimal activity, severe morphological changes and granularity; 0= all worms dead, severe morphological changes and granularity; the granularity is characterised only for schistosomula. The regular movement of both larval and adult schistosomes has proven to be a valuable trait in assessing schistosome viability *in vitro* because lack of movement is a good indicator of death. Worm death is usually defined as no movement observed for at least 2 min of examination (Manneck et al., 2010). In this context, the viability of worm during the culture period is also assessed by motor activity reduction, and it is defined as "slight " or "significant". This subjective criterion is commonly used by several research groups (Braguine et al., 2009; de Melo et al., 2011; de Moraes et al., 2011; Magalhães et al., 2009, 2010; Moraes et al., 2011, Parreira et al., 2010; Pereira et al., 2011; Xiao et al., 2007). Size measurements of parasites are also employed to study phenotypic changes.

In addition to the phenotypic approaches, another *in vitro* drug-screening assay method is based on microcalorimetry. Manneck et al. (2011) analysed the effects of drugs on the metabolic activity of schistosomula and adult *S. mansoni* by comparing their heat flow. In this study, a multi-channel isothermal microcalorimeter equipped with 48 measuring channels was used to monitor the heat production by schistosomes as a result of their metabolism over time. The results show that microcalorimetry can be a valuable tool to study antischistosomal drugs, and the microcalorimetric measurements confirmed, in part, the results of the phenotypic evaluation. However, the level of agreement between microscopy and microcalorimetry data requires further investigation (Manneck et al., 2011). In the following section, other methods are described that are used to determine the effect of drugs on schistosomula and adult *S. mansoni.*

Phenotypic changes are determined as mentioned above. However, because of the lack of standardisation between laboratories, the replication of results obtained by microscopic means is not always possible. In an effort to avoid the subjective nature of quantifying schistosome viability from the microscopic examination of phenotype alone, further adaptations have been developed and are based on the differentiating potential of some colorimetric vital dyes. Diamidinophenylindole (DAPI) has been used as a differential stain of dead schistosomula during microscopy; in addition, the low DAPI concentration (1 μg/ml) in the medium proved not to be toxic to the schistosomula, nor did it cause any background fluorescence (Van Der Linden & Deelder, 1984). Trypan blue has also been shown to be a reliable dye for differentially staining dead schistosomula (Harrop & Wilson, 1993) and by means of a methylene blue dye exclusion test (Gold, 1997). The tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is also a vital dye that has been successfully used to assess the viability of worms. The use of this assay on helminths was pioneered by Comley et al. (1989), and several nematode

species have been assessed. Nare et al. (1991) used the MTT marker to evaluate the viability of adult schistosomes; currently, some *in vitro* bioassays used to study the effects of drugs have demonstrated the ability of MTT to assess the viability of adult worms (Braguine et al., 2009; de Melo et al., 2011; Magalhães et al., 2009, 2010; Parreira et al., 2010; Pereira et al., 2011).

Recently, Peak et al. (2010) validated a high-throughput system for detecting the viability of schistosomula using a microtiter plate-based method. In this study, the authors combined the use of propidium iodide with fluorescein diacetate to allow the easy assessment of the percent of viable schistosomula present in a sample. This helminth fluorescent bioassay was developed into a method of wide-scale application because it is sensitive, relevant to industrial high-throughput (384-well microtiter plate compatibility, 200 schistosomula/well) and academic (96-well microtiter plate compatibility, 1000 schistosomula/well) settings, translatable to drug screening assays, does not require a priori knowledge of schistosome biology or extensive training in parasite morphology, and is objective and quantitative.

The development of high-content screening systems is an important step for the assessment of parasite viability in a high-throughput format. A novel assay for anthelmintic drug screening by real-time monitoring of parasite motility was developed by Smout et al. (2010). This technological advance is based on the detection of changing electrical currents running through mini gold electrodes on the bottom of tissue culture plates. In this assay, the authors assessed the motility of *S. mansoni* using an xCELLigence system (Real Time Cell Assay, RTCA SP instrument), which monitors cellular events in real time without the incorporation of labels by measuring the electrical impedance across interdigitated micro-electrodes integrated on the bottom of tissue culture plates. This technology was applied to adult schistosome using one pair (one coupled male and female worm) in 200 µl per well of an E-plate, which is a 96-well plate for cell-based assays on the RTCA instruments. Because the real-time system measures changes in worm motility with the high level of precision necessary for high-throughput studies, it is widely applicable to a range of helminth species and developmental stages (Smout et al., 2010). This motility assay may provide a superior methodology to microscopy by removing the subjectivity from helminth phenotype characterisation and making available a technology that could allow the direct comparison of results from different laboratories. However, the initial cost of this RTCA system and E-plates may restrict its use, especially in an academic laboratory.

5.3 Assessment of changes in the tegument of parasites

The tegument is the major interface between the schistosome and its external environment. In addition to providing protection, the tegument is an important site of the uptake of nutrients and other molecules. Moreover, the tegument is extremely important for infection success and survival in the host, and it has been a major target for the development of antischistosomal drugs (Skelly & Wilson, 2006; Van Hellemond et al., 2006). Therefore, most of the drugs currently used against schistosome act by damaging the worm tegument (Doenhoff et al., 2008; Fenwick et al., 2003; El Ridi et al., 2010; Keiser, 2010; Manneck et al., 2010, 2011; Mostafa et al., 2011; Xiao et al., 2000).

The schistosome tegument is often approached as a drug target in schistosomiasis and is associated with the subjective assessment of parasite viability described here. The

morphological alterations of the tegument of *S. mansoni* are also assessed by methods that involve microscopic analysis. Indeed, during the assay, the parasite is manipulated *in vitro*, and the effect of such manipulation is assessed by bright-field examination of the morphology of the parasite (Figure 2). The criteria used to assess morphological changes induced by a drug require visual scoring by skilled operators and is assessed subjectively. Morphological changes are usually defined qualitatively as "partial" or "extensive" (Braguine et al., 2009; de Melo et al., 2011; de Moraes et al., 2011; Magalhães et al., 2009, 2010; Moraes et al., 2011; Parreira et al., 2010; Pereira et al., 2011; Xiao et al., 2007). Therefore, the effect of anthelmintic drugs on the tegument of schistosome cannot be evaluated in a dose-dependent manner. In addition, a question remains: is the death of the parasite is associated with damage to the coat?

The inherent subjectivity of this qualitative analysis led Moraes et al. (2011) and de Moraes et al. (2011) to develop a quantitative method for evaluating the effect of drugs on the tegument of *S. mansoni* using confocal microscopy. In this quantitative analysis, areas of the tegument of male worms are assessed, and the numbers of tubercles are counted. Briefly, the parasites are fixed in Formalin-Acetic-Alcohol solution (FAA) and analysed under a confocal microscope at 488 nm (excitation) and 505 nm (emission), as described by Moraes et al. (2011) and de Moraes et al. (2011). During the microscopic analysis of the three-dimensional images captured using LSM Image Browser software (Zeiss), areas of the tegument of parasite are assessed, and the numbers of tubercles on the dorsal surface of male helminths are counted in a 20,000 μm^2 area, which is calculated using the same software image capture. Importantly, the area chosen is in the dorsal region of the male adult worm, close to the ventral sucker (acetabulum) region, because there is no significant variation in the number of tubercles in this region. Furthermore, schistosome and others trematodes are self-fluorescent (Moraes et al., 2009), and this fluorescence is increased when the parasites are in the FAA solution. This is advantageous because it allows images to be captured without using a fluorescent fluorophore. The FAA solution consists of a 2:9:30:59 mixture of acetic acid, formaldehyde, ethanol (95%) and distilled water. This methodology is summarised in Figure 3.

As previously mentioned, drug effects are currently assessed by observing morphological changes in parasites using light microscopy methods. However, these techniques do not allow the microscopic analysis of the tegument in detail. The quantitative analysis described by Moraes et al. (2011) and de Moraes (2011) must be performed with high-resolution microscopy, such as confocal or scanning electron microscopy. Therefore, the drug effects assessed by phenotypic changes, such as tegumental alterations, cannot be trusted. For example, Moraes et al. (2011) used confocal microscopy to evaluate the *in vitro* schistosomicidal activity of piplartine, an amide isolated from *Piper tuberculatum*, and demonstrated that the tegumental damage occurs after incubation with doses higher than the lethal concentrations, suggesting that worm death is caused by different mechanisms. Thus, tegumental damage may not always result in death, and a quantitative assessment technique is needed to understand the mechanisms of action of newly discovered antischistosomal drugs. In addition, the advent of screening methods that allow high-throughput, scalable, automated, and objective assays for helminth viability, combined with knowledge of the molecular biology of the schistosome to identify possible new drug targets, will make drug development for schistosomiasis easier.

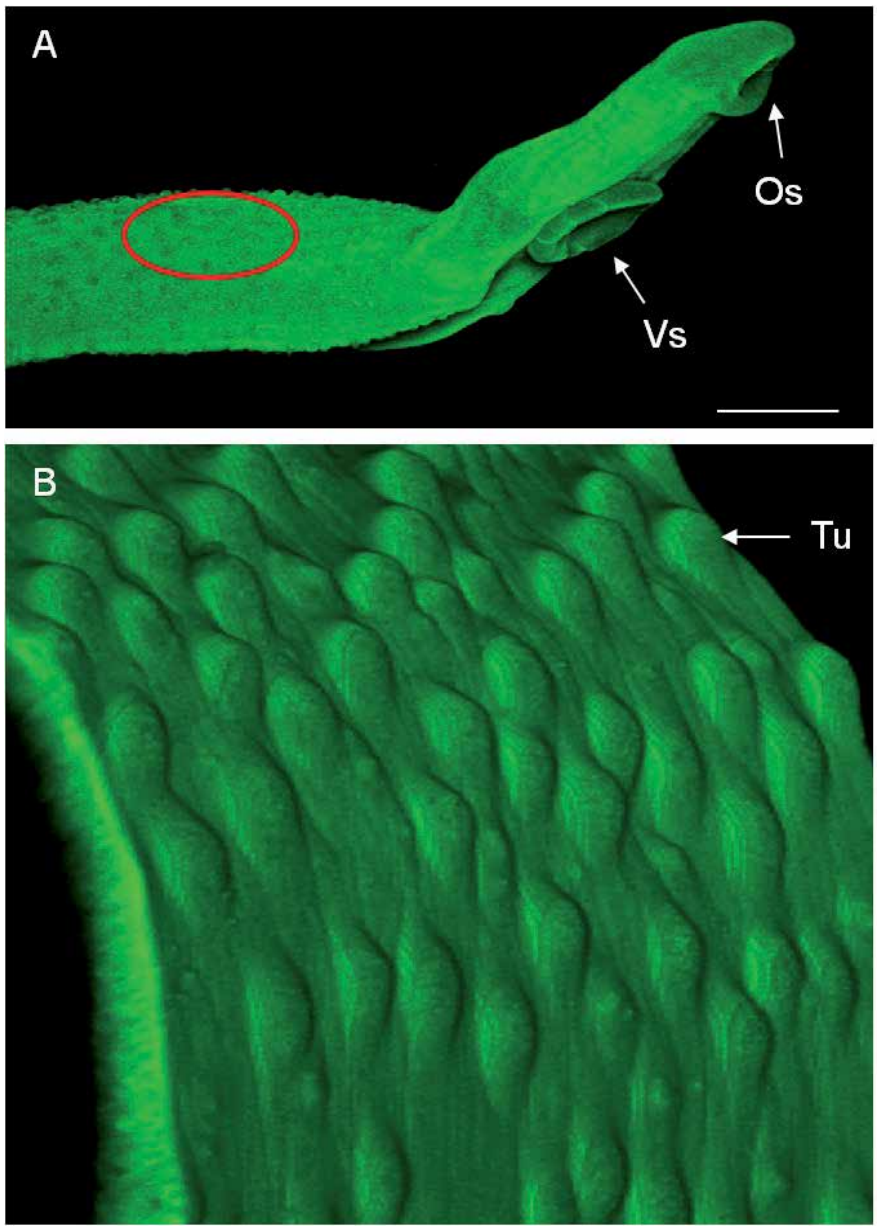


Fig. 3. Dorsal region of a *Schistosoma mansoni* adult male worm, on which the effect of antischistosomal compounds on the tegument is evaluated quantitatively. The parasite was fixed in FAA solution, and fluorescent images were obtained using a confocal microscope. A: General view of the anterior helminth region showing, in red, the location where tubercles were counted. Bar = 500 μm. B: View of an area of 20,000 μm², calculated with the Zeiss LSM Image Browser software, showing the tubercles. This image is a higher magnification of the dorsal region of the *S. mansoni* adult worm marked in red in panel A. X and Y: three-dimensional images obtained from laser scanning confocal microscopy. Os: oral sucker; Vs: ventral sucker; Tu: tubercles

5.4 In vitro assessment of the reproductive fitness of adult worms

The effects of natural or synthetic products on the reproductive fitness of *S. mansoni* have been previously reported in several studies (Braguine et al., 2009; de Moraes et al., 2011; Magalhães et al., 2009, 2010; Mohamed et al., 2005; Moraes et al., 2011; Sanderson et al., 2002). To evaluate drug effects on schistosome during *in vitro* screening drug assays, cultures are continually monitored to assess the sexual fitness of worms treated with sub-lethal concentrations of drug. In this case, the following parameters are assessed: (1) changes in the pairing, an indicator of the mating process; (2) egg production, an indicator of egg output per worm; and (3) egg development.

In the experiments, adult worm pairs (male and female coupled) are incubated in a 24-well culture plate, as previously described here, and parasites are monitored on daily basis for 5 days using an inverted microscope and a stereomicroscope (Figure 2). Therefore, it is important that, after collection by the perfusion technique, the parasites are carefully washed to prevent the separation of the worm pairs.

Schistosome egg output *in vitro* is usually determined by counting the number of eggs. Egg development can be analysed quantitatively and scored as developed or undeveloped on the basis of the presence or absence of the miracidium (de Melo et al., 2011; Magalhães et al., 2009, 2010). This is a simple and recommended method because conventional light microscopy is able to distinguish morphologic differences in eggs. However, the characterisation of the viability of immature eggs is very difficult. Alternatively, the analysis of egg viability, distinguishing live immature eggs from dead immature ones, can be performed using a fluorescent label, as described by Sarvel et al. (2006). In this assay, the eggs obtained in culture are stained with the Hoescht 33258 probe and observed with fluorescent microscopy. The authors evaluated fluorescent labels and vital dyes, aiming at differentiating live and dead eggs, and showed the only the fluorescent Hoechst 33258 can be considered a useful tool to differentiate between dead and live eggs.

5.5 Cytotoxicity assays

Finding a new compound capable of killing a parasite is not difficult. However, it is difficult to find a substance that can kill the parasite without affecting the host. Therefore, early *in vitro* studies of new compounds must include comparative cytotoxicity data from human or animal cells in tissue culture to establish that the compound has selective antischistosomal activity and may be a realistic prospect for future clinical use in humans. In our operating procedures for antischistosomal drug screening, mammalian cells are exposed to concentrations of at least two times higher than what is needed to elicit a schistosomicidal effect. Thus, the *in vitro* schistosomicidal activity of compounds cannot be associated with cytotoxic effects.

General toxicity tests can be conducted in many cell types (e.g., fibroblasts and epithelial and hepatoma cells). Peripheral blood mononuclear cells and erythrocytes are widely used in *in vitro* studies to detect cytotoxicity or cell viability following exposure to antischistosomal compounds. Vero mammalian cells (African green monkey kidney fibroblasts) are also commonly used to examine whether natural or synthetic antiparasitic compounds are tolerated by mammalian cells (da Silva Filho et al., 2009; Moraes et al., 2011; Parreira et al., 2010).

The crystal violet staining method and the neutral red and MTT assays are the most common methodologies used to detect cytotoxicity or cell viability following exposure to toxic substances. In our *in vitro* cytotoxicity assays with cultured cells, the crystal violet

staining method is routinely used because it is rapid and inexpensive. This method measures the effects of compounds on cell growth through the colorimetric evaluation of fixed cells stained with crystal violet. Briefly, cells maintained in culture medium are seeded into 96-well culture microplates in the presence of different concentrations of extracts, fractions or isolated compounds. After different timepoints of incubation (e.g., 2, 24, 48, 72, and 96 h), the supernatants are removed and the remaining live cells are assessed by fixing and staining them with crystal violet (0.2% in 20% methanol). Viable cells attach to the bottom of the well plate, and the absorbance is measured by reading each well at 595 nm in a microplate reader (Moraes et al., 2011).

Neutral red is another cell viability assay often used to determine cytotoxicity following exposure to toxic substances (Borenfreund & Puerner, 1985). It has been used as an indicator of cytotoxicity in cultures of primary hepatocytes and other cell lines (Fautz et al., 1991; (Fotakis & Timbrell, 2006; Morgan et al., 1991). Living cells take up the neutral red, which is concentrated within the lysosomes of cells.

The MTT assay is also used to measure cell viability. MTT is a water-soluble tetrazolium salt, which is converted to an insoluble purple formazan upon the cleavage of the tetrazolium ring by succinate dehydrogenase within the mitochondria. The formazan product is impermeable to the cell membranes, and therefore, it accumulates in healthy cells. The MTT assay has been tested for its validity in various cell lines (Fotakis & Timbrell, 2006; Mossmann, 1983).

Alternatively, the lactate dehydrogenase (LDH) leakage assay and protein assay are also used to detect cytotoxicity, despite the fact that they have low sensitivity when compared to the methods already described. The LDH leakage assay is based on the measurement of LDH activity in the extracellular medium. The loss of intracellular LDH and its release into the culture medium is an indicator of irreversible cell death due to cell membrane damage (Decker & Lohmann-Matthes, 1988; Fotakis & Timbrell, 2006). The protein assay is an indirect measurement of cell viability because it measures the protein content of viable cells. Despite the existence of several protocols to establish total protein concentration (e.g., biuret, bicinchoninic acid, Lowry and Bradford protocols), the two most commonly used methods for protein quantification are the Lowry and Bradford assays (Bradford et al., 1976; Lowry et al., 1951).

Finally, tritiated thymidine-based methods, which act through the incorporation of tritium into the DNA of cells, have also been used currently to detect cytotoxicity, especially in immune cells (Pechhold et al., 2002).

6. Conclusions

Schistosomiasis is a neglected disease that is one of the most common chronic infections among the poorest people in the world. Most chemotherapeutic-based programs attempting to eradicate schistosomiasis in the developing world rely on the effectiveness of a single drug, praziquantel; therefore, there is an urgent need to identify new parasite targets and effective antischistosomal compounds. Secondary plant metabolites have attracted the attention of many researchers over the years as a result of the variety of their chemical structures and their broad range of biological activities that may provide lead structures for the development of new drugs. Recently, marine organisms have also been recognised as an attractive source of antiparasitic compounds, and it can be expected that other living organisms, such as insects and amphibians, will emerge as additional sources in the future.

Discovering untapped natural sources of new anthelmintic compounds remains a major challenge and a source of novelty in the era of combinatorial chemistry and genomics. To find new anthelmintics, all sources of natural, synthetic and semi-synthetic lead compounds must be investigated. *In vitro* bioassays using parasitic worms have played a central role in the early pre-clinical stages of most research on potential natural anthelmintics. The identification of the antiplasmodial and antischistosomal activity of the sesquiterpene lactone artemisinin has stimulated interest in natural products, and soon, promising leads will be identified with new chemical types and active agents against schistosomiasis. Therefore, bioprospecting programmes related to the isolation of bioactive compounds must be rewarded, and the screening *in vitro* of chemical constituents belonging to different classes must be evaluated on the blood fluke *S. mansoni*.

The literature regarding antischistosomal compounds contains a large number of natural products screened for their schistosomicidal properties. However, only a few of these may be promising drug leads in the development of a therapeutic reserve for schistosomiasis. Therefore, it is important to continue to identify new drugs and to explore alternative strategies to improve screening efficacy. Most of the extracts or natural compounds were only evaluated with *in vitro* studies; it is expected that they will be evaluated using *in vivo* experimental models. Further, it must be mentioned that the results of *in vitro* assays with many drugs do not correspond to what is observed *in vivo*; however, *in vitro* screening could identify novel anthelmintics that could eventually translate into practical applications. Thus, while *in vitro* tests are recommended initially, the assessment of therapeutic activity using *in vivo* models should be performed.

The analysis of the *S. mansoni* genome and transcriptome offers great possibilities for identifying possible new drug targets and will facilitate further exploration of differences between host and parasite metabolic pathways. In addition to the isolation and structural determination of new drugs from natural products and information from the originating plant, the integration of the pharmacological properties of natural products with the functional genomic and proteomic studies in schistosome and *in vitro* screening methods with improved automatic high-content screening will be important tools to identify possible new drugs in the future and shed light on the approaches of helminth chemotherapy. Attempting new combinations of natural or synthetic drugs will be also important in discovering alternative drugs to replace the use of praziquantel.

7. Acknowledgments

I thank Dr. Eliana Nakano and Mr. Alexsander S. Souza (Laboratório de Parasitologia, Instituto Butantan, São Paulo, SP, Brazil) for assistance with confocal microscopy. I also thank Ms. Edinéia C. Moraes for drawing the lifecycle diagram and Ms. Aline A. L. Carvalho for comments and suggestions.

8. References

Abdul-Ghani, R.A.; Loutfy, N. & Hassan, A. (2009). Myrrh and trematodoses in Egypt: an overview of safety, efficacy and effectiveness profiles. *Parasitology International*, Vol.58, pp. 210-214, ISSN 1383-5769

Abdulla, M.H.; Ruelas, D.S.; Wolff, B.; Snedecor, J.; Lim, K.C.; Xu, F.; Renslo, A.R.; Williams, J.; Mckerrow, J.H. & Caffrey, CR. (2009). Drug discovery for schistosomiasis: hit

and lead compounds identified in a library of known drugs by medium-throughput phenotypic screening. *PLoS Neglected Tropical Disease*, Vol.3, pp. e478, ISSN 1935-2735

Araújo, R.V.; Melo-Júnior, M.R.; Beltrão, E.I.; Mello, L.A.; Iacomini, M.; Carneiro-Leão, A.M.; Carvalho, L.B. Jr & Santos-Magalhães, N.S. (2011). Evaluation of the antischistosomal activity of sulfated α-D-glucan from the lichen *Ramalina celastri* free and encapsulated into liposomes. *Brazilian Journal of Medical and Biological Research*, Vol.44, pp. 311-318, ISSN 0100-879X

Badria, F.; Abou Mohamed, G.; El-Mowafy, A.; Massoud, A. & Salama, O. (2001). Mirazid: a new schistosomicidal drug, *Pharmaceutical Biology*, Vol.39, pp. 127–131. ISSN 1388-0209

Baldé, A.M.; Van Marck, E.A.; Kestens, L.; Gigase, P.L. & Vlietinck, A.J. (1989). Schistosomicidal effects of *Pavetta owariensis* and *Harrisonia abyssinica* in mice infected with *Schistosoma mansoni*. *Planta Medica*, Vol.55, pp. 41-43, ISSN 0032-0943

Barth, L.R.; Fernandes, A.P.; Ribeiro-Paes, J.T. & Rodrigues, V. (1997). Effects of goyazensolide during *in vitro* cultivation of *Schistosoma mansoni*. *Memórias do Instituto Oswaldo Cruz*, Vol.92, pp. 427–429, ISSN 0074-0276

Bergquist, R.; Utzinger, J. & McManus, D.P. (2008). Trick or treat: the role of vaccines in integrated schistosomiasis control, *PLoS Neglected Tropical Disease*, Vol.2, pp. e244, ISSN 1935-2735

Basch, P.F. (1981) Cultivation of *Schistosoma mansoni in vitro*. I. Establishment of cultures from cercariae and development until pairing. *The Journal of Parasitology*, Vol.67, p. 179-185, ISSN 0022-3395

Borenfreund, E. & Puerner, J. (1985). Toxicity determined *in vitro* morphological alterations and neutral red absorption. *Toxicology letters*, Vol.24, pp. 119-124, ISSN 0378-4274

Botros, S.; Sayed, H.; El-Dusoki, H.; Sabry, H.; Rabie, I.; El-Ghannam, M.; Hassanein, M.; El-Wahab, Y.A. & Engels, D. (2005). Efficacy of mirazid in comparison with praziquantel in Egyptian *Schistosoma mansoni*-infected school children and households. *The American Journal of Tropical Medicine and Hygiene*, Vol.72, pp. 119-123, ISSN 0002-9637

Botros, S.; William, S.; Ebeid, F.; Cioli, D.; Katz, N.; Day, T.A.; Bennett, J.L. (2004). Lack of evidence for an antischistosomal activity of myrrh in experimental animals. *The American Journal of Tropical Medicine and Hygiene*, Vol.71, pp. 206-210, ISSN 0002-9637

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Analytical Biochemistry*, Vol.72, pp. 248–254, ISSN 0003-2697

Braguine, C.G.; Costa, E.S.; Magalhães, L.G.; Rodrigues, V.; da Silva Filho, A.A.; Bastos, J. K.; Silva, M.L.; Cunha, W.R.; Januário, A.H. & Pauletti, P.M. (2009). Schistosomicidal evaluation of *Zanthoxylum naranjillo* and its isolated compounds against *Schistosoma mansoni* adult worms. *Zeitschrift für Naturforschung. C, Journal of biosciences*, Vol.64, pp. 793-797, ISSN 0939-5075

Broadhead, C.L. & Bottrill, K. (1997). Strategies for replacing animals in biomedical research. *Molecular Medicine Today*, Vol.3, pp. 483-487, ISSN 1357-4310

Caffrey, C.R. (2007). Chemotherapy of schistosomiasis: present and future. *Current Opinion in Chemical Biology*, Vol.11, pp. 433–439, ISSN 1367-5931

Chai, M.; McManus, D.P.; McInnes, R.; Moertel, L.; Tran, M.; Loukas, A.; Jonesa, M.K. & Gobert, G.N. (2006) Transcriptome profiling of lung schistosomula,in vitro cultured

schistosomula and adult *Schistosoma japonicum*. *Cellular and Molecular Life Sciences*, Vol.63, pp. 919-929, ISSN 1420-682X

Cioli, D. (2000). Praziquantel: is there real resistance and are there alternatives? *Current Opinion in Infectious Diseases*, Vol.13, pp. 659-663, ISSN 0951-7375

Cioli, D.; Pica-Mattoccia, L. & Archer, S. (1995). Antischistosomal drugs: past, present ... and future?. *Pharmacology & therapeutics*, Vol.68, pp. 35-85, ISSN 0163-7258

Cioli, D. (1998). Chemotherapy of schistosomiasis: an update. *Parasitology Today*, Vol.14, pp. 418-422, ISSN 0169-4758

Colley, D.G. & Wikel, S.K. (1974). *Schistosoma mansoni*: simplified method for the production of schistosomules. *Experimental Parasitology*, Vol.35, pp. 44-51, ISSN 0014-4894

Comley, J.C.W.; Rees, M.J.; Turner, C.H. & Jenkins, D.C. (1989). Colorimetric quantitation of filarial viability. *International Journal for Parasitology*, Vol.19, pp. 77–83, ISSN 0020-7519

de Melo, N.I.; Magalhaes, L.G.; de Carvalho, C.E.; Wakabayashi, K.A.; de P Aguiar, G.; Ramos, R.C.; Mantovani, A.L.; Turatti, I.C.; Rodrigues, V.; Groppo, M.; Cunha, W.R.; Veneziani, R.C. & Crotti. A.E. (2011). Schistosomicidal activity of the essential oil of *Ageratum conyzoides* L. (Asteraceae) against adult *Schistosoma mansoni* worms. *Molecules*, Vol.16, pp. 762-773, ISSN 1420-3049

de Moraes, J.; Nascimento, C.; Miura, L.M.; Leite, J.R. ; Nakano, E. & Kawano, T. (2011). Evaluation of the *in vitro* activity of dermaseptin 01, a cationic antimicrobial peptide, against *Schistosoma mansoni*. *Chemistry & Biodiversity*, Vol.8, pp. 548-558, ISSN 1612-1872

da Silva Filho, A.A.; Resende, D.O.; Fukui, M.J.; Santos, F.F.; Pauletti, P.M.; Cunha, W.R.; Silva, M.L.; Gregório, L.E.; Bastos, J.K. & Nanayakkara, N.P. (2009). *In vitro* antileishmanial, antiplasmodial and cytotoxic activities of phenolics and triterpenoids from *Baccharis dracunculifolia* D. C. (Asteraceae). *Fitoterapia*, Vol.80, pp. 478–482, ISSN 0367-326X

Decker, T.; Lohmann-Matthes, M.L. (1988). A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. *Journal of Immunological Methods*, Vol.115, pp.61-69, ISSN 0022-1759

Doenhoff, M.; Cioli, D. & Utzinger, J. (2008). Praziquantel: mechanisms of action, resistance and new derivatives for schistosomiasis. *Current Opinion in Infectious Diseases*, Vol.21, pp. 659–667, ISSN 0951-7375

El-Ansary, A.K. Ahmed, S.A. & Aly, S.A. (2007). Antischistosomal and liver protective effects of *Curcuma longa* extract in *Schistosoma mansoni* infected mice. *Indian Journal of Experimental Biology*, Vol.45, pp. 791–801, ISSN 0019-5189

El-Banhawey, M.A.; Ashry, M.A.; El-Ansary, A.K. & Aly, S.A. (2007). Effect of *Curcuma longa* or praziquantel on *Schistosoma mansoni* infected mice liver—histological and histochemical study. *Indian Journal of Experimental Biology*, Vol.45, pp. 877–889 ISSN 0019-5189

El Ridi, R.; Aboueldahab, M.; Tallima, H.; Salah, M.; Mahana, N.; Fawzi, S.; Mohamed, S.H.; Fahmy, O.M. (2010). *In vitro* and *in vivo* activities of arachidonic acid against *Schistosoma mansoni* and *Schistosoma haematobium*. *Antimicrobial Agents and Chemotherapy*, Vol.54, pp. 3383-3389, ISSN 0066-4804

El Shenawy, N.S.; Soliman, M.F. & Reyad, S.I. (2008). The effect of antioxidant properties of aqueous garlic extract and *Nigella sativa* as antischistosomiasis agents in mice. *Revista do Instituto de Medicina Tropical de São Paulo*, Vol.50, pp. 29-36 ISSN 0036-4665

Fautz, R.; Husein, B. & Hechenberger, C. (1991). Application of the neutral red assay (NR assay) to monolayer cultures of primary hepatocytes: rapid colorimetric viability determination for the unscheduled DNA synthesis test (UDS). *Mutation Research*, Vol.253, pp. 173-179, ISSN 0027-5107

Fenwick, A.; Savioli, L.; Engels, D.; Robert Bergquist, N. & Todd, M.H. (2003). Drugs for the control of parasitic diseases: current status and development in schistosomiasis. *Trends in Parasitology*, Vol.19, pp.509-515, ISSN 1471-4922

Fenwick, A. & Webster, J.P. (2006). Schistosomiasis: challenges for control, treatment and drug resistance. *Current Opinion in Infectious Diseases*, Vol.19, pp. 577–582, ISSN 0951-7375

Ferreira, J.F.; Peaden, P. & Keiser, J. (2011). *In vitro* trematocidal effects of crude alcoholic extracts of *Artemisia annua*, *A. absinthium*, *Asimina triloba*, and *Fumaria officinalis*: Trematocidal plant alcoholic extracts. *Parasitology Research*, ISSN 0932-0113, doi: 10.1007/s00436-011-2418-0

Fotakis, G. & Timbrell, J.A. (2006). *In vitro* cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicology Letters*, Vol.160, pp. 171-177, ISSN 0378-4274

Gobert, G.N.; Chai, M. & McManus, D.P. (2007). Biology of the schistosome lung-stage schistosomulum. *Parasitology*, Vol.134, pp. 453-460, ISSN 0031-1820

Gold, D. (1997). Assessment of the viability of *Schistosoma mansoni* schistosomula by comparative uptake of various vital dyes. *Parasitology Research*, Vol.83, pp. 163-169, ISSN 0932-0113

Golenser, J.; Waknine, J.H.; Krugliak, M.; Hunt, N.H. & Grau, G.E (2006). Current perspectives on the mechanism of action of artemisinins. *International Journal for Parasitology*, Vol.36, pp. 1427-1441, ISSN 0020-7519

Gryseels, B.; Polman, K.; Clerinx, J. & Kestens, L. (2006). Human schistosomiasis. *Lancet*, Vol.368, pp. 1106–1118, ISSN 0140-6736

Hagan, P.; Appleton, C.C.; Coles, G.C.; Kusel, J.R. & Tchuem-Tchuenté, L.A. (2004). Schistosomiasis control: keep taking the tablets. *Trends in Parasitology*, Vol.20, pp. 92–97, ISSN 1471-4922

Harrop, R. & Wilson, R.A. (1993). Protein synthesis and release by cultured schistosomula of *Schistosoma mansoni*. *Parasitology*, Vol.107, pp. 265-274, ISSN 0031-1820

Jaramillo, M.A. & Manos, P.S. (2001). Phylogeny and patterns of floral diversity in the genus *Piper* (Piperaceae). *American Journal of Botany*, Vol.88, pp. 706–716, ISSN 0002-9122

Jatsa, H.B.; Ngo Sock, E.T.; Tchuem Tchuente, L.A. & Kamtchouing, P. (2009). Evaluation of the *in vivo* activity of different concentrations of *Clerodendrum umbellatum* Poir against *Schistosoma mansoni* infection in mice. *African Journal of Traditional, Complementary, and Alternative Medicines*, Vol.6, pp. 216-221, ISSN 0189-6016

Kato, M.J. & Furlan, M. (2007). Chemistry and evolution of the Piperaceae. *Pure and Applied Chemistry*, Vol.79, pp. 529–538, ISSN 0033-4545

Kayser, O.; Kiderlen, A.F.; Croft, S.L. (2003). Natural products as antiparasitic drugs. Parasitology Research, Vol.90 (Suppl 2), pp. S55-S62, ISSN 0932-0113

Keiser, J. (2010). *In vitro* and *in vivo* trematode models for chemotherapeutic studies. *Parasitology*, Vol.137, pp. 589-603, ISSN 0031-1820

Keiser, J. & Utzinger, J. (2007). Advances in the discovery and development of trematocidal drugs. *Expert Opinion on Drug Discovery*, Vol.2 (Suppl 1), pp. S9–23, ISSN 1746-0441

Lazdins, J.K.; Stein, M.J.; David, J.R. & Sher, A. (1982). *Schistosoma mansoni*: rapid isolation and purification of schistosomula of different developmental stages by centrifugation on discontinuous density gradients of Percoll. *Experimental Parasitology*, Vol.53, pp. 39-44, ISSN 0014-4894

Lee, S. (2007). Artemisinin, promising lead natural product for various drug developments. *Mini Reviews in Medicinal Chemistry*, Vol.7, pp. 411-422, ISSN 1389-5575

Leite, J.R.S.A.; Miura, L.M.C.V.; Lima, D.F. Carneiro, S.M.P.; Carvalho, F.A.A.; Moraes, J. & Batista, M.C.S. (2011). Processo de obtenção da epiisopiloturina e sua aplicação no combate à infecções parasitárias. PI 0904110-9 A2 *Revista de Propriedade Industrial, Instituto Nacional de Prodiedade Industrial*, No 2108, pp. 132

Lima, C.M. ; Freitas, F.I.; Morais, L.C.; Cavalcanti, M.G.; Silva, L.F.; Padilha, R.J.; Barbosa C.G.; Santos, F.A.; Alves, L.C. & Diniz, M.D. (2011). Ultrastructural study on the morphological changes to male worms of *Schistosoma mansoni* after *in vitro* exposure to allicin. *Revista da Sociedade Brasileira de Medicina Tropical*, ISSN 0037-8682, doi: 10.1590/S0037-86822011005000023

Lowry, O.H.; Rosebrough, N.J.; Farr, A.L. & Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. *The Journal of Biological Chemistry*, Vol.193, pp. 265-275, ISSN 0021-9258

Lyddiard, J.R.; Whitfield, P.J. & Bartlett, A. (2002). Antischistosomal bioactivity of isoflavonoids from *Millettia thonningii* (Leguminosae). *The Journal of Parasitology*, Vol.88, pp. 163-170. ISSN 0022-3395

Magalhães, L.G.; Kapadia, G.J.; da Silva Tonuci, L.R.; Caixeta, S.C.; Parreira, N.A.; Rodrigues, V. & Da Silva Filho (2010). *In vitro* schistosomicidal effects of some phloroglucinol derivatives from *Dryopteris* species against *Schistosoma mansoni* adult worms. *Parasitology Research*, Vol.106, pp. 395-401, ISSN 0932-0113

Magalhães, L.G.; Machado, C.B.; Morais, E.R.; Moreira, E.B.; Soares, C.S.; da Silva, S.H.; Da Silva Filho, A.A.; Rodrigues, V. (2009). *In vitro* schistosomicidal activity of curcumin against *Schistosoma mansoni* adult worms. *Parasitology Research*, Vol. 104, pp. 1197-1201, ISSN 0932-0113

Mahmoud, M.R.; El-Abhar, H.S. & Saleh, S. (2002). The effect of *Nigella sativa* oil against the liver damage induced by *Schistosoma mansoni* infection in mice. *Journal of Ethnopharmacology*, Vol.79, pp. 1-11, ISSN 0378-8741

Manneck, T.; Haggenmüller, Y. & Keiser, J. (2010). Morphological effects and tegumental alterations induced by mefloquine on schistosomula and adult. *Parasitology*, Vol.137, pp. 85-98, ISSN 0031-1820

Manneck, T.; Braissant, O.; Ellis, W. & Keiser J. (2011). *Schistosoma mansoni*: antischistosomal activity of the four optical isomers and the two racemates of mefloquine on schistosomula and adult worms *in vitro* and *in vivo*. *Experimental Parasitology*, Vol.127, pp. 260-269, ISSN 0014-4894

Mohamed, A.M.; Metwally, N.M.; Mahmoud, S.S. (2005). Sativa seeds against *Schistosoma mansoni* different stages, *Memórias do Instituto Oswaldo Cruz*, Vol.100, pp. 205-211, ISSN 0074-0276

Mølgaard,P.; Nielsen, S.B.; Rasmussen, D.E.; Drummond, R.B.; Makaza, N.; & Andreassen, J. (2001). Anthelmintic screening of Zimbabwean plants traditionally used against schistosomiasis. *Journal of Ethnopharmacology*, Vol. 74, pp. 257-264, ISSN 0378-8741

Moraes, J. ; Nascimento, C. ; Lopes, P.O. ; Nakano, E. ; Yamaguchi, L.F.; Kato, M.J. & Kawano T. (2011). *Schistosoma mansoni*: *In vitro* schistosomicidal activity of piplartine. *Experimental Parasitology*, Vol.127, pp. 357-364, ISSN 0014-4894

Moraes, J.; Silva, M.P.; Ohlweiler, F.P. & Kawano, T (2009). *Schistosoma mansoni* and other larval trematodes in *Biomphalaria tenagophila* (Planorbidae) from Guarulhos, São Paulo State, Brazil. *Revista do Instituto de Medicina Tropical de São Paulo*, Vol.51, pp. 77-82, ISSN 0036-4665

Morgan, D.C.; Mills, C.K.; Lefkowitz, L.D. & Lefkowitz, S.S. (1991). An improved colorimetric assay for tumor necrosis factor using WEHI 164 cells cultured on novel microtiter plates. *Journal of Immunological Methods*. Vol.145, pp. 259–262, ISSN 0022-1759

Mossmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*. Vol.65, pp. 55–63, ISSN 0022-1759

Mostafa, O.M.; Eid, R.A. & Adly, M.A. (2011). Antischistosomal activity of ginger (*Zingiber officinale*) against *Schistosoma mansoni* harbored in C57 mice. Parasitology Research, ISSN 0932-0113 doi 10.1007/s00436-011-2267-x

Nare, B.; Smith, J.M. & Prichard, R.K. (1991). Differential effects of oltipraz and its oxy-analogue on the viability of *Schistosoma mansoni* and the activity of glutathione S-transferase. *Biochemical Pharmacology*, Vol.42, pp. 1287-1292, ISSN 0006-2952

Ndamba, J.; Nyazema, N.; Makaza, N.; Anderson, C. & Kaondera, K.C (1994). Traditional herbal remedies used for the treatment of urinary schistosomiasis in Zimbabwe. *Journal of Ethnopharmacology*, Vol.42, pp. 125-132, ISSN 0378-8741

Newman, D.J. & Cragg, G.M. (2007). Natural products as sources of new drugs over the last 25 years *Journal of Natural Products*, Vol.70, pp. 461-477, ISSN 0163-3864

Newman, D.J.; Cragg, G.M. & Snader, K.M. (2003). Natural products as sources of new drugs over the period 1981-2002. *Journal of Natural Products*, Vol.66, pp. 1022-1037, ISSN 0163-3864

Ogboli, A. (2000) Medicinal application of *Vernonia amygdalina* del leaf extracts in the treatment of schistosomiasis in mice. *Nigerian Journal of Natural Products and Medicine*, Vol.4, pp. 73–75, ISSN 1118-6267

Parmar, V.S.; Jain, S.C.; Bisht, K.S.; Jain, R.; Taneja, P.; Jha, A.; Tyagi, O.M.; Prasad, A.K.; Wengel, J.; Olsen, C.E. & Boll, P.M. (1997). Phytochemistry of the genus *Piper*. *Phytochemistry*, Vol.46, pp. 597–673, ISSN 0031-9422

Parreira, N.A.; Magalhães, L.G.; Morais, D.R.; Caixeta, S.C.; de Sousa, J.P.; Bastos, J.K.; Cunha, W.R.; Silva, M.L.; Nanayakkara, N.P.; Rodrigues, V. & da Silva Filho A.A. (2010). Antiprotozoal, schistosomicidal, and antimicrobial activities of the essential oil from the leaves of *Baccharis dracunculifolia*. *Chemistry & Biodiversity*, Vol.7, pp. 993-1001, ISSN 1612-1872

Pereira, A.C.; Magalhães, L.G.; Gonçalves, U.O.; Luz, P.P.; Moraes. A.C.; Rodrigues, V.; da Matta Guedes, P.M.; da Silva Filho, A.A.; Cunha, W.R.; Bastos, J.K.; Nanayakkara, N.P. & E Silva, M.L. (2011). Schistosomicidal and trypanocidal structure-activity relationships for (±)-licarin A and its (-)- and (+)-enantiomers. *Phytochemistry*, Vol.72, pp. 1424-1430, ISSN 0031-9422

Peak, E. Chalmers, I.W. & Hoffmann, K.F. (2010). Development and validation of a quantitative, high-throughput, fluorescent-based bioassay to detect schistosoma viability, *PLoS Neglected Tropical Disease*, Vol.4, pp. e759, ISSN 1935-2735

Pechhold, K.; Craighead, N.; Wesch, D. & Kabelitz, D. (2002). Measurement of cellular proliferation. *Methods in Microbiology*, Vol.32, pp. 77-97, ISSN 0580-9517

Prasad, A. K.; Kumar, V.; Arya, P.; Kumar, S.; Dabur, R.; Singh, N.; Chhillar, A. K.; Sharma, G.; Ghosh, B.; Wengel, J.; Olsen, C. E. & Parmar, V. S. (2005). Investigations toward new lead compounds from medicinally important plants. *Pure and Applied Chemistry*, Vol.77, pp. 25-40, ISSN 0033-4545

Ramalho-Pinto, F.J.; Gazzinelli, G.; Howells, R.E.; Mota-Santos, T.A.; Figueiredo, E.A. & Pellegrino, J. (1974). *Schistosoma mansoni*: defined system for stepwise transformation of cercaria to schistosomule *in vitro*. *Experimental Parasitology*, Vol.36, pp. 360-372, ISSN 0014-4894

Ramirez, B.; Bickle, Q.; Yousif, F.; Fakorede, F.; Mouries, M.A. & Nwaka, S (2007). Schistosomes: Challenges in compound screening. *Expert Opinion on Drug Discovery*, Vol.2, pp. 53-61, ISSN 1746-0441

Reich, M.R. & Fenwick, A. (2001). *Schistosoma haematobium*. *The New England Journal of Medicine*, Vol.344, pp. 1170, ISSN 0028-4793

Riad, N.H.; Fares, N.H.; Mostafa, O.M.; Mahmoud, Y.I. (2007). The effect of garlic on some parasitological parameters and on hepatic tissues reaction in experimental schistosomiasis mansoni. *Journal of Applied Sciences Research*, Vol.3, pp. 949-960, ISSN 1819-544X

Ribeiro-dos-Santos. G.; Verjovski-Almeida, S. & Leite, L.C. (2006). Schistosomiasis – a century searching for chemotherapeutic drugs. *Parasitology Research*, Vol.99, pp. 505-521, ISSN 0932-0113

Sanderson, L.; Bartlett, A. & Whitfield, P.J. (2002). *In vitro* and *in vivo* studies on the bioactivity of a ginger (*Zingiber officinale*) extract towards adult schistosomes and their egg production. *Journal of Helminthology*, Vol.76, pp. 241–247, ISSN 0022-149X

Sarvel, A.K.; Kusel, J.R.; Araujo, N.; Coelho, P.M.Z. & Katz, N. (2006). Comparison between morphological and staining characteristics of live and dead eggs of *Schistosoma mansoni*. *Memórias do Instituto Oswaldo Cruz*, Vol.101, Suppl. I, pp. 289-292, ISSN 0074-0276

Sheir, Z.; Nasr, A.A.; Massoud, A.; Salama, O.; Badra, G.A.; El-Shennawy, H.; Hassan, N. & Hammad, S.M. (2001). A safe, effective, herbal antischistosomal therapy derived from myrrh. *The American Journal of Tropical Medicine and Hygiene*, Vol.65, pp. 700-704, ISSN 0002-9637

Skelly, P.J. & Alan Wilson, R. (2006). Making sense of the schistosome surface, *Advances in Parasitology*, Vol.63, pp. 185-284, ISSN 0065-308X

Smithers, S.R. & Terry, R.J. (1965). The infection of laboratory hosts with cercariae of *Schistosoma mansoni* and the recovery of adults worms. *Parasitology*, Vol.55, pp. 695-700, ISSN 0031-1820

Smout, M.J.; Kotze, A.C.; McCarthy, J.S. & Loukas, A. (2010). A novel high throughput assay for anthelmintic drug screening and resistance diagnosis by real-time monitoring of parasite motility. *PLoS Neglected Tropical Disease*, Vol.4, pp. e885, ISSN 1935-2735

Steinmann, P.; Keiser, J.; Bos, R.; Tanner, M. & Utzinger, J. (2006). Schistosomiasis and water resources development: systematic review, meta-analysis, and estimates of people at risk. *The Lancet Infectious Diseases*, Vol.6, pp. 411–425, ISSN 1473-3099

Tagboto, S. & Townson, S. (2001). Antiparasitic properties of medicinal plants and other naturally occurring products. *Advances in Parasitology*, Vol.50, pp. 199-295, ISSN 0065-308X

Utzinger, J.; Keiser, J.; Shuhua, X.; Tanner, M. & Singer, B.H. (2003). Combination chemotherapy of schistosomiasis in laboratory studies and clinical trials. *Antimicrobial Agents and Chemotherapy*, Vol.47, pp. 1487-1495, ISSN 0066-4804

Utzinger, J.; Xiao, S. N'Goran, E.K.; Bergquist, R. & Tanner, M (2001). The potential of artemether for the control of schistosomiasis. *International Journal for Parasitology*, Vol.31, pp. 1549-1562, ISSN 0020-7519

Utzinger J, Xiao, S.H.; Tanner, M. & Keiser, J. (2007). Artemisinins for schistosomiasis and beyond. *Current Opinion in Investigational Drugs*, Vol.8, pp. 105-116, ISSN 1472-4472

Van der Linden, P.W. & Deelder, A.M. (1984). *Schistosoma mansoni*: a diamidinophenylindole probe for *in vitro* death of schistosomula. *Experimental Parasitology*, Vol.57, pp.125-131, ISSN 0014-4894

van der Werf, M.J.; de Vlas, S.J.; Brooker, S.; Looman, C.W.; Nagelkerke, N.J.; Habbema, J.D. & Engels, D. (2003). Quantification of clinical morbidity associated with schistosome infection in sub-Saharan Africa. *Acta Tropica*, Vol.86, pp. 125-139, ISSN 0001-706X

Van Hellemon, J.J.; Retra, K.; Brouwers, J.F.; van Balkom, B.W.; Yazdanbakhsh, M.; Shoemaker, C.B. & Tielens, A.G. (2006). Functions of the tegument of schistosomes: clues from the proteome and lipidome. *International Journal for Parasitology*, Vol.36, pp. 691-699, ISSN 0014-4894

Xiao, S.; Tanner, M.; N'Goran, E.K.; Utzinger, J.; Chollet, J.; Bergquist, R.; Chen, M. & Zheng, J. (2002). Recent investigations of artemether, a novel agent for the prevention of schistosomiasis japonica, mansoni and haematobia. *Acta Tropica*, Vol.82, pp. 175-181, ISSN 0001-706X

Xiao, S.H.; Keiser, J.; Chollet, J.; Utzinger, J.; Dong, Y.; Endriss, Y.; Vennerstrom, J.L. & Tanner, M. (2007). *In vitro* and *in vivo* activities of synthetic trioxolanes against major human schistosome species. *Antimicrobial Agents and Chemotherapy*, Vol.51, pp. 1440–1445 ISSN 0066-4804

Xiao, S.H.; Binggui, S.; Chollet, J. & Tanner, M. (2000). Tegumental changes in 21-day-old *Schistosoma mansoni* harboured in mice treated with artemether. *Acta Tropica*, Vol.75, pp. 341–348, ISSN 0001-706X

Yousif, F.; Hifnawy, M.S.; Soliman, G.; Boulos, L.; Labib, T.; Mahmoud, S.; Ramzy, F.; Yousif M.; Hassan, I.; Mahmoud, K.; El-Hallouty, S.M.; El-Gendy, M.; Gohar, L.; El-Manawaty, M.; Fayyad, W. & El-Menshawi, B.S. (2007). Large-scale *in vitro* screening of Egyptian native and cultivated plants for schistosomicidal activity. *Pharmaceutical Biology*, Vol. 45, pp. 501–510, ISSN 1388-0209

21

Control of Schistosomiasis and Soil-Transmitted Helminthiasis in Sub-Saharan Africa: Challenges and Prospects

Louis-Albert Tchuem Tchuenté
University of Yaoundé I, Laboratory of Parasitology and Ecology, Centre for Schistosomiasis and Parasitology, Yaoundé Cameroon

1. Introduction

Schistosomiasis and soil-transmitted helminthiasis (STH) are the most common types of parasitic infections in the world. These diseases have major health and socio-economic repercussions, and constitute an important public health problem in developing countries. Human schistosomiasis is caused by six species of schistosomes, i.e. *Schistosoma haematobium*, *Schistosoma mansoni*, *Schistosoma japonicum*, *Schistosoma mekongi*, *Schistosoma intercalatum* and *Schistosoma guineensis*; and is endemic in 78 countries, where 779 million people are at risk of infection. *S. haematobium* is responsible for urogenital schistosomiasis, and the other species cause intestinal schistosomiasis. It is estimated that 207 million people are infected (WHO, 2002; Steinmann et al. 2006). STH, also known as intestinal worm infection, is caused by four main species of worms commonly known as roundworms (*Ascaris lumbricoides*), whipworms (*Trichuris trichiura*) and hookworms (*Ancylostoma duodenale* and *Necator americanus*). It is estimated that STH affects more than 2 billion people worldwide, and the greatest numbers of infections occur in sub-Saharan Africa, the Americas, China and east Asia (WHO, 2006; Hotez et al., 2006; Brooker et al., 2006; Awasthi et al., 2003).

These diseases affect the poorest of the poor and infections are particularly abundant among people living in rural or deprived urban settings with low socio-economic status, lack of clean water and poor sanitation (Hotez et al., 2006). The morbidity caused by these worms is commonly associated with heavy infection intensities. Compared with any other age group, school-aged children and pre-school children are the most vulnerable group and they harbor the greatest numbers of intestinal worms. As a result, they experience growth stunting and diminished physical fitness as well as impaired memory and cognition (Crompton and Nesheim, 2002; Stephenson et al., 2000; Bethony et al., 2006). These adverse health consequences combine to impair childhood educational performance and reduce school attendance (Miguel & Kremer, 2004; Hotez et al., 2008). Studies have demonstrated that children may acquire helminth infections early in life (Sousa-Figueiredo et al., 2008; Stothard et al., 2008); which causes initial organ damage that can remain subclinical for years and manifest overtly only later, in adulthood (WHO, 2006; Odogwu et al., 2006).

Despite the existence of tools in the 1970s and 1980s, control was sustained for a prolonged period only in few countries and almost no progress was made in sub-Saharan African

countries, the most affected part of the world. In the 1990s, interest in the control of these diseases in Africa waned. Therefore, as with other neglected tropical diseases (NTDs), schistosomiasis and STH control has been overshadowed by other health priorities. The highest priority of the international health community was given to the 'big three', i.e. HIV/AIDS, tuberculosis and malaria, with less attention to other infections related to poverty (Molyneux et al., 2005).

Recent years have witnessed an increased interest in the control of NTDs, and today there exists a global momentum for the control of these diseases. The control of NTDs has become a priority on the agenda of many governments, donors and international agencies. The World Health Organization (WHO) has played a major role in this prospect. Indeed, under the aegis of WHO, all member states of WHO (over 200 countries) have endorsed in May 2001 the World Health Assembly resolution WHA 54.19, with as a major objective the regular treatment of at least 75% of all school-aged children at risk of morbidity by 2010. The renewed impetus for schistosomiasis and STH control has generated a greater political commitment, as well as an unprecedented opportunity for cost-effective action (Molyneux et al., 2005). This momentum has encouraged many countries to establish national action plans and programmes to control schistosomiasis, STHs and other NTDs (Hotez et al., 2009; Tchuem Tchuenté & N'Goran, 2009). Within the past decade, significant progress has been made on large scale treatments through integrated control of schistosomiasis, STH and other NTDs, thanks to a number of international organizations, donor foundations, bilateral institutions and non-governmental organizations that responded to the 2001 WHO's call for action (Savioli et al., 2009). Today, treatment is cost-effective and the 'preventive chemotherapy' is currently the strategy of choice (WHO, 2006). With a support from the American (USAID) and British (DFID) governments, as well as the Bill and Melinda Gates Foundation, the pharmaceutical industry, and many not-for profit organizations, millions of children are regularly treated for schistosomiasis, STH and other NTDs. However, the control of these diseases is a long-term undertaking which involves several challenges. This paper highlights the progress made and also focuses on some main challenges that are reviewed and discussed.

2. Epidemiology and burden of schistosomiasis and STH

Schistosomiasis and STH transmission are intimately associated with poverty and poor sanitation. For schistosomiasis, infecion is caused by penetration of the skin by larvae in water; whereas for STH, infection is caused by the ingestion of parasite eggs from contaminated food or dirty hands – in the case of *A. lumbricoides* and *T. trichiura* – or by active penetration of the skin by larvae in the soil – in the case of hookworms. People who get infected carry parasite eggs in the feces or in the urine (in the case of urinary schistosomiasis), and in areas where there is no latrine systems the soil and water around the villages and communities are contaminated with feces or urine containing worm eggs. Although schistosomiasis and STH infections occur predominantly in rural areas, the social and environmental conditions in many unplanned slums and squatter settlements of developing countries are ideal for their persistence (Crompton & Savioli, 1993). In endemic populations, infections are aggregated: most infected individuals in a community will have infections of a light or moderate intensity, while a few will be heavily infected. Heavily infected individuals suffer most of the clinical consequences of the infections and are the major source of infection for the rest of the community (WHO, 2002).

The epidemiology of helminth infections is influenced by several key determinants, including environment, population heterogeneity, age, household clustering, genetics and polyparasitism (Hotez et al., 2008). In recent years, considerable progress has been made in the use of geographical information system (GIS) and remote sensing (RS) to better understand helminth ecology and epidemiology, and to develop low-cost ways to identify target populations for treatment. GIS and RS were used to describe the global distribution of schistosomiasis and STH infections and to estimate the number of infections in school-age children in sub-Saharan Africa.

There is considerable geographical variation in the occurrence of infections (Brooker et al., 2009). In general, changes with age in the average intensity of helminth infections tend to be convex, rising in childhood and declining in adulthood. For schistosomiasis, *A. lumbicoides* and *T. trichiura*, the heaviest and most frequent infections are in children aged 5–15 years, with a decline in intensity and frequency in adulthood (Gilles, 1996). In contrast, hookworm frequently exhibits a steady rise of intensity of infection with age, peaking in adulthood (Bethony et al., 2002). Household clustering of infected individuals has been demonstrated for STH (Forrester et al., 1988), and this can persist through time, as shown by familial predisposition to heavy infection with *A. lumbricoides* and *T. trichiura* (Forrester et al., 1990).

Because morbidity is associated with worm burden rather than the absence or presence of infection, prevalence is commonly combined with worm burden (intensity of infection) to assess the epidemiological situation for schistosomiasis and STH infections. Worm burden is commonly measured by the number of eggs per gram (EPG) of feces or eggs per 10ml of urine (Anderson, 1982; Montresor et al., 1998). Prevalence and intensity of infections are used to classify communities into transmission categories, which enable to determine the appropriate type of mass treatment a community should receive (WHO, 2006). Both should be assessed in monitoring the impact of deworming campaigns.

3. Control of schistosomiasis and STH

3.1 Progress towards the 2010 global target

Progress in implementing schistosomiasis and STH control programmes has been slow but steady. Since 2006, there has been an overall increase in the number of people treated for schistosomiasis and STH. The increase in treatments has occurred entirely in the African Region, where the number of people treated more than doubled from 2006 to 2009 (WHO, 2011). This number increased by 93% in 2010. The increase in the number treated suggests that both governments and their donor partners are now investing in schistosomiasis control (WHO, 2012). In 2010, 18 over 42 schistosomiasis endemic countries in the African region and 34/46 STH endemic countries or territories reported their treatment data to WHO. Overall, 27,983,327 people were treated for schistosomiasis, and 91,025,863 children for STH (Table 1).

Characteristics	Schistosomiasis	STH
No. endemic countries	42	46
No. countries reporting MDA data	18	34
No. people requiring treatment	220 578 484	283 800 000
No. people treated	27 983 327	91 025 863
No. pre-school aged children treated	-	42 711 551
No. school aged children treated	-	48 314 312

Table 1. African Regional summary of children treated for schistosomiasis and STH, 2010

Figure 1 illustrates the progress in Africa ten years after the 2001 WHA resolution. Although significant progress has been made over the past years to significantly reduce schistosomiasis and STH infection prevalence below low risk, or to regularly implement mass drug administration (MDA) in several countries, the global achievement is still very far from the WHO's target of regular deworming of at least 75% of school-age children at risk. Indeed, from the data of epidemiological coverage of STHs, it was estimated that only 15% of school-aged children at risk of infection have been treated with preventive chemotherapy in 2008 (WHO, 2010). School-based deworming interventions still cover only a minority of children considered to be at risk despite the low cost of these interventions and their significant impact on health. More worrying, the number of people treated for schistosomiasis in Sub-Saharan Africa is estimated to be only 6.71% of the people infected (WHO, 2011). The major constraint to controlling schistosomiasis continues to be the limited access to praziquantel (Hotez et al., 2010). In the African Region, only few countries (18 in 2008) have achieved the 75% treatment target.

3.2 Taking advantage of integrated control of NTDs

In the developing world, polyparasitism is the norm rather than the exception (Molyneux et al., 2005; Fleming et al., 2006; Tchuem Tchuenté et al., 2003). In large parts of the world, particularly in Africa, most children are infected by more than one species of helminth. These NTDs frequently overlap geographically and they impose a great burden on poor populations, affecting the same individuals. Therefore, the current strategy for NTD control is to integrate interventions for multiple diseases (Molyneux et al., 2005). This integrated approach is the basis for cost-effectiveness and streamlined efficiency. Also, because many of the drugs used for mass treatment are provided free of charge by major multinational pharmaceutical companies, the MDA approach is the most cost-effective global public health control measure (Hotez et al., 2007). Schistosomiasis and STH infections are the most prevalent and widespread of the common NTDs, and they overlap in many parts with many of the other NTDs. Therefore, an integration of schistosomiasis and STH control with other helminth control programmes and a good coordinated use of (donated) drugs would be highly beneficial for their control. This would indeed allow to take advantage of drug donation and co-administration, and to optimize the preventive chemotherapy.

Within the past decade, significant progress has been made on large-scale treatment of schistosomiasis and STH through integrated control with other NTDs, thanks to a number of international organizations, donor foundations, bilateral institutions and non-governmental organizations that responded to the 2001 WHO's call for action (Savioli et al., 2009). With a support from the American (USAID) and British (DFID) governments, as well as the Bill and Melinda Gates Foundation, the pharmaceutical industry, and many not-for profit organizations, millions of children are regularly treated for schistosomiasis, STH and other NTDs. Today, an integrated control of NTD using the preventive chemotherapy is operating in more than 15 countries. Within the first three years (2006-2009) of implementation of the USAID NTD Control Program, the number of persons reached each year increased progressively, with a cumulative total of 98 million persons receiving 222 million treatments (Linehan et al., 2011). In West Africa, nearly 13.5 million doses of albendazole have been administered against STH between 2004 and 2006 in Burkina Faso, Mali and Niger, with coverage rates varying between 67.0% and 93.9% (Garba et al., 2009). Monitoring and evaluation activities after large-scale administration of praziquantel for schistosomiasis and albendazole for STH showed a significant decrease in the intensity of infections. Also, there was a significant increase in haemoglobin concentration after 1 and 2

(A)

(B)

Countries likely to have achieved interruption of transmission or towards elimination
Countries with on-going large-scale schistosomiasis control programme
Countries with limited or no schistosomiasis control programme

Fig. 1. Comparative status of schistosomiasis control in Africa at the end of the 20th century (A) and in 2010 (B).

years of treatment (Fenwick et al., 2009). Combination of ivermectin and albendazole, and co-administration of praziquantel and albendazole or mebendazole have been proven to be safe, with no side effects outside those commonly associated with each of these drugs (Horton et al., 2000; Olds et al., 1999). Though WHO does not formally recommends combination of praziquantel and ivermectin yet, recent studies have demonstrated the safety of triple co-administration of praziquantel, albendazole and ivermectin in areas where schistosomiasis, STH and LF or/and onchocerciasis are co-endemic and where several rounds of treatment with one or two drugs have been implemented in the past (Mohammed et al., 2008).

3.3 A country example: Cameroon

In Cameroon, schistosomiasis and STH are important parasitic diseases. Recent estimates indicate that at least 2 million people are infected with schistosomiasis, 5 million are at risk and more than 10 million are infected with gastrointestinal helminths (Minsante, 2005). In 1983, a pilot project for schistosomiasis control, funded by USAID, was set up. Within this framework a national epidemiological survey of schistosomiasis and STH was conducted between 1985 and 1987. Overall, 49 divisions, 504 schools and 23 850 schoolchildren were investigated. The results showed the occurrence of *S. haematobium, S. mansoni, S. guineensis, A. lumbricoides, T. trichiura* and *N. americanus* as the major helminth species. The highest transmission levels of schistosomiasis occured in the northern part, whereas STH were more prevalent in the southern part of the country (Ratard et al., 1990, 1991; Brooker et al., 2000). When considering all these helminthic diseases, no region of the country is spared. However, this pilot project stopped in 1989 when the USAID support ended.

Taking advantage of the renewed momentum for NTDs, the national programme for the control of schistosomiasis and STH was created in March 2003. There is a strong political commitment from the Ministries of Public Health and Basic Education. These two ministries work in close collaboration and this inter-sectorial engagement is consolidated by the fact that the national steering committee for the control is co-chaired by the Minister of Public Health and the Minister of Basic Education, as president and vice-president, respectively. The Cameroonian proposal for support to SCI was presented by the Minister of Public Health himself who attended the SCI advocacy meeting in London in July 2003. Therefore, the non-selection of Cameroon for SCI support came both as a surprise and a disappointment. Beyond the main ranking criteria, including the existence of a strategic plan for control, the strong political commitment and the quality of the proposal, the SCI selection was finally made on a regional combination of three East African countries and three West African countries, with emphasis on country-regional collaboration and consortium. In spite of this, the Cameroonian government made necessary efforts to ensure the success of the schistosomiasis and STH control, as it was among the priority programmes of the country. Hence, the national control programme was officially launched on 25 March 2004.

The action of the programme during the past few years was intense and multifaceted, with a number of key achievements. Based on the limited resources available, the priority activities were centred on three major activities: (1) the production of various strategic documents necessary for the implementation of the activities and advocacy; (2) the advocacy and the mobilisation of partners and funding; and (3) the implementation of activities in selected areas. A strong emphasis was put on advocacy, the results of which were encouraging for future activities and plans. The most important was the selection of Cameroon as the first

start-up country for mebendazole donation in Africa. Indeed, in 2005 Johnson & Johnson established a partnership with the Task Force for Child Survival and Development (currently The Task Force for Global Health) to develop a programme to donate mebendazole via a multi-disciplinary initiative designed to address intestinal worm infections in the most at-risk children of the world. Cameroon was selected as the start-up country for this drug donation programme because of its leadership and commitment to eliminating infections as a major public health problem (http://www.janssenpharmaceutica.be/download_Cameroen_N.asp).
With regard to control, a pilot phase was completed in February 2006 in one health district, where approximately 20,000 school-aged children were treated with praziquantel and albendazole. Subsequently, the activities were extended to one entire region of the ten regions in Cameroon, i.e. the Adamawa region in the northern part of the country. This was implemented with support from partners, including the World Food Programme (WFP), the Canadian Co-operation, the United Nations International Children's Emergency Fund (UNICEF) and SCI/Medpharm (which donated the drugs praziquantel and albendazole). Deworming was conducted in all 500 primary schools in this region, and approximately 150,000 school-aged children were treated in May 2006. Overall, 700 head teachers, 500 representatives of parent teacher associations, and 2500 teachers were trained. In addition, parasitological surveys for schistosomiasis and STH were conducted in 40 selected schools where stool and urine samples were collected from a total of 1830 children.
The mebendazole donation enabled the national control programme to scale-up activities rapidly. As a result, deworming activities were increased to encompass all ten regions. In 2007, Cameroon launched a nationwide deworming campaign, and 4 million school-aged children were treated. The launching ceremony allowed the government and partners to further reaffirm their commitment and to galvanise communities, international development agencies, non-governmental organisations (NGOs) and other stakeholders to join in the effort to implement fundamental improvements in disease control and prevention. The country has in place school-based and community-directed channels and in the programme teachers and community drug distributors administer the drugs to children along with health and hygiene education. The major activities conducted are: (1) training of health and education personnel, (2) sensitization and education of communities about the disease, the risks of infection and measures for prevention, (3) promotion of hygiene, safe water and sanitation systems in communities, and (4) deworming of children. Since 2007, 4 million school-age children are treated annually with mebendazole, with the involvement of over 75,000 trained teachers and head teachers. Control of STH is primarily implemented through school-based distribution of mebendazole, co-administered with praziquantel where schistosomiasis is endemic. In addition, albendazole is co-administered with ivermectin during the community-directed treatment for LF. Furthermore, over 2.7 million children aged 1–5 years are dewormed during child health week campaigns implemented twice a year (Tchuem Tchuenté and N'Goran, 2009).
Moreover, parasitological surveys were conducted in selected schools in all 63 health districts of the Centre, East and West regions of Cameroon, in order to update the disease distribution map, to assess the impact of previous deworming campaigns, and to determine where treatment with PZQ should be extended. The results showed significant variation of schistosomiasis and STH prevalence between schools, villages, districts and regions. In comparison to previous mapping data collected 25 years ago, the results showed an increase of schistosomiasis transmission in several health districts, where PZQ MDA was not implemented so far. On the contrary, there was a significant decline of STH infection

prevalence and intensities in all three regions, with an overall decline of prevalence from 90.06% (95% CI: 89.45-90.63%) to 24.11% (95% CI: 23.37-24.86%). Based on the prevalence data, the continuation of annual or bi-annual MDA for STH was recommended, as well as an extension of PZQ treatment in identified moderate and high risk communities for schistosomiasis (Tchuem Tchuenté et al., 2012). These results show the positive impact of annual deworming campaigns, and illustrate the progressive success of the national programme for the control of schistosomiasis and STH in Cameroon. This is illustrated in Figure 2. Parasitological surveys are in progress in the remaining regions of Cameroon for the update mapping.

4. Challenges

The control of schistosomiasis and STHs is a long-term undertaking, which involves several challenges. The first challenge is to mobilize the funds required for successful actions. Unlike some of the stigmatising NTDs with 'visible' morbidity such as onchocerciasis and LF which have successfully raised funding for the implementation of control activities, little funding are globally available for the implementation of schistosomiasis and STH control alone. Therefore, for their schistosomiasis and STH control programmes, endemic countries should take advantage of the opportunities resulting from the global growing financial resources generated by the new partnerships and impetus for NTD control. The recent changes in the international agenda and the increasing funding opportunities provide leverage for the control of schistosomiasis and STHs. The piggyback and integrated control approaches – based on a complementary co-administration of relevant drugs – are the basis for cost-effectiveness and streamlined efficiency. However, pigging back on other interventions or integration with other health programmes requires the setting up of clear efficient coordination mechanisms in order to optimize the use of resources mobilized from various partners. Among other challenges, the most important are the co-ordination at various levels, the country ownership and leadership, the scale up of control, the sustainability of control programmes, the strengthening of partnerships and institutional capacities, the implementation of operational research, monitoring and evaluation, and the development of new diagnostic tools.

4.1 Co-ordination

The NTD control programmes involve many partners and stakeholders, different diseases, several funding sources, and multiform resources. The main challenge is to set up clear efficient coordination mechanisms in order to optimize the resources mobilised from all partners. To ensure transparency in all processes, there is a need for a coordination mechanism for funding, drug supplies and integrated control of NTDs to enable all stakeholders to share information. The coordination mechanism should be at global, regional and country levels. The precise form of this co-ordination mechanism needs to be explored and agreed amongst stakeholders. At national level, the Ministry of Health should play the central role in this co-ordination.

4.2 Country ownership and leadership

In order to ensure the sustainability and the success of NTD control programmes, country leadership in the coordination of activities is essential. Each partner may have its own vision and own interest, and the ones who bring the money also sometimes decide what to do.

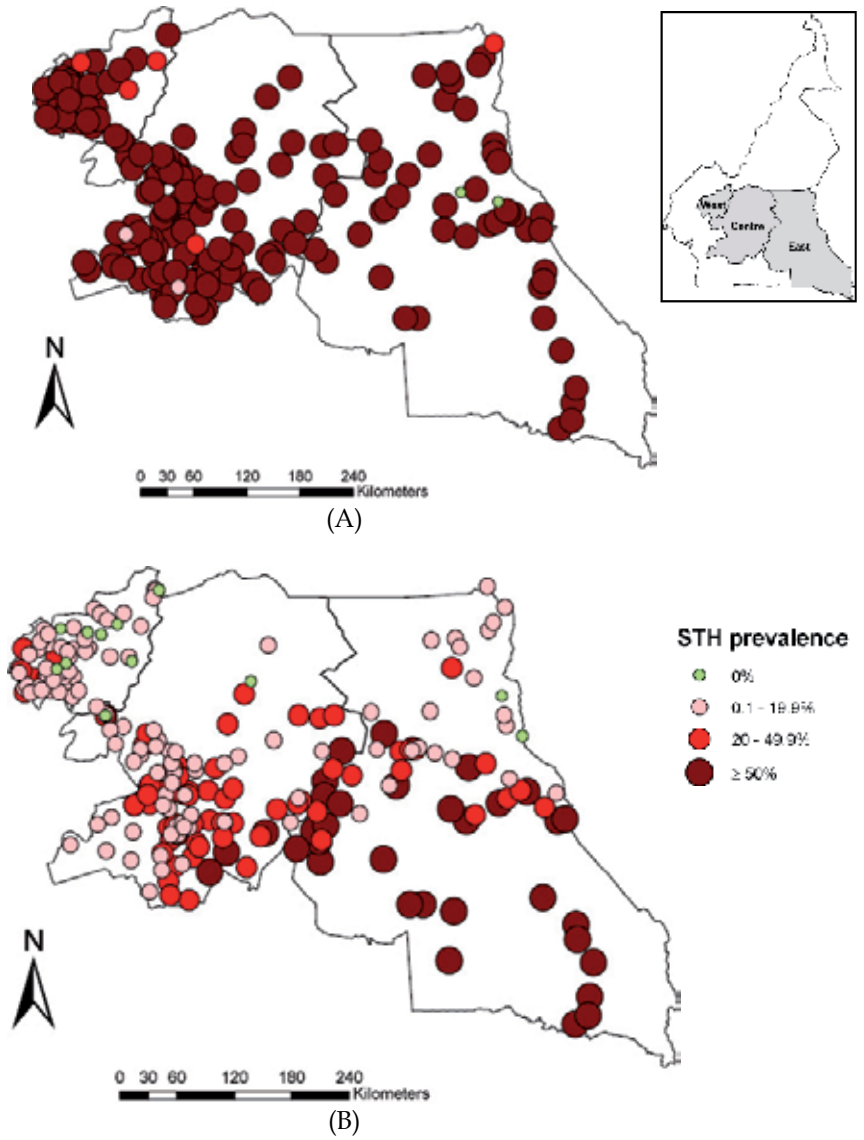


Fig. 2. Comparative maps of the overall soil-transmitted helminthiasis prevalence between the years 1985-1987 (A) and 2010 (B) in the Centre, East and West regions of Cameroon.

Sometimes, many people in a country are working for the same thing, but with different targets because there are different donors. Country ownership brings many gains, including development of local capacity and expertise. There should be a strong political commitment to tackle NTDs in endemic countries. Indeed, examples of success stories are from those countries (e.g. Japan, Morocco and China) that had clear ambitious goal, with high government and national commitment, funding mobilization and leadership. For example, in early 1963 STH occurred in Okinawa, Japan at high prevalence up to 40% in adult population. Through the development of an ambitious 'zero parasites' campaign a successful control was conducted and within a period less than 10 years STHs and other

parasites were eradicated in Okinawa. In contrary, lack of ambitious control is remarkable in Sub-Saharan Africa where many countries have passive attitude, waiting for partners to decide and to tell them what to do. African governments should develop more positive and ambitious approach for the control of NTDs, and donors and agencies beyond disease endemic countries—the agents of aid to developing countries—will need to accept that countries must have ownership of their health systems and total control over decisions about the health of their people.

4.3 Scale up of control

In order reach the target set by WHA for the different NTDs, there is a need to accelerate the extent of treatment. The extension of regular deworming coverage as a public health intervention to reach all individuals at risk remains a challenge. There is also a challenge to tackle the big countries such as Nigeria, Democratic Republic of Congo, Ethiopia and Tanzania which count for 60% of at risk population not covered by preventive chemotherapy yet. In the context of multi-disease integrated control, the current tendency in sub-Saharan Africa is to integrate helminth control within the community-directed interventions, taking advantages of the long experience and gains of onchocerciasis and LF control programmes through the establishment of community-directed treatment with ivermectin (CDTI) in all countries supported by the African Programme for Onchocerciasis Control – APOC (Sékétéli et al., 2002; Amazigo et al., 2002). Yet, in the majority of countries, STHs are more widely distributed than both onchocerciasis and LF. Therefore, the challenge will be to extend MDA coverage in those areas where CDTI does not exist. Countries should thus act cautiously and anticipate to avoid to neglect STH and schistosomiasis control in those endemic areas. School-based deworming is probably the simple, cost-effective and best sustainable way to expand coverage of children. With more schools than clinics, and more teachers than health workers, the existing and extensive education infrastructure provides the most efficient way to reach the highest number of school-age children and to reach the WHO target of covering at least 75% of all school-aged children in need. The recent increases of donated anthelminthic medicines by Merck KGaA (praziquantel), Johnson & Johnson (mebendazole) and GlaxoSmithKline (albendazole) constitute a real boost towards the achievement of this goal.

4.4 Sustainability

To roll back the NTDs, it is necessary to continue and maintain the implementation of control activities during very long period. The programme should be long-term, and financial and implementation plans should be made accordingly. This would avoid one of the major errors of the past where most of the programmes were supported by short-term external funds and stopped immediately when funds ceased. To ensure sustainability, countries should, from the beginning, envisage strategies to: (1) keep the momentum, interest and enthusiasm for the control of the diseases; (2) organize and finance in a sustained way the distribution of drugs to those who need them; (3) mobilize funding for operational costs for drug distribution, health education materials, baseline data collection, monitoring and evaluation, etc.; (4) ensure the availability and affordability of the drugs at community level in endemic areas; (5) integrate programme activities within the existing health structures and networks; and (6) strengthen the health system and national capacity at all levels, especially in the peripheral and remote areas which manage the greater number of communities and infected persons. One of the key questions is how to guarantee the continuation of control after the decline or interruption of external funds.

4.5 Strengthening of partnership

An efficient control requires multisector collaboration and multilateral funding. No single organization can hope to achieve this goal alone. School-aged children are the target group for schistosomiasis and STH, so the collaboration between the Ministry of Health and the Ministry of Education should be reinforced. The target group for the different filarial infections is the entire population and the control strategy is based on community-directed interventions, which requires ownership by the communities and strong partnership with NGOs. Overall, partnership with other government departments and NGOs concerned with water supply, sanitation and development projects should be developed and reinforced.

4.6 Strengthening of institutional capacities

The integration of control activities within the health services is fundamental for the sustainability of the programme. However, most of the existing structures do not have the capacity to ensure the implementation of these activities, leading to programme failure. Therefore, the strengthening of the health system is a requirement for the long-term viability of the control programme. Furthermore, an efficient participation of schools and communities in the disease control requires a reinforcement of capacity of the school system and the community ownership.

4.7 Operational research

It is important to support operational research to fulfill gaps and improve the implementations of the control. For example, the understanding of some key issues, such as baseline epidemiology, morbidity assessment, drug efficacy testing and monitoring of drug resistance, is of vital importance.

4.8 Monitoring and evaluation

Regular monitoring and evaluation are necessary to ensure that programmes are efficiently implemented and that the beneficiaries gain the maximum benefit. This is particularly challenging given the diversity of tools and indicators. Substantial progress has been made towards the development of standardized tools for monitoring and evaluating NTD control programmes. WHO is currently developing guidelines based on the experience to date with integrated preventive chemotherapy interventions.

4.9 Better diagnostic tools

Because of its simplicity and relatively low-cost, the Kato–Katz technique (Katz et al., 1972) is widely used for epidemiological field surveys and is recommended by the WHO for surveillance and monitoring of schistosomiasis and STH control programmes (WHO, 1991; Montresor et al., 1998). Though the specificity is very high, the sensitivity of Kato–Katz in single stool sample examination is limited by day-to-day variation in egg excretion leading to measurement error in estimating the presence of infection. This is particularly accentuated in areas with high proportions of light intensity infections (Hall, 1981; Booth et al., 2003; Tarafder et al., 2009). In the current era of preventive chemotherapy, the intensification of large-scale interventions and repeated mass deworming will significantly reduce the prevalence and intensities of schistosomiasis and STH infections (Savioli et al., 2004; Kabatereine et al., 2007). As consequence of the increase of low-intensity infections, more light infections will be often missed if single stool samples are examined by Kato–Katz method, resulting in high underestimation rates. Therefore, there is a need to develop and

validate more sensitive diagnostic tools for accurate surveillance and monitoring of schistosomiasis and STH control programmes, and for monitoring of drug efficacy. Some studies recommended multiple stool samples in order to avoid underestimating the 'true' prevalence and transmission potential of schistosomiasis and STH infections. Indeed, it was demonstrated that Kato–Katz examination of 3 instead of 1 stool specimen increased the sensitivity of helminth diagnosis, most notably for hookworm (Steinmann et al., 2008; Knopp et al., 2008) and intestinal schistosomiasis (De Vlas and Gryseels, 1992; Lin et al., 2008). Recent studies suggested that urine circulating cathodic antigen (CCA) assays maybe an appropriate test for the diagnosis of *S. mansoni* in moderate transmission zones (Shane et al., 2011; Stothard et al., 2011; Tchuem Tchuenté et al., 2012). Several alternative stool examination techniques have been tested for the detection of STH infections. FLOTAC, a new technique mainly used in the veterinary field, was suggested as a suitable diagnostic tool particularly in situations of low parasite infection intensities (Cringoli, 2006). Recent studies found that a single FLOTAC examination was more sensitive than triplicate Kato–Katz thick smears for the diagnosis of low-intensity STH infections (Knopp et al., 2009). In particular, the FLOTAC technique improves the ability to diagnose human hookworm infections accurately (Utzinger et al., 2008), which is generally underestimated when using Kato–Katz thick smears due to a rapid disintegration of hookworm eggs and the constraint to read the slides very shortly (within 30 min) after preparation (Dacombe et al., 2007). FLOTAC was thus suggested as a suitable method for a rigorous surveillance of helminth control programmes, monitoring of STH transmission and verification of local elimination (Knopp et al., 2009). McMaster, another flotation technique commonly used in veterinary parasitology, is an alternative diagnostic tool. The results of a comparative study of four techniques, i.e. ether-based concentration, Parasep Solvent Free, McMaster and FLOTAC, showed that despite the fact that McMaster was less sensitive than FLOTAC, the former technique was the most feasible and easy to perform under field conditions. McMaster appeared as a promising technique of choice when using fecal egg counts for monitoring of drug efficacy against STHs (Levecke et al., 2009). Overall, several techniques are available for the detection of STH infections, with significant difference in the cost, sensitivity, simplicity and field applicability. Though a true 'gold standard' test with 100% accuracy does not exist, Kato–Katz thick smears is so far commonly and widely used as the basic and 'default' technique for helminth epidemiology, despite some limitations.

Further efforts should be made to validate other detection tools (Tchuem Tchuenté, 2011). The choice of a specific diagnostic assay should be governed by the objective of the activity, and according to the stage of helminth control (Berquist et al., 2009). As the accuracy of a given diagnostic technique may vary significantly according to helminth transmission level, tools should be adapted when moving from morbidity control to elimination of infection. Moving toward the surveillance and elimination phases requires more sensitive techniques such as antibody detection. However, sero-diagnostic tools for detection of helminth infections require blood sample collection (invasive), access to affordable, high-quality reagents; which are important limiting factors for their integration into large-scale national control programmes. These are probably some of the reasons why today only a few countries have adopted antibody detection as a key strategy in helminth diagnosis (Berquist et al., 2009).

4.10 New paradigm shift: Moving from control to elimination

In the past years, the costs of PZQ and the lack of resources were major constraints for the control of schistosomiasis. Today, there exists new impetus for the control, with increasing funding opportunity and donated PZQ by pharmaceutical companies. Therefore, there is an

opportuny to take up the challenge, to be more ambitious and to move from control to elimination where feasible. To do this there is a need to adapt the current threshold for intervention (i.e. prevalence > 10%) and to carefully define the implementation unit for PZQ mass administration. Treatment algorithms should be re-defined based on current knowledge and experiences.

5. Conclusion

During the past several decades, many attempts have been made to control schistosomiasis, STH and other NTDs. Important advances have been made such as the development of praziquantel, albendazole and mebendazole, the current drugs of choice for these NTDs, which amplified treatment possibilities to the majority of people who need it. Although few control successes were achieved, the global objective is long way off and these diseases remain important public health problems in developing countries. Fulfilling the mandate of the World Health Assembly resolution 54.19 will require the regular treatment of hundreds of millions of children over decades. A successful control of schistosomiasis and STHs across the endemic regions calls for strengthening health system and interventions, ensuring access to anthelminthic drugs in all health services, co-implementation and the coordinated use of the different anthelminthic drugs, promotion of access to safe water, adequate sanitation and health education, and mobilization of resources to sustain control activities. The challenge also includes an improvement of drug efficacy for a much better rapid impact. Indeed, the low cure rate of the recommended drugs for STH, at the single dose as commonly used, highlights the need for an alternative strategy based either on a multiple-days treatment regimen, an alternating use of albendazole and mebendazole from one deworming round to another, or the use of an alternative drug. A multiple day treatment would obviously increase the costs, but the immediate and long-term benefits would be priceless. Today, there exist new impetus to global helminth control and a series of favourable factors for implementing successful control programmes. There are several and increasing funding opportunities, and we have a great opportunity to properly take up the challenge and relieve poor communities from disease burden that jeopardise their development. There is an urgent need for well co-ordinated and transparent use of multisource funds to increase efficiency and to avoid duplication of efforts. We should act cautiously and address the dilemma between costs and efficacy of interventions, and between control and elimination. Time is right to act more ambitiously and to set the agenda for schistosmiasis and STH elimination.

6. Acknowledgements

The author gratefully acknowledges all partners and stakeholders who contribute towards the successful control of schistosomiasis and STH in Cameroon.

7. References

Amazigo, U.V., Obono, O.M., Dadzie, K.Y., Remme, J., Jiya, J., Ndyomugyenyi, R., Roungou, J.B., Noma, M., Sékétéli, A. (2002). Monitoring community-directed treatment programmes for sustainability: lessons from the African Programme for Onchocerciasis Control (APOC). *Ann. Trop. Med. Parasitol*. 96 (Suppl. 1), S75–S92.

Anderson, R.M. (1982). Population Dynamics of Infectious Diseases: *Theory and Applications*. Chapman and Hall, London, United Kingdom, 377 pp.

Awasthi, S., Bundy, D.A., Savioli, L. (2003). Helminth infections. *British Medical Journal* 327, 431-433.

Berquist, R., Johansen, M.V., Utzinger, J. (2009). Diagnostic dilemmas in helminthology: what tools to use and when? *Trends Parasitol*. 25, 151–156.

Bethony, J., Brooker, S., Albonico, M., Geiger, S.M., Loukas, A., Diemert, D., Hotez, P.J. (2006). Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm. *Lancet* 367, 1521–1532.

Bethony, J., Chen, J., Lin, S., Xiao, S., Zhan, B., Li, S., Xue, H., Xing, F., Humphries, D., Yan, W., Chen, G., Foster, V., Hawdon, J.M., Hotez, P.J. (2002). Emerging patterns of hookworm infection: influence of aging on the intensity of Necator infection in Hainan Province, People's Republic of China. *Clin. Infect. Dis*. 35, 1336–1344.

Booth, M., Vounatsou, P., N'Goran, E.K., Tanner, M., Utzinger, J. (2003). The influence of sampling effort and the performance of the Kato–Katz technique in diagnosing *Schistosoma mansoni* and hookworm co-infections in rural Côte d'Ivoire. *Parasitology* 127, 525–531.

Brooker, S., Clements, A.C.A., Bundy, D.A.P. (2006). Global epidemiology, ecology and control of soil-transmitted helminth infections. *Advance in Parasitology* 62, 221-261.

Brooker, S., Donnelly, C.A. and Guyatt, H.L. (2000). Estimating the number of helminthic infections in the Republic of Cameroon from data on infection prevalence in schoolchildren. *Bulletin of the World Health Organization* 78, 1456–1465.

Brooker, S., Kabatereine, N.B., Smith, J.L., Mupfasoni, D., Mwanje, M.T., Ndayishimiye, O., Lwambo, N.J.S., Mbotha, D., Karanja, P., Mwandawiro, C., Muchiri, E., Clements, A.C.A., Bundy, D.A.P., Snow, R.W. (2009). An updated of human helminth infections: the example of East Africa. *Int. J. Health Geogr*. 8, 42.

Cringoli, G. (2006). FLOTAC, a novel apparatus for a multivalent faecal egg count technique. *Parasitologia* 48, 381–384.

Crompton, D.W., Savioli, L. (1993). Intestinal Parasitic Infections and Urbanization. *Bull. World Health Organ*. 71, 1–7.

Crompton, D.W.T., Nesheim, M.C. (2002). Nutritional impact of intestinal helminthiasis during the human life cycle. *Ann. Rev. Nutr*. 22, 35-59.

Dacombe, R.J., Crampin, A.C., Floyd, S., Randall, A., Ndhlovu, R., Bickle, Q., Fine, P.E.M. (2007). Time delays between patient and laboratory selectively affect accuracy of helminth diagnosis. *Trans. R. Soc. Trop. Med. Hyg*. 101, 140–145.

De Vlas S.J., Gryseels B. (1992). Underestimation of *Schistosoma mansoni* prevalences. *Parasitology Today* 8: 274-277.

Fenwick, A., Webster, J.P., Bosque-Oliva, E., Blair, L., Fleming, F.M., Zhang, Y., Garba, A., Stothard, J.R., Gabrielli, A.F., Clements, A.C.A., Kabatereine, N.B., Toure, S., Denbele, R., Nyandindi, U., Mwansa, J., Koukounari, A. (2009). The Schistosomiasis Control Initiative (SCI): rationale, development and implementation from 2002–2008. *Parasitology* 136, 1719–1730.

Fleming, F.M., Brooker, S., Geiger, S.M., Caldas, I.R., Correa-Oliveira, R., Hotez, P.J., Bethony, J.M. (2006). Synergistic associations between hookworm and other helminth species in a rural community in Brazil. *Trop. Med. Int. Health* 11, 56–64.

Forrester, J.E., Scott, M.E., Bundy, D.A., Golden, M.H. (1988). Clustering of *Ascaris lumbricoides* and *Trichuris trichiura* infections within households. *Trans. R. Soc. Trop. Med. Hyg*. 82, 282–288.

Forrester, J.E., Scott, M.E., Bundy, D.A.P., Golden, M.H.N. (1990). Predisposition of individuals and families in Mexico to heavy infection with *Ascaris lumbricoides* and *Trichuris trichiura*. *Trans. R. Soc. Trop. Med. Hyg*. 84, 272–276.

Garba, A., Touré, S., Dembelé, R., Boisier, P., Tohon, Z., Bosqué-Oliva, E., Koukounari, A., Fenwick, A. (2009). Present and future schistosomiasis control activities with support from the Schistosomiasis Control Initiative in West Africa. *Parasitology* 136, 1731–1737.

Gilles, H. (1996). Soil-transmitted helminths (geohelminths). In: *Tropical Diseases.* Cook, G. (Ed.), Manson's. W.B. Saunders, London, United Kingdom, pp. 1369–1412.

Hall, A. (1981). Quantitative variability of nematode egg counts in faeces: a study among rural Kenyans. *Trans. R. Soc. Trop. Med. Hyg*. 75, 682–687.

Horton, J., Witt, C., Ottensen, E.A., Lazdins, J.K., Addiss, D.G., Awadzi, K., Beach, M.J., Belizario, V.Y., Dunyo, S.K., Espinel, M., Gyapong, J.O., Hossain, M., Ismail, M.M., Jayakody, R.L., Lammie, P.J., Makunde, W., Richard-Lenoble, D., Selve, B., Shenoy, R.K., Simonsen, P.E., Wamae, C.N., Weerasooriya, M.V. (2000). An analysis of the safety of single dose, two drug regimens used in programmes to eliminate lymphatic filariasis. *Parasitology* 121, S147–S160.

Hotez, P.J., Brindley, P.J., Bethony, J.M., King, C.H., Pearce, E.J., Jacobson, J. (2008). Helminth infections: the great neglected tropical diseases. *The Journal of Clinical Investigation* 118, 1311-1321.

Hotez, P.J., Bundy, D.A.P., Beegle, K., Brooker, S., de Silva, N., Montresor, A., Engels, D., Drake, L., Chitsulo, L., Michaud, C., Bethony, J.M., Oliveira, R., Xiao, S.H., Fenwick, A., Savioli, L. (2006). Helminth infections: soil-transmitted helminth infections and schistosomiasis. In: *Disease Control Priorities in Developing Countries*, 2nd Edition. D. Jamison, M. Claeson, J. Breman and A. Meacham, eds. New York: Oxford University Press.

Hotez, P.J., Fenwick, A., Savioli, L., Molyneux, D.H. (2009). Rescuing the bottom billion through control of neglected tropical diseases. *Lancet* 373, 1570-1575.

Hotez, P.J., Molyneux, D.H., Fenwick, A., Kumaresan, J., Ehrlich Sachs, S., Sachs, J.D., Savioli, L. (2007). Control of neglected tropical diseases. *N. Engl. J. Med*. 357, 1018–1027.

Hotez, P.J., Engels, D., Fenwick, A., Savioli, L. (2010). Africa is desperate for praziquantel. *Lancet*, 376: 496–498.

Kabatereine, N.B., Brooker, S., Koukounari, A., Kazibwe, F., Tukahebwa, E.M., Fleming, F.M., Zhang, Y.B., Webster, J.P., Stothard, J.R., Fenwick, A. (2007). Impact of a national helminth control programme on infection and morbidity in Ugandan schoolchildren. *Bull. World Health Organ*. 85, 91–99.

Katz, N., Chaves, A., Pelligrino, J. (1972). A simple device for quantitative stool thicksmear technique in schistosomiasis mansoni. *Rev. Inst. Med. Trop. Sao Paulo* 14, 397–400.

Knopp, S., Mgeni, A.F., Khamis, I.S., Steinmann, P., Stothard, J.R., Rollinson, D., Marti, H., Utzinger, J. (2008). Diagnosis of soil-transmitted helminths in the era of preventive chemotherapy: effect of multiple stool sampling and use of different diagnostic techniques. *PLoS Negl. Trop. Dis*. 2, e331.

Knopp, S., Rinaldi, L., Khamis, I.S., Stothard, J.R., Rollinson, D., Maurelli, M.P., Steinmann, P., Marti, H., Cringoli, G., Utzinger, J. (2009). A single FLOTAC is more sensitive than triplicate Kato–Katz for the diagnosis of low-intensity soil-transmitted helminth infections. *Trans. R. Soc. Trop. Med. Hyg*. 103, 347–354.

Levecke, B., De Wilde, N., Vandenhoute, E., Vercruysse, J. (2009). Field validity and feasibility of four techniques for the detection of Trichuris in Simians: a model for monitoring drug efficacy in public health? *PLoS Negl. Trop. Dis*. 3, e366.

Lin D.D., Liu J.X., Liu Y.M., Hu F., Zhang Y.Y., et al. (2008). Routine Kato-Katz technique underestimates the prevalence of *Schistosoma japonicum*: a case study in an endemic area of the People's Republic of China. *Parasit. Int.* 57, 281-286.

Linehan, M., Hanson, C., Weaver, A., Baker, M., Kabore, A., Zoerhoff, K.L.; Sankara, D., Torres, S., Ottensen, E. (2011). Integrated implementation of programs targeting naglected tropical diseases through preventive chemotherapy: proving the feasibility at national scale. *American Journal of Tropical Medicine and Hygiene*, 84, 1: 5-14.

Miguel, E., Kremer, M. (2004). Worms: Identifying impacts on education and health in the presence of treatment externalities. *Econometrica* 72, 159–217.

Minsante (2005). Programme National de Lutte contre la Schistosomiase et les Helminthiases Intestinales: Plan stratégique 2005-2010. Ministère de la Santé Publique, Cameroun.

Mohammed, K.A., Haji, H.J., Gabrielli, A.F., Mubila, L., Biswas, G., Chitsulo, L., Bradley, M.H., Engels, D., Savioli, L., Molyneux, D.H. (2008). Triple co-administration of ivermectin, albendazole and praziquantel in Zanzibar: a safety study. *PLoS Negl. Trop. Dis*. 2 (1), e171.

Molyneux, D. H., Hotez, P., Fenwick, A. (2005). Rapid impact interventions: how a policy of integrated control for Africa's neglected tropical diseases could benefit the poor. *PLoS Medicine* 2, 1064–1070.

Molyneux, D.H., Hotez, P.J., Fenwick, A. (2005). Rapid impact interventions: how a policy of integrated control for Africa's neglected tropical diseases could benefit the poor. *PLoS Medecine* 2, 1064-1070.

Montresor, A., Crompton, D.W.T., Hall, A., Bundy, D.A.P., Savioli, L. (1998). Guidelines for the Evaluation of Soil-transmitted Helminthiasis and Schistosomiasis at Community Level: A Guide for Managers of Control Programmes. *World Health Organization*, Geneva.

Odogwu, S.E., Ramamurthy, N.K., Kabatereine, N.B., Kazibwe, F., Tukahebwa, E.,Webster, J.P., Fenwick, A., Stothard, J.R. (2006). Intestinal schistosomiasis in infants (3 years of age) along the Ugandan shoreline of Lake Victoria. *Ann. Trop. Med. Parasitol*. 100, 315–326.

Olds, G.R., King, C., Hewlett, J., Olveda, R., Wu, G., Ouma, J., Peters, P., McGarvey, S., Odhiambo, O., Koech, D., Liu, C.Y., Aligui, G., Gachihi, G., Kombe, Y., Parraga, I., Ramirez, B., Whalen, C., Horton, R.J., Reeve, P. (1999). Double blind, placebocontrolled study of concurrent administration of albendazole and praziquantel in schoolchildren with schistosomiasis and geohelminths. *J. Infect. Dis*. 179, 996–1003.

Ratard, R. C., Kouemeni, L. E., Ekani Bessala, M. M., Ndamkou, C. N., Greer, G. J., Spilsburg, J., Cline, B. L. (1990). Human schistosomiasis in Cameroon. I.

Distribution of schistosomiasis. *American Journal of Tropical Medicine and Hygiene* 42, 561–572.

Ratard, R. C., Kouemeni, L. E., Ekani Bessala, M. M., Ndamkou, C. N., Sama, M. T., Cline, B. L. (1991). Ascariasis and trichuriasis in Cameroon. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 85, 84–88.

Savioli, L., Albonico, M., Engels, D., Montresor, A. (2004). Progress in the prevention and control of schistosomiasis and soil-transmitted helminthiasis. *Parasitol. Int*. 53, 103–113.

Savioli, L., Gabrielli, A.F., Montresor, A., Chitsulo, L., Engels, D. (2009). Schistosomiasis control in Africa: 8 years after World Health Assembly Resolution 54.19. *Parasitology* 136, 1677-1681.

Sékétéli, A., Adeoye, G., Eyamba, A., Nnoruka, E., Drameh, P., Amazigo, U.V., Noma, M., Agboton, F., Aholou, Y., Kale, O.O., Dadzie, K.Y. (2002). The achievements and challenges of the African Programme for Onchocerciasis Control (APOC). *Ann. Trop. Med. Parasitol*. 96 (Suppl. 1), S15–S28.

Shane H.L.L., Verani J.R., Abudho B., Montgomery S.P., Blackstock A.J., Mwinzi P.N.M., Butler S.E., Karanja D.M.S., Secor W.E. (2011). Evaluation of urine CCA assays for detection of *Schistosoma mansoni* infection in Western Kenya. *PLoS Negl. Trop. Dis.* 5(1): e951. doi:10.1371/journal.pntd.0000951.

Sousa-Figueiredo, J.C., Basanez, M.G., Mgeni, A.F., Khamis, I.S., Rollinson, D., Stothard, J.R. (2008). A parasitological survey, in rural Zanzibar, of pre-school children and their mothers for urinary schistosomiasis, soil-transmitted helminthiases and malaria, with observations on the prevalence of anaemia. *Ann. Trop. Med. Parasitol*. 102, 679–692.

Steinmann, P., Du, Z.W., Wang, L.B., Wang, X.Z., Jiang, J.Y., Li, L.H., Marti, H., Zhou, X.N., Utzinger, J. (2008). Extensive multiparasitism in a village of Yunnan Province, People's Republic of China, revealed by a suite of diagnostic methods. *Am. J. Trop. Med. Hyg*. 78, 760–769.

Steinmann, P., Keiser, J., Bos, R., Tanner, M., Utzinger, J. (2006). Schistosomiasis and water resources development: systematic review, meta-analysis, and estimates of people at risk. *Lancet Infectious Diseases* 6, 411-425.

Stephenson, L.S., Latham, M.C., Ottesen, E.A. (2000). Malnutrition and Parasitic Helminth Infections. *Parasitology* 121 (Suppl.), S23–28.

Stothard, J.R., Imison, E., French, M.D., Sousa-Figueiredo, J.C., Khamis, I.S., Rollinson, D. (2008). Soil-transmitted helminthiasis among mothers and their pre-school children on unguja island, zanzibar with emphasis upon ascariasis. *Parasitology* 135, 1447–1455.

Stothard J.R., Sousa-Figuereido J.C., Betson M., Adriko M., Arinaitwe M., et al. (2011). *Schistosoma mansoni* infections in young children: when are schistosome antigens in urine, eggs in stool and antibodies to eggs first detectable? *PLoS Negl. Trop. Dis.* 5(1): e938. doi:10.1371/journal.pntd.0000938

Tarafder, M.R., Carabin, H., Joseph, L., Balalong Jr., E., Olveda, R., McGarvey, S.T. (2009). Estimating the sensitivity and specificity of Kato–Katz stool examination technique for detection of hookworms, *Ascaris lumbricoides* and *Trichuris trichiura* infections in humans in the absence of a 'gold standard'. *Int. J. Parasitol*., doi:10.1016/j.ijpara.2009.09.003.

Tchuem Tchuenté, L.A. (2011). Control of soil-transmitted helminths in sub-Saharan Africa: Diagnosis, drug efficacy concerns and challenges. *Acta Tropica* 120S, S4– S11.

Tchuem Tchuenté, L.A., N'Goran, E.K. (2009). Schistosomiasis and soil-transmitted helminthiasis control in Cameroon and Côte d'Ivoire: implementing control on a limited budget. *Parasitology* 136, 1736-1745.

Tchuem Tchuenté, L.A., Behnke, J.M., Gilbert, F., Southgate, V.R., Vercruysse, J. (2003). Polyparasitism with *Schistosoma haematobium* and soil-transmitted helminth infections among school children in Loum, Cameroon. *Tropical Medicine and International Health* 8, 975–986.

Tchuem Tchuenté, L.A., Kamwa Ngassam, R.I., Sumo, L., Ngassam, P., Dongmo Noumedem, C., Luogbou, D.G.O., Dankoni, E., Kenfack, C.M., Feussom Gipwe, N., Akame, J., Tarini, A., Zhang, Y., Angwafo III, F.F. (2012); Mapping of schistosomiasis and soil-transmitted helminthiasis in the regions of Centre, East and West Cameroon. *PLoS Negl. Trop. Dis*. (in press).

Tchuem Tchuenté L.A., Kueté Fouodo C.J., Kamwa Ngassam R.I., Sumo L., Dongmo Noumedem C., Kenfack C.M., Feussom Gipwe N., Dankoni Nana E., Stothard J.R., Rollinson D. (2012). Evaluation of circulating cathodic antigen (CCA) urine-tests for diagnosis of *Schistosoma mansoni* infection in Cameroon. *PLoS Negl. Trop. Dis.* (in press).

Utzinger, J., Rinaldi, L., Lohourignon, L.K., Rohner, F., Zimmermann, M.B., Tschannen, A.B., N'Goran, E.K., Cringoli, G. (2008). FLOTAC: a new sensitive technique for the diagnosis of hookworm infections in humans. *Trans. R. Soc. Trop. Med. Hyg*. 102, 84–90.

World Health Organization (2002). Prevention and control of schistosomiasis and soil-transmitted helminthiasis. Report of a WHO Expert Committee. WHO *Technical Report Series* No. 912. World Health Organization, Geneva.

World Health Organization (2006). Preventive chemotherapy in human helminthiasis: coordinated use of anthelminthic drugs in control interventions : a manual for health professionals and programme managers. World Health Organization Press, Geneva.

World Health Organization (2010). Soil-transmitted helminthiasis: number of children treated 2007-2008: update on the 2010 global target. *Weekly Epidemiological Record* 16, 85: 141-148.

World Health Organization (2011). Schistosomiasis: number of people treated, 2009. *Weekly Epidemiological Record* 9, 86: 73-80.

World Health Organization (2012). Schistosomiasis: population requiring preventive chemotherapy and number of people treated in 2010. *Weekly Epidemiological Record* 4, 87: 37–44.

World Health Organization (1991). Basic Laboratory Methods in Medical Parasitology. World Health Organization, Geneva.

22

Hyperinfection Syndrome in Strongyloidiasis

Cristiane Tefé-Silva[1,*], Eleuza R. Machado[2],
Lúcia H. Faccioli[2] and Simone G. Ramos[1]
[1]*Department of Pathology, Faculty of Medicine of Ribeirão Preto*
[2]*Department of Clinical Analyses, Toxicology and Bromatology Faculty of Pharmaceutics Sciences of Ribeirão Preto, University of São Paulo*
Brazil

1. Introduction

Strongyloidiasis is an intestinal parasitosis found in tropical and subtropical areas, where the warm climates are suitable for parasite survival (Barr, 1978). It is a common cause of morbidity and mortality, particularly in developing countries, and infects over one-quarter of the world's population (Genta, 1989). Approximately 52 species are known to infect mammals, birds, reptiles and amphibians (Speare, 1989). The most common globally distributed human pathogen of clinical importance is *Strongyloides stercoralis* (Schad, 1989). Another species, *Strongyloides fuelleborni,* is a zoonotic parasite that infects primates and is found sporadically in humans in Africa (Pampiglione & Ricciardi, 1972). *S. stercoralis* is a ubiquitous soil-transmitted intestinal nematode that was first reported in 1876 in French soldiers working in Vietnam. It is unique among helminths in that it completes its life cycle inside a single human host. A unique feature of strongyloidiasis is the ability of the parasite to autoinfect the host, which makes *S. stercoralis* a significant public health problem (Grove, 1989).

2. Epidemiology

The epidemiology of *Strongyloides* infection is poorly understood because it is difficult to detect and can be underestimated (Albonico et al., 1999; Viney & Lok, 2007). However, it is estimated that from 30 a 100 million people are infected worldwide with *Strongyloides*, and can range from asymptomic to multiorgan failure (Genta, 1989). *Strongyloides* is found in tropical and subtropical areas and requires specific soil and climate conditions for its development. In North America, Latin America, Africa and Southeast Asia, the infection is endemic (Roxby et al., 2009). The risk of acquiring strongyloidiasis is higher in rural areas, among people who work with soil, and among lower socioeconomic groups *(*Vadlamudi et al., 2006; Viney & Lok, 2007*).* Walking barefoot in areas where human faeces containing the parasite are deposited increases the probability of acquiring the infection (Grove, 1994).

3. Parasite

S. stercoralis has free-living and parasitic life cycles, and the morphology of each differs. Parasitic worms are female adults that reproduce by parthenogenesis and measure approximately 1 - 10 mm in length by 27 -95 µm in width. Free-living adults are

approximately 1 mm, live in the soil, and reproduce sexually; females are slightly larger than males (Speare, 1989). Embryonated eggs are thin-shelled and measure approximately 55 - 60 µm in length and 28 - 32 µm in width. Rhabditiform larvae are the first-stage larvae (210 µm) and develop into free-living larvae or third-stage infective larvae that measure approximately 490-630 µm and are capable of infecting the host (Schad, 1989).

4. Life cycle

The life cycle of *S. stercoralis* includes a parasitic cycle (within human hosts) and an environmental cycle (free-living larvae). The parasitic cycle occurs when the infective filariform larvae penetrate the skin and secrete metalloproteases that facilitate penetration. The main larval route is via the bloodstream to the lungs, where they break into the alveolar spaces within hours after infection, promote haemorrhage, ascend the respiratory tree, are swallowed, and migrate to the intestine. Alternatively, the larvae may migrate directly through connective tissues (Grove, 1994, 1996).

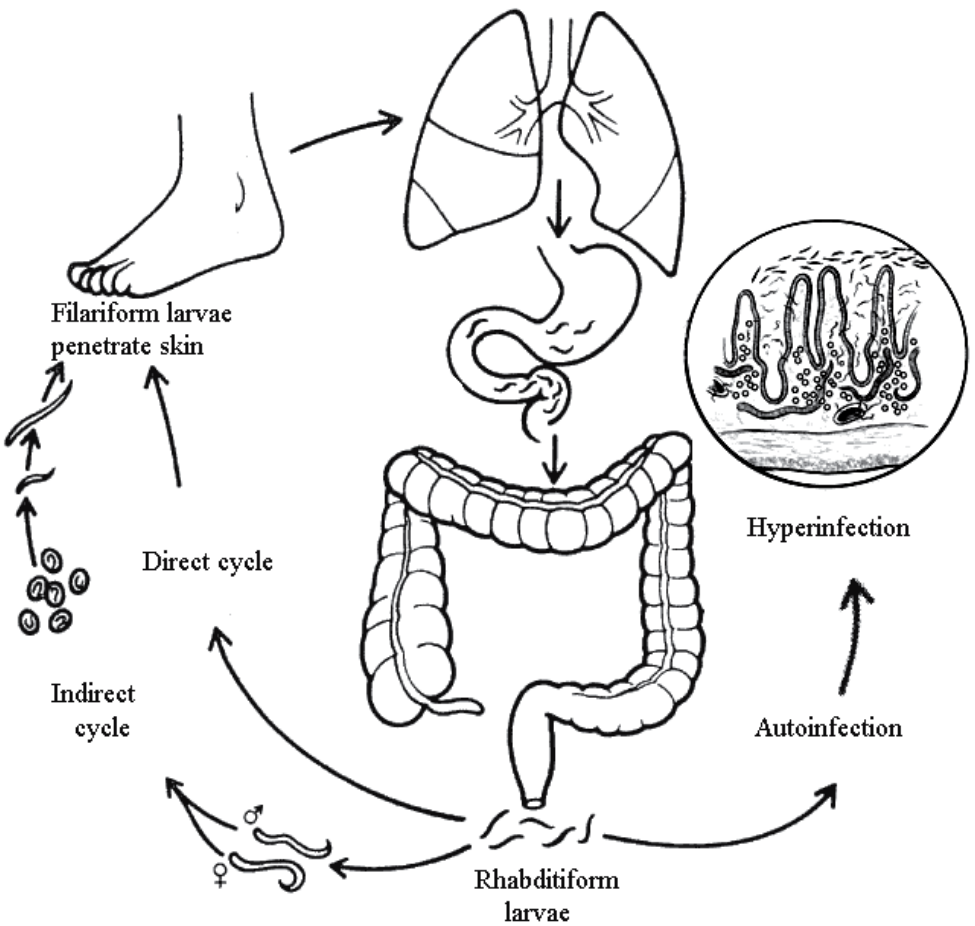


Fig. 1. Life cycle of *Strongyloides stercoralis* (modified from Carvalho & Da Fonseca Porto, 2004, and http://emedicine.medscape.com/article/229312-overview#a0104).

The infective larvae reach the small intestine, where they moult twice to become female adult worms. These females reproduce in the absence of males by parthenogenesis. The females are embedded in the intestinal mucosa and produce eggs in the duodenum. The rhabditiform larvae emerge from the hatching eggs and migrate into the intestinal lumen, then pass into the external environment with the faeces. Depending on temperature and humidity, the rhabditiform larvae may have two different life cycles in the environment: an indirect (heterogonic) life cycle, in which the rhabditiform larvae differentiate into free-living adults and release eggs that hatch and transform into infective larvae, or a direct (homogonic) life cycle, in which the rhabditiform larvae may moult directly into filariform larvae and repenetrate the host skin, restarting the cycle (Schad, 1989).
One characteristic that differentiates *S. stercoralis* from almost all other worms is its capacity to replicate within the host. Rhabditiform larvae in the bowel lumen transform into filariform larvae before excretion and invade the intestinal wall or the perianal skin, permitting ongoing cycles of autoinfection, an important feature of strongyloidiasis (Concha et al., 2005).

5. Clinical manifestation

The clinical presentation of strongyloidiasis varies with the status of the host´s immune system, and the infection is classified as acute, chronic or severe. Acute infections of strongyloidiasis manifest as a wide spectrum of clinical features ranging from asymptomatic disease to cutaneous (larva currens and urticaria), pulmonary (cough and tracheal irritation), and gastrointestinal symptoms (diarrhoea and constipation), although the majority of *S. stercoralis* infections are resolved (Mahmoud, 1996).
The ability of *S. stercoralis* to establish a cycle of autoinfection within the host results in chronic infections that can persist in an individual for decades. Chronic infections are often asymptomatic, but when symptoms occur they are usually mild, episodic and prolonged, including nausea, vomiting, diarrhoea, constipation, weight loss or cutaneous reactions (Grove, 1989).
Uncontrolled autoinfection of *S. stercoralis* is more likely to occur in immunosuppressed patients, leading to hyperinfection syndrome. The pulmonary phase of hyperinfection due to migrating larvae resembles Löffler syndrome with coughing and wheezing, asthma-like symptoms, haemorrhaging and pneumonia. In the intestine, symptoms include diarrhoea, nausea, vomiting, abdominal pain, and weight loss (Concha et al., 2005; Viney & Lok, 2007).
Bacteremia is a common complication of hyperinfection syndrome and is caused by filariform larvae that may lead bacteria from the bowel to the bloodstream with subsequent secretion into the host circulation (Bamias et al., 2010). Pathogens such as *Streptococcus bovis, Escherichia coli, Klebsiella pneumonia* or *Enterobacter cloacae* are found during fatal complications of strongyloidiasis (Link & Orenstein, 1999). The mortality rate of dissemination associated with bacterial infections can reach approximately 90% (Igra-Siegman et al., 1981).
Dissemination occurs upon larval migration to organs beyond the range of the pulmonary phase, such as the liver, heart, lymph nodes, gallbladder, kidneys, pancreas, and brain (Keiser & Nutman, 2004). Petechiae and purpura have also been reported in disseminated cases as a result of larval migration through vessel walls, which promotes haemorrhage (Basile et al., 2010). Others complications of disseminated strongyloidiasis can occur and include syndromes such as cholecystitis, pancreatitis, paralytic ileus, intestinal perforation

or infarction, peritonitis, and sepsis (Krishnan et al., 2006). Although unusual, brain involvement can occur in disseminated infections, with symptoms including headache, focal seizures, altered mental state, secondary bacterial meningitis and coma (Dutcher et al., 1990).

6. Hyperinfection syndrome

Since 1966, studies have reported that autoinfection may result in the dissemination of worms; denoted hyperinfection syndrome, the number of worms increases significantly, and worms are detectable in extraintestinal regions, with a mortality rate above 80% (Siddiqui & Berk, 2001). High-risk factors for hyperinfection and dissemination include corticosteroid therapy, malignancies, transplantation, malnutrition, hypogammaglobulinemia, and viral infections such as HIV (Human Immunodeficiency Virus) and HTLV-1 (Human T-Lymphotropic Virus Type 1) (Concha et al., 2005).

6.1 Corticosteroids

In recent decades, hyperinfection syndrome has increased significantly with the use of immunosuppressive drug therapy. Corticosteroids are widely prescribed drugs with potent immunosuppressive effects and are a major risk factor for the transformation of chronic strongyloidiasis into hyperinfection, which has a higher index of mortality (Armignacco et al., 1989; Al Maslamani et al., 2009). Corticosteroids are involved in the treatment of several diseases that are considered immunological abnormalities, such as lymphoma, rheumatoid arthritis, leprosy, chronic obstructive pulmonary disease (COPD), and polymyositis, leading to fatal hyperinfection in many cases (Keiser & Nutman, 2004).

However, the role of corticosteroids in susceptibility to severe *S. stercoralis* infection is poorly understood. One hypothesis is that both endogenous and exogenous corticosteroids promote immunosuppression by decreasing the number of inflammatory cells, such as eosinophils and mast cells, and suppressing the transcription of several cytokines. In addition, corticosteroids increase the apoptosis of Th2 lymphocytes (Genta, 1989). Corticosteroids may also have a direct effect on female worms by increasing the production of ecdysteroid-like molecules, hormones that control moulting in insects and possibly helminths (Genta, 1992). An increase in these molecules increases the moulting rate and transforms rhabditiform larvae into filariform larvae, increasing the worm burden and promoting hyperinfection and dissemination (Genta, 1992; Siddiqui et al., 2000).

6.2 Hematologic and others malignancies

Patients with hematologic malignancies have a high prevalence of *S. stercoralis* infection when compared with the global index. The reported cases of hematologic malignancies and *S. stercoralis* hyperinfection syndrome are associated with glucocorticoid treatment. The malignancy usually associated with *S. stercoralis* is lymphoma that is being treated with chemotherapy. Moreover, lung cancer has been associated with hyperinfection during the administration of immunosuppressive chemotherapy (Keiser & Nutman, 2004).

6.3 Transplantation

Hyperinfection syndrome is associated with transplants, and the progression of chronic intestinal infection before transplantation appears to be the most common mechanism.

Hyperinfection cases following organ transplant principally occur during the initial months after transplantation, but the infection was acquired before transplantation in the majority of cases (Roxby et al., 2009). Higher mortality rates occur from extraintestinal strongyloidiasis, which in most of these cases are related to corticosteroid therapy to treat rejection (Keiser & Nutman, 2004).

Renal transplants are most commonly associated with hyperinfection, which is related to immunosuppressive treatments (Devault et al., 1990; Rajapurkar et al., 2007; Valar et al., 2007). Cases of hyperinfection have been described in transplant recipients of other organs, such as the liver (Vilela et al., 2008; Rodrigues-Hernandez et al., 2009), heart (Schaeffer et al., 2004), pancreas (Ben-Yousseff et al., 2005), lung (Balagopal et al., 2009), and intestine (Patel et al., 2008). Hyperinfection in hematopoietic stem cell transplant patients may be due to immunosuppressive therapies (Dulley et al., 2008; Wirk & Wingard, 2008).

6.4 Malnutrition

An important cause of immunodeficiency that is related to hyperinfection is malnutrition, particularly in developing countries. Malnutrition promotes disruption of the intestinal mucosa, impairing the host's ability to expel the parasite from the gut (Olsen et al., 2009).

6.5 Hypogammaglobulinemia

Patients with immunodeficient conditions, such as hypogammaglobulinemia, may develop fatal hyperinfection. Case reports show that hypogammaglobulinemia is refractory to prolonged anthelmintic therapy (Brandt de Oliveira et al., 1981; Seet et al., 2005).

6.6 HIV

Although HIV infection predisposes a patient to hyperinfection due to immunosuppression, few cases of *S. stercoralis* and AIDS have been described (Marcos et al., 2008). The association between *S. stercoralis* and HIV principally occurs in endemic areas (Siddiqui & Berk, 2001). The hyperinfection syndrome can occur in patient with HIV with immune reconstruction syndromes increased after starting of highly active antiretroviral therapy (Brown et al., 2006). On the other hand, the infection with *Strongyloides* may contribute to serious nutritional deficiencies in HIV-infected individuals, such as anorexia and malabsorption (Lindo et al., 1998). However, the immunobiological and immunoregulatory mechanisms involving HIV and strongyloidiasis remain unclear.

6.7 HTLV-1

HTLV-1 is a virus that infects T cells and induces lymphocyte proliferation with the production of a Th1-type immune response in humans. The genome of the HTLV-1virus is diploid and, following interaction with the immune system, HTLV-1 enables the transcription of the viral DNA by integrating into the host genome effectively evading immune surveillance without killing the host (Iriemenan et al., 2010). Strongyloidiasis is strongly associated with HTLV-1, which predisposes patients to severe infections by depressing cell-mediated immunity or IgE responses (Grove, 1996; Carvalho & Da Fonseca Porto, 2004). *Strongyloides* and HTLV-1 may promote the Th1-type response in patients, increasing interferon levels and decreasing Th2-type responses, such as interleukin 4 (IL-4), IL-5, IL-13, and IgE, important host defences against helminths, and a decrease in this response allows not only an increasing in autoinfection but also decreased parasite killing.

In addition, this association reduces the efficacy of anthelmintic drugs, increasing the prevalence of infection (Montes et al., 2009; Iriemenam et al., 2010). Stool examinations should be performed with special attention to detect *S. stercoralis* larvae in all patients infected by HTLV-1 (Carvalho & Da Fonseca Porto, 2004).

7. Host-parasite interaction

The relationship between *S. stercoralis* and its host is complex, and little is known about the immunomodulatory mechanisms that regulate this interaction. Different factors are involved, including the capacity of the parasite to replicate, the adequacy of the host immune response, and the ability of the parasite to evade those responses (Grove, 1994; Trajman et al., 1997).

7.1 Cellular immune response

During helminthic infection, Th2-type cell-dependent host defences that involve CD4 cells are developed (Maizels & Yazdanbakhsh, 2003; Anthony et al., 2007). In human hosts and animal models, the cellular immune response to *Strongyloides* infection is characterised by intraepithelial and tissue eosinophils, neutrophils and mast cells with Th2-type production of cytokines such as IL-4, IL-5 and IL-13. Conversely, Th1-type responses are down-regulated during nematode infection (El-Malky et al., 2003; Paterson et al., 2008; Iriemenam et al., 2010).

7.1.1 Eosinophils

Eosinophils are essential against nonphagocytosable parasites, such as *Strongyloides,* that cannot be ingested because of their large size. Eosinophils defend the host by attacking the parasite via the FcεRI receptor, capturing antigens from the worms and presenting the antigens to T cells to initiate an antigen-specific immune response (Galioto et al., 2006; Padigel et al., 2006; Iriemenam et al., 2010). Others mechanisms may be involved, including antibody-dependent cellular cytotoxicity (ADCC) mediated by eosinophils on the parasite surface, which releases toxic molecules in an attempt to eliminate the parasite (Ligas et al., 2003; Klion & Nutman, 2004).

7.1.2 Mast cells

Mast cells have an important role in the defence against *S. stercoralis* by inhibiting the invasion of the adult worm into the intestinal epithelium, promoting the stimulation of gut motility, mucus release and expulsion of the parasites. In addition, mast cells induce the attraction and modulation of eosinophils (Kobayashi et al., 1998; Concha et al., 2005).

7.2 Cytokines

Strongyloidiasis promotes the robust Th2-type immune response with the production of cytokines, such as IL-3, IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13. Contrarily, Th1-type responses are reduced during nematode infection (Wilkes et al., 2007; Patel et al., 2009).
IL-3 is important during *Strongyloides* infection stimulates the synthesis of potent mast cells and basophils enhancing the function of these cells (Abe et al., 1993). In addition, IL-3 can enhance the levels of intra-cellular IL-4 upon activating basophils, with anti-IgE and IL-3 contributing to an increase in eosinophils (Kimura et al., 2006; Lantz et al., 2008).

IL-4 has multiple immunoregulatory functions, including T-cell growth factor activity, B-cell regulation, serum IgE level enhancement, and stimulation of the growth and/or differentiation of macrophages, hematopoietic cells, and mast cells (Urban et al., 1991; Negrão-Correa et al., 2006; Wilkes et al., 2007). IL-4 decreases the fecundity and survival of adult worms and increases intestinal smooth muscle contraction, facilitating the expulsion of the parasite (Concha et al., 2005).
IL-5 regulates the production of eosinophil myelocyte precursors in bone marrow, the development of mature eosinophils after helminth infection and, in most instances, the production of a number of other cytokines, including IL-4 and IL-13, and chemokines such as RANTES and eotaxin (Herbert et al., 2000; Klion & Nutman, 2004; Mir et al., 2006).
IL-13 also participates in the defence mechanisms against helminths, promoting an increase in the intestinal fluid content and increased smooth muscle contractility, a phenomenon that may contribute to worm expulsion (Porto et al., 2001; Shea-Donohue & Urban, 2004; Patel et al., 2009).

7.3 Humoral immune response

The humoral immune response complements defence mechanisms against strongyloidiasis with the production of immunoglobulins by plasma cells. Several immunoglobulins, such as IgE, IgG and IgM, are essential for the elimination of the parasite (Ligas et al., 2003; Machado et al., 2005).
IgE antibodies can mediate the activation of accessory cells and the recognition of parasite antigens, promoting goblet cell mucus secretion and the degranulation of mast cells that release mediators affecting parasite survival (Machado et al., 2009). IgG and IgM can transfer immunity against the human parasite in the presence of the complement system and neutrophils (Abraham et al., 1995; Vadlamudi et al., 2006)
Laboratory models have suggested that both T and B cells mediate the immune response through an increase in immunoglobulins, eosinophils and mast cells and hyperplasia of goblet cells, which require interleukins and chemokines for their development and activation. In strongyloidiasis, dexamethasone seems to primarily suppress cytokines such as IL-1β, IL-4, VEGF, TNF-α, IFN-γ, IL-3, IL-4, IL-5, IL-10 and IL-12 and decreases the production of IgG and IgE antibodies during *S. venezuelensis* infection (Machado et al., 2011; Tefé-Silva et al., 2012).

7.4 Other responses

The complement system activates both classical and alternative pathways with chemoattraction and binding of granulocytes in association with effector cells, which are essential against *S. stercoralis* (Vadlamudi et al., 2006). Studies have reported that complement component C3 is required during *S. stercoralis* infection and facilitates eosinophil degranulation and larval death during the innate immune response (Kerepesi et al., 2006).
Strongyloides infection induces the production of leukotrienes, which are required to invoke the protective expulsion of parasites. Leukotrienes play an important role in controlling parasite burdens, as well as in altering the parasite reproductive cycle and eliminating the *S. venezuelensis* parasite (Machado et al., 2005).

8. Pathology

The pathology of strongyloidiasis differs in different stages of infection.

8.1 Acute infection

The obligate pulmonary phase of the parasite's life cycle typically occurs within hours after infection. During larval passage through the lungs, the parasite induces haemorrhage in the alveolar spaces, inflammatory infiltrate, and, occasionally, granuloma (Kinjo et al., 1998). Histopathological analyses of human intestines have shown that *S. stercoralis* eggs and adult females colonise the duodenum and upper jejunum. Studies have also demonstrated the presence of oedema, duodenal villous atrophy, and crypt hyperplasia with disrupted epithelium due to the inhibition of cell proliferation and apoptosis (Coutinho et al., 2006; Werneck-Silva et al., 2006). Surface damage, ulceration, an increase in mucus secretion and functional changes in the intestine have also been reported. In many cases, the eosinophil infiltrates are associated with the intensity of the infection (Rivasi et al., 2006; Kishimoto et al., 2008).

8.2 Hyperinfection syndrome

The histology of lungs affected by hyperinfection syndrome revealed alveolar haemorrhage with large numbers of larvae in the alveoli, septa, pleurae and blood vessels. Many larvae were present throughout the walls of the tracheobronchial tree, with an increase in number toward the upper respiratory tract. Larvae in the lungs provoked inflammatory infiltrate

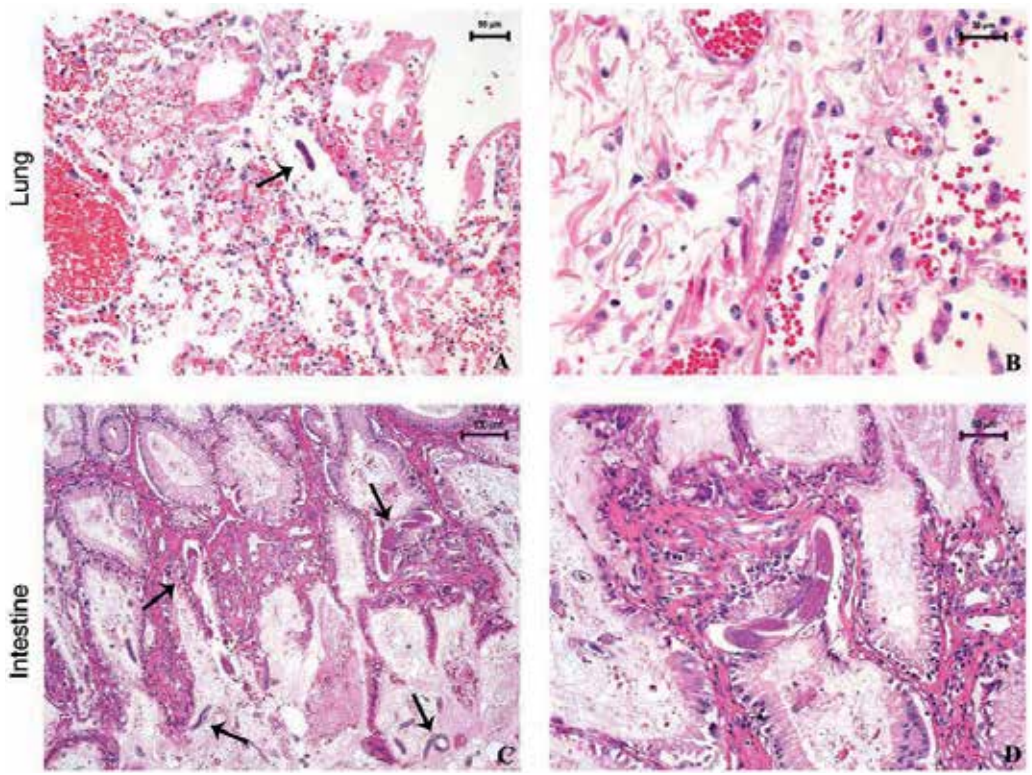


Fig. 2. Histopathology of *Strongyloides stercoralis* in the lungs and intestine of a 48-year-old woman with a gastric carcinoid tumor treated with chemotherapy. A and B: Pulmonary parenchyma. Note the presence of larvae in alveolar space (arrow). C and D: Female worms in the duodena (arrows). HE stain.

and were occasionally walled off by granulomas. Bronchopneumonia is probably a consequence of tissue damage inflicted by the invading larvae (Zumla & James, 2002).
In the human intestine, hyperinfection results in mucosal oedema, acute inflammation, mucosal haemorrhage, and focal ulceration with numerous *S. stercoralis* larvae, adult worms and ova embedded within the small bowel villi (Sathe &Madiwale, 2006; Al Maslamani et al., 2009).

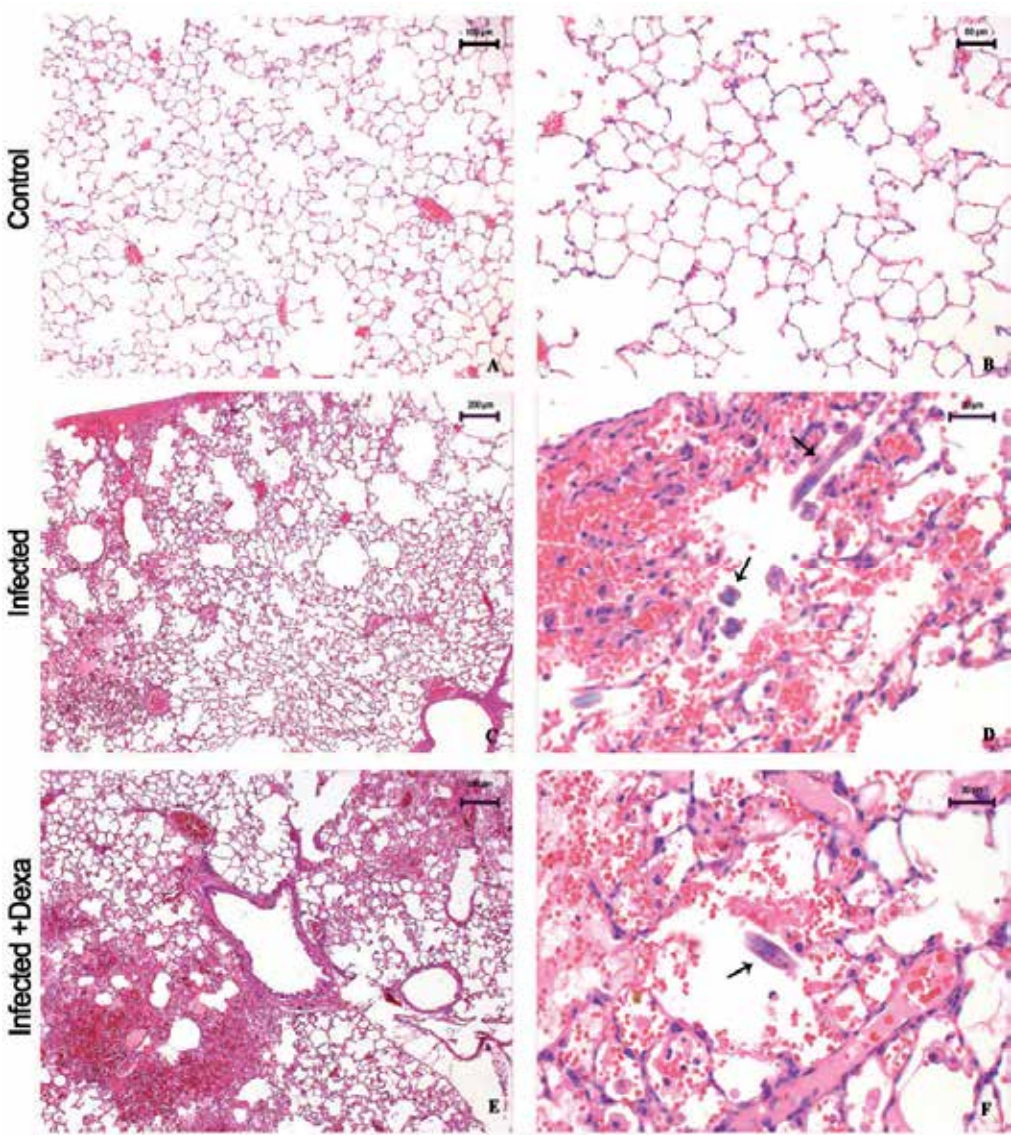


Fig. 3. Histopathology of the lungs of rats in an experimental model of strongyloidiasis on day 3 post-infection: A and B: Controls; C and D: Infected with *S. venezuelensis.* Note the scarce hemorrhagic foci with larvae in the alveolar spaces (arrows); E and F: Infected with *S. venezuelensis* and treated with dexamethasone. Note the prominent hemorrhagic foci showing larvae in the alveolar spaces (arrow). HE stain.

8.3 Hyperinfection syndrome in experimental models

Animal models are important for understanding the mechanism of hyperinfection. Studies in experimental models of *S. venezuelensis* infection have reported that filariform larvae were surrounded by inflammation mediated by eosinophils and mast cells in the lungs. The infection also promoted an important granulomatous response, sometimes entrapping the larvae, which is probably an attempt by the host to contain the parasite. In addition, airway

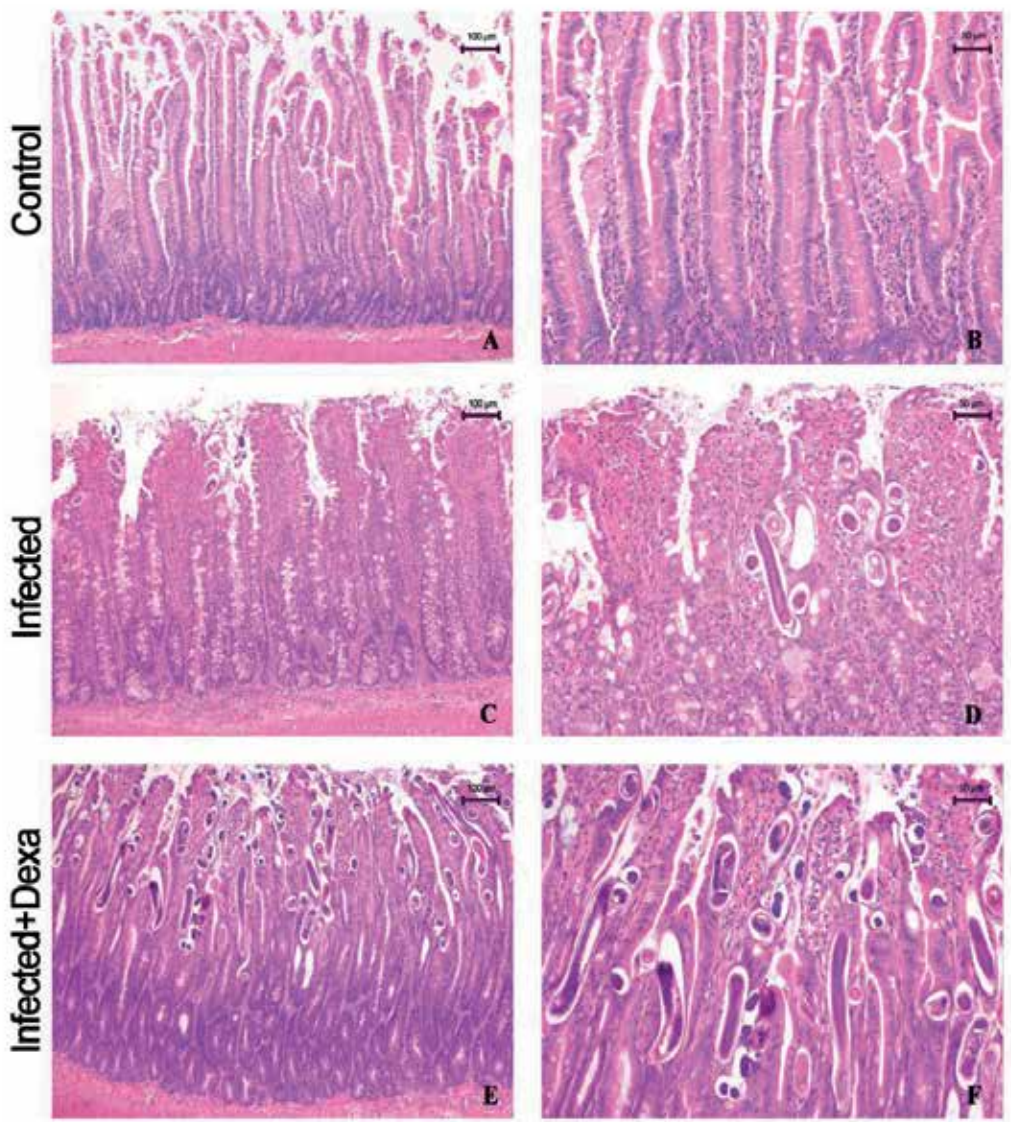


Fig. 4. Histopathology of the duodena of rats in an experimental model of strongyloidiasis on day 14 post-infection: A and B: Controls; C and D: Infected with *S. venezuelensis.* E and F: Infected with *S. venezuelensis* and treated with dexamethasone on day 14 post-infection. Note the massive mucosal invasion of fertile eggs and adult parasites, accompanied by erosion of the intestinal epithelial layer. HE stain.

remodelling similar to asthma, characterised by hyperplasia of goblet cells and increased bronchiolar wall thickness caused by oedema, hypertrophy of smooth muscle cells, neovascularisation and collagen deposition, was reported. In contrast, immunosuppression with dexamethasone interferes with the pulmonary cycle of *Strongyloides venezuelensis* infection and promotes greater haemorrhage, which is provoked by the substantial quantities of larvae that pass into the alveolar spaces, accompanied by a decrease in eosinophil and mast cell migration and impaired formation of granulomas (Tefé-Silva et al., 2008). In addition, dexamethasone treatment inhibited airway remodelling, contributing to the dissemination of the parasite (Tefé-Silva et al., 2012).

In the small intestine of rodents infected with *S. venezuelensis*, females and fertile eggs were observed in the wall of the gastrointestinal tract and invading the intestinal mucosa, with increased inflammatory exudate and eosinophils (Machado et al., 2005). Dexamethasone treatment promoted increased mucus production, which progressed to a massive mucosal invasion of fertile eggs and adult parasites that was accompanied by the erosion of the intestinal epithelial layer. Interestingly, the inflammatory response was relatively inconspicuous. Proliferative activity increased in the crypts and the villous fusion, resulting in an apparent reduction in the number of intestinal epithelial cells. In addition, dexamethasone enhanced parasite fertility and proliferation, with dissemination of the larvae to other visceral organs, such as the spleen, kidneys, heart, liver and brain (Machado et al., 2011).

Mice infected with *S. venezuelensis* and treated with dexamethasone showed increased blood neutrophil numbers and a reduction in eosinophil and mononuclear cell numbers in the blood, bronchoalveolar cells, and peritoneum when compared to *S.* venezuelensis infection in the absence of dexamethasone. In addition, dexamethasone impaired the host immune response, decreasing the production of cytokines such as tumour necrosis factor (TNF), interferon (IFN), interleukin-3 (IL-3), IL-4, IL-5, IL-10, and IL-12 in the lungs and circulating antibodies such as IgG, and IgE but increasing the overall parasite burden in the intestines and faeces (Machado et al., 2011).

9. Diagnosis

Strongyloidiasis is diagnosed on the basis of suspicion in patients with clinical signs and symptoms of the disease; however, in approximately 50% of cases, the infection is asymptomatic, complicating diagnosis. In some cases, diagnosis is difficult despite a low intestinal worm load and larval excretion in the faeces (Rajapurkar et al., 2007).

The classic triad of urticaria, abdominal pain and diarrhoea is suggestive of a diagnosis of strongyloidiasis. Parasites are usually found in the faeces; they are sometimes also seen in other body fluids or in tissue samples (Basile et al., 2010). The parasitological diagnosis is usually made after an examination of the faeces, and several diagnostic methods can be used to detect *S. stercoralis*, including stool examination, a modified Baermann technique, and stool culture on a blood agar plate. Enzyme-linked immunosorbent assays (ELISA) are used for serological diagnosis and have proven valuable in detecting both symptomatic and asymptomatic strongyloidiasis infection, with a high specificity for detecting IgG antibodies to *S. stercoralis* (Basile et al., 2010).

In patients with a disseminated infection, the diagnosis is relatively straightforward, given the high numbers of larvae that exist in the stool and, usually, in the sputum. White blood

cell numbers may be elevated. Although an increase in eosinophils frequently occurs during infection, studies have shown that an absence of eosinophilia does not exclude a diagnosis of strongyloidiasis (Krishnan et al., 2006). Diagnosis through imaging is usually possible. Chest radiographs of some patients have shown infiltrate consistent with Loeffler's syndrome. Methods such as bronchoalveolar lavage and sputum culture are used to diagnose disseminated strongyloidiasis (Williams et al., 1988, Yassin et al., 2010). Duodenal fluid aspiration and intestine biopsy or the use of Enterotest ® may be required to detect the *Strongyloides* parasite (Yassin et al., 2010).

10. Treatment

Early identification of the disease and anthelminthic treatment results in a better prognosis for strongyloidiasis and, in many cases, prevents a fatal infection (Basile et al., 2010). *S. stercoralis* is resistant to anthelmintic drugs, and the parasite has the capacity to replicate and increase the worm burden again (Grove, 1996).

Thiabendazole, albendazole, and mebendazole are effective drugs against *S. stercoralis.* Thiabendazole was the drug of choice for treatment of strongyloidiasis, with a cure rate of up to 80%. Albendazole has variable therapeutic efficacy but has been used in hyperinfection syndrome and remains a viable treatment alternative to ivermectin. Mebendazole can be used to treat strongyloidiasis but is not recommended because of an association with liver dysfunction (Rajapurkar et al., 2007). Recently, there has been a change in the treatment of strongyloidiasis, as more studies support the choice of the drug ivermectin, which is effective at killing worms in the intestine. In patients with hyperinfection syndrome, ivermectin is considered the first-line therapy, and longer courses of treatment are indicated (Roxby et al., 2009).

Efficient treatment of strongyloidiasis depends on several factors that can decrease the efficacy of the drugs used for treatment, such as immunodeficiency, corticosteroid use, or co-infection with HTLV-1 (Vadlamudi et al., 2006). Prolonged or repeated treatment may be required in patients receiving immunosuppressive drugs.

Other measures, including decreasing the dosage of corticosteroids, discontinuing immunosuppressive therapies and treating bacterial infections, are essential elements in the treatment of these patients. In all cases, patients with strongyloidiasis, regardless of the severity of symptoms, must be treated to prevent long-term complications (Montes et al., 2010).

11. Control

Like other soil-transmitted nematode infections, strongyloidiasis can be controlled by improving sanitary conditions and properly disposing of faeces. Infected patients should be treated, even if they are asymptomatic, to preclude the possible onset of autoinfection. Immunosuppressants are contraindicated in these patients. Personal hygienic measures like proper protection of the skin to prevent contact with infected soil, community education about protective and hygienic measures, and prompt treatment of diagnosed cases would help prevent the disease (Vadlamudi et al., 2006).

12. Conclusion

Strongyloidiasis is an infection with a tendency to become chronic with an indolent course. However, in immunocompromised patients, especially those treated with corticosteroids,

hyperinfection syndrome can compromise the prognosis of the patient. The mortality rates of hyperinfection are high, making *Strongyloides* infection an important global health problem. It is important to understand the biology and immunology of infection with *S. stercoralis* and the altered courses of infection that may occur when immune regulation is compromised. Clinicians who are aware of the possibility of hyperinfection are better equipped to diagnose, treat, or altogether prevent the fatal consequences of this lethal nematode.

13. Acknowledgments

We wish to thank Elaine Medeiros Floriano for excellent technical assistance and Mara R. Celes and Marcela S. Oliveira for photography assistance.
Financial support: This study was supported by the FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico). Cristiane Tefé-Silva is supported by a scholarship through CNPq. Simone G. Ramos and Lúcia H. Faccioli are investigators at CNPq.

14. References

Abe, T., Sugaya, H., &Yoshimura, K. (1998). Analysis of T cell populations and IL-3 mRNA expression in mesenteric lymph node cells and intestinal intraepithelial lymphocytes in *Strongyloides ratti*-infected mice. *Journal of Helminthology,* Vol.72, No.1, (March), pp. 1-8, ISSN 0022-149X

Abraham, D., Rotman, H.L., Haberstroh, H.F., Yutanawiboonchai, W., Brigandi, R.A., Leon, O., Nolan, T.J., & Schad, G.A. (1995). *Strongyloides stercoralis*: protective immunity to third-stage larvae inBALB/cByJ mice. *Experimental Parasitology*, Vol.80, No.2, (March), pp. 297-307, ISSN 1090-2449

Al Maslamani, M.A., Al Soub, H.A., Al Khal, A.L., Al Bozom, I.A., Abu Khattab, M.J.,& Chacko, K.C. (2009). *Strongyloides stercoralis* hyperinfection after corticosteroid therapy: a report of two cases. *Annals of Saudi of Medicine,* Vol.29, No.5, (September-October), pp. 397-401, ISSN 0975-4466

Albonico, M., Crompton, D.W.T., & Savioli, L. (1999). Control strategies for human intestinal nematode infections. *Advances in Parasitology*, Vol.42, pp. 277–341, ISSN 0065-308X

Anthony, R.M., Rutitzky, L.I., Urban, J.F. Jr., Stadecker, M.J., & Gause, W.C. (2007). Protective immune mechanisms in helminth infection. *Nature Reviews Immunology,* Vol.7, pp. 975-987, ISSN 1474-1733

Armignacco, O., Capecchi, A., De Mori, P., & Grillo, L.R. (1989). *Strongyloides stercoralis* hyperinfection and the acquired immunodeficiency syndrome. *American Journal of Medicine*, Vol.86, No.2, (February), pp. 258, ISSN 0002-9343

Balagopal, A., Mills, L., Shah, A., & Subramanian, A. (2009). Detection and treatment of *Strongyloides* hyperinfection syndrome following lung transplantation. *Transplant Infectious Disease,* Vol.11, No.2, (April), pp. 149-154, ISSN 1399-3062

Bamias, G., Toskas, A., Psychogiou, M., Delladetsima, I., Siakavellas, S.I., Dimarogona, K., & Daikos, G.L. (2010). *Strongyloides* hyperinfection syndrome presenting as

enterococcal meningitis in a low-endemicity area. *Virulence*, Vol.1, No.5, (September-October), pp. 468-470, ISSN 2150-5608

Barr, J.R. (1978). *Strongyloides stercoralis. Canadian Medical Association Journal,* Vol.118, No.8, (April), pp. 933-935, ISSN 1488-2329

Basile, A., Simzar, S., Bentow, J., Antelo, F., Shitabata, P., Peng, S.K., & Craft, N. (2010). Disseminated *Strongyloides stercoralis*: hyperinfection during medical immunosuppression. *Journal of the American Academy of Dermatology*, Vol.63, No.5, (November), pp. 896-902, ISSN 0190-9622

Ben-Youssef, R., Baron, P., Edson, F., Raghavan, R., & Okechukwu, O. (2005). *Strongyloides stercoralis* infection from pancreas allograft: case report. *Transplantation,* Vol.80, No.7, (October), pp. 997-998, ISSN 1534-0608

Brandt de Oliveira, R., Voltarelli, J.C., & Meneghelli, U.G. (1981). Severe strongyloidiasis associated with hypogammaglobulinaemia. *Parasite Immunology*, Vol.3, No.2, (Summer), pp. 165-169, ISSN 1365-3024

Brown, M., Cartledge, J.D., & Miller, R.F. (2006). Dissemination of Strongyloides stercoralis as an immune restoration phenomenon in an HIV-1-infected man on antiretroviral therapy. *International Journal of STD & AIDS,* Vol.15, No.8, (August), pp.560-661, ISSN 0956-4624

Carvalho, E.M. & Da Fonseca Porto, A. (2004). Epidemiological and clinical interaction between HTLV-1 and *Strongyloides stercoralis*. *Parasite Immunology*, Vol.26, No.11-12, (November-December), pp. 487-497, ISSN 1365-302

Concha, R., Harrington, W.Jr., & Rogers, A.I. (2005). Intestinal strongyloidiasis: recognition, management, and determinants of outcome. *Journal of Clinical Gastroenterology,* Vol.39, No.3, (March), pp. 203-211, ISSN 1539-2031

Coutinho, HB., Robalinho, T.I., Coutinho, V.B., Almeida, J.R., Filho, J.T., King, G., Jenkins, D., Mahida, Y., Sewell, H.F., & Wakelin, D. (1996). Immunocytochemistry of mucosal changes in patients infected with the intestinal nematode *Strongyloides stercoralis*. *Journal of Clinical Pathology*, Vol.49, No.10, (October), pp. 717-720, ISSN 1472-4146

DeVault, G.A.Jr., King, J.W., Rohr, M.S., Landreneau, M.D., Brown, S.T.III., & McDonald, J.C. (1990). Opportunistic infections with *Strongyloides stercoralis* in renal transplantation. *Reviews of Infectious Diseases,* Vol.12, No.4, (July-August), pp. 653-671, ISSN 0162-0886

Dulley, F.L., Costa, S., Cosentino, R., Gamba, C., & Saboya, R. (2009). *Strongyloides stercoralis* hyperinfection after allogeneic stem cell transplantation. *Bone Marrow Transplantation*, Vol.43, No.9, (May), pp. 741-742, ISSN 1476-5365

Dutcher, J.P., Marcus, S.L., Tanowitz, H.B., Wittner, M., Fuks, J.Z., & Wiernik, P.H. (1990). Disseminated strongyloidiasis with central nervous system involvement diagnosed antemortem in a patient with acquired immunodeficiency syndrome and Burkitts lymphoma. *Cancer,* Vol.66, No.11, (December), pp. 2417-2420, ISSN 1097-0142

El-Malky, M., Maruyama, H., Hirabayashi, Y., Shimada, S., Yoshida, A., Amano, T., Tominaga, A., Takatsu, K., & Ohta, N. (2003). Intraepithelial infiltration of eosinophils and their contribution to the elimination of adult intestinal nematode,

Strongyloides venezuelensis in mice. *Parasitology International,* Vol.52, No.1, (March), pp. 71-79, ISSN 1383-5769

Galioto, A.M., Hess, J.A., Nolan, T.J., Schad, G.A., Lee, J.J., & Abraham, D. (2006). Role of eosinophils and neutrophils in innate and adaptive protective immunity to larval *Strongyloides stercoralis* in mice. *Infection and Immunity,* Vol.74, No.10, (October), pp. 5730-5738, ISSN 1098-5522

Genta, R.M. (1989). Global prevalence of strongyloidiasis: critical review with epidemiologic insights into the prevention of disseminated disease. *Reviews of Infectious Diseases*, Vol.11, No.5, (September-October), pp. 755-767, ISSN 0162-0886

Genta, R.M. (1992). Dysregulation of strongyloidiasis: a new hypothesis. *Clinical Microbiology Reviews,* Vol.5, No.4, (October), pp. 345-355, ISSN 1098-6618

Grove, D.I. (1994). Strongyloidiasis: a conundrum for gastroenterologists. *Gut,* Vol.35, No.4, (April), pp. 437-440, ISSN 1468-3288

Grove, D.I. (1996). Human strongyloidiasis. *Advances in Parasitology,* Vol.38, pp. 251-309, ISBN 0-12-031738-9, London United Kingdom

Grove, D.I. (1989a). Clinical manifestations. In: Strongyloidiasis a major roundworm infection of man, D.I. Grove (Ed.), 155–174, Taylor and Francis, ISBN 0-85066-732-1, London, United Kingdom

Herbert, D.R., Lee, J.J., Lee, N.A., Nolan, T.J., Schad, G.A., & Abraham, D. (2000) Role of IL-5 in innate and adaptive immunity to larval *Strongyloides stercoralis* in mice. *Journal of Immunology*, Vol.165, No.8, (October), pp. 4544-4551, ISSN 1550-6606

Igra-Siegman, Y., Kapila, R., Sem, P., Kaminski, Z.C., & Louria, D.B. (1981). Syndrome of hyperinfection with *Strongyloides stercoralis*. *Reviews of Infectious Diseases*, Vol.3, No.3, (May-June), pp. 397-407, ISSN 0162-0886

Iriemenam, N.C., Sanyaolu, A.O., Oyibo, W.A., & Fagbenro-Beyioku, A.F. (2010). *Strongyloides stercoralis* and the immune response. *Parasitology International*, Vol.59, No.1, (March), pp. 9-14, ISSN 1383-5769

Keiser, P.B. & Nutman, T.B. (2004). *Strongyloides stercoralis* in the immunocompromised population. *Clinical Microbiology Reviews,* Vol.17, No.1, (January), pp. 208-217, ISSN 1098-6618

Kerepesi, L.A., Hess, J.A., Nolan, T.J., Schad, G.A., & Abraham, D. (2006). Complement component C3 is required for protective innate and adaptive immunity to larval *Strongyloides stercoralis* in mice. *The Journal of Immunology,* Vol. 176, No.7, (April), pp. 4315-4322, ISSN 1550-6606

Kimura, K., Song, C.H., Rastogi, A., Dranoff, G., Galli, S.J., & Lantz, C.S. (2006). Interleukin-3 and c-Kit/stem cell factor are required for normal eosinophil responses in mice infected with *Strongyloides venezuelensis*. *Laboratory Investigation,* Vol.86, No.10, (October), pp. 987-996, ISSN 1530-0307

Kinjo, T., Tsuhako, K., Nakazato, I., Ito, E., Sato, Y., Koyanagi, Y., & Iwamasa, T. (1998). Extensive intra-alveolar haemorrhage caused by disseminated strongyloidiasis. *International Journal for Parasitology*, Vol.28, No.2, (February), pp. 323-330, ISSN 1879-013

Kishimoto, K., Hokama, A., Hirata, T; Ihama, Y., Nakamoto, M., Kinjo, N., Kinjo, F., & Fujita, J. (2008). Endoscopic and histopathological study on the duodenum of *Strongyloides*

stercoralis hyperinfection. *World Journal of Gastroenterology*, Vol.14, No.11, (March), pp. 1768-1773, ISSN 1007-9327

Klion, A.D. & Nutman, T.B. (2004). The role of eosinophils in host defense against helminth parasites. *Journal of Allergy and Clinical Immunology,* Vol.113, No.1, (January), pp. 30-37, ISSN 0091-6749

Krishnan, S., Barger, G., & Chandrasekar, P. (2006). Recurrent S*trongyloides stercoralis* hyperinfection syndrome case report and a brief review. *Infectious Diseases in Clinical Practice,* Vol.14, No.4, (July), pp. 240–243, ISSN 1056-9103

Kobayashi, T., Tsuchiya, K., Hara, T., Nakahata, T., Kurokawa, M., Ishiwata, K., Uchiyama, F., & Nawa, Y. (2008). Intestinal mast cell response and mucosal defence against *Strongyloides venezuelensis* in interleukin-3-hyporesponsive mice. *Parasite Immunology,* Vol.20, No.6, (June), pp. 279-284, ISSN 1365-3024

Lantz, C.S., Min, B., Tsai, M., Chatterjea, D., Dranoff, G., & Galli, S.J. (2008). IL-3 is required for increases in blood basophils in nematode infection in mice and can enhance IgE-dependent IL-4 production by basophils in vitro. *Laboratory Investigation,* Vol.88, No.11, (November), pp. 1134-1142, ISSN 1530-0307

Ligas, J.A., Kerepesi, L.A., Galioto, A.M., Lustigman, S., Nolan, T.J., Schad, G.A., & Abraham, D. (2003). Specificity and mechanism of immunoglobulin M (IgM)- and IgG-dependent protective immunity to larval *Strongyloides stercoralis* in mice. *Infection and Immunity,* Vol.71, No.12, (December), pp. 6835-6843, ISSN 1098-5522

Lindo, J.F., Dubon, J.M., Ager, A.L., de Gourville, E.M., Solo-Gabriele, H., Klaskala, W.I., Baum, M.K., & Palmer, C.J. (1998). Intestinal parasitic infections in human immunodeficiency virus (HIV)-positive and HIV-negative individuals in San Pedro Sula, Honduras. *American Journal of Tropical Medicine and Hygiene*. Vol.58, No.4, (April), pp. 431-435, ISSN 0002-9637

Link, K. & Orenstein, R. (1999). Bacterial complications of strongyloidiasis: *Streptococcus bovis meningitis*. *Southern Medical Journal,* Vol.72, No.7, (July), pp. 728-731, ISSN 0038-4348

Machado, E.R., Ueta, M.T., Lourenço, E.V., Anibal, F.F., Sorgi, C.A., Soares, E.G., Roque-Barreira, M.C., Medeiros, A.I., & Faccioli, L.H. (2005). Leukotrienes play a role in the control of parasite burden in murine strongyloidiasis. *Journal of Immunology*, Vol.175, No.6, (September), pp. 3892-3899, ISSN 1550-6606

Machado, E.R., Carlos, D., Lourenço, E.V., Sorgi, C.A., Silva, E.V., Ramos, S.G., Ueta, M.T., Aronoff, D.M., & Faccioli, L.H. (2009). Counterregulation of Th2 immunity by interleukin 12 reduces host defenses against *Strongyloides venezuelensis* infection. *Microbes and Infection,* Vol.11, No.5, (April), pp. 571-578, ISSN 1286-4579

Machado, E.R., Carlos, D., Sorgi, C.A., Ramos, S.G., Souza, D.I., Soares, E.G., Costa-Cruz, .JM., Ueta, M.T., Aronoff, D.M., & Faccioli, L.H. (2011). Dexamethasone effects in the *Strongyloides venezuelensis* infection in murine model. *American Journal of Tropical Medicine and Hygiene,* Vol.84, No.6, (June), pp. 957-966, ISSN 0002-9637

Mahmoud, A.A. (1996). Strongyloidiasis. *Clinical Infectious Diseases,* Vol.23, No.5, (November), pp. 949-952, ISSN 1537-6591

Maizels, R.M. & Yazdanbakhsh, M. (2003). Immune regulation by helminth parasites: cellular and molecular mechanisms. *Nature Reviews Immunology,* Vol.3, (September), pp. 733-744, ISSN 1474-1733

Marcos, L.A., Terashima, A., Dupont, H.L., & Gotuzzo, E. (2008). *Strongyloides* hyperinfection syndrome: an emerging global infectious disease. *Transaction of the Royal Society of Tropical Medicine and Hygiene*, Vol.102, No.4, (April), pp. 314-318, ISSN 0035-9203

Mir, A., Benahmed, D., Igual, R., Borrás, R., O'Connor, J.E., Moreno, M.J., & Rull, S. (2006). Eosinophil-selective mediators in human strongyloidiasis. *Parasite Immunology,* Vol.28, No.8, (August), pp. 397-400, ISSN 1365-3024

Montes, M., Sanchez, C., Verdonck, K., Lake, J.E., Gonzalez, E., Lopez, G., Terashima, A., Nolan, T., Lewis, D.E., Gotuzzo, E., White, & A.C.Jr. (2009). Regulatory T cell expansion in HTLV-1 and strongyloidiasis co-infection is associated with reduced IL-5 responses to *Strongyloides stercoralis* antigen. *PLoS Neglected Tropical Diseases,* Vol.9, No.6, (June), pp. e456, ISSN 1935-2735

Negrão-Corrêa, D., Pinho, V., Souza, D.G., Pereira, A.T., Fernandes, A., Scheuermann, K., Souza, A.L., & Teixeira, M.M. (2006). Expression of IL-4 receptor on non-bone marrow-derived cells is necessary for the timely elimination of *Strongyloides venezuelensis* in mice, but not for intestinal IL-4 production. *International Journal for Parasitology,* Vol.36, No.10-11, *(*September), pp. 1185-1195, ISSN 1879-0135

Olsen, A., van Lieshout, L., Marti, H., Polderman, T., Polman, K., Steinmann, P., Stothard, R., Thybo, S., Verweij, J.J., & Magnussen, P. (2009). Strongyloidiasis-the most neglected of the neglected tropical diseases? *Transaction of the Royal Society of Tropical Medicine and Hygiene,* Vol.103, No.10, (October), pp. 967-972, ISSN 0035-9203

Padigel, U.M., Lee, J.J., Nolan, T.J., Schad, G.A., & Abraham, D. (2006). Eosinophils can function as antigen-presenting cells to induce primary and secondary immune responses to *Strongyloides stercoralis. Infection and Immunity*, Vol.74, No.6, (June), pp. 3232-3238, ISSN 1098-5522

Pampiglione, S. & Ricciardi, M.L. (1972). Geographic distribution of *Strongyloides fuelleborni* in humans in tropical Africa. *Parasitologia,* Vol.14, pp. 329–338

Patel, N., Kreider, T., Urban JF Jr., & Gause, W.C. (2009). Characterisation of effector mechanisms at the host: parasite interface during the immune response to tissue-dwelling intestinal nematode parasites. *International Journal for Parasitology*, Vol.39, No.1, (January), pp. 13-21, ISSN 1879-0135

Paterson, S., Wilkes, C., Bleay, C., & Viney, M.E. (2008). Immunological responses elicited by different infection regimes with *Strongyloides ratti. PLoS One*, Vol.3, No.6, (June), pp. e2509, ISSN 1932-6203

Porto, A.F., Neva, F.A., Bittencourt, H., Lisboa, W., Thompson, R., Alcântara, L., & Carvalho, E.M. (2001). HTLV-1 decreases Th2 type of immune response in patients with

strongyloidiasis. *Parasite Immunology*, Vol.23, No.9, (September), pp. 503-507, ISSN 1365-3024

Rajapurkar, M., Hegde, U., Rokhade, M., Gang, S., & Gohel, K. (2007). Respiratory hyperinfection with *Strongyloides stercoralis* in a patient with renal failure. *Nature Clinical Practice Nephrology*, Vol.3, No.10, (October), pp. 573-577, ISSN 1745-8331

Rivasi, F., Pampiglione, S., Boldorini, R., & Cardinale, L. (2006). Histopathology of gastric and duodenal *Strongyloides stercoralis* locations in fifteen immunocompromised subjects. *Archives of Pathology & Laboratory Medicine,* Vol.130, No.12, (December), pp. 1792-1798, ISSN 1543-2165

Rodriguez-Hernandez, M.J., Ruiz-Perez-Pipaon, M., Cañas, E., Bernal, C., & Gavilan, F. (2009). *Strongyloides stercoralis* hyperinfection transmitted by liver allograft in a transplant recipient. *American Journal of Transplantation,* Vol.9, No.11, (November), pp. 2637-2640, ISSN 1600-6143

Roxby, A.C., Gottlieb, G.S., & Limaye, A.P. (2009). Strongyloidiasis in transplant patients. *Clinical Infection Diseases,* Vol.49, No.9, (November), pp. 1411-1423, ISSN 1537-6591

Sathe, P.A. & Madiwale, C.V. (2006). Strongyloidiasis hyperinfection in a patient with membranoproliferative glomerulonephritis. *Journal of Postgraduate Medicine*, Vol.52, No.3, (July-September), pp. 221-222, ISSN 0022-3859

Schad, G.A. (1989). Morphology and life history of *Strongyloides stercoralis*. In: Strongyloidiasis a major roundworm infection of man, D.I. Grove (Ed.), 85-104, Taylor and Francis, ISBN 0-85066-732-1, London, United Kingdom

Schaeffer, M.W., Buell, J.F., Gupta, M., Conway, G.D., Akhter, S.A., & Wagoner, L.E. (2004). *Strongyloides* hyperinfection syndrome after heart transplantation: case report and review of the literature. *The Journal of Heart and Lung Transplantation*, Vol.23, No.7, (July), pp. 905–911, ISSN 1053-2498

Seet, R.C., Lau, L.G., & Tambyah, P.A. (2005). *Strongyloides* hyperinfection and hypogammaglobulinemia. *Clinical and Diagnostic Laboratory Immunology,* Vol.12, No.5, *(*May), pp. 680-682, ISSN 1098-6588

Shea-Donohue; T.B. & Urban, J.F.Jr. (2004). Gastrointestinal parasite and host interactions. *Current Opinion in Gastroenterology*, Vol.20, No.1 (January), pp. 3-9, ISSN 1531-7056

Siddiqui, A.A. & Berk, S.L. (2001). Diagnosis of *Strongyloides stercoralis* infection. *Clinical Infectious Diseases*, Vol.33, No.7, (October), pp. 1040-1047, ISSN 1537-6591

Siddiqui, A.A., Stanley, C.S., Skelly, P.J., & Berk, S.L. (2000). A cDNA encoding a nuclear hormone receptor of the steroid/thyroidhormone-receptor superfamily from the human parasitic nematode *Strongyloides stercoralis*. *Parasitology Research*, Vol.86, No.1, (January), pp. 24-29, ISSN 1432-1955

Speare, R. (1989). Identification of species of *Strongyloides*. In: Strongyloidiasis a major roundworm infection of man, D.I. Grove (Ed.), 11-84, Taylor and Francis, ISBN 0-85066-732-1, London, United Kingdom

Tefé-Silva, C., Souza, D.I., Ueta, M.T., Floriano, E.M., Faccioli, L.H., & Ramos, S.G. (2008). Interference of dexamethasone in the pulmonary cycle of *Strongyloides venezuelensis*

in rats. *American Journal of Tropical Medicine and Hygiene*, Vol.79, No.4, (October), pp. 571-578, ISSN 0002-9637

Tefé-Silva, C., Beneli, C.T., Celes, M.R., Machado, E.R., Ueta, M.T., Sorgi, C.A., Floriano, E.M., Faccioli, L.H., & Ramos, S.G. (2012). Dexamethasone reduces bronchial wall remodeling during pulmonary migration of *Strongyloides venezuelensis* larvae in rats. P*arasitology International*, Accepted, ISSN 1383-5769

Trajman, A., MacDonald, T.T., & Elia, C.C. (1997). Intestinal immune cells in *Strongyloides stercoralis* infection. *Journal of Clinical Pathology*, Vol.50, No.12, (December), pp. 991-995, ISSN 1472-4146

Urban, J.F.Jr., Madden, K.B., Svetić, A., Cheever, A., Trotta, P.P., Gause, W.C., Katona, .IM., & Finkelman, F.D. (1992). The importance of Th2 cytokines in protective immunity to nematodes. *Immunology Reviews,* Vol.127, (June), pp. 205-220, ISSN 1600-065X

Vadlamudi, R.S., Chi, D.S., & Krishnaswamy, G. (2006). Intestinal strongyloidiasis and hyperinfection syndrome. *Clinical and Molecular Allergy*, Vol.4, No.8, (May), ISSN 1476-7961

Valar, C., Keitel, E., Dal Prá, R.L., Gnatta, D., Santos, A.F., Bianco, P.D., Sukiennik, T.C., Pegas, K.L., Bittar, A.E., Oliveira, K.T., & Garcia, V.D. (2007). Parasitic infection in renal transplant recipients. *Transplantation Proceedings*, Vol.39, No.2, (March), pp. 460–462, ISSN 0041-1345

Vilela, E.G., Clemente, W.T., Mira, R.R., Torres, H.O., Veloso, L.F., Fonseca, L.P, de Carvalho E Fonseca, L.R., Franca, M.C., & Lima, A.S. (2009). *Strongyloides stercoralis* hyperinfection syndrome after liver transplantation: case report and literature review. *Transplant Infectious Disease*, Vol.11, No.2, (April), pp. 132–136, ISSN 1399-3062

Viney, M.E. & Lok, J.B. (2007). *Strongyloides* spp. *WormBook*, Vol.23, No.2, (April), pp. 1-15, ISSN 1551-8507

Werneck-Silva, A.L., Alvares, E.P., Gama, P., Damiao, A.O., Osaki, L.H., Ogias, D., & Sipahi, A.M. (2006). Intestinal damage in strongyloidiasis: the imbalance between cell death and proliferation. *Digestive Diseases Sciences,* Vol.51, No.6, (June), pp. 1063-1069, ISSN 1573-2568

Wilkes, C.P., Bleay, C., Paterson, S., &Viney, M.E. (2007). The immune response during a *Strongyloides ratti* infection of rats. *Parasite Immunology*, Vol.29, No.7, (July), pp. 339-346, ISSN 1365-3024

Williams, J., Dralle, W., Berk, S.L., & Verghese, A. (1988). Diagnosis of pulmonary strongyloidiasis by bronchoalveolar lavage. *Chest*, Vol.94, No.3, (September), pp. 643-644, ISSN 1931-3543

Wirk, B., & Wingard, J.R. (2009). *Strongyloides stercoralis* hyperinfection in hematopoietic stem cell transplantation. *Transplant Infectious Disease*, Vol.11, No.2, (April), pp. 143-148, ISSN 1399-3062

Yassin, M.A., El Omri, H., Al-Hijji, I., Taha, R., Hassan, R., Aboudi, K.A., & El-Ayoubi, H. (2010). Fatal *Strongyloides stercoralis* hyper-infection in a patient with multiple myeloma. *The Brazilian Journal of Infectious Diseases,* Vol.14, No.5, (October), pp. 536-539, ISSN 1678-4391

Zumla, A.I. & James, D.G. (2002). Immunologic aspects of tropical lung disease. *Clinics in Chest Medicine*, Vol.23, No.2, (June), pp. 283-308, ISSN 1557-8216

23

Molecular Diagnosis and Monitoring of Benzimidazole Susceptibility of Human Filariids

Adisak Bhumiratana[1,2,3], Apiradee Intarapuk[3], Danai Sangthong[3], Surachart Koyadun[4], Prapassorn Pechgit[1] and Jinrapa Pothikasikorn[5]

[1]*Department of Parasitology and Entomology, Faculty of Public Health, Mahidol University, Bangkok*

[2]*Center for EcoHealth Disease Modeling and Intervention Development Research, Faculty of Public Health, Mahidol University, Bangkok*

[3]*Environmental Pathogen Molecular Biology and Epidemiology Research Unit, Faculty of Veterinary Medicine, Mahanakorn University of Technology, Bangkok*

[4]*Ministry of Public Health, Department of Disease Control, Office of Disease Prevention and Control 11 Nakhon Si Thammarat*

[5]*Department of Microbiology, Faculty of Science, Mahidol University, Bangkok*

Thailand

1. Introduction

Lymphatic filarial nematode parasites, mainly *Wuchereria bancrofti* and *Brugia malayi,* are causing agents of lymphatic filariasis in humans, which can be effectively treated with antifilarial drugs including diethylcarbamazine (DEC) and ivermectin. Albendazole, an effective benzimidazole compound, acts as a board-spectrum anthelminthic drug, and when combined with either one of antifilarial drugs, it exerts synergistic effects on reduction of peripheral microfilaremia in lymphatic filariasis cases. However, the varying parasite infection levels in those treated with DEC or ivermectin alone or in combination with albendazole are due to differences in drug responses. The additional clearance of infection with albendazole relative to what is observed with DEC or ivermectin alone suggests that albendazole has different parasite target(s). The homologous *β-tubulin* gene of human and veterinary filariids that β-tubulin homologs have conserved domains structurally related to other orthologs among the nematodes, cestodes, trematodes and vertebrate hosts, is responsible for benzimidazole susceptibility. The genetic inheritance of resistance in nematode parasites can undergo under selection of benzimidazole compounds in a way that albendazole resistance mechanism involves one of two single amino acid substitutions from phenylalanine to tyrosine in parasite β-tubulin at position 167 or 200. This genetically-stable marker has shown promise for molecular diagnosis and monitoring of *W. bancrofti* infections that carry responsible genotypes associated with benzimidazole susceptibility or resistance. In particular, this approach can augment the surveillance and monitoring of mass treatment impacts on the parasite populations in target areas where long-running elimination programs for lymphatic filariasis are implemented at a large-scale by using a regionally-adopted combination therapy with antifilarial drugs, recommended by the World Health Organization.

2. Lymphatic filariasis towards elimination

2.1 Factors that favor elimination

Lymphatic filariasis (LF) is a mosquito-borne parasitic disease caused by three main species of thread-like filarial nematode parasites belonging to the superfamily Filarioidea, which are *Wuchereria bancrofti* and *Brugia malayi*, and lesser extent by *Brugia timori*. These endoparasitic nematode lifestyles have a highly conservative life-cycle development sequence in which they develop their metamorphosis in both human and mosquito (Fig. 1). The adult worms that cause clinical manifestations of the disease are dioecious. Male and female worms have separate reproductive system in their pseudocoelomatic body cavity and they live in the

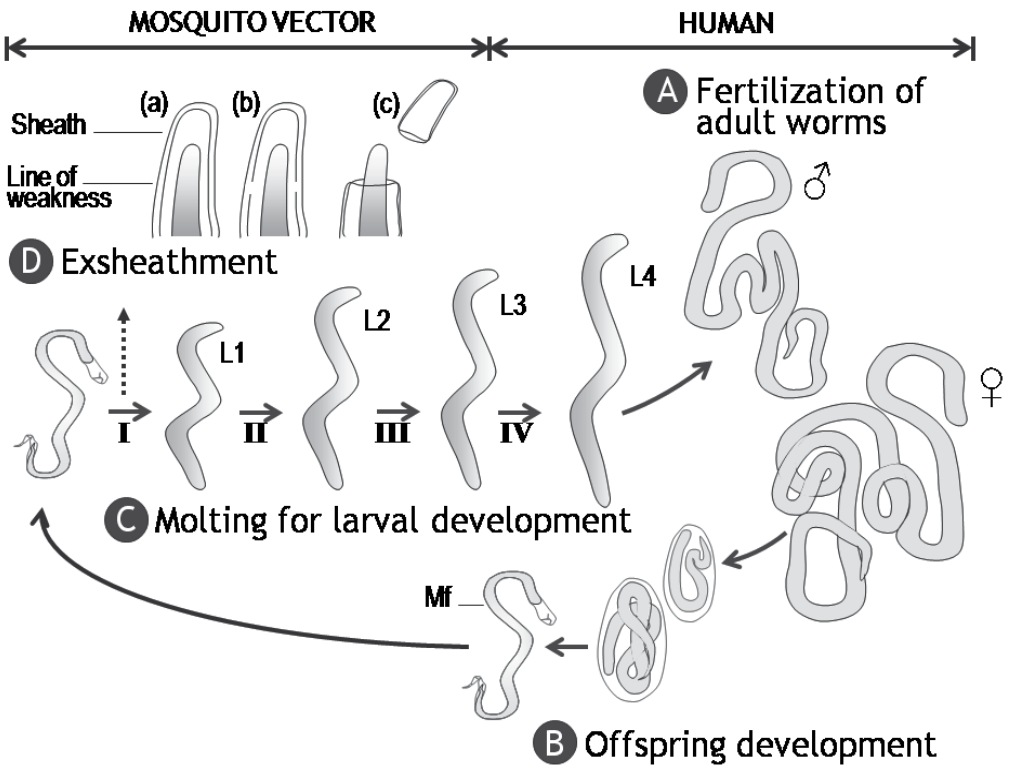


Fig. 1. A development sequence of filarial nematode parasite in human and mosquito (A-D). After the induction of L3 infection in susceptible human host, the mature filarial female worm (A) that possesses fecundicity and fertilization can produce microfilariae (Mf). These offsprings develop from fertilized eggs (B) in the uterus of female worm. They are ingested during the bite of mosquito vector, and consequently, a 5-stage molting progression (B) initially starts after exsheatment of the Mf in mosquito's midgut (C). In this regard, the probable mechanisms involve: (a) proposed anterior of line of weakness, (b) internal digestion of the sheath, and (c) exsheatment of anterior end. Third stage larvae (L3) possess post-infective stage development in mosquito thoracic muscle and then migrate to the proboscis. They are transmitted by infective mosquito during a blood meal, and that they become the L4 (or L5) and mature adult worms in the lymphatic system in human.

human lymphatic system, i.e., lymphatic vessels, with 5-15 years of life expectancy (Table 1). Only when its fecundic lifespan is capable of mating does the lymphatic-dwelling female worm produce advanced stage of sheathed larvae called "microfilariae". These short-living offsprings then penetrate the blood circulation. For a complete life cycle, the microfilariae are ingested by susceptible female mosquito during a blood meal in which they can develop further into larval stages L1-L2 to infective L3 stage. Transmission occurs when the infected mosquito transmits this L3 stage to susceptible persons during other blood meals. The naturally acquired transmission is associated with both intrinsic and extrinsic factors that can regulate the parasitic worm burdens in an endemic population (Fig. 2).

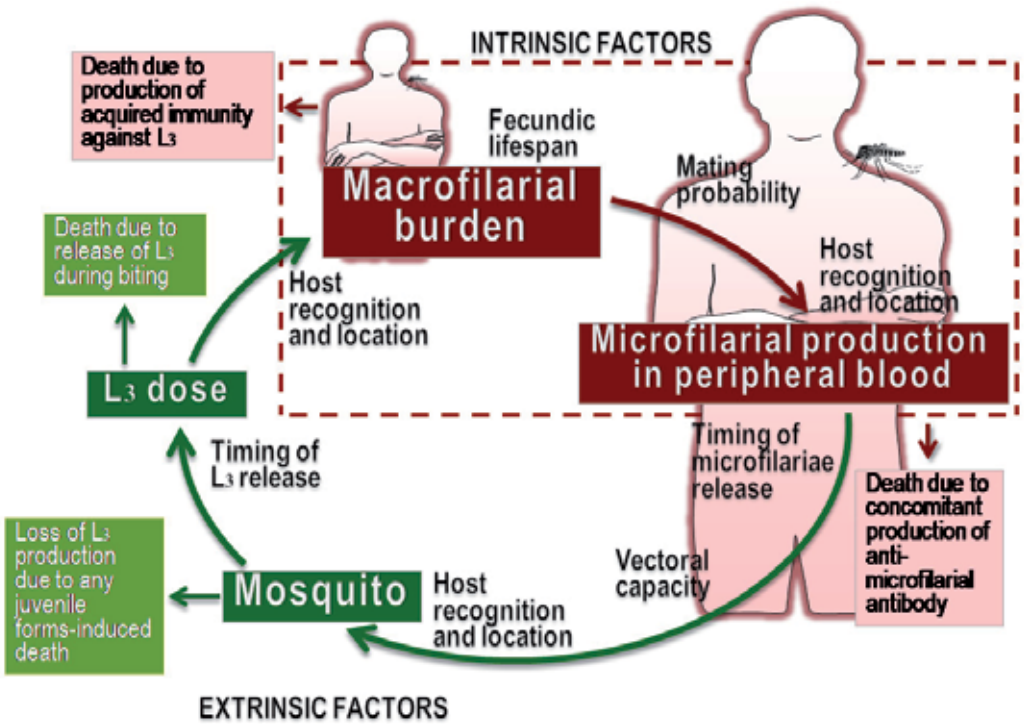


Fig. 2. An explanatory model of human-parasite-vector interactions and favorable factors that influence its adaptation in hosts. The lymphatic filarial infections in humans depend on extrinsic factors of incoming L3 inocula and host recognition and location, and a proportion of adult worms with fecundicity and mating probability. Transmission is influenced by host-vector combination (host location and recognition of mosquito feeding habits), microfilarial loads in blood with timing of microfilariae release, and vectoral capacity (longevity and low refractoriness). Long lifespan of the mosquito and timing of L3 release are favorable factors of transmission.

As such, understanding of how these filarial nematode parasites can be removed from the human hemisphere is to understand basically the biology of their life cycle. How do the parasite taxa succeed their complete life cycle in the certain conditions under which they evoke host exploitation strategies? The biology of the parasitic symbionts can disclosure the parasite diversity and fitness shaped by hosts and environmental constraints that the

parasites can evolve the adaptation, i.e., the ability to control the physiology and behavior of their host for their own benefit. It has been known so far their distribution among hosts and strategies of host exploitation are restricted in number of host species. The *W. bancrofti*, which is highly host-specific parasite, is sessile to human while other zoonotic *Brugia* taxa exploit other non-human reservoir hosts. This host-specificity variation does not account for the origin of the parasitism as they succeed their parasitism and share common transmission pattern and complex life cycle in vertebrate hosts and arthropods. The geographical variation, on the other hand, can be restrained by selective pressures from the hosts, or the physio-chemical environments such as therapeutic agents and insecticides, or due to phylogenetic constraints. Such this selection can be explained by the experimental infections, mainly using *Brugia* species in rodents (e.g., jirds, hamsters, rats and mice), dogs, ferrets, cats and monkeys. All of which were proposed not just for understanding vertebrate immunology, pathology and chemotherapy, but also for exploring the interactions of the parasite and host. Among these host-parasite systems, *B. pahangi* that infects naturally cats in the Southeast Asia is experimentally appropriate for studies of infection and disease dynamics (Table 1).

Parameter	**Cat**	**Human**
Parasite	*Brugia pahangi*	*Wuchereria bancrofti* *B. malayi, B. timori*
Longevity (years)	7-8	5-15
Mature female worm length (cm)	3-7	5-10
Location of :		
Adult	Lymphatics	Lymphatics
Microfilariae	Blood	Blood
Vector	Mosquito	Mosquito
Typical infection/disease sequlae[a]	Infection – Loss of infection – Pathology	Infection – Loss of infection – Pathology

[a]For both host-parasite systems, infection patterns encapsulate a spectrum ranging from asymptomatic persistent microfilaremia to symptomatic amicrofilaremia or complete refractoriness to infection. Adapted from Grenfell et al (1991).

Table 1. Correlation between feline and human lymphatic filariasis

Because of their conservation of complex life-cycle development between feline and human lymphatic filarial parasites, it induces the infection and clinical sequelae in susceptible cats, and hence, resembles that of *Brugia* and *Wuchereria* in humans. However, the parasite does not always succeed their population diversity by increasing its fitness in the hosts (Fig. 2). Its population dynamic is primarily influenced by naturally acquired immunity (i.e., a type of concomitant immunity), which plays a significant role in host selection pressure to restrict a parasitic worm burden (Bundy et al, 1991; Grenfell et al, 1991; Mitchell, 1991; Grenfell and Micheal, 1992). This immunity against incoming L3 cannot remove them from the infection in humans but restrict a number of L3 by a production of concomitant immunity. Variability of adult worm burden results in a proportion of microfilaremic and amicrofilaremic persons in the population. The circulating microfilariae have short life cycle in human blood circulation; a proportion of the microfilariae can be removed by anti-microfilarial antibody

(Simonsen et al, 1996; 2008; Ravindran et al, 2003). They develop two molts (L1 and L2) and induce the infection in susceptible mosquito vectors while the infected mosquitoes can regulate melanization involved in innate immune defense and wound healing to the penetration of filarial nematodes (Zou et al, 2010; Castillo et al, 2011). In blood-engorged mosquitoes that harbor high microfilarial density, they can induce death due to the vector intolerance against development of juvenile forms, and consequently, this leads to loss of L3. Furthermore, release of the L3 does not always permit a passage at an equal number during other blood meal taken by the infective mosquito due to the vector tolerance. Thus, they are naturally killed by host immune, vector barrier, and physical environment; these contributing factors can favor the LF elimination in humans.

As was, LF becomes one of six potentially eradicable diseases of which the criteria for assessing their eradicability (CDC, 1993) are based not only on the scientific feasibility of understanding the biological information mentioned earlier and practical use of the public health interventions and other methods to be applied or used in existing national control program, but also on the political will or popular support of executing the implementation of LF control strategies. First, the scientific feasibility depends basically on the disease vulnerability. Unlike the *W. bancrofti* that exists only in humans, the *B. malayi* spends an enzoonotic life-cycle in which the parasite is transmitted by the potent mosquito and thrive in domestic cats as non-human reservoir, and a *per se* epizoonotic cycle in which the parasite is transmitted and thrive through which the mosquito vector takes blood meal from infected cats, and subsequently, transmit the infective stage to humans during other blood meal. Controversially, the humans carrying *B. malayi* infection can serve as a source for the infection to permit a passage to the animal reservoir through bites of the mosquito vector. Nonetheless, both diseases do not always ease their spread into the population as a result of the naturally-induced immunity and duration of microfilarial production in susceptible individuals or communities (Maizels and Lawrence, 1991; Ottesen, 1992) (Fig. 2). More important, the infections can be both easily diagnosed using advanced tools and effectively treated with the antifilarial drugs that are safe, inexpensive and easily deployed. The feasibility of elimination has been shown that the operation of the pragmatic diagnostic methods or interventions is demonstrable at a large scale in the target populations. Last, the political will/popular support is pivotal for the program manager to capture information required to analyze situations of the perceived burden of LF that is figured out by a large number of suffering and disabled persons worldwide (WHO, 2002; 2008; 2010): the details are available at the websites of the Global Program to Eliminate Lymphatic Filariasis (GPELF) (http://www.who.int/lymphatic_filariasis/disease/en/) and Global Alliance to Eliminate Lymphatic Filariasis (http://www.filariasis.org). Also, the National Program to Eliminate Lymphatic Filariasis (PELF) by which the resource mobilization and funding structure are administered must be allied, by adopting the GPELF's strategies, to practical considerations of interventions, methods, logistic supplies (medical and field-work), delivery processes, expenditures (unit costs and cost-effectiveness), integration of control activities into existing health systems services (or other health development programs), socio-economic impacts on gaining health benefits, community awareness and acceptance, and ecological disturbances to target disease, non-target disease and the environment. For the resource-limited countries as allied nations, a national budget plan that notifies outsources and fund raising needs the subsidy of the internationally collaborative program.

2.2 Mass chemotherapy as elimination strategy

Ideally, the success of controlling the disease depends definitely on the objectives and ultimate goals of disease control spectrum (CDC, 1993); with this regard, the rational management of LF elimination differs with control (Table 2). As recommended by the World Health Organization (WHO), two pillars of global elimination strategies emphasize interruption of transmission and elimination of the infection in humans, and the other large-scale morbidity control to prevent disease and disability (WHO, 1999a; 2002; 2008; 2010). Principle outcomes of the GPELF reduce numbers of microfilaremic persons and disease cases as preventing new infection introduced among the population at risk of, or affected with, the infection. To meet this criteria, the GPELF proposes a mainstay of elimination strategy effectively available for mass drug administration (MDA) in target population; a combined treatment with diethylcarbamzine citrate (DEC) 6 mg/kg plus 400 mg albendazole (in *W. bancrofti* transmssion areas where *Onchocerca volvulus* is not coendemic), or with 200 μg ivermectin plus 400 mg albendazole (in *W. bancrofti* transmssion areas where *O. volvulus* is coendemic) (Ottesen et al, 1997; 1999; Ottesen, 2000; WHO, 1999a; 2000; 2001; 2002). An annual MDA with coverage of 60-80% for 4-5 years is considered to be effective enough to interrupt transmission in control areas in the absence of vector control (Gyapong et al, 2005). Also, new options for mass treatment of at risk population are effectively available: DEC-fortified salt for 1 year; and a combination of single annual dose of albendazole plus DEC, followed by DEC-fortified salt (Weaver et al, 2011). The ample supply and distribution of DEC-fortified salt can be administered in some countries.

2.3 Surveillance and monitoring systems

As for presenting dynamics of the infection and disease in nature (Grenfell et al, 1991; Srividya et al, 1991; Ottesen, 1992; Meyrowitsch et al, 1995), the parasites cause a wide spectrum of clinical manifestations in the affected population that are characterized by asymptomatic microfilaremia, acute lymphatic inflammation and chronic lymphatic pathology (WHO, 1992a; 1992b; 1994). Susceptible persons develop clinically LF as a result of prolonged exposure to multiple infective bites of potent mosquitoes from several months to years. On the other hand, the people living for at least 6 months are at the greatest risk for the infection. The chronic filariasis cases represent a tip of the iceberg, as microfilaremic carriers are the reservoir of the infection to others. The prevalence and intensity of the infection in humans depends mainly upon a number of microfilaremic persons and a geometric mean of microfilarial loads in the affected population. These proximate measures are indicative of the degrees of endemicity. The prevalence and geographical distribution of the disease are important not just for determination of its potential transmission in mosquito, but also for diagnosis and surveillance in different endemic settings. Therefore, the recognition of what is the filarial origin of the disease and where the affected individuals or communities are is fundamental for public health importance to identify the solution and control rationale. Diagnostic approaches that emphasize the detection and specific identification of microfilaremic infection in the individual by using most standard microscopic methods are important component of LF control program, which is aimed to reduce number of microfilaremic persons. The suitable blood collection and microscopic diagnosis in health settings can be effectively available to identify anyone infected, and subsequently, treated with antifilarial drug regimens. However, this specific objective of control is less important as the elimination is desired not just to interrupt transmission and clear human infection on a large-scale, but also to reduce the morbidity attributed to the

disease and hence improve both personal-focused hygiene and health care to all beneficiaries in target population at risk (Table 2).

	Control	Elimination
1. Definition	- Reduction of microfilaremia prevalence	- Reduction of microfilaremia prevalence as arbitrarily qualitative or quantitative level of control as no longer a public health problem
2. Outcome indicators as options for monitoring tools	- Annual infection prevalence; microfilaremia rate (a microfilaremic number surveyed in endemic population) - Disease rate (a number of disease cases surveyed in endemic population) - Annual transmission potential (ATP) (i.e., a number of infective bite per annum which acquire the infection) as mosquito infection rate is not useful indicator	- Degrees to which transmission is interrupted; microfilaremia rate mosquito infection rate and antigenemia rate - Disease rate is not useful indicator
2.1 Objective	- **To assess whether the control program achieves its goals or desired outcomes** (e.g., reduction in microfilaremia and/or disease prevalence)	- **To assess whether the elimination program achieves its goals or desired outcomes†** (e.g., reduction in microfilaremia prevalence, antigenemia prevalence)
3. Process indicators as options for monitoring and evaluation tools	- Coverage of selective treatment and follow-up, as mosquito net utilization, insecticide spraying and reservoir control can be useful indicators	- Mass treatment coverage, drug compliance coverage (e.g., adverse drug reactions prevalence and severe adverse event reports), KAP survey
3.1 Objective	- **To assess how well the various components of the control program are functioning** (e.g., the number of newly and follow-up microfilaremic persons treated with antifilarial drugs)	- **To assess how well the various components of the elimination program are functioning‡** (e.g., the number of new infections, the number of drug tablets distributed)

Adapted from the GPELF (http://www.who.int/lymphatic_filariasis/disease/en/).
†,‡Outcome indicators can be optionally used for monitoring systems; longitudinal surveillance of populations in sentinel sites, cross-sectional "spot checks" in other sites, and auxiliary "background" surveillance.
KAP - Knowledge, attitudes and practices.

Table 2. Rational approaches to lymphatic filariasis control and elimination

Tools	Advantages for the applications	Chief obstacles when applied or used in demonstrated areas	References
ICT Filariasis	• Simple-to-use rapid diagnostic test kit commercially available for use in qualitative detection of *W. bancrofti* adult worm circulating antigens present in whole blood/serum/plasma samples; stronger positive test line equivalent to higher antigen levels; • Sensitive and specific for anytime-of-day determination of active infection (antigenemic) whether microfilaremia is present, or the treatment is given; • Highly reproducible and practical when lots of large-scale blood samples (100 µl each) are analyzed either under the field conditions by not-well-trained field workers or in laboratory settings (with 100 µl each of serum/plasma samples freshly prepared or frozen) by laboratory personnel in order to assess the human infection rates in areas known as endemic for *W. bancrofti* or respective areas of emergence/reemergence; • Suitable for rapid assessment survey to detect the early infection in endemic carriers including migrants, refugees, any persons who work in endemic areas (mine in rainforest and border) for years, or visitors to the areas; • Suitable to monitor and evaluate the infection in humans inhabiting in risk areas in initial surveillance before MDA; drug responses during MDA; and the new infection in post-MDA areas whether they are certified as eliminated areas	• Costly; • Indicate, but not quantify, the level of the circulating antigens; somehow, provides false-negative identifications with the infections harboring very low antigen titers; • Cannot differentiate the status between infection and disease, occurrence and recurrence, or sensitivity and resistance • Primarily requires standardization and quality control of lots of samples (finger-prick blood) that are collected and analyzed	Weil & Liftis, 1987 Weil et al, 1987; 1996; 1997 Freedman et al, 1997 Ramzy et al, 1999 Bhumiratana et al, 1999; 2002; 2004; 2005 Nguyen et al, 1999 Phantana et al, 1999 Simonsen et al, 1999 Omar et al, 2000 Pani et al, 2000; 2004 Sunish et al, 2002; 2003 Braga et al, 2003 Engelbrecht et al, 2003 Koyadun et al, 2003 Nuchprayoon et al, 2003 Siriaut et al, 2005 Ruberanziza et al, 2009 Foo et al, 2011

Tools	Advantages for the applications	Chief obstacles when applied or used in demonstrated areas	References
Og4C3 ELISA	• Commercially available diagnostic test kit, and principally the same when use in qualitative and quantitative detection of active *W. bancrofti* infection, but more sensitive and specific than the ICT Filariasis; • Highly reproducible when lots of large-scale serum/plasma samples (100 µl each of freshly prepared or frozen samples) are analyzed in the public health reference laboratory or research institutes in order to assess the human infection rates, and to monitor and evaluate the infection or drug responses in individuals or in the target population under the circumstances described above;	• Costly, labor-intensive and intrusive • Cannot differentiate the status between infection and disease, occurrence and recurrence, or sensitivity and resistance • Primarily requires standardization and quality control of lots of venous blood samples (subsequently prepared for sera or plasma) that are collected, transported, stored and analyzed; • Also requires well-trained field workers and laboratory personnel	More & Copeman, 1990 Turner et al, 1993 Chanteau et al, 1994a; 1994b Lammie et al, 1994 McCarthy et al, 1995 Rocha et al, 1996 Nicolas, 1997 Ismail et al, 1998 Eberhard, 1997 Simonsen and Dunyo, 1999 Nuchprayoon et al, 2003 Bhumiratana et al, 2004; 2005
Polymerase chain reaction (PCR)	• Very highly sensitive and specific for the microfilaremic infections with *W. bancrofti* and *B. malayi* in humans and mosquitoes distinguishable from other filarial nematode parasites such as *O. volvulus*, *D. immitis*, *D. repens* and *B. pahangi*; • Highly reproducible and practical when lots of samples as low as 20 µl human blood, individual mosquito (dissected or whole body), or mosquito pool, are analyzed in the public health reference laboratory or research institutes in order to assess the human and	• Too costly, labor-intensive, intrusive, in-house developed tool • Primarily requires standardization and quality control of lots of samples (finger-prick or venous blood, or pooled wild-caught mosquitoes) that are collected, transported, stored and analyzed; • Also requires well-trained field workers or	Chanteau et al, 1994c Lizotte et al, 1994 McCarthy et al, 1996 Siridewa et al, 1996 Williams et al, 1996 Zhong et al, 1996 Ramzy et al, 1997 Nicolas et al, 1999 Cox-singh et al, 2000 Thanomsub et al, 2000 Pradeep Kumar et al, 2002

Tools	Advantages for the applications	Chief obstacles when applied or used in demonstrated areas	References
	mosquito infection rates, and to monitor and evaluate the infections or drug responses in individuals or in the target population under the circumstances described above; • Can differentiate the status between infection (microfilaremia) and disease, occurrence (new infection) and recurrence, or sensitivity and resistance; • Detection limit of as low as one Mf per blood volume tested (up to 1 ml), or one juvenile larva (L1, L2 or L3) per pooled mosquitoes (up to 100)	laboratory personnel and accessory equipments for DNA preparation and analysis of PCR products; • Cannot differentiate larval stages (L1, L2 or L3) in infected mosquitoes	Fischer et al, 2003 Kanjanavas et al, 2005 Nuchprayoon et al, 2005; 2007 Rao et al, 2006 Mishra et al, 2007 WHO, 2009 Bhumiratana et al, 2010 Pechgit et al, 2011 Takagi et al, 2011

Table 3. Direct determination tools for use as part of the Global Program to Eliminate Lymphatic Filariasis

To meet this objective, a large-scale transmission control requires a current magnitude and geographical distribution of the disease in the at-risk population. To understand the extent to which the target population needs to be designed for MDA and monitored whether the MDA implementation is effective, such surveillance and monitoring systems are required. To identify the communities with, or at risk of, the infection, for instance, the direct assessment techniques (Table 3) are required for practical use both in initial surveillance for filarial infection and in monitoring and evaluating the effectiveness of mass treatment, as part of the GPELF (WHO, 1999a; 1999b; Ottesen, 2000). In this regard, the mass treatment with more effective antifilarial drug regimens as well as the availability of other existing and alleviating control measures has been deliberately implemented to meet such these highly achievable objectives of the elimination. Nonetheless, in addition to what is recommended by WHO, the GPELF requires for ground-breaking development of systems, protocols and tools that will be able to be convincingly applied to or routinely used in the PELF to fix undesirable events of mass treatment impacts in different complex epidemiological settings (Kyelem et al, 2008; Ottesen et al, 2008; WHO, 2008; 2010).

3. Parasite infection and drug responsiveness

3.1 Microfilaremia and drug-responsive microfilaremia

In an endemic population that represents the infection and disease dynamics in nature, the asymptomatic microfilaremia is a stage of the active infection with filarial adult worms (Srividya et al, 1991; Meyrowitsch et al, 1995), which regulate host immune responses (hypoimmune responsiveness) in infected individuals (Bundy et al, 1991; Grenfell et al, 1991; Maizels and Lawrence, 1991; Mitchell, 1991; Grenfell and Micheal, 1992; Ottesen, 1992;

Simonsen et al, 1996; 2008; Ravindran et al, 2003). This phenomenon results in immunotolerance, i.e., a prolonged induction of the balance of immune defense to the parasites stimulated by the adult worms, in most asymptomatic microfilaremic persons. The female adult worm involves in regulation of the host microfilaremia. The fecundicity (a period of pregnancy) allows its fertilization to produce a diverse number of the offspring microfilariae. Although the proportion of microfilariae can be removed from the blood circulation in patients, there are the plenty of microfilariae, which circulate in the peripheral blood and show the appearance both nocturnally and diurnally. This microfilarial periodicity or circadian cycle of the parasite in humans is clinically unimportant for treatment but very important for its epidemiologic implication, which plays a significant role in diagnosis, surveillance and epidemiology. The parasite infection is the foundation for the processes that not only determine the infection prevalence but also monitor and evaluate the effectiveness of the treatment with the antifilarial drugs in infected individuals. In this regard, the amount of microfilaremia seems to be a function of naturally-acquired infection loads, which refers to as the most viable microfilariae, and drug-responsive microfilaremia refers to as the affected parasite population that harbors a diverse range of viable and non-viable microfilariae (Pechgit et al, 2011). These outcome indicators are useful for monitoring and evaluating the benzimidazole susceptibility of the filarial nematode parasites in the population in areas of the PELF implementing the MDA 2-drug regimen either albendazole plus DEC or albendazole plus ivermectin.

However, the MDA 2-drug regimen is not the only factor that shapes the parasite population under complex epidemiological settings. Of note, the *W. bancrofti* populations have ability to provoke the genetic variability that shows the important implications in the endemic populations targeted by the MDA (Pradeep Kumar, 2000). The existence of genetic diversity of *W. bancrofti* populations that has greater heterogeneity under DEC therapy and vector control gives rise to questioning about the development of drug resistance in LF, which possibly occurs in the target populations. The selection pressure is an intensity of selection affecting the frequency of genes in a parasite population. The selection that increases or decreases the susceptibility of the parasite population depends on the frequency of the alleles involved. The genetic polymorphism occurring in the *W. bancrofti* population under the selection pressure(s) may evoke gradually under specified conditions to yield the fitness, which can be determined by a genotype in the parasite population. The increase in the parasite fitness can be estimated by the equilibrium frequencies of the alleles (genotypes) at heterozygote advantage in a hypothethical population. That is, rapid establishment of advantageous alleles in the *W. bancrofti* population, called "selection sweep", may evoke with advantageous drug-resistant genotypes epidemiologically linked to other factors shaped by the host and environment (Schwab et al, 2006: 2007; Churcher et al, 2008). Eventually, it may reduce the genetic variation in the population.

3.2 Benzimidazole-susceptibility of the parasite

This chapter emphasizes microfilaremia responsiveness in the population under the suppression of the PELF implementing MDA 2-drug regimen, 6 mg/kg DEC plus 400 mg albendazole. The microfilaremia responses against the DEC are the foundations of understanding how the albendazole exerts the effects on the parasite population in addition to what is observed by DEC alone. The DEC is known as the oldest of the antifilarial drugs used in the LF control. The single-dose drug acts as microfilaricide as does the effective ivermectin (de Silva et al, 1997; Ottesen, 2000; Molynuex et al, 2003; Ottesen et al, 2008)

while its macrofilaricidal activity is not definitely effective against adult worms (Eberhard et al, 1991; Norões et al, 1997; Dreyer et al, 1998; Rajendran et al, 2002; 2004; Oliveira-Menezes et al, 2007). The adult worm loads that are age-dependent (Lammie et al, 1994; Rajendran et al, 2002) are susceptible to treatment with the DEC alone or even combination with albendazole (Norões et al, 1997; Rajendran et al, 2002). It was seen that DEC alone disturbs the microfilarial sheath of some filarial species while it has effects on the oogenesis and fertilization of female adult worms. Nonetheless, little is known about the filarial nematode parasites whether they evoke resistance mechanism against DEC due to the lack of deepening its mechanism of action, particularly the availability in parasite tissues and the selectivity on parasite targets. Oliveira-Menezes et al (2007) demonstrated that DEC has minor effects on alterations of the cuticle or surface of both male and female adult worms; these responsible parasites were collected from the *W. bancrofti*-infected patients treated with DEC, as compared to those isolated from the untreated patients. Additionally, such alterations are seen in adult worms recovered from the patients treated with DEC plus albendazole. The possible explanation is that the potential adulticidal effect of albendazole relative to what is observed by the DEC alone. The subtle alterations imply the distinct morphologic characteristics of the parasite itself or the complex host-parasite interactions, and if implemented and continuously prolonged, the annual mass treatment with DEC plus albendazole has not yet become an apparent issue, particularly the impacts on the parasite population adaptation. Of note, the *O. volvulus* parasite develops the mechanism involved in resistance to ivermectin (Awadzi et al, 2004). Do the filarial nematode parasites have mimicry in the resistance mechanisms to ivermectin and albendazole? However, detailed study of the parasite resistance to albendazole has not been established.

Focus is on the MDA 2-drug that acts as effective microfilaricide while its aberrant activity that influences microfilaremia response to its efficacy in microfilaricidal activity. Provided this phenomenon occurs, the expected outcomes of such drug failure will impact on solving the solutions and paving the implications of how they will adapt under the certain circumstances and how we will also mitigate their adaptation. Most studies enlightened the understanding of this effective deworming MDA 2-drug, which plays the significance of reduction of the infection prevalence. A single-dose combined treatment with DEC plus albendazole has short- and long-term effects on *W. bancrofti* microfilaremics in the endemic populations (Ismail et al, 1998; Ottesen et al, 1999; El Setouhy et al, 2004; Rajendran et al, 2004). Compared to those receiving DEC alone, an additional benefit of the combined drugs results in decline in annual cyclic infection prevalence due to progressive reduction in density of *W. bancrofti* microfilaremia. Although its macrofilaricidal effect on clearance of *W. bancrofti* antigenemia has been reported (McCarthy et al, 1995; Eberhard, 1997; Rajendran et al, 2002; 2004; Koyadun et al, 2003; Bhumiratana et al, 2004; Siriaut et al, 2005; Bhumiratana et al, 2005; Yongyuth et al, 2006), the DEC alone or co-administered with the albendazole does not clear rapidly the antigenemia. The MDA with the DEC alone will recover an increase in the antigenemia prevalence of *W. bancrofti* unless there is yearly-round MDA in the population (Rajendran et al, 2002). A 400 mg single oral-dose albendazole regimen is broad-spectrum effective against helminthiases (Albonico, 1994; de Silva et al, 1997; Beach et al, 1999; Ottesen et al, 1999; Horton, 2000) and, as co-administered orally with DEC, a synergistic and long-term effect on geohelminths has been proven useful for 'beyond-lymphatic filariasis' elimination program (Ottesen et al, 1997; 1999; Ismail et al, 1998; Horton et al, 2000; Ottesen, 2000; Mani et al, 2002; 2004; Molynuex et al, 2003; Yongyuth et al, 2006).

Few reports established the evidence that the human filarial nematode parasites provoke molecular mechanism involved in benzimidazole sensitivity/resistance until recently findings of the genetically-induced resistance against benzimidazole compounds have been well documented in veterinary nematode parasites (Beech et al, 1994; Kwa et al, 1995; Humbert et al, 2001; Bennett et al, 2002; Drogemuller et al, 2004; Robinson et al, 2004; Cole et al, 2006; Ghisi et al, 2007). Resistance to albendazole in veterinary nematodes is known to be caused by either one of two single amino acid substitutions from phenylalanine to tyrosine in parasite β-tubulin at position 167 or 200. The genetically stable *W. bancrofti* β-tubulin gene responsible for a molecular mechanism of drug resistance has been proposed as that of the veterinary helminth parasites is performed under selection of albendazole and ivermectin. The *W. bancrofti* population isolated from the patients treated with a combination of albendazole and ivermectin had significantly higher genotypic frequencies associated with resistance at position 200 (Schwab et al, 2005). A resistance mutation was not detected at position 167. Hoti et al (2003, 2009) reported that the polymorphism in the codon of this residue in *W. bancrofti* populations representing geographically distant areas of India, through sequencing exon 5 region of *β-tubulin* isotype 1 gene. The nucleotide sequence data showed that *W. bancrofti* isolates from wide geographic areas of India had codon for Phe (TTC) at position 200, suggesting that the parasite might be genetically sensitive to benzimidazole. Similarly, Bhumiratana et al (2010) and Petchgit et al (2011) demonstrated that the *W. bancrofti* population recovered from the dynamic cross-border migrant population from areas that have been targeted by the MDA 2-drug regimen (300 mg DEC plus 400 mg albendazole) elicits the genetic background of benzimidazole susceptibility; a resistance mutation has not been observed at position 167 or 200. However, the albendazole, anthelmintic benzimidazole, is being co-administered with an antifilarial drug such as DEC, part of the PELF implementing in many endemic countries. But this drug is known to result in the faster development of drug resistance in the veterinary nematode parasites and hence it is necessary to monitor drug sensitivity among the responsible *W. bancrofti* populations.

4. Molecular diagnosis and monitoring of benzimidazole susceptibility

4.1 Parasite beta-tubulin encoding gene as molecular marker

Molecular mechanisms of benzimidazole resistance in nematode parasites are hypothesized. However, detailed study of benzimidazole resistance in trichostrongylids found that the β-tubulin encoding gene involved in benzimidazole susceptibility is responsible for the genetic inheritance of resistance in the veterinary nematode parasites under selection with benzimidazole that involves one of two single amino acid substitutions from phenylalanine (Phe) to tyrosine (Tyr) in parasite β-tubulin at position 167 or 200 (Beech et al, 1994; Kwa et al, 1993; 1994; 1995; Roos et al, 1990; Elard et al, 1996; Elard and Humbert, 1999; Humbert et al, 2001; von Samson-Himmelstjerna et al, 2002; Winterrowd et al, 2003; Drogemuller et al, 2004; Cole et al, 2006; Ghisi et al, 2007). The potential point mutation occurs at the DNA level by nucleotide substitution for the codon for amino acid position 200 of the *β-tubulin* gene, a substitution of TTC (Phe) with TAC (Tyr). This irreversible change brings about distinguishment of the responsible parasite population between benzimidazole-sensitive and -resistant nematodes. This principal mechanism for benzimidazole resistance is postulate to involve changes in the selectivity of the benzimidazoles on the primary structure of β-tubulin molecules, a building block of the microtubule in the parasites (Lacey, 1988; Lacey and Gill, 1994; Robinson et al, 2004).

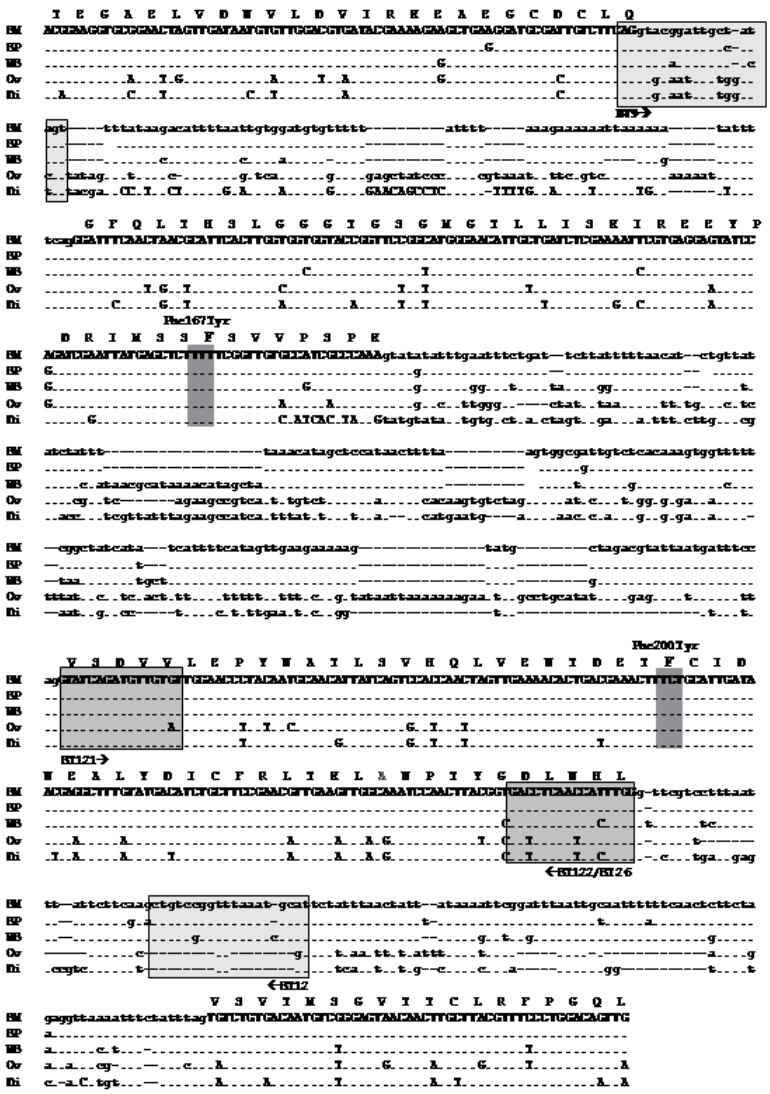


Fig. 3. ClustalW alignment of the filarial *β-tubulin* gene. The partial nucleotide sequence representatives (accession no. and positions): *Brugia malayi* (BRQD553TR, 3-789), *Brugia pahangi* (M36380, 2267-3054), *Wuchereria bancrofti* (AY705383, 109-916), *Onchocerca volvulus* (AF019886, 1582-2400) and *Dirofilaria immitis* (HM596854, 1462-2244) are shown as coding (upper case) and non-coding (lower case) sequences. The deduced amino acid sequences for the conserved domains are shown for all taxa aligned; *D. immitis* and *O. volvulus* have one amino acid substituted at position Ala218Thr. The gap is performed on the maximum homology (insertion/deletion), which represents conserved (•) and degenerate nucleotide residues and the regions designed to amplify specifically the target sequences based on the Wb*tubb* primer sets (light-gray boxes), both forward (→) and reverse (←). Hypothetically, two amino acid substitutions at positions Phe167Tyr and Phe200Tyr (dark-gray boxes) retained in DNA fragments (141 and 174 bp) could be identified using the PCR detection system described by Bhumiratana et al (2010) and Pechgit et al (2011).

Intriguingly, such mimicry in molecular mechanism for benzimidazole resistance in the filarial nematode parasites has been increasingly investigated, based basically on the molecular characterization of the homologous β-*tubulin* gene retained in their genome and the advantageous fitness of benzimidazole-resistant genotypes in the population (Roos et al, 1995; Elard et al, 1998; Elard et al, 1999; Silvestre et al, 2001; Silvestre and Humbert, 2002).

The nematode parasites possess the single-copy homologous *β-tubulin* (*tubb*) gene that encodes a β-tubulin polypeptide, 448 amino acids (Met1 to Glu448). Hypothetically similar to that of trichostrongylids, the binding of benzimidazoles to conserved domains (of the exons 4 to 6) leads to blocking an assembly of tubulin (TUBB), and thus disrupting structural formation of microtubule (cytoskeleton protein) in the nematode parasites. The nucleotide sequences of the homologous *β-tubulin* gene as molecular marker and other related TUBB gene family of the nematode parasites can be retrieved from the genome databases: the GenBank at the National Center for Biotechnology Information (NCBI), http://www.ncbi.nlm.nih.gov/genbank/, the European Molecular Biology Laboratory (EMBL) http://www.ebi.ac.uk/, and the DNA DataBank of Japan (DDBJ) http://www.ddbj.nig.ac.jp/. The website of nematode and neglected genomics (http://www.nematodes.org/fgn/index.html) establishes genome database, especially for the filarial genome project (FGP), which includes published complete *B. malayi* genome. Meanwhile, the homologous sequences of *B. malayi β-tubulin* gene can be obtained from the TIGR genome database (http://www.tigr.org/tdb/e2k1/bma1).

The structural organization of homologous *tubb* genes of two filarial nematode parasites, *B. pahangi* (Guenette et al, 1991) and *D. immitis* (Bourguinat et al, 2011), has been shown for the establishment of complete coding sequences that span 9 discrete exons: exon 1 (Met1 to Lys19), exon 2 (Phe20 to Asp55), exon 3 (Gly56 to Gln131), exon 4 (Gly132 to Lys174), exon 5 (Val175 to Leu228), exon 6 (Val229 to Gln292), exon 7 (Met293 to Arg324), exon 8 (Glu325 to Thr386) and exon 9 (Ala387 to Glu448). The homology is 78% at DNA level due to bias of codon usage and insertion/deletion of intron sequences (Fig. 3). Among these, the exons 4 and 5 confer hypothetical point mutation at amino acid positions Phe167Tyr (or TTT/TAT) and Phe200Tyr (or TTC/TAC), based only on the second nucleotide base changed in the codons. In the homologous segment of its closely related taxa, *W. bancrofti β-tubulin* (Wb*tubb*) gene that possesses two distinct exons, 4 (Gly132 to Lys174) and 5 (Val175 to Leu228), with flanking intron sequences (Fig. 3) shares the homology at DNA level with *B. malayi* and *B. pahangi* (93% similarity), compared to *O. volvulus* and *D. immitis* (76% similarity) (Bhumiratana et al, 2010; Pechgit et al, 2011). This target DNA has been proved useful for designing Wb*tubb* locus-specific primers to discriminate between Wb*tubb* and other homologs of human and animal filariids. Based on its usefulness in molecular diagnosis and monitoring of the infection carrying the benzimidazole-sensitive or resistant phenotypes, the PCR applications of this molecular marker for *W. bancrofti* have been well documented (Hoti et al, 2003; 2009; Schwab et al, 2005; Bhumiratana et al, 2010; Pechgit et al, 2011).

4.2 Polymerase chain reaction-based approaches

In contrary to the antigen detection methods such as ICT Filariasis and Og4C3 ELISA that provide the proof of *W. bancrofti* antigenemic infection in human blood, the microfilarial DNA detection by PCR provides the evidence of *W. bancrofti* microfilaremic infection in human blood and mosquito (Table 3). As a result of the existence of genetically stable

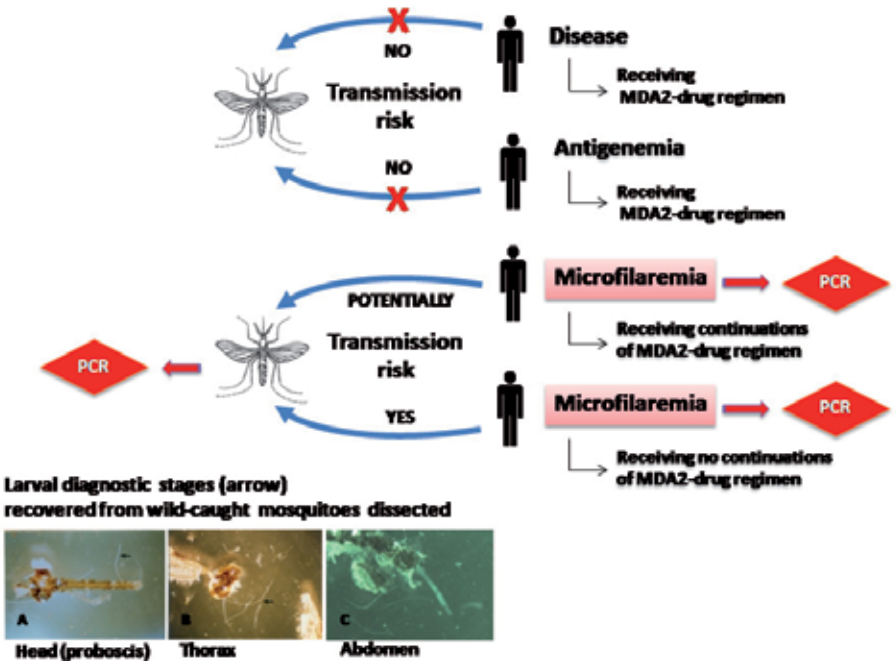


Fig. 4. A purposed scheme for PCR detection of *W. bancrofti* benzimidazole-susceptible isolates in human blood and mosquito.

Primer Name	Sequence (5′ to 3′)	Direction	Length (bases)	Expected amplicon size (bp)	Hypothetical nucleotide positions	Susceptible(S)/ resistant(R) genotypes investigated	Reference
BT9	CAGGTACAGATT GCTACAGT	Forward	20	607[c]	(TTT)167(TAT) (TTC)200(TAC)	S/Phe167 S/Phe200	Bhumiratana et al (2010)
BT12	GCGATTTAAACC CGACAGC	Reverse	19				
BT121[a]	GGATCCGTATCA GATGTTGTG	Forward	21	174[d]	(TTC)200(TAC)	S/Phe200	Bhumiratana et al (2010)
BT122[b]	GAATTCCAAGTG GTTGAGGTCG	Reverse	22				
Wt2F	GTATCAGATGTT GTGTTG	Forward	18	475[e]	(TTC)200(TAC)	S/Phe200	Hoti et al (2003)
Wt2R	ACGACTTGAATG AGTTGTC	Reverse	19				
Wbbt2 F	TATCAGATGTTG TGTTGG	Forward	18	475[f]	(TTC)200(TAC)	S/Phe200	Hoti et al (2009)
Wbbt2 R	CTGTTGAG AAGTTCAGCA	Reverse	19				

5′modifications with additional recognition sequences: [a]*Bam*H I (GGATCC) and [b]*Eco*R I (GAATTC).
[c d]Retrieved *Wuchereria bancrofti* genome accession nos.: AY705383 and GU190718–24.
[e]Retrieved *Brugia pahangi* genome accession nos.: M36380.
[f]Retrieved *Wuchereria bancrofti* genome accession nos.: EF190199-190209, EF492870-492878.

Table 4. The *β-tubulin* isotype 1 gene-specific primers used in the PCR amplification of *W. bancrofti* benzimidazole-susceptible isolates

W. bancrofti β–tubulin gene, the nested PCR amplification can work well with the microfilaremic infection that responds to treatment with MDA 2-drug regimen (DEC plus albendaozle) (Pechgit et al, 2011). This newly developed PCR assay in addition to promising advanced tool (Hoti et al, 2003; 2009; Bhumiratana et al, 2010) has the potential benefits in the molecular diagnosis and monitoring of the infection, as compared to the other PCR amplification methods previously described elsewhere (Table 3). The concepts for PCR assays based on the Wb*tubb* locus-specific primers (Table 4) have been proposed in two applicable formats: the locus-specific nested PCR and allele-specific nested PCR. These applications have established the advantages on how to circumvent some common counterintuitive problems of conventional PCR with regards to both parasite genome analysis and low-copy gene detection; such detailed study has been well established by Pechgit et al (2011). The *W. bancrofti* microfilarial DNA detection methods depends much on the purity and quantity of the microfilariae recovered from different blood sample preparations. The purified aggregate parasite number in the absence of human host white blood cells, for example, are ideal for the quality of DNA extract, which serves as target sequences in the PCR reactions. In general, most PCR methods for the detection of *W. bancrofti* distinguishable from other filarial nematode parasites in human and mosquito is based on the repetitive *Ssp* I sequences, which are highly copy number per haploid genome. However, PCR amplification based on this *Ssp* I locus provides the positive identifications of the parasite infection existed in specimens of choice. The assay does not determine the infection that responds to benzimidazole sensitivity/resistance; such responsible *W. bancrofti* parasite population is amplified based on the *β–tubulin* gene which is single copy in haploid genome. Therefore, the amplification is performed using the Wb*tubb* locus-specific nested PCR and allele-specific nested PCR that provides the proof of the *W. bancrofti* infection carrying benzimidazole-sensitive/resistant phenotypes; methodologically, the technical requirements for their applications have been described by Pechgit et al (2011) and Hoti et al (2009). More specific, based on our experience, the Wb*tubb* locus-specific nested PCR with thermocycling modifications using touchdown and touchup cycles has been applied or used in detection and characterization of *W. bancrofti* infection both in human blood from patients untreated or treated with DEC plus albendazole and in wild-caught mosquito, provided such infections carrying benzimidazole-sensitive/resistant strains are the same source of the parasite population (Fig. 4). Hypothesis is that whether the parasite infection is genetically predisposed to the MDA 2–drug regimen (DEC plus albendazole) in areas under suppression of PELF, it will have frequencies of benzimidazole-susceptible homozygous allele (*SS*) greater than benzimidazole-susceptible heterozygous allele (*Sr*) and homozygous resistant allele (*rr*), which are associated with albendazole resistance, unless the parasite fitness is increased. This also permit the monitoring and evaluation of the parasite fitness to better understand theoretically and hypothetically evolutionary biology and ecology of the parasite, by which the human hosts play a key as a major source of selective pressures on the adaptation of parasite population constrained by environmental conditions.

5. Future perspectives

The GPELF has been deployed into the endemic countries implementing MDA 2-drug regimes (i.e., single annual doses of albendazole in combination with DEC or ivermectin) to reduce microfilaremia prevalence to levels low enough (principally lower than transmission threshold) to interrupt transmission of the disease in the absence of vector control. Based on scientific information on drug resistance to anthelmintics, the issue of albendazole resistance

in the *W. bancrofti* parasite has assumed increasing importance since the GPELF is implemented on a large-scale in at-risk populations in different complex epidemiological settings, and predictably, the implementation phrase of the program will increase. Many studies have shown some vulnerability in how the parasite has the ability to evoke molecular mechanisms for resistance to anthelminthics as the nematodes of veterinary importance have developed resistance against both albendazole and ivermectin. Furthermore, there have been lines of evidence that the vulnerability of helminthiasis control programs that employ the MDA with these drugs is associated with several factors that facilitate the promotion of drug-resistant strains. At the same time, the factors are considered concerning the new options of drug combinations with different parasite targets or modes of actions to keep active shelf-life of DEC because the filarial nematode parasites have long life-span and complex life-cycle development. Likewise, to better understand what is relative to achieve MDA's goal to confront growing trend of drug resistance, it is essential for the development of molecularly diagnosing and monitoring the benzimidazole sensitivity/resistance in areas of long-running PELF program implementation using albendazole plus DEC or albendazole plus ivermectin. More applicable tools which will be validated in effective manner are also required to explore the genetic basis for resistance to anthelminthics.

6. References

Albonico, M. (1994). A randomized controlled trial comparing mebendazole and albendazole against *Ascaris*, *Trichuris* and hookworm infections. *Trans R Soc Trop Med Hyg* 88, 585–589.

Awadzi, K., Boakye, D.A., Edwards, G., Opoku, N.O., Attah, S.K., Osei-Atweneboana, M.Y. et al. (2004). An investigation of persistent microfilaridermias despite multiple treatments with ivermectin, in two onchocerciasis-endemic foci in Ghana. *Ann Trop Med Parasitol* 98, 231–249.

Beach, M.J., Streit, T.G., Addiss, D.G., Prospere, R., Roberts, J.M., Lammie, P.J. (1999). Assessment of combined ivermectin and albendazole for treatment of intestinal helminth and *Wuchereria bancrofti* infections in Haitian schoolchildren. *Am J Trop Med Hyg* 60, 479–486.

Beech, R.N., Prichard, R.K., Scott, M.E. (1994). Genetic variability of the beta-tubulin genes in benzimidazole-susceptible and resistant strains of *Haemonchus contortus*. *Genetics* 138, 103–110.

Bennett, A.B., Anderson, T.J., Barker, G.C., Michael, E., Bundy, D.A. (2002). Sequence variation in the *Trichuris trichiura* beta-tubulin locus: implications for the development of benzimidazole resistance. *Int J Parasitol* 32, 1519–1528.

Bhumiratana, A., Koyadun, S., Suvannadabba, S., Karnjanopas, K., Rojanapremsuk, J., Buddhirakkul, P., et al. (1999). Field trial of the ICT Filariasis for diagnosis of *Wuchereria bancrofti* infections in an endemic population of Thailand. *Southeast Asian J Trop Med Public Health* 30, 562–568.

Bhumiratana, A., Wattanakull, B., Koyadun, S., Suvannadabba, S., Rojanapremsuk, S., Tantiwattanasup, W. (2002). Relationship between male hydrocele and infection prevalences in clustered communities with uncertain transmission of *Wuchereria*

bancrofti on the Thailand-Myanmar border. *Southeast Asian J Trop Med Public Health* 33, 7–17.

Bhumiratana, A., Siriaut, C., Koyadun, S., Satitvipawee, P. (2004). Evaluation of a single oral dose of diethylcarbamazine 300 mg as provocative test and simultaneous treatment in Myanmar migrant workers with *Wuchereria bancrofti* infection in Thailand. *Southeast Asian J Trop Med Public Health* 35, 591–598.

Bhumiratana, A., Koyadun, S., Srisuphanunt, M., Satitvipawee, P., Limpairojn, N., Gaewchaiyo, G. (2005). Border and imported bancroftian filariases: baseline seroprevalence in sentinel populations exposed to infections with *Wuchereria bancrofti* and concomitant HIV at the start of diethylcarbamazine mass treatment in Thailand. *Southeast Asian J Trop Med Public Health* 36, 390–407.

Bhumiratana, A., Pechgit, P., Koyadun, S., Siriaut, C., Yongyuth, P. (2010). Imported bancroftian filariasis: Diethylcarbamazine response and benzimidazole susceptibility of *Wuchereria bancrofti* in dynamic cross-border migrant population targeted by the National Program to Eliminate Lymphatic Filariasis in South Thailand. *Acta Trop* 113, 121–128.

Bourguinat, C., Keller, K., Prichard, R.K., Geary, T.G. (2011). Genetic polymorphism in *Dirofilaria immitis*. *Vet Parasitol* 176, 368–373.

Braga, C., Dourado, M. I., Ximenes, R. A. D. A., Alves, L., Brayner, F., Rocha, A., et al. (2003). Field evaluation of the whole blood immunochromatographic test for rapid bancroftian filariasis diagnosis in the northeast of Brazil. *Rev Inst Med Trop Sao Paulo,* 45, 125–129.

Bundy, D.A.P., Grenfell, B.T., Rajagopwlan, P.K. (1991). Immunoepidemiology of Lymphatic filariasis: the relationship between infection and disease. *Immunol Today* 12, A71–75.

Castillo, J.C., Reynolds, S.E., Eleftherianos, I. (2011). Insect immune responses to nematode parasites. *Trends Parasitol* (in press), doi:10.1016/j.pt.2011.09.001.

CDC. (1993). Recommendations of the International Task Force for Disease Eradication. *Morb Mortal Wkly Rep* 42(RR16), 1–38.
[http://www.cdc.gov/mmwr/preview/mmwrhtml/00025967.htm]

Chanteau, S., Moulia-Pelat, J.P., Glaziou, P., Nguyen, N.L., Luquiaud, P., Plichart, C., et al. (1994a). Og4C3 circulating antigen: a marker of infection and adult worm burden in *Wuchereria bancrofti* filariasis. *J Infect Dis* 170, 247–250.

Chanteau, S., Glaziou, P., Luquiaud, P., Plichart, C., Moulia-Pelat, J.P., Cartel, J.L. (1994b). Og4C3 circulating antigen, anti-*Brugia malayi* IgG and IgG4 titers in *Wuchereria bancrofti* infected patients, according to their parasitological status. *Trop Med Parasitol* 45, 255–257.

Chanteau, S., Luquiaud, P., Failloux, A.B., Williams, S.A. (1994c). Detection of *Wuchereria bancrofti* larvae in pools of mosquitoes by the polymerase chain reaction. *Trans R Soc Trop Med Hyg* 88, 665–666.

Churcher, T.S., Schwab, A.E., Prichard, R.K., Basáñez, M.G. (2008). An analysis of genetic diversity and inbreeding in *Wuchereria bancrofti*: implications for the spread and detection of drug resistance. *PLoS Negl Trop Dis* 2, e211.

Cole, G.C., Jackson, F., Pomroy, W.E., Prichard, R.K., von Samson-Himmeltjerna, G., Silvestre, A., et al. (2006). The detection of anthelminthic resistance in nematodes of veterinary importance. *Vet Parasitol* 136, 167–185.

Cox-Singh, J., Pomrehnb, A.S., Wolfec, N.D., Rahmand, H.A., Lua, H., Singha, B. (2000). Sensitivity of the nested-polymerase chain reaction (PCR) assay or *Brugia malayi* and significance of 'free' DNA in PCR-based assays. *Int J Parasitol* 30, 1177–1179.

de Silva, N., Cuyatt, H., Bundy, D.A. (1997). Anthelminthics: a comprehensive review of their clinical pharmacology. *Drugs* 53, 769–788.

Dreyer, G., Addiss, D., Santos, A., Figueredo-Silva, J., Norões, J. (1998). Direct assessment in vivo of the efficacy of combined single-dose ivermectin and diethylcarbamazine against adult *Wuchereria bancrofti*. *Trans R Soc Trop Med Hyg* 92, 219–222.

Drogemuller, M., Schieder, T., von Samson-Himmelstjerna, G. (2004). Beta-tubulin complementary DNA sequence variations observed between cyathostomins from benzimidazole-susceptible and -resistant populations. *J Parasitol* 90, 868–870.

Eberhard, M.L. (1997). Clearance of *Wuchereria bancrofti* antigen after treatment with diethylcarbamazine or ivermectin. *Am J Trop Med Hyg* 57, 483–486.

Eberhard, M.L., Lammie, P.J., Dickinson, C.M., Roberts, J.M. (1991). Evidence of nonsusceptibility to diethylcarbamazine in *Wuchereria bancrofti*. *J Infect Dis* 163, 1157–1160.

Elard, L., Cabaret, J., Humbert, J.F. (1999). PCR diagnosis of benzimidazole-susceptibility or -resistance in natural populations of the small ruminant parasite, *Teladorsagia circumcincta*. *Vet Parasitol* 80, 231–237.

Elard, L., Comes, A.M., Humbert, J.F. (1996). Sequences of beta-tubulin cDNA from benzimidazole-susceptible and -resistant strains of *Teladorsagia circumcincta*, a nematode parasite of small ruminants. *Mol Biochem Parasitol* 79, 249–253.

Elard, L., Humbert, J.F. (1999). Importance of the mutation of amino acid 200 of the isotype 1 β-tubulin gene in the benzimidazole resistance of the small-ruminant parasite *Teladorsagia circumcincta*. *Parasitol Res* 85, 452–456.

Elard, L., Sauve, C., Humbert, J.F. (1998). Fitness of benzimidazole-resistant and -susceptible worms of *Teladorsagia circumcincta*, a nematode parasite of small ruminants. *Parasitology* 117, 571–578.

El Setouhy, M., Ramzy, R.M.R., Ahmed, E.S., Kandil, A.M., Hussain, O., Farid. H.A., et al. (2004). A randomized clinical trial comparing single- and multi-dose combination therapy with diethylcarbamazine and albendazole for treatment of bancroftian filariasis. *Am J Trop Med Hyg* 70, 191–196.

Engelbrecht, F., Oettl, T., Herter, U., Link, C., Philipp, D., Edeghere, H., et al. (2003). Analysis of *Wuchereria bancrofti* infections in a village community in northern Nigeria: Increased prevalence in individuals infected with *Onchocerca volvulus*. *Parasitol Int* 52, 13–20.

Fischer, P., Boakye, D., Hamburger, J. (2003). Polymerase chain reaction-based detection of lymphatic filariasis. *Med Microbiol Immunol* 192, 3–7.

Foo, P.K., Tarozzic, A., Mahajan, A., Yoong, J., Krishnan, L., Kopf, D., et al. (2011). High prevalence of *Wuchereria bancrofti* infection as detected by immunochromatographic

card testing in five districts of Orissa, India, previously considered to be non-endemic. *Trans R Soc Trop Med Hyg* 105, 109–114.

Freedman, D.O., de Almeida, A., Miranda, J., Plier, D.A., Braga, C. (1997). Field trial of a rapid card test for *Wuchereria bancrofti*. *Lancet* 350, 1681.

Ghisi, M., Kaminsky, R., Maser, P. (2007). Phenotyping and genotyping of *Haemonchus contortus* isolates reveals a new putative candidate mutation for benzimidazole resistance in nematodes. *Vet Parasitol* 144, 313–320.

Grenfell, B.T., Micheal, E., Denham, D.A. (1991). A model for the dynamics of human lymphatic filariasis. *Parasitol Today* 7, 318–323.

Grenfell, B.T., Micheal, E. (1992). Infection and disease in lymphatic filariasis: an epidemiological approach. *Parasitology* 104(suppl), S81–90.

Guenette, S., Prichard, R.K, Klein, R.D., Matlashewski, G. (1991). Characterization of a beta-tubulin gene and a beta-tubulin gene products of *Brugia pahangi*. *Mol Biochem Parasitol* 44, 153–164.

Gyapong, J.O., Kumaraswami, V., Biswas, G., Ottesen, E.A. (2005). Treatment strategies underpinning the global programme to eliminate lymphatic filariasis. *Expert Opin Pharmacother* 6, 179–200.

Horton, J. (2000). Albendazole: a review of anthelminthic efficacy and safety in humans. *Parasitology* 121(suppl), S113–132.

Horton, J., Witt, C., Ottesen, E.A., Lazdins, J.K., Addiss, D.G., Awadzi, K., et al. (2000). An analysis of the safety of the single dose, two drug regimens used in programmes to eliminate lymphatic filariasis. *Parasitology* 121, S147–160.

Hoti, S.L., Subramaniyan, K., Das, P.K. (2003). Detection of codon of amino acid 200 in isotype 1 beta-tubulin gene of *Wuchereria bancrofti* isolates, implicated in resistance to benzimidazole in other nematodes. *Acta Trop* 88, 77–81.

Hoti, S.L., Dhamodharan, R., Subramaniyan, K., Das, P.K. (2009). An allele specific PCR assay for screening for drug resistance among *Wuchereria bancrofti* populations in India. *Indian J Med Res* 130, 193–199.

Humbert, J.F., Cabaret, J., Elard, L., Leignel, V., Silvestre, A. (2001). Molecular approaches to studying benzimidazole resistance in trichostrongylid nematode parasites of small ruminants. *Vet Parasitol* 101, 405–414.

Ismail, M.M., Jayakody, R.L., Weil, G.J., Nirmalan, N., Jayasinghe, K.S.A., Abeyewickrema, W., et al. (1998). Efficacy of single dose combinations of albendazole, ivermectin and diethylcarbamazine for the treatment of bancroftian filariasis. *Trans R Soc Trop Med Hyg* 92, 94–97.

Kanjanavas, P., Tan-ariya, P., Khawsak, P., Pakpitcharoena, A., Phantana, S., Chansiri, K. (2005). Detection of lymphatic *Wuchereria bancrofti* in carriers and long-term storage blood samples using semi-nested PCR. *Mol Cell Probes* 19, 169–172.

Koyadun, S., Bhumiratana, A., Prikchu, P. (2003). *Wuchereria bancrofti* antigenemia clearance among Myanmar migrants after biannual mass treatments with diethylcarbamazine, 300 mg oral-dose FILADEC tablet, in Southern Thailand. *Southeast Asian J Trop Med Public Health* 34, 758–767.

Kwa, M.S.G., Veenstra, J.G., Van Dijk, M., Roos, M.H. (1995). *β-Tubulin* genes from the parasitic nematode *Haemonchus contortus* modulate drug resistance in *Caenorhabditis elegans*. *J Mol Biol* 246, 500–510.

Kwa, M.S.G., Veenstra, J.G., Roos, M.H. (1993). Molecular characterisation of beta-tubulin genes present in benzimidazole-resistant populations of *Haemonchus contortus*. *Mol Biochem Parasitol* 60, 133–143.

Kwa, M.S.G., Veenstra, J.G, Roos, M.H. (1994). Benzimidazole resistance in *Haemonchus contortus* is correlated with a conserved mutation at amino acid 200 in beta-tubulin isotype 1. *Mol Biochem Parasitol* 63, 299–303.

Kwa, M.S.G., Veenstra, J.G., Van Dijk, M., Roos, M.H. (1995). *β-Tubulin* genes from the parasitic nematode *Haemonchus contortus* modulate drug resistance in *Caenorhabditis elegans*. *J Mol Biol* 246, 500–510.

Kyelem, D., Biswas, G., Bockarie, M.J., Bradley, M.H., El-Setouhy, M., Fischer, P.U., et al. (2008). Determinants of success in national programs to eliminate lymphatic filariasis: a perspective identifying essential elements and research needs. *Am J Trop Med Hyg* 79, 480–484.

Lacey, E. (1988). The role of the cytoskeletal protein, tubulin, in the mode of action and mechanism of drug resistance to benzimidazoles. *Int J Parasitol* 18, 885–936.

Lacey, E., Gill, J.H. (1994). Biochemistry of benzimidazole resistance. *Acta Trop* 56, 245–262.

Lammie, P.J., Hightower, A.W., Eberhard, M.L. (1994). Age specific prevalence of antigenemia in a *Wuchereria bancrofti*-exposed population. *Am J Trop Med Hyg* 51, 348–355.

Lizotte, M., Supali, T., Partono, F., Williams, S.A. (1994). A polymerase chain reaction assay for the detection of *Brugia malayi* in blood. *Am J Trop Med Hyg* 51, 314–321.

Maizels, R.M., Lawrence, R.A. (1991). Immunological tolerance: the key feature in human filariasis? *Parasitol Today* 7, 271–276.

Mani, T.R., Rajendran, R., Munirathinam, A., Sunish, I.P., Abdullah, S.M., Augustin, D.J. et al. (2002). Efficacy of co-administration of albendazole and diethylcarbamazine against geohelminthiases: a study from South India. *Trop Med Int Health* 7, 541–548.

Mani, T.R., Rajendran, R., Sunish, I.P., Munirathinam, A., Arunachalam, N., Satyanarayana, K. et al. (2004). Effectiveness of two annual, single-dose mass drug administrations of diethylcarbamazine alone or in combination with albendazole on soil-transmitted helminthiasis in filariasis elimination programme. *Trop Med Int Health* 9, 1030–1035.

McCarthy, J.S., Guinea, A., Weil, G.J., Ottesen, E.A. (1995). Clearance of circulating filarial antigen as a measure of the macrofilaricidal activity of diethylcarbamazine in *Wuchereria bancrofti* infection. *J Infect Dis* 172, 521–526.

McCarthy, J.S., Zhong, M., Gopinath, R., Ottesen, E.A., Williams, S.A., Nutman, T.B. (1996). Evaluation of a polymerase chain reaction-based assay for diagnosis of *Wuchereria bancrofti* infection. *J Infect Dis* 173, 1510–1504.

Meyrowitsch, D.W., Simonsen, P.E., Makunde, W.H. (1995). Bancroftian filariasis: analysis of infection and disease in five communities of north-eastern Tanzania. *Ann Trop Med Parasitol* 89, 653–663.

Mishra, K., Raj, D.K., Hazra, R.K., Dash, A.P., Supakar, P.C. (2007). The development and evaluation of a single step multiplex PCR method for simultaneous detection of *Brugia malayi* and *Wuchereria bancrofti*. *Mol Cell Probes* 21, 355–362.

Mitchell, G.F. (1991). Co-evolution of paarsites and adaptive immune responses. *Immunol Today* 12, A2–5.

Molynuex, D.H., Bradley, M., Hoeraul, A., Kyelem, D., Taylor, M.J. (2003). Mass drug treatment for lymphatic filariasis and onchocerciasis. *Trends Parasitol* 19, 516–522.

More, S.J., Copeman, D.B. (1990). A highly specific and sensitive monoclonal antibody-based ELISA for the detection of circulating antigen in bancroftian filariasis. *Trop Med Parasitol* 41, 403–406.

Nguyen, N.L., Plichart, C., Esterre, P. (1999). Assessment of immunochromatographic test for rapid lymphatic filariasis diagnosis. *Parasite* 6, 355–358.

Nicolas, L. (1997). New tools for diagnosis and monitoring of bancroftian filariasis parasitism: the Polynesian experience. *Parasitol Today* 13, 370–375.

Nicolas, L., Luquiaud, P., Lardeux, F., Mercer, D.R. (1999). A polymerase chain reaction assay to determine infection of *Aedes polynesiensis* by *Wuchereria bancrofti*. *Trans R Soc Trop Med Hyg* 90, 136–139.

Norões, J, Dreyer, G, Santos, A, Mendes, VG, Mendeiros, Z, Addiss, D. (1997). Assessment of the efficacy diethylcarbamazine on adult *Wuchereria bancrofti in vivo*. *Trans R Soc Trop Med Hyg* 91, 78-81.

Nuchprayoon, S., Porksakorn, C., Junpee, A., Sanprasert, V., Poovorawan, Y. (2003). Comparative assessment of an Og4C3 ELISA and an ICT filariasis test: A study of Myanmar migrants in Thailand. *Asian Pac J Allerg Immunol* 21, 253–257.

Nuchprayoon, S., Junpee, A., Poovorawan, Y., Scott, A.L. (2005). Detection and differentiation of filarial parasites by universal primers and polymerase chain reaction-restriction fragment length polymorphism analysis. *Am J Trop Med Hyg* 73, 895–900.

Nuchprayoon, S., Junpee, A., Poovorawan, Y. (2007). Random amplified polymorphic DNA (RAPD) for differentiation between Thai and Myanmar strains of *Wuchereria bancrofti*. *Filaria J* 6:6, doi:10.1186/1475–2883–6–6.

Oliveira-Menezes, A., Lins, R., Norões, J., Dreyer, G. (2007). Comparative analysis of a chemotherapy effect on the cuticular surface of *Wuchereria bnacrofti* adult worms in vivo. *Parasitol Res* 101, 1311–1317.

Omar, M.S., Sheikha, A.K., Al-Amari, O.M., Abdalla, S.E., Musa, R.A. (2000). Field evaluation of two diagnostic antigen tests for *Wuchereria bancrofti* infection among Indian expatriates in Saudi Arabia. *Southeast Asian J Trop Med Public Health* 31, 415–418.

Ottesen, E.A. (1992). Infection and disease in lymphatic filariasis: an immunological perspective. *Parasitology* 104(suppl), S71–79.

Ottesen, E.A. (2000). The global programme to eliminate lymphatic filariasis. *Trop Med Int Health* 5, 591–594.

Ottesen, E.A., Duke, B.O.L., Karam, M., Behbehani, K. (1997). Strategies and tools for the control/elimination of lymphatic filariasis. *Bull World Health Org* 75, 491–503.

Ottesen, E.A., Ismail, M.M., Horton, J. (1999). The role of albendazole in programmes to eliminate lymphatic filariasis. *Parasitol Today* 15, 382–386.

Ottesen, E.A., Hooper, P.J., Bradley, M., Biswas, G. (2008). The global programme to eliminate lymphatic filariasis: health impact after 8 years. *PLoS Negl Trop Dis* 2, e317. doi:10.1371/journal.pntd.0000317.

Pani, S.P., Hoti, S.L., Elango, A., Yuvaraj, J., Lall, R., Ramaiah, K.D. (2000). Evaluation of the ICT whole blood antigen card test to detect infection due to nocturnally periodic *Wuchereria bancrofti* in South India. *Trop Med Int Health* 5, 359–363.

Pani, S.P., Hoti, S.L., Vanamail, P., Das, L.K. (2004). Comparison of an immunochromatographic card test with night blood smear examination for detection of *Wuchereria bancrofti* microfilaria carriers. *Natl Med J India,* 17, 304–306.

Pradeep Kumar, N., Patra, K.P., Hoti, S.L., Das, P.K. (2002). Genetic variability of the human filarial parasite, *Wuchereria bancrofti* in South India. *Acta Trop* 82, 67–76.

Pechgit, P., Intarapuk, A., Pinyoowong, D., Bhumiratana, A. (2011). Touchdown-touchup nested PCR for low-copy gene detection of benzimidazole-susceptible *Wuchereria bancrofti* with a *Wolbachia* endosymbiont imported by migrant carriers. *Exp Parasitol* 127, 559–568.

Phantana, S., Sensathein, S., Songtrus, J., Klagrathoke, S., Phongnin, K. (1999). ICT filariasis test: a new screening test for Bancroftian filariasis. *Southeast Asian J Trop Med Public Health* 30, 47–51.

Rajendran, R., Sunish, I.P., Mani, T.R., Munirathinam, A., Abdullah, S.M., Arunachalarm, N. et al. (2002). The influence of the mass administration of diethylcarbamazine, alone or with albendazole, on the prevalence of filarial antigenaemia. *Ann Trop Med Parasitol* 96, 595–602.

Rajendran, R., Sunish, I.P., Mani, T.R., Munirathinam, A., Abdullah, S.M., Arunachalarm, N. et al. (2004). Impact of two annual single-dose mass drug administrations with diethylcarbamazine alone or in combination with albendazole on *Wuchereria bancrofti* microfilaraemia and antigenaemia in South India. *Trans R Soc Trop Med Hyg* 98, 174–181.

Ramzy, R.M.R., Farid, H.A., Kamal, I.H., Ibrahim, G.H., Morsy, Z.S., Faris, R., et al. (1997). A polymerase chain reaction-based assay for detection of *Wuchereria bancrofti* in human blood and *Culex pipiens*. *Trans R Soc Trop Med Hyg* 91, 156–160.

Ramzy, R.M.R., Helmy, H., El-Lethy, A.S.T., Kandil, A.M., Ahmed, E.S., Weil, G.J., et al. (1999). Field evaluation of a rapid format kit for the diagnosis of bancroftian filariasis in Egypt. *East Mediterr Health J* 5, 880–887.

Rao, R.U., Atkinson, L.J., Ramzy, R.M.R., Helmy, H., Farid, H.A., Bockarie, M.J., et al. (2006). A real-time PCR-based assay for detection of *Wuchereria bancrofti* DNA in blood and mosquitoes. *Am J Trop Med Hyg* 74, 826–832.

Ravindran, B., Satapathy, A.K., Sahoo, P.K., Mohanty, M.C. (2003). Protective immunity in human lymphatic filariasis: problems and prospects. *Med Microbiol Immunol* 192, 41–46.

Robinson, M.W., Mcferran, N., Trudgett, A., Hoey, L., Fairweather, I. (2004). A possible model of benzimidazole binding to beta-tubulin disclosed by invoking an inter-domain movement. *J Mol Graph Model* 23, 275–284.

Rocha, A., Addiss, D., Ribeiro, M.E., Norões, J., Baliza, M., Medeiros, Z., et al. (1996). Evaluation of the Og4C3 ELISA in *Wuchereria bancrofti* infection: infected persons with undetectable or ultra-low microfilarial densities. *Trop Med Int Health* 1, 859–864.

Roos, M.H., Boersema, J.H., Borgsteede, F.H.M., Cornelissen, J., Taylor, M., Ruitenberg, E.J. (1990). Molecular analysis of selection for benzimidazole resistance in the sheep parasite *Hemonchus contortus*. *Mol Biochem Parasitol* 43, 77–88.

Roos, M.H., Kwa, M.S.G., Grant, W.N. (1995). New genetic and practical implications of selection for anthelmintic resistance in parasitic nematodes. *Parasitol Today* 11, 148–150.

Ruberanziza, E., Mupfasoni, D., Karibushi, B., Rujeni, N., Kabanda, G., Kabera, M., et al. (2009). Mapping of lymphatic filariasis in Rwanda. *J Lymphoedema* 4, 20–23.

Schwab, A.E., Boakye, D.A., Kyelem, D., Prichard, R.K. (2005). Detection of benzimidazole resistance-associated mutations in the filarial nematode *Wuchereria bancrofti* and evidence for selection by albendazole and ivermectin combination treatment. *Am J Trop Med Hyg* 73, 234–238.

Schwab, A.E., Churcher, T.S., Schwab, A.J., Basáñez, M.G., Prichard, R.K. (2007). An analysis of the population genetics of potential multi-drug resistance in *Wuchereria bnacrofti* due to combination chemotherapy. *Parasitology* 134(Pt 7), 1025–1040.

Schwab, A.E., Churcher, T.S., Schwab, A.J., Basáñez, M.G., Prichard, R.K. (2006). Population genetics of concurrent selection with albendazole and ivermectin or diethylcarbamazine on the possible spread of albendazole resistance in *Wuchereria bancrofti*. *Parasitology* 133(Pt 5), 589–601.

Silvestre, A., Cabaret, J., Humbert, J.F. (2001). Effect of benzimidazole under-dosing on the resistant allele frequency in *Teladorsagia circumcincta* (Nematoda). *Parasitology* 123, 103–111.

Silvestre, A., Humbert, J.F. (2002). Diversity of benzimidazole-resistance alleles in populations of small ruminant parasites. *Int J Parasitol* 32, 921–928.

Simonsen, P.E., Dunyo, S.K. (1999). Comparative evaluation of three new tools for diagnosis of bancroftian filariasis based on detection of specific circulating antigens. *Trans R Soc Trop Med Hyg* 93, 278–282.

Simonsen, P.E., Lemnge, M.M., Msangeni, H.A., Jakobsen, P.H., Bygbjerg, I.C. (1996). Bancroftian filariasis: The patterns of filarial-specific immunoglobulin G1 (IgG1), IgG4, and circulating antigens in an endemic community of northeastern Tanzania. *Am J Trop Med Hyg* 55, 69–75.

Simonsen, P.E., Meyrowitsch, D.W., Jaoko, W.G., Malecela, M.N., Michael, E. (2008). Immunoepidemiology of *Wuchereria bancrofti* infection in two East African communities: Antibodies to the microfilarial sheath and their role in regulating host microfilaremia. *Acta Trop* 106, 200–206.

Siriaut, C., Bhumiratana, A., Koyadun, S., Anurat, K., Satitvipawee, P. (2005). Short-term effects of a treatment with 300 mg oral-dose diethylcarbamazine on nocturnally periodic *Wuchereria bancrofti* microfilaremia and antigenemia. *Southeast Asian J Trop Med Public Health* 36, 832–840.

Siridewa, K., Karunanayake, E.H., Chandrasekharan, N.V. (1996). Polymerase chain reaction based technique for the detection of *Wuchereria bancrofti* in human blood samples, hydrocele fluid, and mosquito vectors. *Am J Trop Med Hyg* 54, 72–76.

Srividya, A., Pani, S.P., Rajagopalan, P.K., Bundy, D.A.P., Grenfell, B.T. (1991). The dynamics of infection and disease in Bancroftian filariasis. *Trans R Soc Trop Med Hyg* 85, 255–259.

Sunish, I.P., Rajendran, R., Mani, T.R., Gajanana, A., Reuben, R., Satyanarayana, K. (2003). Long-term population migration: an important aspect to be considered during mass drug administration for elimination of lymphatic filariasis. *Trop Med Int Health* 8, 316–321.

Sunish, I.P., Rajendran, R., Mani, T.R., Munirathinam, A., Tewari, S.C., Hiriyan, J., et al. (2002). Resurgence in filarial transmission after withdrawal of mass drug administration and the relationship between antigenaemia and microfilaraemia - A longitudinal study. *Trop Med Int Health* 7, 59–69.

Takagi, H., Itoh, M., Kasai, S., Yahathugoda, T.C., Weerasooriya, M.V., Kimura, E. (2011). Development of loop-mediated isothermal amplification method for detecting *Wuchereria bancrofti* DNA in human blood and vector mosquitoes. *Parasitol Int* (in press), doi:10.1016/j.parint.2011.08.018.

Thanomsub, B.W., Chansiri, K., Sarataphan, N., Phantana, S. (2000). Differential diagnosis of human lymphatic filariasis using PCR-RFLP. *Mol Cell Probes* 14, 41–46.

Turner, P., Copeman, D.B., Gerisi, D., Speare, R. (1993). A comparison of the Og4C3 antigen capture ELISA, the knott test, an IgG4 assay and clinical sign, in the diagnosis of bancroftian filariasis. *Trop Med Parasitol* 44, 45–48.

von Samson-Himmelstjerna, G., Pape, M., von Witzendorff, C., Schnieder, T. (2002). Allele-specific PCR for the beta-tubulin codon 200 TTC/TAC polymorphism using single adult and larval small strongyle (Cyathostominae) stages. *J Parasitol* 88, 254–257.

Weaver, A., Brown, P., Huey, S., Magallon, M., Bollman, E. B., Mares, D., et al. (2011). A low-tech analytical method for diethylcarbamazine citrate in medicated salt. *PLoS Negl Trop Dis* 5, e1005, doi: 10.1371/journal.pntd.0001005.

Weil, G.J., Liftis, F. (1987). Identification and partial characterization of a parasite antigen in sera from humans infected with *Wuchereria bancrofti. J Immunol* 138, 3035–3041.

Weil, G.J., Jain, D.C., Santhanam, S., Malhotra, A., Kumar, H., Sethumadhavan, K.V.P. et al. (1987). A monoclonal antibody-based enzyme immunoassay for detecting parasite antigenemia in bancroftian filariasis. *J Infect Dis* 156, 350–355.

Weil, G.J., Ramzy, R.M.R., Chandrashekar, R., Gad, A.M., Lowrie Jr., R.C., Faris, R. (1996). Parasite antigenemia without microfilaremia in bancroftian filariasis. *Am J Trop Med Hyg* 55, 333–337.

Weil, G.J., Lammie, P.J., Weiss, N. (1997). The ICT Filariasis Test: a rapid-format antigen test for diagnosis of bancroftian filariasis. *Parasitol Today* 13, 401–404.

Who. (1992a). Lymphatic filariasis: disease and its control. Fifth Report of the WHO Expert Committee on Filariasis. Geneva: World Health Oragnization. *WHO Tech Rep Ser* 821, p. 1–71.

Who. (1992b). Informal consultation on evaluation of morbidity in lymphatic filariasis, Tuberculosis Research Centre, Madras 10-11 February 1992. Geneva: World Health Organization. Mimeographed document WHO/TDR/FIL/92.3.

Who. (1994). Lymphatic filariasis infection and disease: control strategies. Report of a consultative meeting held at the University Sains Malaysia, Penang, Malaysia, August 1994. Geneva: World Health Organization. Mimeographed document WHO/TDR/CTD/FIL/PENANG/94.1.

Who. (1999a). Informal consultation on epidemiologic approaches to lymphatic filariasis elimination: initial assessment, monitoring, and certification, Atlanta, Georgia, USA 2-4 September 1998. Geneva: World Health Organization. Mimeographed documented WHO/FIL/99.195.

Who. (1999b). Guidelines for certifying lymphatic filariasis elimination (including discussion of critical issues and rationale), following from Informal consultation on epidemiologic approaches to lymphatic filariasis elimination: initial assessment, monitoring, and certification, Atlanta, Georgia, USA 2-4 September 1998. Geneva: World Health Organization. Mimeographed documented WHO/FIL/99/197.

Who. (2000). Elimination of lymphatic filariasis. Report of informal consultative meeting on lymphatic filaraisis in SEA region, Bhubaneswar, Orissa, India, 23-25 February 2000.

Who. (2001). Regional strategic plan for elimination of lymphatic filariasis (2000-2004). South-East Asia Regional Office, New Delhi: World Health Organization. Mimeographed documented SEA/FIL/28.corr.1.

Who. (2002). Global programme to eliminate lymphatic filariasis: Annual report on lymphatic filariasis 2001. Geneva: World Health Organization. Mimeographed document WHO/CDS/CPE/CEE/2002.28.

Who. (2008). Global programme to eliminate lymphatic filariasis. *Wkly Epidemiol Rec* 83, 333–341.

Who. (2009). Report: The role of polymerase chain reaction technique for assessing lymphatic filariasis transmission. Geneva: World Health Organization, p.1–55. Mimeographed document WHO/HTM/NTD/PCT/2009.1.

Who. (2010). Progress report 2000–2009 and strategic plan 2010–2020 of the global programme to eliminate lymphatic filariasis: halfway towards eliminating lymphatic filariasis. Geneva: World Health Organization, p.1–93. Mimeographed document WHO/HTM/NTD/PCT/2010.6

Williams, S.A., Nicolas, L., Lizotte-Waniewski, M., Plichart, C., Luquiaud, P., Nguyen, L.N., et al. (1996). A polymerase chain reaction assay for the detection of *Wuchereria bancrofti* in blood samples from French Polynesia. *Trans R Soc Trop Med Hyg* 90, 384–387.

Winterrowd, C.A., Pomroy, W.E., Sangster, N.C., Johnson, S.S., Geary, T.G. (2003). Benzimidazole-resistant β-tubulin alleles in a population of parasitic nematodes (*Cooperia oncophora*) of cattle. *Vet Parasitol* 117, 161–172.

Yongyuth, P., Koyadun, S., Jaturabundit, N., Sampuch, A., Bhumiratana, A. (2006). Efficacy of a single-dose treatment with 300 mg diethylcarbamazine and a combination of 400 mg albendazole in reduction of *Wuchereria bancrofti* antigenemia and

concomitant geohelminths in Myanmar migrants in Southern Thailand. *J Med Assoc Thai* 89, 1237–1248.

Zhong, M., McCarthy, J.S., Bierwert, L., Lizotte-Waniewski, M., Chanteau, S., Nutman, T.B., et al. (1996). A polymerase chain reaction assay for detection of the parasite *Wuchereria bancrofti* in human blood samples. *Am J Trop Med Hyg* 54, 357–363.

Zou, Z., Shin, S.W., Alvarez, K.S., Kokoza, V., Raikhel, A.S. (2010). Distinct melanization pathways in the mosquito *Aedes aegypti*. *Immunity* 32, 41–53.

24

Lymphatic Filariasis Transmission and Control: A Mathematical Modelling Approach

Asep K. Supriatna and N. Anggriani
Padjadjaran University
Indonesia

1. Introduction

Lymphatic filariasis has an effect on almost 120 million individuals all over the world. The disease may cause a chronic morbidity if the persons who are infected are left untreated. It is endemic in many parts of tropical countries. To prevent worldwide parasite transmission, the World Health Organization initiated the Global Programme to Eliminate Lymphatic Filariasis (GPELF) by eliminating filarial parasites from their human hosts (Molyneux & Zagaria, 2002). Various GPELF implementations are done in many participating countries. In 2004 alone there were more than thirty countries have started elimination program and this number is still rising. Various degrees of success have emerged as a result of the implementation of this program. Although it was reported that in some places the program has interrupted the transmission, in many other places the program could not stop the transmission of the disease (WHO, 2005). It has been argued that strategic choices and operational or biological factors contribute to the success or failure of the program. In general, it is difficult to evaluate the success or the failure of a health program, especially in the beginning of the program.

A mathematical model provides useful tools for planning and evaluation of control program in disease elimination (Goodman, 1994). In our earlier work (Supriatna *et al*., 2009) we develop a mathematical model for the transmission of Lymphatic Filariasis disease in Jati Sampurna, Indonesia. In Indonesia, the disease is already alarming. For example, the incidence of filariasis in Jati Sampurna (a district in the West Java province) is more than 1%. Within less than five years since the date of the publication confirming that Jati Sampurna is an endemic area, almost all regions nearby Jati Sampurna, and other relatively far distance areas are affected by the disease, and some of them are also categorized as endemic areas. Other cases of filarial prevalence are reported outside Java island, such as in Alor islands (the province of Nusa Tenggara Timur). On Alor islands, both *B. timori* and *W. bancrofti* are circulated, with a prevalence of up to 20% (Supali *et al.*, 2002). Indonesia joined the GPELF since 2001 and implemented administration of a single dose regimen of diethylcarbamazine (DEC) and albendazole in endemic areas (Krentel *et al.*, 2006). Our previous model tries to capture the effectiveness of this scenario in the attempt of controlling the spread of the disease, inspired by the transmission of the disease in Jati Sampurna.

The model assumes that acute infected humans are infectious and treatment is given to a certain number of acute infected humans found from screening process. The screening is

done every time a new chronic reported. The treated acute individuals are assumed to be remains susceptible to the disease. The model is analyzed and it is found a condition for the existence and stability of the endemic equilibrium. A well known rule of thumb in epidemiological model, that is, the endemic equilibrium exists and stable if the basic reproduction number is greater than one, is established. Moreover, it is also shown that if the level of screening is sufficiently large, current medical treatment strategy will be able to reduce the long-term level of incidences. However, in practice it is not realistic for the following reasons.

One important concept in mathematical epidemiology regarding transmission of a disease is the basic reproduction number. It measures the number of new infections caused by an infective during the life time of the infective. Although our previous model is able to gain some insights on how the provision of a medical treatment can reduce the level of disease incidence, however it is worth to note that the basic reproduction number does not depend on the level of the treatment. It means that the treatment, no matter how large it is, will not be able to annihilate the endemicity of the disease. This is some what surprising and unexpected, because normally, in many epidemiological models, any medical treatment should reduce the basic reproduction number.

Our earlier work shows that the medical treatment given in the model scenario cannot eliminate the disease, in terms of reducing the basic reproduction number. Our previous model has also ignored an important factor in the transmission stage, namely the time delay. The model has assumed that once an individual infected, he/she become infectious without any delay. Nonetheless, the reproduction number can be reduced by giving additional treatments, such as reducing the biting rate and mosquito's density. This suggests that there should be a combination of treatment to eliminate the disease. In this chapter we review our earlier model of the filariasis transmission and a new model based on the earlier work is developed and analysed. The chapter gives a step by step improvement of our previous model. We do not carry out a heavy mathematical analysis instead some simulations of the models are presented. Finally, some interpretations are derived from the results.

2. Mathematical model with no time delay in infection period

To formulate the model we use the assumptions that initially the human population is virgin, *i.e.* there is no infection, and the total population of human is constant. We assume that there is an invasion by few infective individuals of either human or mosquitoes. There is only one species of worm and one species of mosquito, and there is no vertical transmission of the disease, either in human or mosquitoes populations. The human population is divided into three subpopulations, susceptible S_H, infected-carrier A and infected-chronic K, with the total number of the population given by N_H. We assume that once a human individual is infected then without any delay the individual becomes infectious. However, we strictly assume that transmission to the mosquitoes is only from the acute population. All chronic individuals are isolated perfectly. This strict assumption will be relaxed in some simulation later on. The mosquitoes are divided in two subpopulations, susceptible S_V and infected I_V mosquitoes, with the total number N_V. Related parameters in the model are the human recruitment rate R_H, human death rate μ_H, successful rate of transmission from mosquitoes to susceptible human p_H, mosquitoes biting rate on human

b , symptomatic rate δ , mosquitoes recruitment rate R_V , mosquitoes death rate μ_V and successful rate of filarial transmission from human to susceptible mosquitoes p_V . If the medical treatment is quantified by n number of people screened by the health authority, for every single chronic found, with the successful probability of the treatment p_0 , then the governing differential equations describing the mathematical model of the disease transmission are given by the following equations:

$$\frac{dS_H}{dt} = R_H - \frac{bp_H I_V S_H}{N_H} - \mu_H S_H + \frac{p_0 n \delta A^2}{N_H}, \tag{1}$$

$$\frac{dA}{dt} = \frac{bp_H I_V S_H}{N_H} - \mu_H A - \delta A - \frac{p_0 n \delta A^2}{N_H}, \tag{2}$$

$$\frac{dK}{dt} = \delta A - \mu_H K , \tag{3}$$

$$\frac{dS_V}{dt} = R_V - \frac{bp_V A S_V}{N_H} - \mu_V S_V , \tag{4}$$

$$\frac{dI_V}{dt} = \frac{bp_V A S_V}{N_H} - \mu_V I_V . \tag{5}$$

We can evaluate the effectiveness of the medical treatment *n* in managing the disease within the presumed policy, by inspecting its appearance in the endemic equilibrium and in the basic reproduction number. From the model, by assuming the host and vector populations are constant, so that $N_H = \frac{R_H}{\mu_H}$ and $N_V = \frac{R_V}{\mu_V}$, we found the endemic and non-endemic equilibria of the model related to the basic reproduction number

$$R_0 = \frac{\sqrt{b^2 R_H R_V \mu_H p_H p_V (\delta + \mu_H)}}{R_H \mu_V (\delta + \mu_H)} . \tag{6}$$

We also establish a theorem saying that "if $R_0 > 1$ then the endemic equilibrium of the system is locally asymptotically stable, otherwise it is unstable". The details of the derivation can be seen in Supriatna *et al*. (2009). In terms of controlling the disease it means that we should keep the basic reproduction number as low as possible so that it is lower than the unity by adjusting the level of the treatment *n*. The basic reproduction number is obtained using the next generation matrix (see Diekmann & Heesterbeek, 2000). It is worth to note that the basic reproduction number does not depend on the level of screening *n*, and hence, current presumed method of treatment does not annihilate the endemicity of the disease. This is partially because of the re-susceptibility of the treated population. However, our earlier work show that it indeed reduces the number of the acute population in the long-term as shown in the following section.

2.1 Numerical examples for the model with no delay time in infection period

To facilitate some interpretation regarding the results in our previous work, we present numerical examples using the parameters shown in Table 1. The simulation uses Powersim Constructor Ver. 2.5d with the program listing equivalent to basic model of equations (1) to (5) is provided in the Appendices. Powersim code for other models in the preceding section can be easily modified from this basic model. We give two examples: the first example assumes that a virgin population is invaded by acute infected human (via human immigration) and the second example assume that a virgin population is invaded by infected mosquitoes (e.g. a container un-intentionally transporting infected mosquitoes from an endemic area).

Parameter	Value
R_H	2,500
μ_H	1/70
δ	0.25
p_H	0.01
n	0

Parameter	Value
R_V	1,000,000
μ_V	365 (1/30)
b	250
p_V	0.1
p_0	0.75

Table 1. The main values of parameters used in the numerical examples

Figure 1 depicts the following scenario. Suppose that a population is initially virgin and stays at its equilibrium. We assume that it is then invaded by 10 acute infected human individual, with all the mosquitoes are also virgin. Using the parameter values given in Table 1, we obtain the value of the basic reproduction number is 3.02, which means that the disease will increase if there is no intervention. Figure 1 shows the dynamics when there is no treatment (n=0). The effect of the values of the parameters on the basic reproduction number is clear from equation (6). However its effect on the dynamics and the endemic equilibrium is not so obvious. Figure 2 shows the same dynamics as in Figure 1, with an addition that in the 25th year after the invasion of infective individuals there is a medical treatment with n=200. Figure 3 shows the same dynamics as in Figure 1, but here the treatment is carried out as early as the 5th year after the invasion with only 100 screening (n=100). These figures reveal that an early average treatment is better than a late huge treatment.

The scenario in Figures 1 to 3 assumes that the medical treatment given to the infected persons does not affect the transmission parameters given in Table 1 other than the screening parameter n. The screening parameter n does not appear in the basic reproduction number formula (6). Hence, this treatment does not affect the endemic status of the disease. In reality, there are some treatments that could alter the values of the disease transmission parameters. For example, if we assume that some portion of the population is treated by giving them some insect repellent, then the biting rate b could be altered. Let us assume that an effective insect repellent could decrease the biting rate to 50% of its current level. Figure 4 shows the dynamic when there exist this effective insect repellent, and used from the 5th year in the absence of the medical treatment (n=0) and Figure 5 shows the same scenario as in the previous figure but in the presence of the medical treatment with n=100 given by the same time as the insect repellent provision. Compared to the case when there is no insect repellent (Figure 1), the introduction of the insect repellent is significantly reduces the level

of the disease outbreak (Figure 4) and in the same time reduces the endemic level of the disease (changing the value of the basic reproduction number from 3.02 to 1.51). Meanwhile, if we also apply the medical treatment with only average treatment (n=100), then the level of the outbreak is relatively the same, but apparently with a shorter period of the outbreak (Figure 5).

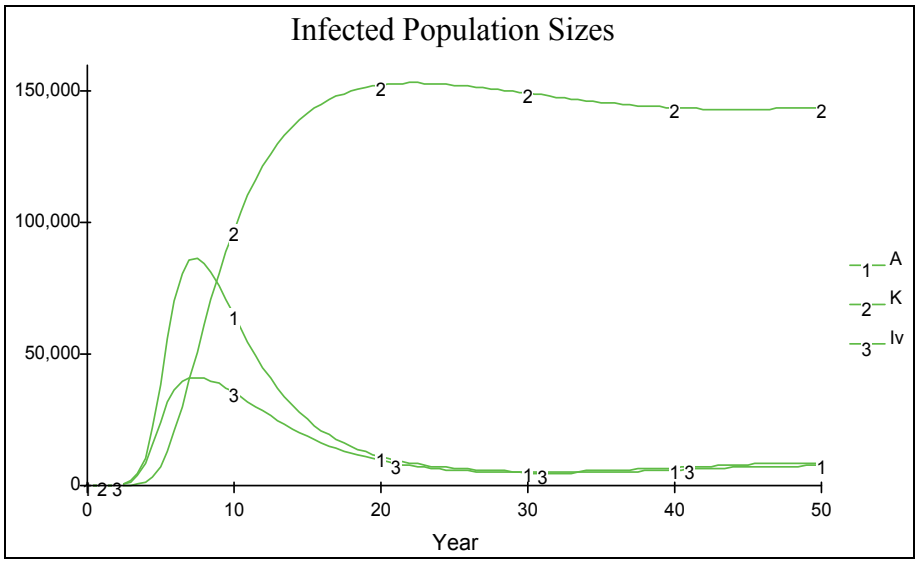


Fig. 1. The dynamics of infected population when there is no medical treatment after the invasion of 10 infected human.

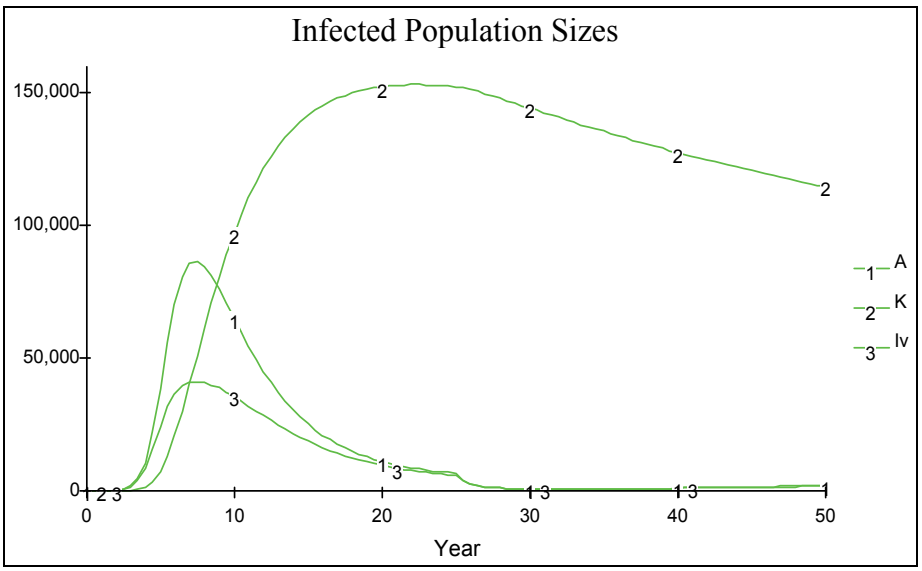


Fig. 2. The dynamics of infected population when there is a medical treatment in the 25th year with n=200.

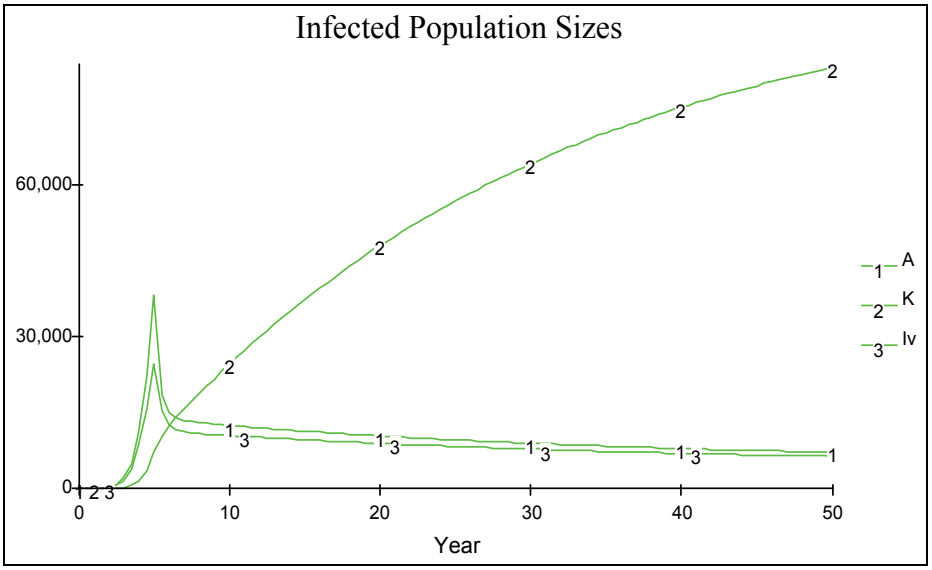


Fig. 3. The dynamics of infected population when there is a medical treatment in the 5th year with n=100.

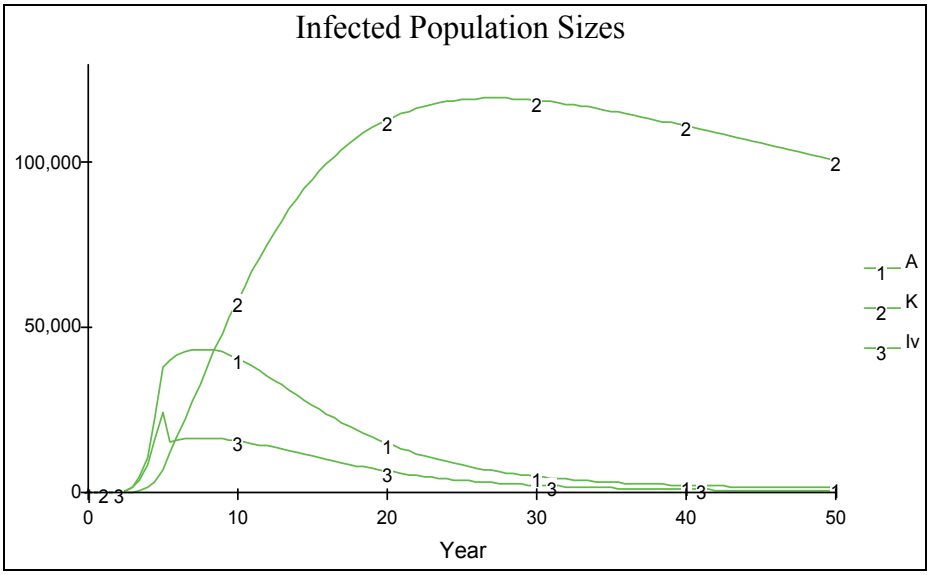


Fig. 4. The dynamics of infected population when there is an effective insect repellent which changes the biting rate to its 50% of the current level with no medical treatment in the 5th year after the disease invasion (n=0).

Other scenarios could also be considered. Some are already known to be ineffective if only applied solely, such as fogging (Soewono & Supriatna, 2002) and other still unexplored, such as newly developed method for shortening mosquitoes life expectancy (Turley *et al.*, 2009). Supposed that with some ways we can reduce the mosquito life expectancy down to 50 % of the existing level (from 30 days as in Table 1 to 15 days). Figure 6 shows its

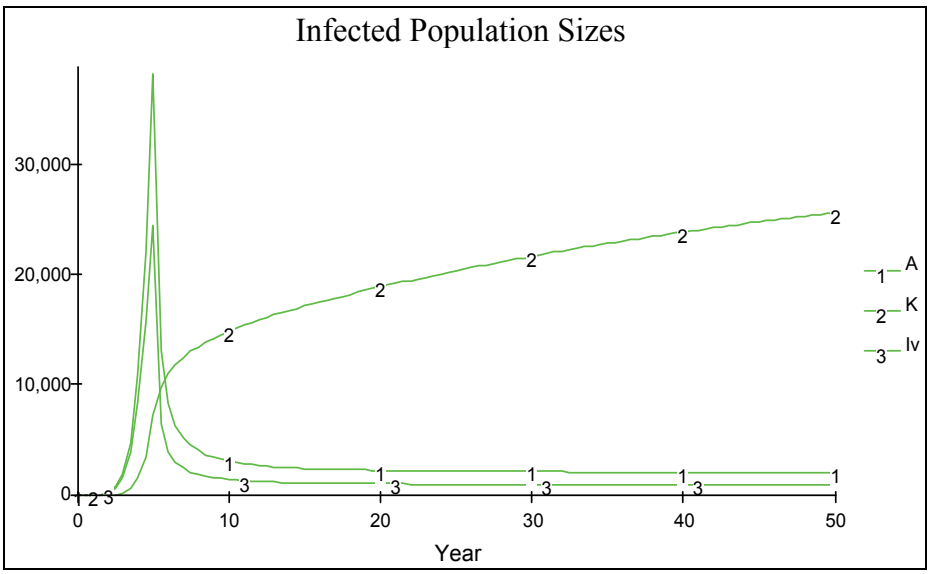


Fig. 5. The dynamics of infected population when there is an effective insect repellent which changes the biting rate to its 50% of the existing level combining with average medical treatment in the 5th year after the disease invasion (n=100).

dynamics which is the same as the dynamics in Figure 4. This is not surprising considering the form of the basic reproduction number (equation (6)), in which the decrease of biting rate acts the same as the decrease of the mosquitoes life expectancy (equivalently the increase of the mosquitoes mortality rate μ_V). If we decrease both values, *i.e.* the values of the biting rate and the life expectancy, then their effect in reducing the basic reproduction number doubled, such as shown by Figure 7, resulting in the value of the basic reproduction number to be less than one (only 0.755), which means the disappearance of the disease is guaranteed. Even in the absence of medical treatment, Figure 8 shows that if we do this strategy before one year has elapsed then the disease does not have any chance to grow. This suggests that preventive action is better than curative action.

In the previous example we assume that invasion is done by infected human. Next in the following example we assume that invasion is done by infected mosquitoes from an endemic area. Considering the short distance of the mosquito flight, we can assume that this invasion happens un-deliberately, for example via container and other transportation modes. However, considering the stability theorem of the endemic equilibrium point in our previous work (Supriatna *et al.*, 2009), we expect that the long term behaviour of the disease transmission dynamics would be the same as in the first example. In other words, there is an independence of initial values, such as illustrated by Figure 9, in which we assume that there are 100 infected mosquitoes invades the virgin population as described in the first example (Figure 1).

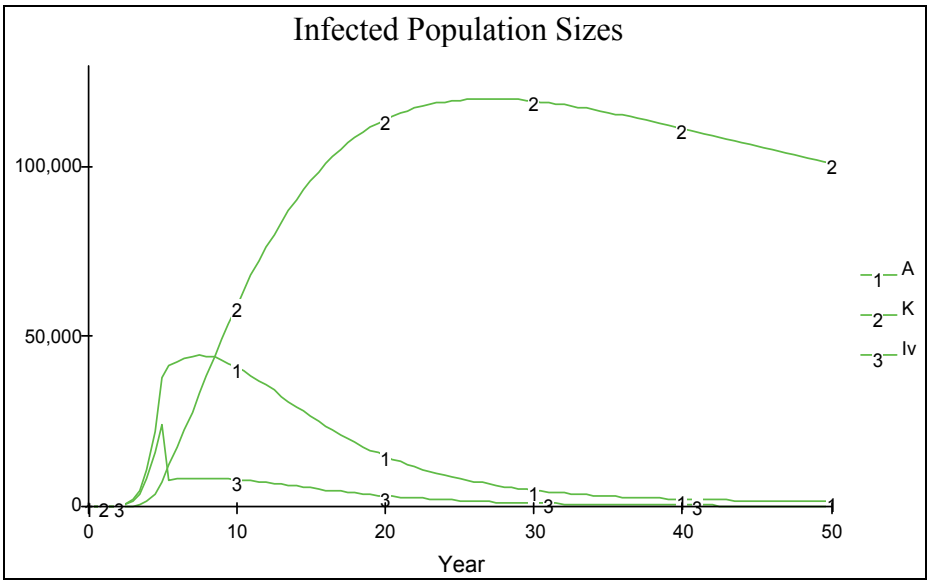


Fig. 6. The dynamics of infected population when there is an intervention which changes the mosquitoes life expectancy to its 50% of the current level with no medical treatment in the 5th year after the disease invasion (n=0).

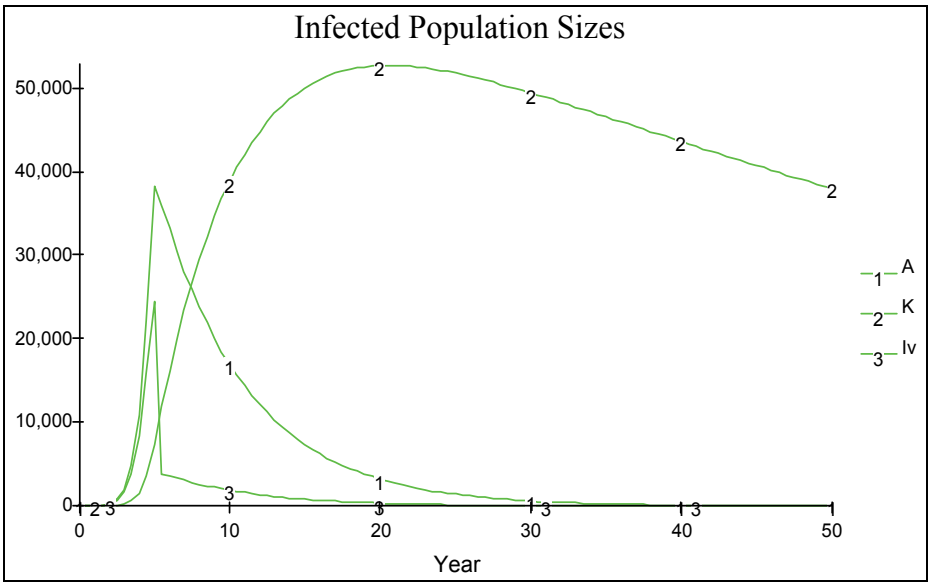


Fig. 7. The dynamics of infected population when there is an intervention which changes both the mosquitoes life expectancy and the biting rate to their 50% level with no medical treatment in the 5th year after the disease invasion (n=0).

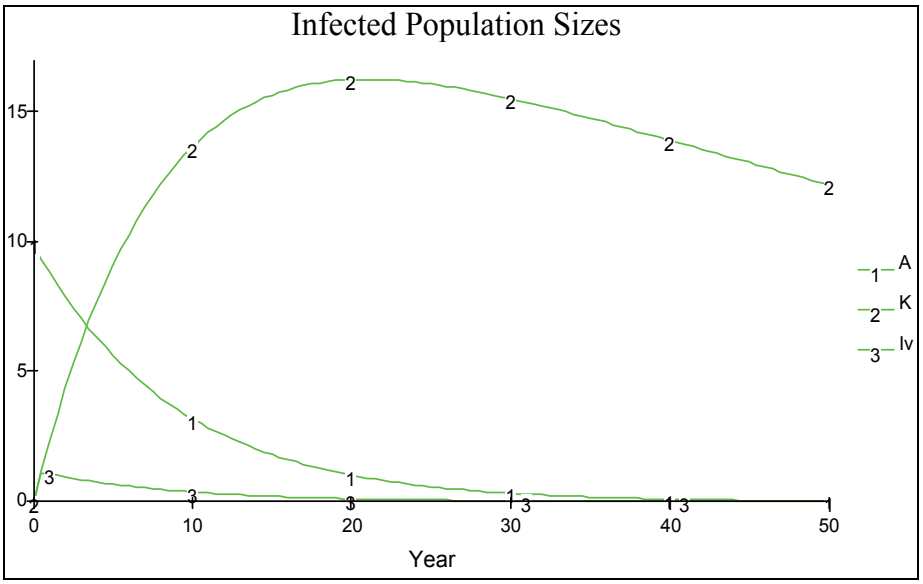


Fig. 8. The dynamics of infected population when there is an intervention which changes both the mosquitoes life expectancy and the biting rate to their 50% level done before one year after the disease invasion has elapsed (n=0).

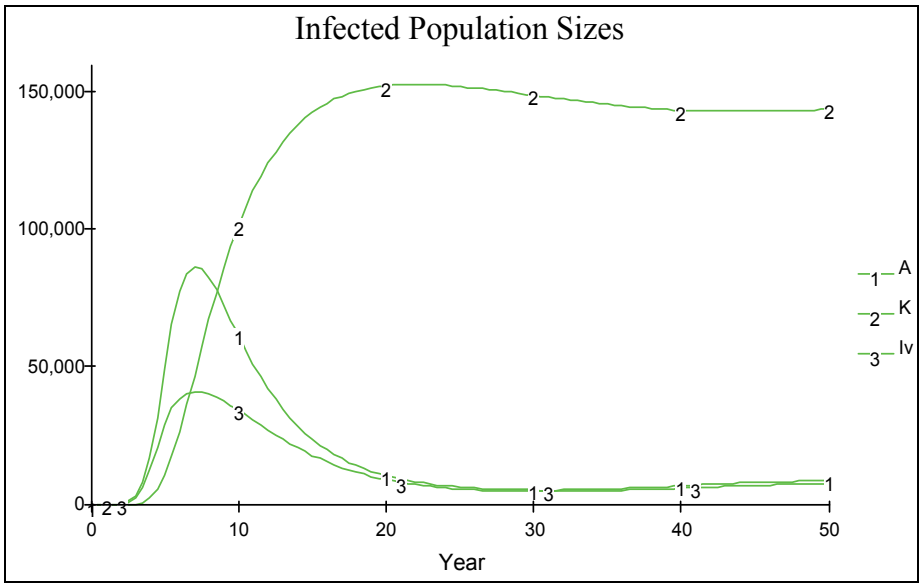


Fig. 9. The dynamics of infected population when there is no medical treatment after the invasion of 100 infected mosquitoes into a totally virgin population. The figure is similar to Figure 1 in which invasion is done by 10 infected human.

3. Mathematical model with delay time in infection period

In this section, we take a delay time into account and re-analysed the resulting model by introducing a new compartment to mimic the presence of the delay time. This is done by adding a sub acute or minor acute compartment A_m into the previous system. The sub acute population has a lower force of infection than the acute population considering their worm burden status, and it might be not infectious yet. This is reflected by a lower successful rate of filarial transmission p_{V2} from the sub acute to susceptible mosquito population, compared to the successful rate of filarial transmission from the acute population p_{V1}. In this case, we can consider the sub acute compartment consist of exposed or latent individuals. Individuals stay in sub acute compartment with the sojourn time $1/\gamma$ before they leave to the acute compartment. The system of equations takes form as the following,

$$\frac{dS_H}{dt} = R_H - \frac{bp_H I_V S_H}{N_H} - \mu_H S_H + \frac{p_0 n\delta A}{N_H}(A + A_m), \tag{7}$$

$$\frac{dA_m}{dt} = \frac{bp_H I_V S_H}{N_H} - \mu_H A_m - \gamma A_m - \frac{p_0 n\delta A A_m}{N_H}, \tag{8}$$

$$\frac{dA}{dt} = \gamma A_m - \mu_H A - \frac{p_0 n\delta A^2}{N_H} - \delta A, \tag{9}$$

$$\frac{dS_V}{dt} = R_V - \frac{b(p_{V1}A + p_{V2}A_m)S_V}{N_H} - \mu_V S_V, \tag{10}$$

$$\frac{dI_V}{dt} = \frac{b(p_{V1}A + p_{V2}A_m)S_V}{N_H} - \mu_V I_V. \tag{11}$$

3.1 Numerical examples for the model with delay time in infection period

As in the previous section, we provide a simulation for the model of equations (7) to (11) to gain some insights. The parameters are the same as before unless it is stated explicitly.

Compared to Figure 1, in which there are 10 acute infected human initially, Figure 10 shows that the present of time delay, by assuming that the sojourn time in the sub acute compartment is 5 years (hence γ is 1/5) with the probability of transmission to the mosquitoes is only 10% of the probability of the acute compartment (hence p_{V2} is 0.01), has an effect on significantly delaying the accumulation of the chronic and reducing the number of acute human population. However, the total infectious ($A + A_m$) in Figure 10 is slightly greater than the total infectious (A) in Figure 1.

We can also simulate if in fact we were unable to perfectly isolate the chronic individuals, hence there is a transmission from a portion of them to the mosquitoes. We would expect the transmission rate from the chronic is far greater than the one from the acute population, say the transmission is more certain considering the worm burden carried by them. One of

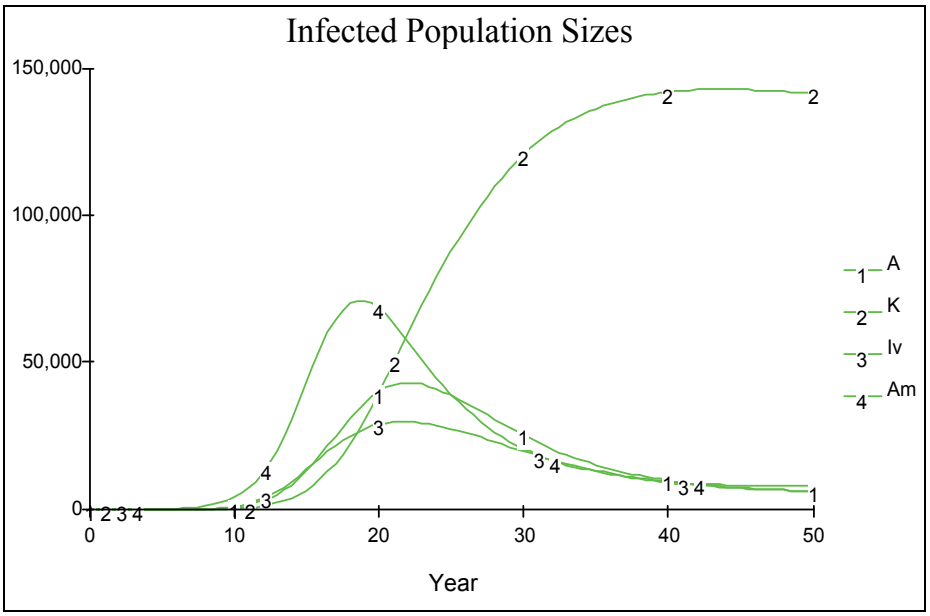


Fig. 10. The dynamics of infected population when there is no medical treatment after the invasion of infected human comprising of 10 acute individuals. Here we assume that there is no sub acute individual, initially.

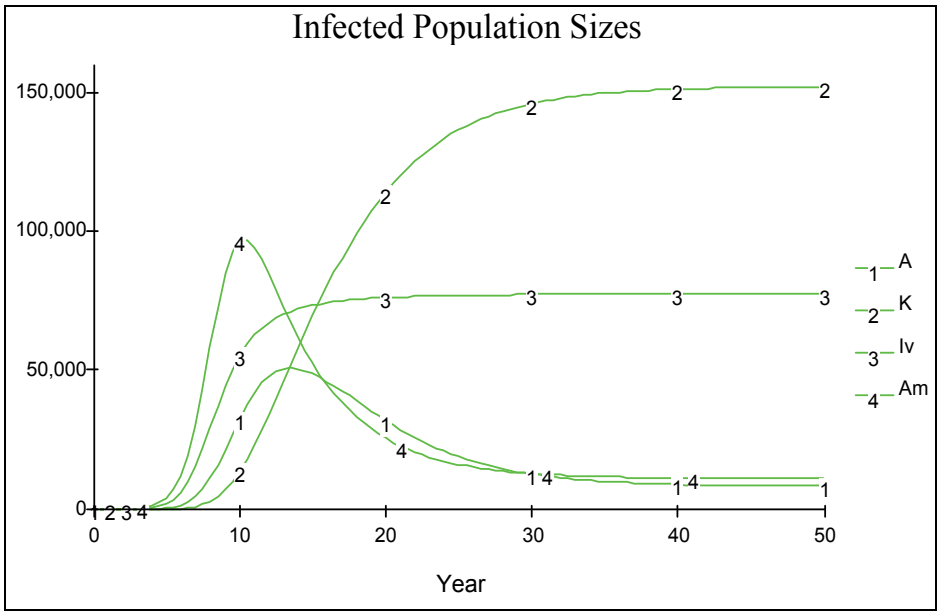


Fig. 11. The dynamics of infected population as with all parameters as in Figure 10, with an addition that infection also occurs from the chronic by assuming there is no perfect isolation.

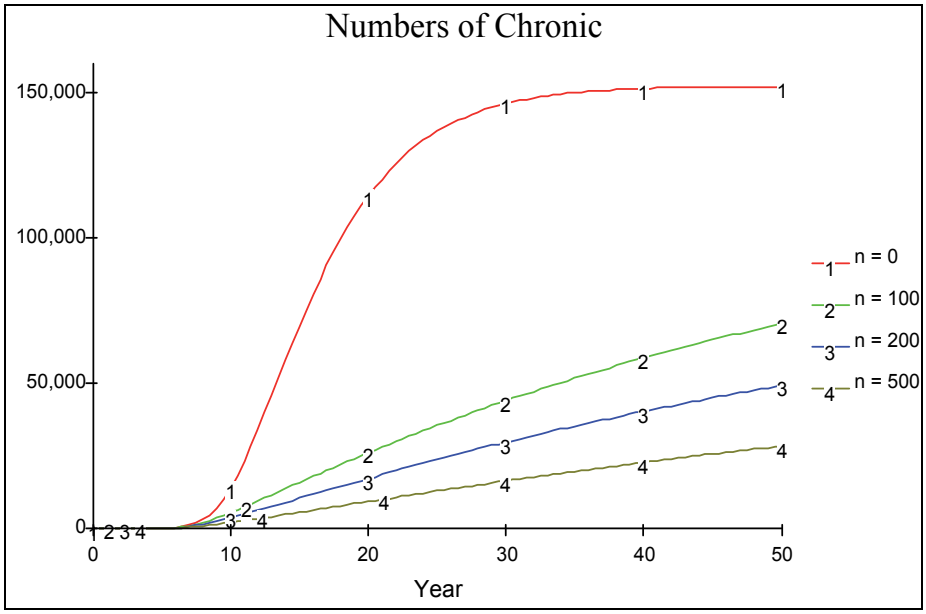


Fig. 12. The dynamics of chronic population when there is an early medical treatment with various values of *n*.

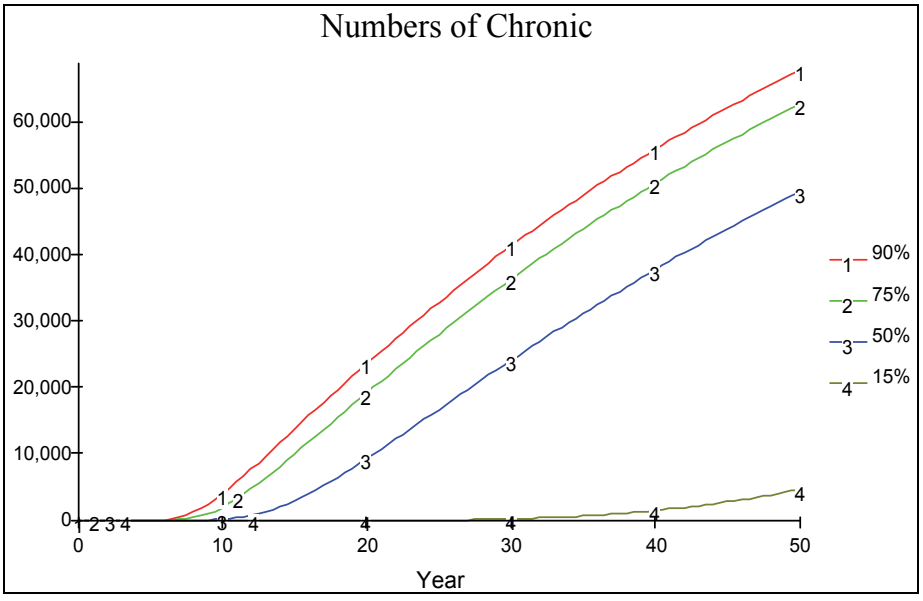


Fig. 13. The dynamics of chronic population when there is an early medical treatment with *n*=100 together with the various reduction of the biting rate up to a certain level.

the realisations is shown in Figure 11. The figure reveals that the peak of the outbreak is higher and reached earlier compared to that in Figure 10. Note that in the early years, there is an iceberg phenomenon, in which the number of chronic is far less than the number of

acute. This indicates that early treatment is better than late treatment. Suppose that we administer a medical treatment as in the previous section, measured by the number of screening *n*. Figure 12 shows various regimes of treatment done continuously since the beginning of the course of the epidemic. Figure 13 shows that a low level of medical treatment combined with the high reduction of biting rate (e.g. up to 15% of the original biting rate) performs better than that resulting from high level of medical treatment with no reduction in biting rate.

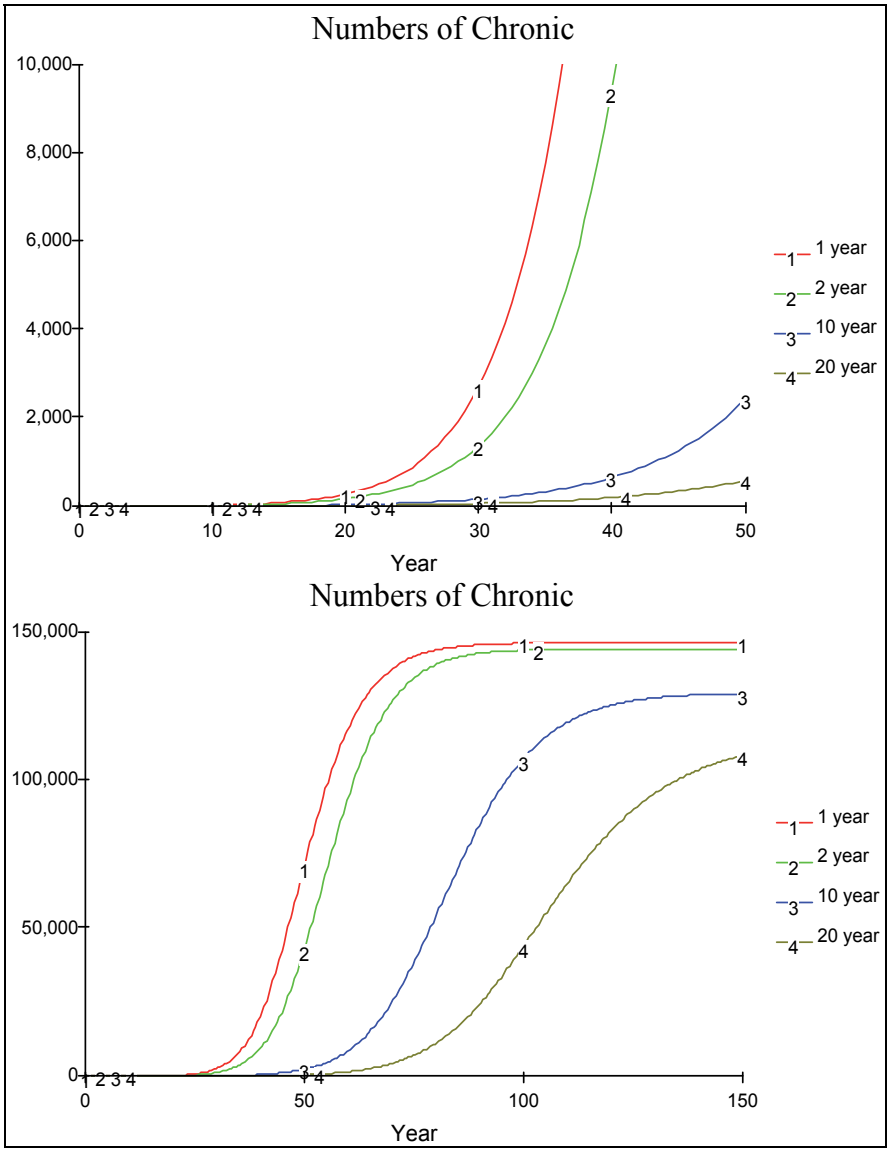


Fig. 14. The dynamics of chronic population when prophylaxis is given to the whole population with various effects to the sub acute sojourn time (equivalent to the reciprocal value of the recruitment rate from sub acute to acute population).

Suppose now that we have another scenario of treatment, that is giving prophylaxis to all the populations (set n=0, simply to evaluate the effectiveness of this prophylaxis). The prophylaxis works by inhibiting the growth of the worms inside human, say by delaying the recruitment into the acute population A from the sub acute population A_m. Technically this is done by varying the values of the transition rate γ (or equivalently the sub acute sojourn time $1/\gamma$) in the model. Figure 14 shows the effect of delay for various sojourn time due to the effect of the prophylaxis application. It seems that all the graphs increase exponentially (upper figure), but in fact at the end they end up to their stable equilibrium (lower figure) with different speed and different peak. This indicates that controlling the density of worm inside the body of infective human is effective in reducing the number of filarial infection. The model assumes that the delivery of prophylaxis has a result in a constant effect over time, which doesn't reflect the reality. To increase the realism, we should consider the decrease of prophylaxis effectiveness by modifying or refining the model. Nevertheless, we still can apply the current model by only believing the short-term prediction given by the model, say only in one to two years prediction and use it as guidance in a periodic delivery of a mass drug administration program.

The introduction of a single exposed compartment is not without a problem. Getz and Lloyd-Smith (2006) showed that a single exposed compartment will produce an exponentially-distributed sojourn time in the exposed stage. Referring to our delay model (equations (8) and (9)), this distribution has mean at $1/\gamma$ while its modus is at 0, which is a poor match to the real distribution of latent periods. Plant and Wilson (1986) pointed out that the drawback can be resolved by introducing a distributed delay or staging delay time approach comprising of k classes of sub acute or exposed individual. This approach gives a gamma-distributed total time of individuals staying in the exposed class with mean $1/\gamma$ and variance $1/(k\gamma^2)$. Note that a fixed time delay $1/\gamma$ is obtained whenever the number of delay stages k approaches the infinity.

In this part we use this approach (see also Getz and Lloyd-Smith (2006)) to our delay model by introducing multiple exposed compartments which is more appropriate to the disease like filariasis which has more than one different exposed stages. The general model is the same as equations (7) to (11) except that equations (8) and (9) are replaced by

$$\frac{dA_{m1}}{dt} = \frac{bp_H I_V S_H}{N_H} - k\gamma A_{m1} - \mu_H A_{m1} - \frac{p_0 n\delta A A_{m1}}{N_H}, \tag{12}$$

$$\frac{dA_{mi}}{dt} = k\gamma(A_{mi-1} - A_i) - \mu_H A_{mi} - \frac{p_0 n\delta A A_{mi}}{N_H}, \qquad i = 2,...,k, \tag{13}$$

$$\frac{dA}{dt} = k\gamma A_{mk} - \mu_H A - \frac{p_0 n\delta A^2}{N_H} - \delta A. \tag{14}$$

The system is much more complex since it consists of 15 differential equations compared to just 6 differential equations in the previous model. However, numerical example in Figure 15 shows that for $k = 10$ (and also for any $k > 1$), the simpler model of equations (7) to (11), qualitatively, is a good approximation of the more realistic model of the same equations but

with equations (8) and (9) are replaced by equations (12) to (14). Initially, the prediction of simpler model ($K1$ in Figure 15) slightly overestimates, but then after a certain years it begins to underestimate, the "true" numbers of chronic individuals ($K10$ in Figure 15). However in the long-term both model produce the same equilibrium point (not shown in the Figure).

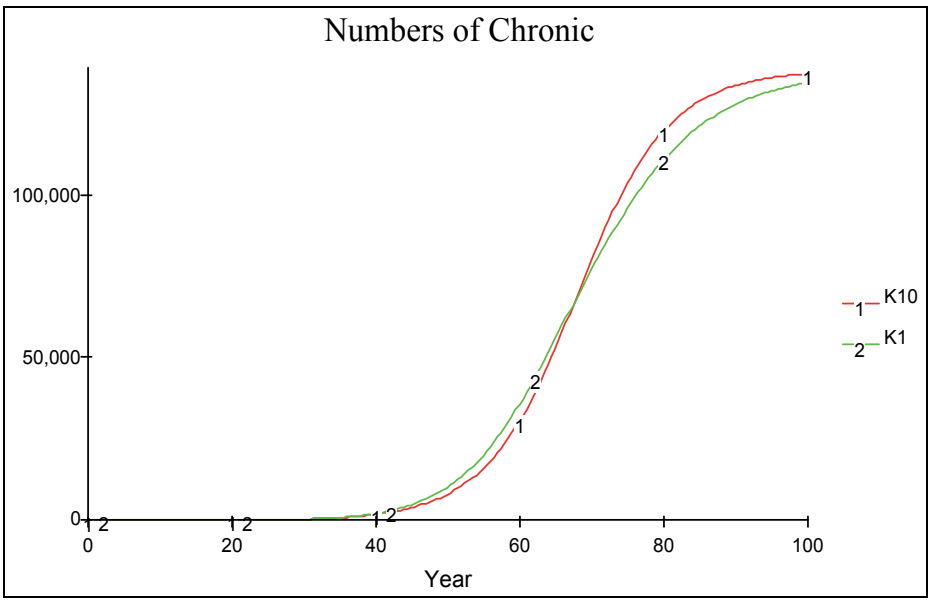


Fig. 15. The dynamics of chronic population predicted by the simple model of equations (7) to (11) and the more realistic staging delay time model of the same equations but with equations (8) and (9) are replaced by equations (12) to (14).

4. Conclusion

In this chapter we review a mathematical model of filarial transmission in human and in mosquitoes. Some simulations are carried out to obtain some insights regarding the transmission and possible actions to control the transmission. Some refinement of the model could be done in many directions to increase the realism of the model and to obtain a more accurate prediction. New directions may include the evolutionary, sosio-economics, and climatology aspects of the disease (Levin, 2002).

In the evolutionary issues of epidemiology, some agents of diseases may develop resistance to certain drug. It is worth to explore how this affects the transmission of the diseases. In many situations, especially in developing countries, there always competing interests related to limited resources and budget. There are many other important diseases, other than filariasis, needs for attention. Choosing the right priorities are among the concerns of health managers and authorities. In the absence of sufficient health budget it is important to address questions like the long term consequences when the treatment is terminated, either purportedly, e.g. because the budget is re-allocated to a higher priority health problem (to other endemic places of the same disease or to other disease problems) or inadvertently (due to the decreasing compliance of the program implementation). This is an example of sosio-

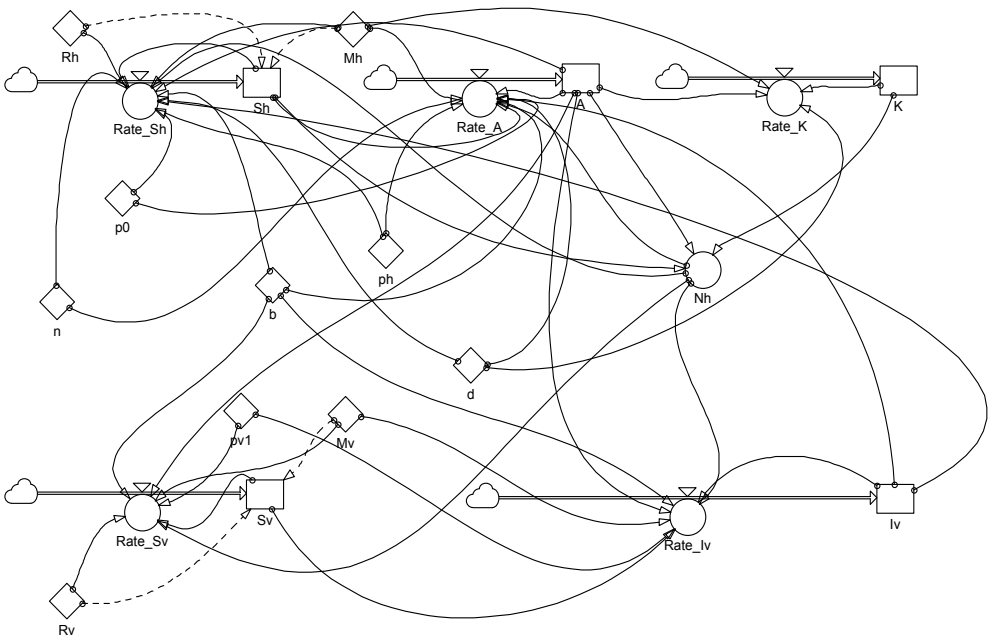
economics issues in epidemiology (Supali *et al*., in prep.). Climate change also regarded as a factor contributes to current emerging and re-emerging infectious diseases. For example, since the global temperature is rising then suitable habitat for mosquitoes becomes wider. It is reported that many parts in the globe of previously free from mosquito is now invaded by incoming mosquitoes. To obtain a better prediction of global filarial transmission, this climatology aspect also should be considered. We believe that there are many other venues are possible for future research in mathematical aspect of filariasis transmission.

5. Acknowledgment

Part of the research is funded by the Indonesian Government through the scheme of Penelitian Hibah Kompetensi 2012.

6. Appendices

6.1 Powersim diagram of the basic filariasis model



6.2 Powersim listing program of the basic filariasis model

```
init    A = 10
flow    A = +dt*Rate_A
init    Iv = 0
flow    Iv = +dt*Rate_Iv
init    K = 0
flow    K = +dt*Rate_K
init    Sh = Rh/Mh
flow    Sh = +dt*Rate_Sh
```

```
init    Sv = Rv/Mv
flow    Sv = +dt*Rate_Sv
aux     Rate_A = ((b*Iv*Sh*ph)/Nh)-Mh*A-((p0*n*A*d*A)/Nh)-d*A
aux     Rate_Iv = ((b*Sv*A*pv1)/Nh)-Mv*Iv
aux     Rate_K = d*A-Mh*K
aux     Rate_Sh = Rh-((b*Iv*Sh*ph)/Nh)-Mh*Sh+((p0*n*A*d*A)/Nh)
aux     Rate_Sv = Rv-((b*Sv*A*pv1)/Nh)-Mv*Sv
aux     Nh = Sh+A+K
const   b = 250
const   d = 0.25
const   Mh = 1/70
const   Mv = 365/30
const   n = 0
const   p0 = 0.75
const   ph = 0.01
const   pv1 = 0.1
const   Rh = 2500
const   Rv = 1000000
```

7. References

Diekmann, O. & Heesterbeek, J.A.P. (2000). *Mathematical Epidemiology of Infectious Diseases*, John Wiley & Son, ISBN 978-0471492412 , New York, USA

Getz, W.M. & Lloyd-Smith, J.O. (2006). Basic Method for Modeling the Invasion and Spread of Contagious Diseases, In: *Disease Evolution: Models, Concepts, and Data Analyses*, Z. Feng; U. Dieckmann & S. Levin (Eds.), 87-105, AMS, ISBN 0-8218-3753-2, USA

Goodman, B. (1994). Models Aid Understanding, Help Control Parasites [News]. *Science* 264: 1862-1863

Krentel, A.; Fischer, P.; Manoempil, P.; Supali, T.; Servais, G. & Ruckert, P. (2006). Using Knowledge, Attitudes and Practice (KAP) Surveys on Lymphatic Filariasis to Prepare a Health Promotion Campaign for Mass Drug Administration in Alor District, Indonesia. *Tropical Medicine and International Health*. 11: 1731–1740

Levin, S.A (2002). New Directions in the Mathematics of Infectious Disease. In: *Mathematical Approaches for Emerging and Reemerging Infectious Diseases: Models, Methods, and Theory,* Castillo-Chavez, C *et al.* (Eds.), 1-5, Springer, ISBN 0-387-95355-8, New-York, USA

Molyneux, D. & Zagaria, N. (2002). Lymphatic Filariasis Elimination: Progress in Global Programme Development. *Ann. Trop. Med. Parasitol*. 96 (Suppl 2): S15-40

Plant, R.E. & Wilson, L.T. (1986). Models for Age-Structured Population with Distributed Maturation Rates. *J. Math. Biol.* 23, 247-262.

Soewono, E. & Supriatna, A.K. (2002). A Two-Dimensional Model for the Transmission of Dengue Fever Disease. *Bulletin of Malaysian Mathematical Sciences Society* 24(1): 49-57

Supali, T.; Wibowo, H.; Ruuckert, P; Fischer K.; Ismid, I.S.; Purnomo; Djuardi, Y. & Fischer P. (2002). High prevalence of Brugia timori infection in the Highland of Alor Island, Indonesia. *Am. J. Trop. Med. Hyg*., 66(5), 2002, pp. 560–565

Supali, T.; Tasman, H.; Supriatna, A.K.; Soewono, E. & Nuraini, N. Long Term Effects of Mass Drug Administration on Lymphatics Filariasis Transmision, In Prep.

Supriatna, A. K.; Serviana, H. & Soewono, E. (2009). A Mathematical Model to Investigate the Long-Term Effects of the Lymphatic Filariasis Medical Treatment in Jati Sampurna, West Java. *ITB Journal of Science* Vol: 41 Issue: 1 Pages/record No.: 1-14

Turley A.P.; Moreira, L.A.; O'Neill, S.L. & McGraw, E.A. (2009). Wolbachia Infection Reduces Blood-Feeding Success in the Dengue Fever Mosquito, Aedes aegypti. *PLOS Neglected Tropical Diseases*. 3(9): e516

World Health Organization (2005). Global Programme to Eliminate Lymphatic Filariasis - Progress Report For 2004. Wkly. *Epidemiol. Rec.* 80: 202-212

Part 3

Other Tropical Infectious and Non-Infectious Conditions

25

Novel Molecular Diagnostic Platform for Tropical Infectious Diseases

Yasuyoshi Mori, Norihiro Tomita,
Hidetoshi Kanda and Tsugunori Notomi
Eiken Chemical Co., Ltd.
Japan

1. Introduction

Infectious disease is one of the most concerning health issues worldwide. To provide patients with effective medical treatment and prevent the spread of diseases and emergence of drug-resistant strains, quick and reliable diagnostic techniques are in high demand. However, lack of accessibility to such diagnostic systems has resulted in the deterioration of the situation in most developing countries, especially in sub-Saharan tropical countries (Rodrigues et al., 2010). Diagnostics using molecular technologies have emerged as a promising methodology because of their remarkable high sensitivity, and therefore, they have been applied as diagnostic tools for detecting various kinds of pathogens in clinical settings in developed countries. However, resources essential for molecular assays, such as bio-safety cabinets, a stable supply of electricity, and well-experienced technicians, are scarce in most of the peripheral laboratories in developing countries. In this chapter, we would like to describe a recently developed novel diagnostic platform and discuss its application for realizing molecular diagnostics for infectious diseases within resource-limited settings.

Molecular diagnostics comprise the following 3 steps: sample preparation, amplification, and detection. To develop a molecular diagnostic platform with the desired simplicity and performance, it is necessary to introduce element technologies for all the 3 steps, which are less complicated and can be used in peripheral laboratories with limited resources. Of the abovementioned 3 steps, amplification of target DNA/RNA is the most important. Therefore, the loop-mediated isothermal amplification (LAMP) method involving the calcein detection method has been applied to the platform as a key technology. LAMP, using the calcein method, enables recognition of small quantities of DNA/RNA of pathogens present in clinical specimens by means of the fluorescence emitted from the LAMP solutions after amplification.

The next important step is sample processing, for which we have developed a simple and easy-to-use technology, namely, procedure for ultra rapid extraction (PURE). The combination of both these technologies can be considered a novel platform for molecular diagnostics, which can be applied to resource-limited settings. The fundamental characteristics of these element technologies and application of the novel platform to diagnostics for evaluation of certain tropical diseases are discussed below.

2. Steps involved in molecular diagnostics

2.1 Amplification – LAMP

Since the publication of the first report regarding LAMP in 2000 (Notomi et al., 2000), LAMP has been used to detect different kinds of pathogens (Mori & Notomi, 2009), including viruses (Kubo et al., 2010), bacteria (Iwamoto et al., 2003), and protozoa (Spencer et al., 2010), and thus far, approximately 500 reports have been published regarding the application of LAMP. Because the LAMP method is simple and quick, it has been considered one of the most ideal nucleic acid amplification methods, which can be applied as an easy-to-use and cost-effective genetic test system (Parida et al., 2008).

2.1.1 Mechanism of LAMP

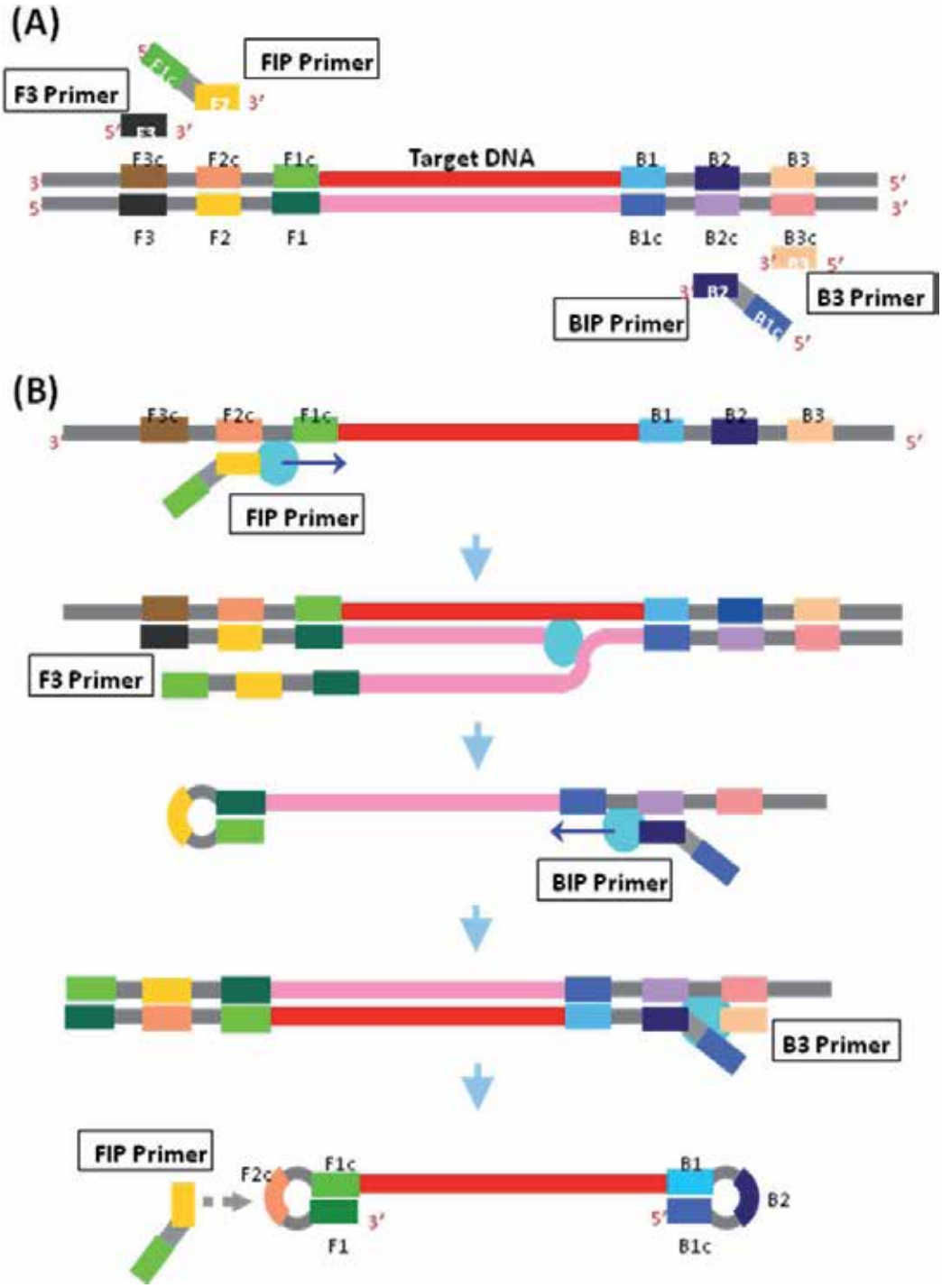
Although the reaction mechanism appears complicated, LAMP is simple to perform—it involves mixing primers (designed as depicted in figure 1-A), DNA polymerase with strand-displacement activity, and dNTPs, in a buffer containing magnesium ions, and maintaining the mixture at a constant temperature of 60–67 °C for 15–60 minutes. If template DNA molecules are present in the sample solution, large quantities of DNA with the target sequence (amplicon) are produced after incubation.

Figures 1-B and C show the schematic representation of the mechanism of LAMP. First, the forward inner primer (FIP) anneals to the template DNA at the F2c sequence and the extension reaction occurs by the enzymatic activity of *Bst* polymerase. Because *Bst* polymerase exhibits strand displacement activity, the product obtained from FIP is displaced by the other extension reaction associated with the F3 primer. Subsequently, the extension reaction occurs from the backward inner primer (BIP) on the product of the FIP, and not on the template DNA with a B2c sequence; the product obtained is also displaced by DNA synthesis associated with the B3 primer. These reactions result in a product with a dumbbell-like structure as shown in figure 1-B. The formation of the dumbbell-like product is essential for LAMP to establish isothermal amplification because the loop structures are always single stranded and can be annealed by FIP or BIP. Thus, formation of the loop structure can lead to the elimination of the denaturing step, which is otherwise essential in PCR for obtaining single-stranded DNA.

After the formation of the dumbbell-like structure, a cyclic reaction is spontaneously established between the dumbbell-like structure and its complementary product, as shown in figure 1-C. Furthermore, in the course of the cyclic reaction, elongated products with various copies of the target sequence are also produced.

The basic characteristics of the LAMP method are summarized below:

1. The whole amplification reaction occurs continuously under isothermal conditions, thus eliminating the need to use a thermal cycler, which is commonly used for PCR.
2. Because LAMP primers recognize 6 distinct regions, the specificity of LAMP is much higher than that of the other commonly used amplification techniques.
3. Amplification can be performed using an RNA template only by the addition of reverse transcriptase to the reaction (one-step RT-LAMP).
4. The LAMP reaction can be accelerated by using additional primers, called "loop primers," which are designed between F1c/B1c and F2c/B2c (Nagamine et al., 2002).


(A)
FIP Primer
F3 Primer
Target DNA
F3c
F2c
F1c
B1
B2
B3
F3
F2
F1
B1c
B2c
B3c
B3 Primer
BIP Primer
(B)
FIP Primer
F3 Primer
BIP Primer
B3 Primer
FIP Primer
F2c
F1c
F1
B1
B2
B1c

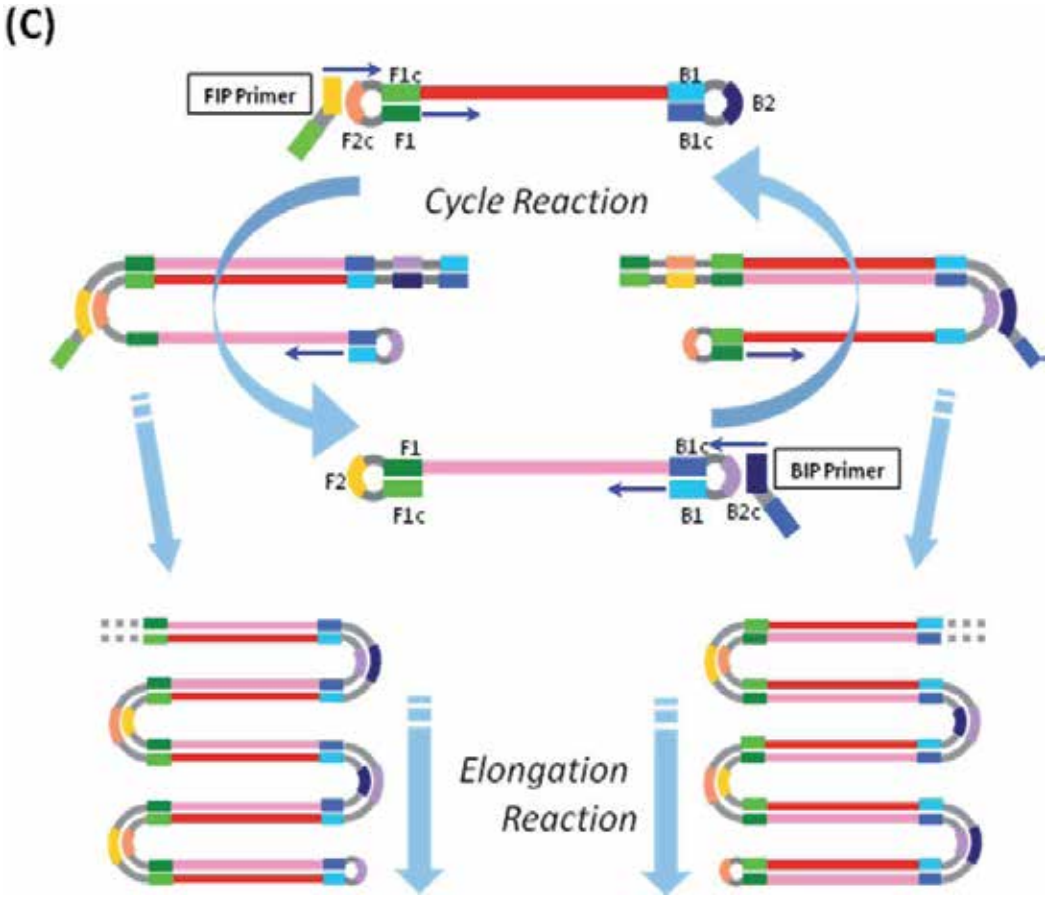


Fig. 1. Schematic representation of the mechanism of the LAMP assay
A) Design of the LAMP primers
B) Formation of a dumbbell-like structure
C) Cyclic and elongation reactions

2.1.2 Strategies that make LAMP simple and cost-effective

Conventional LAMP reagents are supplied in liquid form, and they have to be stored below -20 °C, similar to most PCR reagents. However, because of the lack of a freezer and cold chain transportation system in most of the peripheral laboratories in developing countries, it is essential to formulate LAMP reagents, which can be stably preserved at ambient temperatures (Jorgensen et al., 2006; Aziah et al., 2007). The newly formulated LAMP reagents are dried down into the lid of the reaction tubes, thus obtaining preservation stability at ambient temperatures for more than 12 months. The dried LAMP reagents can be reconstituted quite easily by shaking the tubes after the addition of the purified DNA solution. Because the LAMP reagent for each reaction is deposited on the individual tubes in advance, there is no longer a need to prepare and dispense master-mix solutions to the reaction tubes. Thus, liquid handling using micropipette, one of the most skillful steps, becomes unnecessary in the course of the assay. Moreover, this can contribute to reduced risk of carryover contamination during the assay.

2.2 Detection – Calcein method

The results of the LAMP assay can be detected visually by observing the strength of the green fluorescence emitted after the reaction. Figure 2 represents the mechanism of the calcein method (Tomita et al., 2008). Before LAMP amplification, the metalochrome indicator "calcein" is quenched by the effect of a manganese ion. After the LAMP reaction, pyrophosphate ions (PPi) are produced as a by-product of polymerase reaction; PPi subsequently forms a manganese pyrophosphate complex, causing the removal of the manganese ion from calcein, because the PPi are a stronger base than calcein. Next, free calcein combines with a magnesium ion to produce bright fluorescence. This technology enables the detection of LAMP reactions without the use of fluorescence detectors, which are usually expensive and difficult to manage in resource-limited settings. Other technologies for visual detection using LAMP have also been reported (Tao et al., 2011; Goto et al., 2009; Mori et al., 2006).

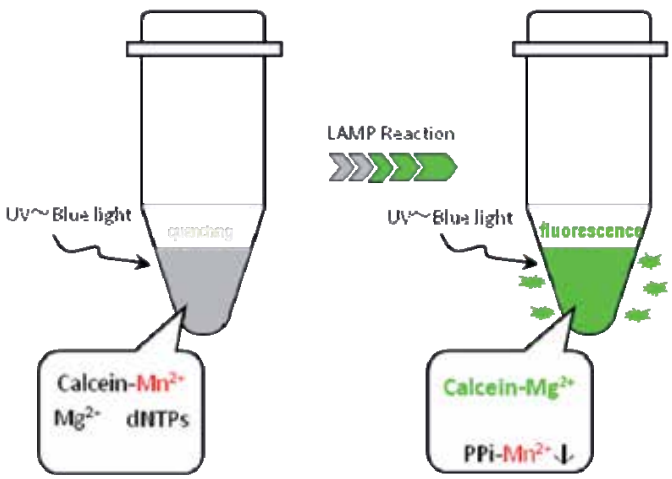


Fig. 2. Mechanism involved using calcein

2.3 Sample preparation – PURE

The sample processing method is the next important step in molecular diagnostics. Silica-based methods are well known and have been applied to a wide variety of samples, including blood and tissue (Bendall, 2002). However, these methods are unsuitable for resource-limited facilities due to the cumbersome procedures involved, including washing with organic solvents using high-speed centrifugation. Therefore, we have developed a simple and swift sample processing method named PURE. Thus far, it has been confirmed that PURE can be successfully applied to sputum, blood, serum, and swab samples. The mechanism of PURE is described below:

1. An aliquot of sample (blood, sputum, etc.) is added to the alkaline-based extraction solution and treated by heat to lyse the pathogens.
2. The sample solution is treated with adsorbent powder to remove inhibitory materials contained in samples and to neutralize the solution without any loss of target DNA.
3. After separating the solution from the powder by filtration, the obtained filtrate containing target DNA molecules can be used for reconstituting dried LAMP reagents, which are deposited to the lids of LAMP reaction tubes.

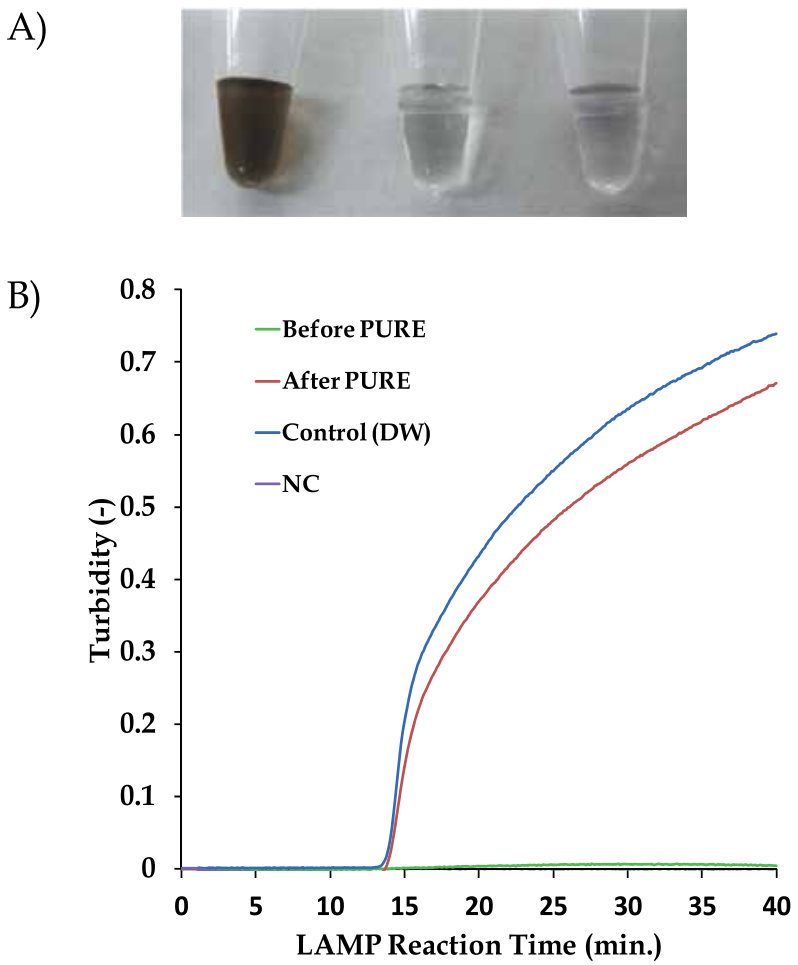


Fig. 3. Performance of the PURE–LAMP system applied for blood processing
A) Pictures of the blood sample solution (3.6% blood in an alkaline-based extraction solution) before and after PURE treatment. Left, Before PURE treatment; Centre, After PURE treatment; Right, distilled water (reference).
B) LAMP kinetics obtained by real-time turbidimetry for the 3 sample solutions with 1,000 copies of a template DNA spiked prior to PURE treatment.Green line, Before PURE treatment (directly added the solution to LAMP reaction); Red line, after PURE treatment; Blue line, control (distilled water); Purple line, negative control.

Figure 3 shows the performance of the PURE method applied for blood processing. An aliquot of blood was mixed with the extraction solution and heated at 70 °C for 5 minutes. Almost colorless solutions have been obtained by mixing the solution with the adsorbent powder (Fig. 3-A). The graph of real-time turbidimetry (Mori et al., 2004) in figure 3-B shows the LAMP kinetics for the 3 samples using 1,000 copies of a spiked template DNA. Untreated blood samples did not provide a positive reaction due to the inhibition from blood and extraction solution. However, PURE-treated blood samples showed almost the same kinetics as those of distilled water, indicating that PURE can remove inhibitory materials quite effectively from the blood samples without any loss of DNA.

Figure 4 shows the overall process of the PURE-LAMP assay system. First, an aliquot of sample is placed in a heating tube and heated at an optimized temperature (70–90 °C) to lyse the target pathogen. Then, the heating tube is attached to an adsorbent tube, and the treated solution is vigorously mixed with adsorbent powder. Next, the injection cap is inserted into the absorbent tube, which is then squeezed to elute the solution containing purified DNA. These processes can be performed in approximately 10 minutes or less for a particular sample. The LAMP reagents deposited in the lid of the tube are reconstituted by shaking the tube several times and then incubating it at around 65 °C for 30–40 minutes. Finally, the results of amplification are detected by simply observing the fluorescence using the LED lights provided in the incubator.
As mentioned above, we have successfully developed simple technologies for all the 3 steps required in molecular diagnostics, that is, the PURE method for sample preparation, the LAMP for amplification, and the calcein method for detection. Therefore, a combination of these technologies can be considered as a platform for a new molecular diagnostic tool with the desired simplicity.

3. Application of the newly developed platform for diagnosing tropical diseases

The developed platform has been applied to the following 3 tropical diseases to evaluate its performance as a practical diagnostic system.

3.1 Malaria and human African trypanosomiasis

Malaria is 1 of the 3 major infectious disease endemics in most tropical countries. More than 500 million people have been infected, and more than 1 million people die from malaria each year, mostly infants and pregnant women. Of the 4 malaria causing species, *Plasmodium falciparum* often causes severe, acute, and fatal malaria. In most developing countries, malaria is confirmed mainly by a blood smear test, although the sensitivity of the test is not sufficient to detect the parasites in patients with early-stage malaria.
Dried LAMP reagents using *P. falciparum* (Pf)-specific primers and pan genus (Pg) primers were developed in this study. The Pg LAMP primers were designed on the basis of the homogeneous sequence shared by all the 4 malaria species, thus providing the same primer specificity for all the 4 species. The Pf-specific LAMP primers were designed based on mutations between the Pf sequence and the other 3 sequences, making the primer specific only to *P. falciparum*. If both Pf and Pg LAMP assays give positive results, it can be interpreted that the patient is infected by *P. falciparum*. On the other hand, if only the Pg LAMP assay gives positive results, the patient can be diagnosed with malaria caused by 1 or more of the other 3 malarial parasites.
The sensitivity of PURE and malaria-LAMP have been evaluated by using of cultured *P. falciparum* parasites obtained from the American Type Culture Collection (ATCC). As shown in figure 5, both Pf and Pg malaria-LAMP assays can detect down to 1 parasite in 1 µl blood, processed by the PURE method. This sensitivity of 1 parasite/µl blood is much higher than that of smear microscopy test (~50 parasites/µl blood for routine tests in an endemic area (Moody, 2002)). It has been reported that the initial malarial symptoms appear after the accumulation of approximately 1,000 parasites/ml of blood (Andrews et al., 2005). Therefore, PURE-malaria-LAMP is sensitive enough to detect parasites in patients who present with the initial symptoms of malaria.
Human African trypanosomiasis (HAT) is one of the most neglected disease endemics in central African countries (Hotez, 2007). HAT is caused by an infection of protozoa

Trypanosoma brucei gambiense or *Trypanosoma brucei rhodesiense* transmitted by tse-tse flies. The symptoms of HAT occur in 2 phases: hemolymphatic phase, followed by the neurological phase. If left untreated, the hemolymphatic phase permits the parasites to invade the central nervous system of the patient, resulting in fatal neurological symptoms such as coma and eventually death. Because no vaccine or preventative drug for HAT is available, and therapeutic drugs for HAT patients in the neurological phase causes severe side effects, a simple and sensitive diagnostic method is in high demand.

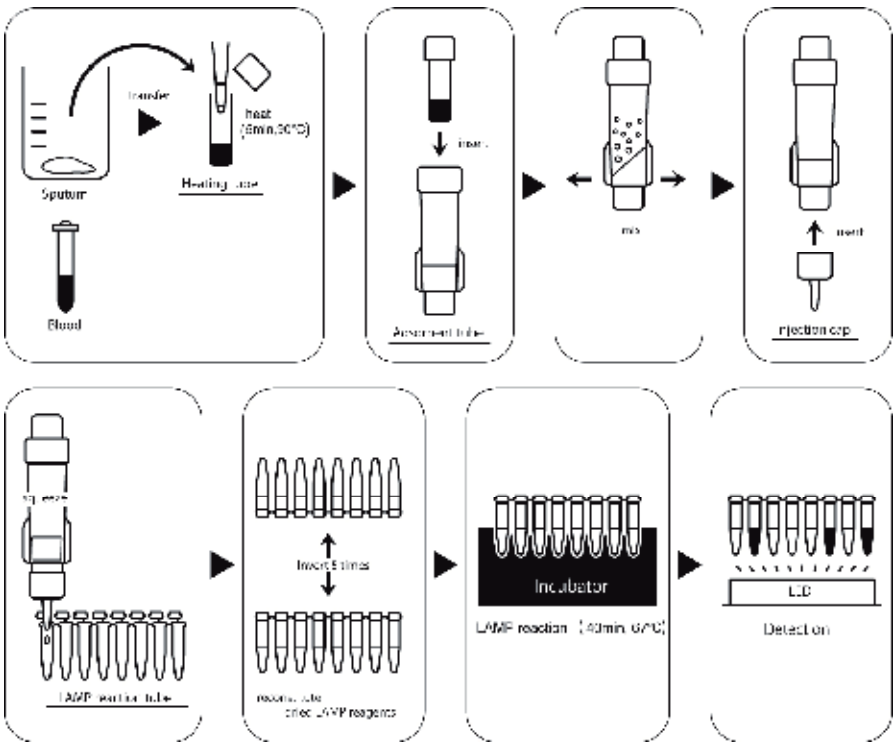


Fig. 4. Diagram of the procedures involved in the PURE–LAMP system

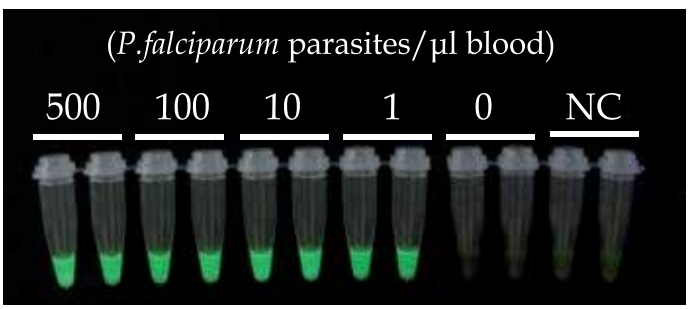


Fig. 5. Sensitivity of PURE-Malaria-LAMP
Thirty-five microliters of control blood spiked with cultured parasites (*P. falciparum*, from ATCC) was treated with PURE and tested by *P. falciparum*-specific and Pan-genus LAMP. Left and right tubes contain the same parasite numbers and are those of Pf-LAMP and Pg-LAMP, respectively.

To enhance sensitivity, the LAMP primers for HAT are designed on a multi-copy gene named RIME, which contains 80–250 copies in a parasite genome (Njiru et al., 2008). The efficacy of the PURE-HAT-LAMP assay was also evaluated by using uninfected control blood spike with cultured parasites. Positive results have been successfully obtained from samples with a parasite density of 100/ml, indicating that the overall analytical sensitivity of PURE-HAT-LAMP can be estimated to be approximately 100 parasites/ml blood. The sensitivities of the currently available smear microscopy tests have been reported to be between 100 and 10,000 parasites/ml blood (Chappuis., 2005). The sensitivity of PURE-HAT-LAMP has been found to be over 10 times higher than that of the simple Giemsa-stained smear microscopy test, which is one of the most common diagnostic tests in HAT endemic areas, and is almost comparable with that of the mini-anion-exchange centrifugation technique, which is quite tedious and time consuming.

3.2 Tuberculosis

Tuberculosis (TB) is one of the most threatening airborne diseases worldwide. One-third of the world's population is thought to be infected with *Mycobacterium tuberculosis* and new infections occur at a rate of about 1/second. Since the TB patients are concentrated in developing counties, including many tropical countries, TB is considered as a major poverty-related disease (Walker et al., 2003).

The sputum direct smear microscopy test is the only diagnostic method available for the detection of TB at peripheral laboratories in developing countries (Keeler et al., 2006). Because of the low sensitivity of the smear test, few patients with a low number of infected TB cells are often misdiagnosed as negative, thus preventing eradication of TB. In order to overcome this situation, we attempted to apply the novel platform PURE-LAMP system for the diagnosis of TB.

As summarized in Table 1, PURE-TB-LAMP provided positive results for both smear- and culture-positive sputum samples collected from patients suspected with TB. Furthermore,

Direct Smear	Culture(Ogawa)		PURE-TB-LAMP (Positive/Total)	% positive
Positive	Positive		34/34	100
Negative	Positive		15/25	60
	Colony Counts	100-200	7/7	100
		20-99	6/8	75
		1-19	2/10	20
Negative	Negative		9/91	9.9

Table 1. Summary of clinical performance of PURE-TB-LAMP
Sixty microliters of sputa obtained from patients suspected with TB in the Pham Ngoc Thach Hospital (PNTH; Vietnam) were analyzed by PURE-TB-LAMP. The performance of PURE-TB-LAMP was compared to those of the direct smear and culture method (Ogawa media) in terms of the positive ratios. Smear and culture tests for each sample were conducted according to the standardized protocols in PNTH.

the PURE-TB-LAMP assay can detect the pathogen with 60% accuracy in smear-negative but culture-positive samples, and with 75% or more accuracy if 20 colonies are detected by the culture test using Ogawa media. This data clearly shows that the PURE-TB-LAMP method is reliable enough to be applied to the targeted peripheral smear centers in developing countries as an alternative method for the direct smear test.

4. Conclusions

As mentioned above, PURE and LAMP system have been newly developed and successfully applied as a novel diagnostic platform for the detection of infectious diseases, which are wildly endemic in the developing world. This platform has the advantage of being simple enough to be applicable in resource-limited facilities and its performances is higher than those of the existing diagnostic methods routinely employed in rural laboratories of most of the developing countries. Recently, a novel idea for performing LAMP without electricity has been proposed (LaBarre et al., 2011). Combination of that technology with the platform mentioned in this chapter would make it possible to realize the use of molecular diagnostics in poorer settings or even in field conditions.

Since the geographical distribution of malaria, HAT, and TB overlap in many of tropical countries (Cook & Zumla, 2003), diagnostic tests for these diseases are often performed at the same rural laboratory in developing countries. The developed platform described in this study is a very useful tool in such laboratories because all the above mentioned diseases can be diagnosed using almost the same technique and the same simple incubator. This new technology can be beneficial as it reduces the initial costs associated with installing new equipments and preparing trained technicians for each target. This platform is potentially applicable to other pathogens, including those causing other neglected diseases such as leishmaniasis and Chagas' disease. The application of this platform could be extended to other diseases that threaten the heath and quality of life of patients in many tropical countries. This can also contribute to distribute them at more affordable rates because of the effect of mass production.

This platform can be considered as a gene point-of-care testing (g-POCT) device, which can also be used in developed countries. In fact, TB-LAMP has been approved as clinical in vitro diagnostics (IVD) in Japan and used along with PURE as a simple and fast screening test for patients suspected with TB. Since NALC-NaOH treatment for sputum is not necessary for PURE-TB-LAMP, turn-around-time of PURE-TB-LAMP is less than that of the decontaminated smear test, which is commonly adopted as the standard screening test for TB in most developed countries. Furthermore, LAMP reagents using similar concepts have been developed for the detection of the influenza virus (Nakauchi, 2011). We hope that the platform will contribute to the improvement of global health and benefit all those under the threat of infectious diseases.

5. Acknowledgments

We sincerely thank Dr. Satoshi Mitarai from the Research Institute of Tuberculosis in Japan, Dr. Noboru Inoue from Obihiro University of Agriculture and Veterinary Medicine in Japan, Dr. C.J. Sutherland and Dr. S.D. Polley from the London School of Hygiene and Tropical Medicine in the UK, and Dr. N.T. Lan from the Pham Ngoc Thach Hospital in Vietnam for their valuable help. This research was supported by the Foundation for Innovative New Diagnostics (FIND) in Switzerland.

6. References

Andrews L, Andersen RF, Webster D, Dunachie S, Walther RM, Bejon P, Hunt-Cooke A, Bergson G, Sanderson F, Hill AV, Gilbert SC. (Jul 2005). Quantitative real-time polymerase chain reaction for malaria diagnosis and its use in malaria vaccine clinical trials. *Am J Trop Med Hyg*. Vol.73, No.1, pp.191-8.

Aziah I, Ravichandran M, Ismail A. (Dec 2007). Amplification of ST50 gene using dry-reagent-based polymerase chain reaction for the detection of Salmonella typhi. *Diagn. Microbiol. Infect. Dis*. Vol.59, No.4, pp. 373-7.

Bendall K.E., Setzke E., Quandt A. (2002). Purification of Viral Nuculeic Acid, In: Nuculeic acids isolation methods, Bowien B. & Dürre P. (Eds.) pp.53-59, American Scientific Publishers, ISBN 1-58883-018-7 2002

Chappuis F, Loutan L, Simarro P, Lejon V, Büscher P. (Jan 2005). Options for field diagnosis of human african trypanosomiasis, *Clin. Microbiol. Rev.* Vol.18, No.1, pp. 133-46.

Cook G.C. & Zumla A. (Eds.) Manson's Tropical Diseases Twenty-first edition Elsevier Limited, ISBN 0-7020-2640-9

Goto M, Honda E, Ogura A, Nomoto A, Hanaki K. (Mar 2009). Colorimetric detection of loop-mediated isothermal amplification reaction by using hydroxy naphthol blue. *Biotechniques*. Vol.46, No.3, pp. 167-72.

Hotez PJ, Molyneux DH, Fenwick A, Kumaresan J, Sachs SE, Sachs JD, Savioli L. (Sep 2007)Control of neglected tropical diseases. *N.Engl. J. Med*. Vol.357, No.10, pp. 1018-27.

Iwamoto T, Sonobe T, Hayashi K. (Jun 2003). Loop-mediated isothermal amplification for direct detection of Mycobacterium tuberculosis complex, M. avium, and M. intracellulare in sputum samples., *J.Clin. Microbiol*. Vol. 41, No.6, pp. 2616-22

Jorgensen P, Chanthap L, Rebueno A, Tsuyuoka R, Bell D. (May 2006). Malaria rapid diagnostic tests in tropical climates: the need for a cool chain. *Am J Trop Med Hyg*. Vol.74, No.5, pp. 750-4.

Keeler E, Perkins MD, Small P, Hanson C, Reed S, Cunningham J, Aledort JE, Hillborne L, Rafael ME, Girosi F, Dye C. (Nov 2006). Reducing the global burden of tuberculosis: the contribution of improved diagnostics. *Nature*. 23;444 Suppl 1 pp. 49-57.

Kubo T, Agoh M, Mai le Q, Fukushima K, Nishimura H, Yamaguchi A, Hirano M, Yoshikawa A, Hasebe F, Kohno S, Morita K. (2010) Development of a reverse transcription-loop-mediated isothermal amplification assay for detection of pandemic (H1N1) 2009 virus as a novel molecular method for diagnosis of pandemic influenza in resource-limited settings. ., *J Clin Microbiol*. Vol.48, No. 3, pp. 728-35.

LaBarre P., Hawkins K.R., Gerlach J., Wilmoth J., Beddoe A., Singleton J., Boyle D., and Weigl B. (2011). A Simple, Inexpensive Device for Nucleic Acid Amplification without Electricity—Toward Instrument-Free Molecular Diagnostics in Low-Resource Settings, *PLoS One*. Vol. 6, No.5, e19738.

Moody A., (Jan 2002). Rapid Diagnostic Tests for Malaria Parasites, *Clinical Microbiology Reviews*. Vol.15, No.1, pp. 66-78

Mori Y, Hirano T, Notomi T. (Jan 2006). Sequence specific visual detection of LAMP reactions by addition of cationic polymers. *BMC Biotechnol*. Vol.10 6:3.

Mori Y., Kitao M., Tomita N., Notomi T., (2004). Real-time turbidimetry of LAMP reaction for quantifying template DNA, *J Biochem Biophys Methods*., Vol.59, No.2, pp. 145-57.

Mori Y. & Notomi T. (2009). Loop-mediated isothermal amplification (LAMP): a rapid. Accurate, and cost-effective diagnositic method for infectious diseases, *J.Infect Chemother*., Vol.15, No.2, pp.62-69.

Nagamine K., Hase T, Notomi T. (Jun 2002).Accelerated reaction by loop-mediated isothermal amplification using loop primers. Mol Cell Probes. Vol.16, No.3, pp. 223-9.

Nakauchi M, Yoshikawa T, Nakai H, Sugata K, Yoshikawa A, Asano Y, Ihira M, Tashiro M, Kageyama T. (Jan 2011). Evaluation of reverse transcription loop-mediated isothermal amplification assays for rapid diagnosis of pandemic influenza A/H1N1 2009 virus. *J Med Virol*. Vol.83, No.1, pp. 10-5.

Notomi T Okayama H., Masubuchi H., Yonekawa T., Watanabe K., Amino N. & Hase T. (2000). Loop-mediated isothermal amplification of DNA, Nucleic Acids Res. Vol.28, No. 12, e63.

Njiru ZK, Mikosza AS, Matovu E, Enyaru JC, Ouma JO, Kibona SN, Thompson RC, Ndung'u JM. (Apr 2008). African trypanosomiasis: sensitive and rapid detection of the sub-genus Trypanozoon by loop-mediated isothermal amplification (LAMP) of parasite DNA. *Int J Parasitol*. Vol.38, No.5, pp. 589-99.

Parida M, Sannarangaiah S, Dash PK, Rao PV, Morita K. (Nov-Dec 2008). Loop mediated isothermal amplification (LAMP): a new generation of innovative gene amplification technique; perspectives in clinical diagnosis of infectious diseases. *Rev Med Virol*. Vol.18, No.6, pp. 407-21.

Polley SD, Mori Y, Watson J, Perkins MD, González IJ, Notomi T, Chiodini PL, Sutherland CJ. (Aug 2010). Mitochondrial DNA targets increase sensitivity of malaria detection using loop-mediated isothermal amplification. *J Clin Microbiol*. Vol.48, No.8., pp. 2866-7.

Rodrigues Ribeiro Teles FS, Pires de Távora Tavira LA, Pina da Fonseca LJ. (May-Jun 2010) Biosensors as rapid diagnostic tests for tropical diseases. *Crit Rev Clin Lab Sci*. Vol.47, No.3, pp. 139-69.

Tao ZY, Zhou HY, Xia H, Xu S, Zhu HW, Culleton RL, Han ET, Lu F, Fang Q, Gu YP, Liu YB, Zhu GD, Wang WM, Li JL, Cao J, Gao Q. (Jun 2011) Adaptation of a visualized loop-mediated isothermal amplification technique for field detection of Plasmodium vivax infection. *Parasit Vectors*. Vol.4, No.1, pp. 115.

Tomita N, Mori Y, Kanda H, Notomi T. (2008). Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. *Nat Protoc*. Vol.3, No.5, pp. 877-82.

Walker D, Stevens W. (2003). The economics of TB control in developing countries. *Expert Opin Pharmacother*. Vol.4, No.3, pp. 359-68.

26

Sexually Transmitted Infections in the Tropics

John C. Meade and Denise C. Cornelius
University of Mississippi Medical Center, Department of Microbiology, Jackson, MS USA

1. Introduction

The burden of sexually transmitted infections (STIs) on the health and well-being of the population in the developing world is considerable. The World Health Organization (WHO) estimates that there are 340 million new cases of curable STIs in the world each year; 174 million new cases of trichomoniasis, 92 million new cases of *Chlamydia* infection, 62 million cases of gonorrhea, and 12 million new cases of syphilis (Table 1). Approximately three quarters of these infections are in countries encompassing tropical regions of the world in Latin America, sub-Saharan Africa, and South and Southeast Asia. The prevalence of viral STIs is even higher; infection with Herpes simplex virus-2 (HSV-2) is the most common STI worldwide and as many as 50% of sexually active individuals will be infected with human papillomavirus (HPV) during their life. The prevalence of STIs is considerably greater than many classical tropical diseases and it is unfortunate that they do not receive more attention and resources from international programs and donor groups. The public health impact of these diseases extends well beyond the immediate effects and morbidities of infection. STIs have been implicated in facilitating acquisition and transmission of HIV, in pregnancy complications such as pre-term births, low birth weight infants, stillbirth, neonatal death and blindness, in the inducement of cervical and prostate cancers, and in increased risk of pelvic inflammatory disease and infertility. Failure to diagnose and treat STIs at an early stage thus increases the already substantial burden these conditions impose on the populations of developing countries. Although effective diagnostic tests and treatments are available for these STIs, they are often unavailable or inaccessible in resource-limited tropical settings. As a consequence, syndromic management of STIs remains the option of choice for individual case management. The inadequate public health response coupled to ongoing socioeconomic and demographic trends have led to an epidemic of STIs in many countries in the developing world. The development of antimicrobial resistance is an on-going problem and new agents are often much more expensive, increasing the burden of control. The economic costs of these diseases and their infection sequelae place a considerable burden on national health budgets and household income. In developing countries, STIs are among the top five reasons for which adults seek medical care. Due to the prevalence and public health implications of STIs in the tropics a discussion of STIs should be included in any compilation of tropical medicine.

This chapter will cover sexually transmitted infections caused by *Trichomonas vaginalis* (trichomoniasis), *Chlamydia trachomatis* (chlamydia and lymphogranuloma venereum), *Neisseria gonorrhoeae* (gonorrhea), *Treponema pallidum* (syphilis), *Haemophilus ducreyi*

(chancroid), *Calymmatobacterium granulomatis* (granuloma inguinale or donovanosis), and the viral STIs, herpes simplex virus and human papilloma virus. Each of these STIs spreads via vaginal, anal, and oral sex, as well as by inoculation of material from infected sores in some cases. All share many common risk factors and higher rates of infection are seen in marginalized populations; persons of low socioeconomic status, commercial sex workers, alcohol abusers, illicit drug users, men who have sex with men, prison populations, uncircumcised men, and those with multiple sex partners or who have partners with multiple sex partners. This chapter will cover both syndromic management of STIs as practiced in most areas of the tropical world (WHO 2003, 2005, 2007b) as well as individual descriptions of these diseases, their clinical presentation, diagnosis, and treatment. The treatment regimens presented are those recommended by the Centers for Disease Control and Prevention (CDC, 2010) and the World Health Organization (WHO, 2005).

	Latin America & the Caribbean	Sub-Saharan Africa	North Africa & Middle East	Southeast & South Asia	World Total
Trichomoniasis	18.5	32	5	76.5	174
Chlamydia	9.5	16	3	43	92
Gonorrhea	7.5	17	1.5	27	62
Syphilis	3	4	0.37	4	12
Total STIs	38.5	69	10	151	340
HIV/AIDS	1.4	22.5	0.46	4.1	33.3

Table 1. World Health Organization estimates of new cases of curable sexually transmitted infections and HIV, in millions (WHO, 2001; UNAIDS, 2010).

2. STIs and HIV

It is estimated that there are 34 million cases of HIV in the world with 68% or 22.5 million cases occurring in sub-Saharan Africa, where the prevalence is highest, and 4.1 million in South and Southeast Asia. The emergence of the HIV epidemic has complicated the control of STIs as HIV induced immunosuppression leads many patients to respond poorly to STI treatment regimens, requiring higher drug dosages and longer treatment schedules to affect cure. Sexually transmitted infections (STIs) also facilitate the transmission of other STIs, including human immunodeficiency virus (HIV). Several observational studies have been conducted that conclude there to be a strong association between STI and increased risk of HIV acquisition. An individual with a co-existing STI has a 2-5 fold greater risk of acquiring and transmitting HIV. Increased risk of HIV transmission has mostly been attributed to ulcerative STIs, mainly HSV-1 and 2, but also syphilis, chancroid, lymphogranuloma venereum, and granuloma inguinale. For instance the population attributable risk percent of HIV acquisition for HSV-1 and 2 varies from 15-30% in Africa. However, studies also show that non-ulcerative STIs, gonorrhea, *Chlamydia*, and trichomoniasis increase the risk of HIV transmission and acquisition as well. Studies that have examined non-ulcerative STIs and risk of HIV seroconversion have found an odds ratio of 1.8-4.8 for gonorrhea, 1.8-3.6 for chlamydia, and 1.9 for trichomoniasis. Therefore, early diagnosis and treatment of treatable

STIs could significantly impact the incidence of HIV transmission and acquisition. The number of individuals co-infected with HIV and an STI are high. Studies show increases in treatment failure of treatable STIs in HIV-positive patients. Studies in several countries show high treatment failure of syphilis in HIV-positive patients and in a trichomoniasis study, 18% of HIV-positive women were *T. vaginalis* positive 1 month after treatment. It is therefore possible that more aggressive treatment of non-ulcerative STIs may be necessary to cure an infection in HIV-positive individuals. STI treatment has been shown to significantly reduce HIV-shedding in both men and women. Follow-up for test of cure is also necessary due to the higher risk of treatment failure due to co-infection.

3. Syndromic management

The diagnosis and management of STIs in the tropics has a dual nature. Sophisticated testing equipment and facilities comparable to those available in developed nations can often be found in large urban centers and popular resort destinations in developing countries. However, in many parts of the developing world, the absence of etiologic diagnostic capacity due to constraints imposed by cost, lack of equipment or trained personnel, and time management has forced health care providers to rely on a syndrome-based approach to STI management. This approach employs clinical algorithms based on an STI syndrome to determine antimicrobial therapy. The following sections discuss management of the most common clinical syndromes encountered in STIs. Sexual partners of the index patient should also be examined for STIs and promptly treated for the same condition as treatment failures are common when partners are not treated. Often, treatment regimens to cover multiple infectious agents are recommended due to the difficulty in distinguishing between the overlapping clinical presentations of different STIs, the high prevalence of mixed infections in many areas, and to ensure adequate therapy in the case of loss to follow-up. Syndromic management enables many STIs to be treated and resolved at local clinics which may lack all but the most rudimentary laboratory capabilities. However, patients that do not respond to therapy or those that show systemic signs indicative of other disease conditions warrant referral to a clinic with more comprehensive facilities.

3.1 Urethral discharge in men

Neisseria gonorrhoeae and *Chlamydia trachomatis* are the major STI pathogens causing urethral discharge. In the syndromic management scheme, treatment of men with urethral discharge should cover both of these organisms. Treatment regimens may be found in the specific sections describing these STIs. Single-dose therapies are preferred. Whenever possible microscopic examination of the urethral smear should be performed; the appearance of more than 5 polymorphonuclear leukocytes per high power field (x1000) is indicative of urethritis. A Gram stain could also demonstrate the presence of gonococci and permit specific treatment. Patients should return in 7 days if symptoms persist. Treatment failure may be due to drug resistance necessitating use of one of the alternative drugs for these STI agents. Patients indicating poor compliance with therapy or the possibility of re-infection can be re-treated with the same drug regimen. *Trichomonas vaginalis* can also be a cause of urethritis in men. In areas of high local *T. vaginalis* prevalence, treatment for this organism should also be given at this time. If symptoms still persist, the patient should be referred to a facility possessing the resources for a more extensive workup.

3.2 Genital ulcers

Five STIs typically produce genital ulcers; herpes, syphilis, chancroid, lymphogranuloma venereum (LGV), and granuloma inguinale or donovanosis (Table 2). Physical examination should focus on the characteristics of the lesion(s): single or multiple, painless or painful, indurated or soft, irregular or regular borders, and how they began, as a papule or a vesicle. The examination should also determine the time since exposure, the presence or absence of lymphadenopathy, and the presence of systemic symptoms which may indicate another etiology. Syphilis ulcers are painless, indurated, sharply demarcated with a red, smooth base. When present, inguinal adenopathy is firm, rubbery, nontender and usually bilateral. Herpes ulcers begin as multiple grouped vesicles on a red base which forms shallow ulcers that may coalesce. Herpes inguinal adenopathy is bilateral, firm, and tender when present. Chancroid ulcers are shallow and often multiple with irregular shape, sharply demarcated borders, and undermined edges. Chancroid inguinal adenopathy is typically unilateral, fixed, and tender, with overlying erythema and may supperate. Granuloma inguinale ulcers are shallow sharply demarcated lesions with a beefy red friable base and usually without inguinal adenopathy. LGV ulcers are usually a single lesion, transient, and frequently not noticed. Inguinal adenopathy in LGV is usually unilateral, firm, tender, fixed, and may supperate or form fistulas. When genital ulcers present as vesicles only, syndromic management recommends treatment for both herpes infection and for syphilis if the patient has a positive RPR syphilis test, or has not received recent syphilis treatment. Patients with ulcers and no vesicles should be treated for syphilis plus either chancroid, granuloma inguinale, or lymphogranuloma venereum dependent on clinical presentation and local prevalence of these agents. In areas where herpes prevalence exceeds 30%, patients with ulcers should also be treated for herpes. Patients whose ulcers do not respond to both initial treatment and follow-up therapy should be referred for more extensive diagnostic testing.

Disease	Lesions	Lymphadenopathy	Systemic symptoms
Herpes	Small, painful, pruritic vesicles lesions shallow, usually multiple, grouped, and may coalesce	Tender, firm, bilateral nonsupperative inguinal adenopathy	Yes, primary infection
Primary Syphilis	Painless, indurated, round red smooth base, usually single, sharply demarcated	Nontender, firm, rubbery, nonsupperative, bilateral	None in primary stage Yes in secondary/tertiary
Chancroid	Tender, erythematous papules ulcers painful, purulent, irregular shape, soft undermined edges, often multiple	Tender, regional, painful, erythematous, supperative nodes, usually unilateral	None
LGV	Small, painless vesicle/papule usually single, heals rapidly often not noticed	Painful, matted, firm, large nodes supperate with fistula tracts, usually unilateral	After genital lesions heal spread to regional lymph nodes
Donovanosis	Small, painless pustules, ulcers shallow, erythematous, sharply demarcated, may expand, deepen, become necrotic, can be dry or nodular	Inguinal adenopathy usually absent	Yes, but rarely Extragenital lesions via inoculation from genital sores may occur

Table 2. Characteristics of Genital Ulcers

3.3 Inguinal bubo

Inguinal buboes are frequently associated with LGV and chancroid. If genital ulcers accompany the buboes, patients should be managed using the genital ulcer syndromic management approach. Inguinal buboes not accompanied by genital ulcer presentation should be treated with a regimen effective against LGV and chancroid. The recommended syndromic treatment is ciprofloxacin, 500 mg orally twice daily for 3 days plus doxycycline, 100 mg orally twice daily for 14 days, or erythromycin, 500 mg orally four times daily for 14 days. Some cases may require longer treatment than 14 days if the buboes are not resolved. Fluctuant lymph nodes can be aspirated through healthy skin. Incision and drainage or excision of nodes may delay healing.

3.4 Scrotal swelling

There are multiple infectious causes for epididymitis as well as non-infectious causes such as trauma, testicular torsion, and tumor. Patients with testis that are rotated or elevated or with a history of trauma should be referred for surgical option. An STI is more likely the cause for men under 35 years of age than for older men. An epididymitis which is accompanied by urethral discharge should be treated with drugs for both gonococcal and chlamydial infection.

3.5 Vaginal discharge

An abnormal vaginal discharge in terms of quantity, color, or odor most commonly results from vaginal infection. *Trichomonas vaginalis* is the most common STI cause of vaginal infection, though bacterial vaginosis (BV) and yeast infections also produce vaginal discharge. All women presenting with vaginal discharge should be treated for trichomoniasis and BV, in the absence of specific diagnosis, with metronidazole, 400-500 mg orally twice daily for 7 days. Metronidazole is not recommended in the first trimester of pregnancy. Pregnant women should be treated with metronidazole, 200-250 mg orally 3 times daily for 7 days. In rare cases, vaginal discharge may result from a mucopurulent cervicitis due to infection with *N. gonorrhoeae* or *C. trachomatis*. Treatment for cervical infection in women presenting with vaginal discharge is dependent on the local prevalence of these STIs. Women in high risk areas for *N. gonorrhoeae* or *C. trachomatis* with vaginal discharge and evidence of cervicitis should be offered treatment for these STIs in addition to treatment for BV and trichomoniasis.

3.6 Lower abdominal pain

There are multiple causes of lower abdominal pain in sexually active women in addition to pelvic inflammatory disease (PID) caused by STIs. Women presenting with lower abdominal pain and a missed or overdue period, pregnant, recent delivery, abortion, or miscarriage, abdominal guarding and/or tenderness, abnormal vaginal bleeding, or abdominal mass, should be referred for surgical or gynecological assessment. In the absence of these signs women with lower abdominal pain accompanied by cervical excitation tenderness or lower abdominal tenderness and vaginal discharge should be managed for PID. The etiologic agents for PID include *N. gonorrhoeae*, *C. trachomatis*, *T. vaginalis*, anaerobic and facultative bacteria, and perhaps *Mycoplasma*. When diagnostic capacity to distinguish these agents is absent the treatment regimen must be effective against all these

pathogens. The recommended syndromic treatment is a single dose therapy for gonorrhea, plus doxycyline, 100 mg orally twice daily, or tetracycline, 500 mg orally 4 times daily for 14 days, plus metronidazole, 400-500 mg orally twice daily for 14 days. Patients who do not respond to therapy within three days should be referred for a more complete diagnostic evaluation.

3.7 Neonatal conjunctivitis

Infants with neonatal conjunctivitis (ophthalmia neonatorum) present with eyes that are red, swollen and accompanied by discharge ("sticky eyes"). *Chlamydia trachomatis* and *N. gonorrhoeae* are the most significant pathogens which cause ophthalmia neonatorum in developing countries although infections from *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Haemophilus* spp., and *Pseudomonas* spp. occur. The oropharynx, urogenital tract, and rectum of neonates may also be affected in *Chlamydia trachomatis* or *N. gonorrhoeae* infection. *Neisseria* conjunctivitis develops within a few days of birth whereas *C. trachomatis* conjunctivitis develops slower, 5-14 days after birth. Neonatal conjunctivitis caused by *N. gonorrhoeae* can lead to blindness when untreated. Coverage should be provided for both of these STIs in settings where definitive diagnosis is not possible, especially where there is evidence of a maternal STI. Treatment should include a single dose therapy for gonorrhea and multiple dose therapy for chlamydia. For gonorrhea a single intramuscular injection of ceftriaxone, 50 mg/kg to a maximum of 125 mg total, should be administered. If ceftriaxone is unavailable, single injections of kanamycin or spectinomycin at 25 mg/kg to a maximum dose of 75 mg total may be used. For chlamydia treatment, erythromycin syrup, 50 mg/kg per day orally, in 4 divided doses for 14 days or trimethoprim, 40 mg with sulfamethoxazole, 200 mg orally twice daily for 14 days are recommended. Gonococcal ophthalmia neonatorum is preventable if a 1% silver nitrate solution or 1% tetracycline ointment is applied at birth as a prophylactic measure.

4. Trichomoniasis

Trichomonas vaginalis infects the urogenital tract of men and women, causing trichomoniasis. *Trichomonas vaginalis* is a member of the Phylum Zoomastigina, Class Parabasalia, Order Trichomonadida, and Family Trichomonadidae. *Trichomonas vaginalis* has only one life stage, trophozoites, which display various shapes including pyriform, ameboid, ellipsoidal, ovoidal, and spherical. The organisms measure between 10 – 30 microns. The organism possesses four anterior flagella and a fifth flagellum located posteriorly along the undulating membrane. These flagella of the organism yield the characteristic quivering motion of *T. vaginalis*.

4.1 Epidemiology

Trichomoniasis is the most common, curable, non-viral sexually transmitted infection worldwide. An estimated 174 million new cases of trichomoniasis occur worldwide each year. The incidence of *Trichomonas vaginalis* infection varies in different countries throughout the world. Incidence in Asian countries varies from 0.7% in rural China to 15.1% in sex workers in Indonesia. In South America, studies report incidence ranging from 4-9%. In Brazil prevalence is thought to have a range of 20 up to 40%. Incidence in African countries ranges from 2-20%. The incidence of trichomoniasis is higher in sexually active women over 25 years of age than in younger women.

4.2 Clinical manifestations

The actual number of new or existing cases of trichomoniasis is not known with complete surety because trichomoniasis is not a reportable disease and because of the significant number of asymptomatic cases. Trichomoniasis is usually asymptomatic in men, although sometimes it can cause non-gonococcal, non-chlamydial urethritis, epididymitis, and prostatitis. Clinical trichomoniasis in women ranges from asymptomatic carriers to flagrant vaginitis. Women have symptomatic disease more often than men. One third of asymptomatic woman will become symptomatic within 6 months of the onset of infection. Symptoms can include a vaginal discharge, vulvovaginal irritation and itching, painful urination or intercourse, foul odor, and lower abdominal pain. The presence or absence and severity of these symptoms determine whether the infection is classified as acute, chronic, or asymptomatic.

4.3 Health sequelae

Trichomoniasis is associated with a higher risk for other infectious diseases and adverse pregnancy outcomes such as preterm birth, premature rupture of placental membranes, and low birth weight infants. One study has also found an association between *T. vaginalis infection* in pregnancy and mental retardation in children. *Trichomonas vaginalis* infection is associated with pelvic inflammatory disease, especially PID leading to sterility. Trichomoniasis is significantly associated with HSV infection. *Trichomonas vaginalis* infection also increases the risk of human immunodeficiency virus (HIV) acquisition and the Centers for Disease Control and Prevention (CDC) estimates that as much as 20% of HIV transmission in the African American population in the United States may be attributable to *T. vaginalis* infection. *Trichomonas vaginalis* infection also increases the risk of cervical neoplasia and prostate cancer. Exposure to *T. vaginalis* results in a 2-fold increase in the risk of diagnosis of extraprostatic prostate cancer and a 3-fold increase in the risk of cancer that led to cancer-specific death. Thus, although trichomoniasis itself is a curable disease, *T. vaginalis* infection may indirectly be a life threatening disease.

4.4 Diagnosis

Because of the high prevalence of trichomoniasis, any woman seeking medical care for vaginal discharge should be tested for *T. vaginalis* infection. Trichomoniasis is traditionally diagnosed microscopically (wet mount) by observing mobile protozoa in vaginal secretions, cervical samples, or from urethral or prostatic swabs. However, this method has a relatively low sensitivity and requires immediate evaluation of a wet preparation slide for optimal results. The low sensitivity of this diagnostic method leads to under-diagnosis. The current gold standard for diagnosis of trichomoniasis is culture in Diamonds media and is widely used. Rapid antigen based point-of-care tests and nucleic acid based diagnostic tools are also available. Both of these techniques have high sensitivity and specificity. Papanicolaou (Pap) smear allows for direct visualization in saline prep and can be performed within 10-20 minutes of sample collection but is not widely used. The Whiff test can be performed by mixing vaginal secretions with 10% potassium hydroxide (KOH) to yield a strong fishy odor. This test has a poor specificity due to the fact that bacterial vaginosis can yield a similar result. All of the above mentioned diagnostic methods are applicable for diagnosis in women. In men, culture testing of urethral swabs, urine, or semen and the nucleic acid amplification tests are more sensitive diagnostic tools.

4.5 Treatment

Metronidazole, 2 g orally in a single dose or 500 mg orally twice a day for 7 days, is the treatment of choice for trichomoniasis. An estimated 2.5-10% of *T. vaginalis* infections show some degree of resistance to treatment; a resistance rate of 17.4% has been reported in Papua New Guinea. Treatment failures are higher in HIV-positive individuals. Recalcitrant cases may be treated with tinidazole at 2 g orally in a single dose. Consumption of alcohol should be avoided during treatment and for 24 hrs after completion of metronidazole therapy or 72 hours after completion of tinidazole therapy.

5. Chlamydia

Chlamydia trachomatis is a small, obligate intracellular bacterium that typically infects non-ciliated epithelial cells of mucous membranes; urethral epithelial cells in males and columnar epithelial cells of the endocervix in women. However in the lymphogranuloma venereum serovars, macrophages appear to be the principal host cell. *Chlamydia* is organized into multiple serovars that cause a diverse variety of human disease. Serotypes A, B, Ba, and C are the agents of classic blinding trachoma. Serotypes D thru K can cause adult inclusion and neonatal conjunctivitis, pneumonia, urogenital infections and Reiter's syndrome. Serotypes L1, L2, and L3 infect tissues deeper to the epithelium and cause lymphogranuloma venereum (LGV).

5.1 Epidemiology

Chlamydia infection is the most common bacterial STI in the world and among STIs, only the prevalences of herpes and trichomoniasis are higher. *Chlamydia* infection is highest in sexually active young adults under 25 years of age. *Chlamydia trachomatis* causes 30-50% of nongonoccal urethritis in men and mucopurulent cervicitis in women. In men less than 35 years of age *Chlamydia* is the principal cause of epididymitis. Although there is no lasting immunity and re-infection is common, women do clear the infection faster with increasing age. Lymphogranuloma venereum (LGV) is an uncommon disease and relatively rare in developed countries. The disease is most common in sub-Saharan Africa and is also reported in areas of the Caribbean, Central America, and Southeast Asia and sporadically in developed nations.

5.2 Clinical manifestations

Symptoms of chlamydial infection typically appear 1-3 weeks post exposure. Asymptomatic infection is common among both men, approximately ~50%, and women, approximately 70-80%. When it occurs, symptomatic infection clears spontaneously about 50% of the time. However, both untreated and asymptomatic infections can persist for years; as many as 10% remain infected after 3 years. Symptomatic men might have a urethral discharge and dysuria with burning and itching around the urethral opening. Epididymitis and prostatitis are sometimes present causing pain and swelling of the testes, fever, and rarely sterility. *Chlamydial* infection of the rectum can cause pruritis, rectal pain, discharge, or bleeding. Chlamydia infected women may experience cervicitis, vaginal discharge and dysuria. Infection and inflammation in the cervix may spread to the fallopian tubes and uterus, leading to pelvic inflammatory disease (PID). *Chlamydia* is among the most frequent pathogens associated with PID and up to 40% of women with untreated chlamydia develop PID. Some women with PID report lower abdominal pain, lower back pain, nausea, fever,

abnormal bleeding, and dysparenia but many women show no signs of infection. Untreated PID can result in chronic pelvic pain, tubal infertility in 10-20% of women, and occasionally potentially fatal ectopic pregnancy. Repeated infections increase the risk of adverse sequelae in both men and women. In rare cases persons with genital chlamydial infection can develop Reiter's syndrome, a triad of reactive arthritis accompanied by conjunctivitis and urethritis. Chlamydial infection, even asymptomatic disease, increases the risk of adverse pregnancy outcomes: premature rupture of membranes, preterm delivery and low birth weight. *Chlamydia* can easily pass to neonates during childbirth causing neonatal conjunctivitis and afebrile pneumonia in approximately 60% of those with infected mothers. The high levels of *Chlamydia* infection worldwide mean that there is substantial neonatal morbidity from perinatally transmitted chlamydial infection.

The *Chlamydia* serotypes which cause LGV are more virulent and more invasive than other chlamydial serotypes. The initial stage is a painless genital papule which heals rapidly and may be unrecognized. The organism then disseminates to regional lymph nodes, usually the inguinal nodes, where they replicate within macrophages and elicit a systemic response. This produces a painful inguinal lymphadenopathy, usually unilateral, by 2-6 weeks after the primary lesion often accompanied by fever, headache, and arthralgias. Rectal infection with LGV is characterized by a severe febrile proctocolitis, mimicking inflammatory bowel disease, with painful defecation, tenesmus, and less commonly a bloody mucopurulent discharge. Untreated LGV results in chronic inflammation with late fibrotic complications such as fistulas of the penis, urethra, and rectum, strictures, and genital lymphoedema and elephantiasis.

5.3 Diagnosis

Empirical evidence of *Chlamydia* infection is based on clinical presentation. The presence of greater than 10 polymorphonuclear leukocytes (PMNs) per 1000X field in vaginal discharge or 5 PMNs/field in urethral discharge is indicative of the cervicitis or urethritis characteristic of *Chlamydia* infection. There are currently no widely available point-of-care tests for *Chlamydia* infections. Most *Chlamydia* infections are detected through screening programs based on nucleic acid amplification testing (NAAT), antigen detection by ELISA, and DNA hybridization. Screening is useful for identifying asymptomatic infected individuals and in confirming symptomatic infections, but the delay in obtaining results means that initial diagnosis will be primarily based on clinical presentation. Traditional diagnostic techniques used for bacterial infections, culture and Gram stain, are of limited value for chlamydial infections. *Chlamydia* is an intracellular pathogen that requires tissue culture to propagate and so this approach is infrequently used even in developed countries. The unique cell wall structure of Chlamydia makes it very difficult to stain, although it is considered Gram negative. Direct fluorescent antibody staining can identify *Chlamydia* in clinical specimens but is not widely available. Where testing is available, all sexually active young adults under 25 years should be screened for *Chlamydia*. All pregnant women should be screened for *Chlamydia* as well.

5.4 Treatment

The recommended regimen for treatment of *Chlamydia* infection is azithromycin, 1 g orally in a single dose, or doxycycline, 100 mg orally twice daily for 7 days. Alternative 7 day regimens are 500 mg erythromycin base orally four times a day, 500 mg levofloxacin orally once daily, or 300 mg ofloxacin orally twice daily. The frequency of *Chlamydia* and

gonococcal co-infection is high in many locales and dual treatment should be considered. The recommended treatment for LGV is doxycycline 100 mg orally twice a day for 21 days or alternatively, erythromycin base, 500 mg orally four times a day for 21 days. Azithromycin, 1 g orally once weekly for 3 weeks, may also be effective but clinical data is lacking. LGV buboes may require aspiration.

6. Gonorrhea

Neisseria gonorrheoae is an intracellular Gram-negative aerobic diplococcus that is the causative agent of gonorrhea. The adjacent sides of the diplococci pairs are flattened giving a characteristic kidney bean shape. Gonococci initially penetrate mucosal columnar epithelial cells and pass thru to establish infection in the subepithelial space. Cell destruction mediated by gonococci and the host inflammatory response is responsible for the disease pathology. Gonococci frequently change their surface antigens and lasting immunity does not develop. Therefore, re-infection is common.

6.1 Epidemiology

Gonorrhea is the second most common bacterial STI in the world with 62 million cases annually and is most prevalent in south and Southeast Asia with 27 million cases annually, and sub-Saharan Africa with 17 million cases annually. Gonococcal infection is most common among young persons, particularly those 15-24 years old. Women have a 60-80% risk of acquiring gonorrhea from a single act of vaginal intercourse with an infected man; men have only a 20-50% chance of acquiring infection from intercourse with infected women. Transmission among men who have sex with men is more efficient than a man's risk during heterosexual sex and gonorrhea prevalence is several fold higher in this demographic group. Pharyngeal and rectal gonococcal infection is also especially prevalent in this group. Co-infection with *Chlamydia* is common, occurring in up to 50% of gonococcal infections in some countries.

6.2 Clinical manifestations

Symptoms of infection in men usually appear 2-5 days after exposure with a range of 1-30 days. Women are less likely to have symptomatic infection, up to 70% are subclinical, but those who develop symptoms do so within 10 days of infection. The majority of men with gonococcal infection develop urethritis with a white, yellow, or greenish urethral discharge, dysuria, and sometimes painful and swollen testes. Erythema of the meatus is sometimes observed. Non gonococcal urethritis is usually characterized by less purulent and less copious discharge with little erythema of the meatus. The endocervical canal is the primary site of infection in women. Females with endocervicitis and urethritis experience dysuria, a purulent vaginal discharge, pelvic pain, and pain and bleeding brought on by sexual intercourse. Symptoms of rectal infection include itching, mucopurulent discharge, bleeding, tenesmus, and painful bowel movements. Pharyngeal infection is characterized by exudative pharyngitis and cervical lymphadenopathy. Untreated gonorrhea can lead to severe complications in both men and women. Gonorrhea can spread from the cervix and vagina to the fallopian tubes and uterus leading to chronic salpingitis or pelvic inflammatory disease, ectopic pregnancy, and infertility from scaring of the fallopian tubes. Pregnant women may experience chorioamnionitis and septic abortion. In men epididymitis, usually accompanied by unilateral testicular pain and swelling with fever, is

relatively rare but can cause sterility. However a more likely cause of epididymitis in sexually active young men is *C. trachomatis*. Posterior urethritis, urethral stricture and prostatitis in men and Bartholin gland abscesses in women are additional complications of genital infection. In approximately 1- 3% of infected adults, with a higher occurrence in women, gonococci disseminates via the bloodstream to produce characteristic papulopustular lesions, and to infect joints, typically in fingers, wrists, toes, and ankles, causing septic arthritis. These manifestations are accompanied by fever and can range from mild to severe. Other less common complications of disseminated infection include a purulent conjunctivitis from autoinoculation, fatal septic shock, meningitis, perihepatitis, osteomyelitis, rapidly progressing endocarditis, especially of the aortic valve, and adult respiratory distress syndrome. Neonatal gonococcal infections are now an infrequent occurrence in developed countries but remain a serious problem in developing countries. Newborns infected during birth can develop conjunctivitis, known as ophthalmia neonatorum, which may lead to blindness. Neonates can also acquire pharyngeal or rectal infection and, rarely, develop gonococcal sepsis or pneumonia.

6.3 Diagnosis

There are currently five available tests for detection of gonorrhea; Gram stain, culture, nucleic acid amplification tests (NAAT), gonorrhea antigen detection tests, and nucleic acid hybridization tests. Clinical signs and symptoms of cervicitis or urethritis and the presence of Gram-negative intracellular diplococci within polymorphonuclear neutrophils from urethral, or less commonly, cervical discharge, are diagnostic for gonorrhea. The sensitivity of gram stain is very high in symptomatic men with urethritis but less so in infected women and in rectal infection. Stained smears are not recommended for diagnosis of pharyngeal gonococcal infection. Culture on specialized media can be used for urethral, cervical, pharyngeal, and rectal infection. This is the only testing technique that permits determination of gonococcal antibiotic sensitivity. In resource rich countries, diagnosis using very sensitive NAAT, gonorrhea antigen detection tests via immunoassay, and nucleic acid hybridization tests has become widespread. This has permitted screening of at risk populations and self referred testing in developed countries. NAAT tests are the most sensitive, and can be used on urine samples as well, but require hours to days to yield results. Rapid, point-of-care gonorrhea antigen detection tests and nucleic acid hybridization tests are in use, but are relatively expensive for settings in developing countries. Both of these tests are less sensitive than NAAT and are primarily designed for testing with cervical and urethral material. Some available NAAT, gonorrhea antigen detection tests, and nucleic acid hybridization tests can detect both *N. gonorrhoeae* and *Chlamydia* in the same sample and the NAAT test can be combined with Pap smears.

6.4 Treatment

The recommended treatment for gonococcal infections is ceftriaxone in a single 250 mg dose administered intramuscularly (IM). If unavailable cefixime, 400 mg orally in a single dose, or a single dose injectible cephalosporin plus azithromycin, 1 g orally in a single dose, or doxycycline, 100 mg orally twice a day for 7 days, may be used. Resistance to oral third generation cephalosporins has emerged recently and has been reported throughout Asia and in Australia and some European countries. The recent emergence in Japan of a strain, H041, which is extremely resistant to all cephalosporin-class antibiotics will pose a considerable public health challenge as this strain spreads throughout Asia and beyond.

Therapeutic use of sulfonamides, penicillin, erythromycin, and fluoroquinolones has been largely discontinued due to the development of widespread resistance to these agents. Azithromycin, 2 g orally, is effective but concerns over the prior ease of development of macrolide resistance in *N. gonorrhoeae* should limit its use to special circumstances. Gonococcal infections of the pharynx are more difficult to eliminate and are treated with ceftriaxone, 250 mg IM in a single dose, plus azithromycin, 1 g orally in a single dose, or doxycycline, 100 mg orally twice a day. Neonates born to infected mothers are given erythromycin ointment to the eyes to prevent blindness. Patients infected with *N. gonorrhoeae* are frequently co-infected with *Chlamydia*, and additional treatment for this infection may be appropriate, dependent on local prevalence of these STIs.

7. Syphilis

Treponema pallidum, a thin (0.1-0.18 μm by 6-15 μm) flagellated spirochete, is the etiologic agent of syphilis. *Treponema* spirochetes invade mucous membranes or penetrate through breaks in the skin. Although syphilis is typically acquired via sexual contact the disease can also be transmitted transplacentally and by exposure to blood or lesion exudates from infected persons in the primary and secondary stages of disease.

7.1 Epidemiology

Prior to the antibiotic era, syphilis was a very prevalent disease, particularly in large urban areas. Since then, the incidence has been steadily declining but there are still 12 million new cases each year around the world. Unlike many other STIs, the incidence of syphilis is higher in older individuals and is highest in men aged 30-45. Globally, congenital syphilis is a significant problem and it is estimated that neonatal mortality from syphilis exceeds that of neonatal tetanus, neonatal HIV infection, and mortality from malaria in pregnancy. There is no lasting immunity to syphilis and patients can be re-infected.

7.2 Clinical manifestations

Syphilis presents a wide spectrum of clinical manifestations as it progresses through the different stages of the disease: primary, secondary, latency, and tertiary. Syphilis, particularly the secondary stage, mimics many other infections and has been given the moniker, the "great imitator". The primary stage of syphilis is usually characterized by the appearance of a single sore (chancre) at the site of syphilis entry, although multiple lesions can be present. The chancre appears 10-90 days after infection, approximately 2-3 weeks on average. The chancre is typically a firm, round, and painless ulcer, 1-2 cm in size, which is highly infectious and will spontaneously resolve in 1-6 weeks. Chancres can also be present at non-genital sites, the anus, mouth, or perineum. Regional lymphadenopathy that is rubbery, painless, and bilateral is usually present.

Without treatment the systemic skin rash and mucocutaneous lesions of secondary syphilis appear 4-6 weeks after the primary lesion in approximately 25% of patients following dissemination of the disease throughout the body. Occasionally the symptoms of secondary syphilis will occur prior to resolution of the initial chancre. The red macropapular rash is symmetrical, non-pruritic, and present throughout the body including the palms of hands and soles of feet and may lead to hair loss. White, patchy, raised lesions on mucocutaneous surfaces, known as condylomata latum may also be present. The rash and lesions are accompanied by fever, malaise, and generalized lymphadenopathy. Rare

manifestations of secondary syphilis include hepatitis, glomerulonephritis, and keratitis. Neurosyphilis can occur at any stage of syphilis but is classically associated with tertiary syphilis. Clinical manifestations of early neurosyphilis include acute syphilitic meningitis that typically involves cranial nerves III, VI, VII and VIII; or meningovascular syphilis, a stroke-like syndrome with seizures. Secondary syphilis is usually the fist clinical presentation in persons practicing receptive vaginal or anal intercourse as the primary lesions are often not noticed.

Whereas some secondary syphilis can spontaneously resolve, if untreated, approximately two thirds of secondary syphilis cases enter into a prolonged period of latency where symptoms of infection are absent. Relapses of secondary symptoms may occur in up to 25% of untreated patients, usually within the first year of infection. The latent stage can last for up to 25-30 years but if untreated, about one third of latent infections will progress to tertiary syphilis. Tertiary syphilis is rare in developed countries due to early diagnosis and treatment of syphilis. Tertiary syphilis is characterized by destructive lesions known as gummas, neurologic involvement, and cardiovascular lesions. Gummas, are highly destructive granulomas, usually in the skin, bone and mucosal areas but are sometimes found in other tissues such as genitals, lung, stomach, liver, spleen, spinal cord, breast, brain, and heart. Onset is 10-15 years after infection. Cardiovascular syphilis generally appears about 20-30 years after infection when lesions in the cardiac vasculature produce ascending aortic aneurysm, aortic insufficiency, or coronary ostial stenosis. In tertiary neurosyphilis focal endoarteritis in the blood vessels of the brain and spinal cord provokes signs and symptoms, usually decades after infection, which may resemble other neurologic diseases. Clinical manifestations typically include general paresis and tabes dorsalis. The presence of oral syphilitic lesions is common, particularly in primary and secondary syphilis, and in regions with a high prevalence of syphilis other health care workers, such as dentists, need to be aware of this risk.

7.3 Congenital syphilis

Worldwide each year over 2 million pregnant women, 1.5% of all pregnancies, test positive for syphilis. *Treponema* spirochetes can cross the placenta to infect the fetus resulting in severe adverse pregnancy outcomes. Untreated maternal syphilis will result in stillbirth, premature birth, neonatal death, or congenital infection in up to 80% of pregnancies in developing countries. An estimated 25% of all stillbirths and 11% of neonatal deaths in developing countries are due to fetal syphilis exposure. Symptoms of early congenital syphilis in children less than 2 year old include cutaneous and mucocutaneous lesions, macropapular rash, hepatosplenomegaly, lymphadenopathy, bone alterations from osteitis and osteochondritis, meningitis, pneumonia, and testicular masses. Hematologic abnormalities such as thrombocytopenia and anemia may occur. Early congenital syphilis is more common than late congenital syphilis. Late congenital syphilis in children >2 year old is characterized by Hutchinson's triad, Saddle nose, and bone deformations such as Saber shins. Hutchinson's triad includes tooth deformations where the crown of the incisors is wider in the cervical portion than at the incisor edge and a crescent-shaped notch is present at the incisor edge, interstitial keratitis which can lead to blindness, and eighth nerve deafness. Saddle nose refers to collapse of the bridge and resulting dorsal depression due to erosion of septal support, giving a saddled appearance. Saber shin is a malformation of the tibia with sharp anterior bowing. Interstitial keratitis is the most common manifestation of

late congenital syphilis. These adverse pregnancy outcomes can be prevented by syphilis screening to identify and treat maternal infections prior to 24 weeks gestation.

7.4 Diagnosis

Initial diagnosis of syphilis is typically based on clinical presentation. *Treponema* spirochetes are Gram negative but they cannot be visualized using conventional light microscopy. Darkfield microscopy and direct fluorescent antibody test of spirochetes from lesion exudates and tissue provide definitive diagnosis of early syphilis. These tests however are not utilized in most settings. Presumptive diagnosis of syphilis relies on two types of testing for antibody in blood serum or cerebrospinal fluid. The Venereal Disease Research Laboratory (VDRL) and Rapid Plasma Reagin (RPR), Toluidine Red Unheated Serum (TRUST), and Unheated Serum Reagin (USR) tests utilize a non-treponemal antigen. The VDRL and RPR tests are the most widely used of these. VDRL and PRP testing is most sensitive in the middle stages of the disease, early syphilis and late stage disease may be missed. Detectable antibody titers are not attained until 1-4 weeks after appearance of the chancre and titers often decline to undetectable levels in latency. Non-treponemal tests are nonspecific and can give false-positive results and occasionally false negative results under conditions of antibody excess which can occur during secondary syphilis. Positive results are confirmed with a test utilizing treponemal antigens, such as the fluorescent treponemal antibody absorbed (FTA-ABS) test, treponemal enzyme immunoassay (EIA), microhemagglutination assay for *T. pallidum* antibodies (TPHA), and direct fluorescent antibody-*T. pallidum* test (DFA-TP). These tests are more sensitive than non-treponemal tests in detecting primary and tertiary syphilis. Non-treponemal test antibody titers usually correlate with disease activity and become non-reactive with time after treatment. Treponemal test antibody titers do not correlate disease activity and most (75-85%) will remain reactive for the rest of their lives. Commercially available point-of care tests for syphilis have been introduced recently although these are too expensive for most situations in the developing world.

7.5 Treatment

Benzathine penicillin G, 2.4 million units by intramuscular injection (IM) in a single dose for adults or 50,000 units/kg IM for children, is the preferred treatment for primary, secondary, and early latent stage syphilis. Alternatively, procaine penicillin at 1.2 million units IM daily for 10 days is used. Penicillin allergic non-pregnant patients may be treated with doxycycline, 100 mg orally twice daily for 2 weeks or tetracycline, 500 mg orally four times daily for 2 weeks. Penicillin allergic pregnant patients may receive erythromycin, 500 mg orally, 4 times daily for 14 days. Late latent stage syphilis and tertiary syphilis is treated with three doses benzathine penicillin G at 1 week intervals, 2.4 million units IM each dose for adults and 50,000 units/kg for children. Alternatively, procaine penicillin at 1.2 million units IM daily for 20 days is used. Penicillin allergic non-pregnant patients with late latent stage or tertiary syphilis may be treated with doxycycline, 100 mg orally twice daily for 4 weeks or tetracycline, 500 mg orally four times daily for 4 weeks. Penicillin allergic pregnant patients may receive erythromycin, 500 mg orally 4 times daily for 4 weeks. Neurosyphilis is treated with intravenous aqueous crystalline penicillin G, 3-4 million units every 4 hours (18-24 million units per day) for 10-14 days, or with daily IM injections of procaine penicillin, plus 500 mg probenecid orally 4 times daily, both for 10-14 days. Penicillin

allergic non-pregnant patients with neurosyphilis are treated with doxycycline, 200 mg orally twice daily for 4 weeks or tetracycline, 500 mg orally four times daily for 4 weeks. Non-penicillin allergic pregnant women diagnosed with syphilis are treated with penicillin according to the stage of infection. Early congenital syphilis should be treated with intravenous aqueous crystalline penicillin G at 50,000 units/kg/dose every 12 hours for the first 7 days and every 8 hours for the next 3 days. Alternatively early congenital syphilis is treated with IM injections of procaine penicillin at 50,000 units/kg daily for 10 days. Late congenital syphilis is treated with intravenous or intramuscular aqueous crystalline penicillin G at 50,000 units/kg/dose every 4-6 hours for 10-14 days. For penicillin allergic children, after the first month of life, administer erythromycin, 7.5-12.5 mg/kg orally, 4 times daily for 4 weeks.

8. Chancroid

Haemophilus ducreyi, a fastidious Gram-negative facultative anaerobic coccobacillus, is the causative agent of chancroid. Chancroid is transmitted by vaginal, anal, or oral sex with an infected individual. The organism enters thru breaks in the epithelium and resides primarily in the extracellular spaces. *Haemophilus ducreyi* can resist phagocytosis and untreated lesions may take months to heal.

8.1 Epidemiology

Globally, chancroid is the most common cause of genital ulcer disease in regions where the disease is endemic. WHO estimates the annual global incidence to be about 6 million cases. Chancroid occurs in parts of Africa, south-east Asia and the Caribbean where it accounts for 23-56% of genital ulcer disease. Chancroid is more common in men than women and more common in areas where HIV prevalence is high (>8%). The incidence of chancroid is much lower in developed countries and sporadic outbreaks there are associated with travel, prostitution, and drug use. Chancroid, as are all genital ulcer producing STIs, is a risk factor for HIV transmission.

8.2 Clinical manifestations

After an incubation period of several days to two weeks, a tender erythematous papule develops at the site of inoculation which progresses to a pustular stage. The pustle ruptures within 2-3 days to form a painful genital ulcer with soft edges. Chancroid ulcerative lesions vary from 3-50 mm across but are typically 10-20 mm. Chancroid ulcers can be irregular, round, or oval in shape, are sharply circumscribed with an undermined edge, and contain a grey or yellow purulent exudate. Lesions will have a surrounding cutaneous erythema. One half of men have only a single ulcer and lesions typically appear on the penis: penile shaft, coronal sulcus, prepuce, urethral meatus, and glans. In women infection is often subclinical. Women have multiple ulcers more frequently than men that may merge to form large ulcers. Ulcers in women occur on the fourchette, labia majora, labia minora, cervix, perianal region, and inner thighs. “Kissing ulcers” may develop on the skin surfaces apposing the initial ulcer. Women may also experience dysuria and dyspareunia. Rectal sores in men or women may bleed or cause pain during defecation. Buboes, swelling of the inguinal lymph nodes, occur in one third to one half of infected individuals 1-2 weeks after the ulcers form and these may rupture, producing draining abscesses. The development of buboes is a more

common occurrence in men than women. Buboes are painful, tender, and fluctuant with underlying erythema, and are typically unilateral. Supperative adenopathy is almost pathognomonic for chancroid. The skin over the bubo does not become thickened and edematous or show furrows as in the adenopathy of LGV. Chancroid can also spread via self inoculation to other anatomical sites. Chancroid in HIV-infected patients may produce a larger number of ulcers, atypical ulcers, extra-genital lesions, and longer lasting ulcers even with treatment.

8.3 Diagnosis

Definitive diagnosis of chancroid requires cultivation of *H. ducreyi* on special culture media, which is not routinely carried in most laboratories. Culture on two media is recommended as not all *H. ducreyi* strains can be cultured using one medium. Culture also requires a humid environment, 5% CO_2, and incubation at 33-35 ^oC. Swabs for culture are collected from the undermined edge of the ulcer and the fastidious nature of *H. ducreyi* necessitates use of transport media if the organisms are not cultured within a few hours. Presumptive diagnosis by microscopy is possible if the organism load in ulcers is high and Gram-negative coccobacillus arranged in chains, paired chains or aggregates ("school of fish" appearance) are visualized. However the value of microscopy for diagnosis is limited by low sensitivity and specificity of this technique. Aspirates from buboes are less likely to yield positive results on microscopy or culture. In many cases chancroid is diagnosed clinically and treated without a definitive diagnosis. The combination of painful genital ulcer and suppurative inguinal lymphadenopathy is also supportive of a diagnosis of chancroid. Nucleic acid amplification tests for diagnosis have been developed but are not widely available.

8.4 Treatment

Azithromycin (1 g orally) or ceftriaxone (250 mg IM) offer the advantage of single-dose therapy. Alternatively ciprofloxacin (500 mg orally twice daily for 3 days) or erythromycin base (500 mg orally three times a day for 7 days) may be used. For reasons of cost, erythromycin is usually used for treatment in developing countries. Isolates with intermediate resistance to ciprofloxacin or erythromycin have been reported but data are rather limited on the current status of *H. ducreyi* drug resistance. Ulcerative lesions should be kept clean to avoid the chance of secondary infections. Fluctuant lymph nodes can be aspirated through healthy skin. Incision and drainage or excision of nodes may delay healing. Uncircumcised men and HIV-positive patients may not respond as well to treatment. Large ulcers may require weeks to resolve after treatment and patients should be followed until there is clear evidence of improvement or cure.

9. Human papilloma virus

Human papillomavirus (HPV) is a member of the papillomavirus family of viruses that infect only humans. HPVs can be divided into two general groups based on their preferred infection site: cutaneous and mucosal. Genital HPV, a member of the mucosal HPVs, is transmitted through sexual contact and infects the anogenital regions. There are more than 40 types of HPV that infect the genital area. Non-oncogenic or low risk HPV types are the causative agents of genital warts and recurrent respiratory papillomatosis. Oncogenic or high risk HPV types are the cause of cervical cancers and are associated with other anogenital cancers in men and women.

9.1 Epidemiology

Genital human papillomavirus is considered to be one of the most prevalent STIs in the world. It is estimated that more than 50% of sexually active individuals become infected at least once in their life. WHO estimates that 14.3% of women in developing regions and 10.3% of women in developed regions with normal cervical cytology are infected with HPV. Incidence of HPV in women increases significantly with severity of abnormal cervical cytology. Prevalence of HPV reaches to greater than 70% in women with cervical cancer.

9.2 Clinical manifestations

Asymptomatic genital HPV infection is common and usually self-limited. Seventy percent of infections are gone in 1 year, and 90% in 2 years. The most common symptom of genital HPV infection is genital warts, also known as condylomata acuminata. Genital warts appear as a small white bump or groups of bumps in the genital area. Genital warts are usually flat, papular, or pedunculated growths. However, they can be small or large, raised or flat. Genital warts are usually themselves asymptomatic, but can sometimes be painful and pruritic, depending on the size and anatomic location. Growths commonly occur around the introitus in women, under the foreskin of the uncircumcised penis, and on the shaft of the circumcised penis. Genital warts can also be found in or on the cervix, vagina, urethra, perineum, perianal skin, and scrotum. Intra-anal warts are most often observed in individuals who have had receptive anal intercourse, but may be present in men or women with no history of anal sexual contact.

9.3 Health sequelae

The correlation between persistent HPV infection and cervical cancer has been well established. Cervical cancer is the 2nd most common cancer among women, worldwide. Eighty-six percent of these cases occur in developing countries, making up 13% of the world female population. There is now increasing evidence linking HPV to anogenital cancers other than cervical cancer. These include anal, vulvar, vaginal, penile, and head and neck cancers. Anal cancer occurs rarely with about 99,000 cases in 2002, sixty percent of cases occurring in women and 40% in men. This type of cancer is more prevalent in populations of men who have sex with men and HIV-positive populations. Vulvar cancers make up about 3% of the gynecological cancers, with 40% of them occurring in developing countries. The majority of these cases occurring in the developed world suggest that HPV screening may not be an effective preventative method. Vaginal cancers make up 2% of gynecological cancers, with a majority of vaginal cancers (68%) occurring in developing countries. Penile cancer represents 0.5% of cancers in men. In western countries, incidence if penile cancer in men is less than 1 per 100,000, however, this rate increases in Latin America, India, and Thailand. Two-thirds of oral cancers occur in developing countries and about 15-20% of oral cancers are associated with HPV infection. Growing evidence suggests that HPV-related oral pharyngeal cancers are associated with the practice of oral sex.

9.4 Diagnosis

The presence of genital warts is a straight forward method for diagnosis of HPV. However, in the case of asymptomatic infections, there is no general diagnostic test used to screen normal patients for HPV. The Papanicolaou test (Pap smear or Pap test) is a cytological examination of cervical tissue sample that is used to screen for cervical cancer or

precancerous lesions. The Pap smear is more commonly used in developed countries. Abnormal results for a Pap smear usually result in screening of the tissue sample for the presence of HPV DNA. HPV DNA testing has been shown to have a higher sensitivity than cytology and a high negative predictive value for detecting cervical precancerous lesions. Other diagnostic strategies include visual inspection with acetic acid (VIA), self-vaginal sampling, and liquid based cytology (LBC). VIA has shown sensitivity similar to that seen with cytology, but has a lower specificity, which could lead to over treatment. However, its use has shown a decrease in the incidence of and mortality from cervical cancer, and may therefore be a useful method in resource poor areas. Developing countries have attempted to implement HPV screening programs with variable success. Successful implementation of these programs in some countries such as Taiwan, Japan, Singapore, and developed African countries has caused a decline in incidence and mortality of cervical cancer. In the remainder of the developing world either no screening programs currently exist or screening has had little success due to poor infrastructure and competing health priorities in these countries.

9.5 Treatment

Treatment is not recommended for subclinical genital HPV because these infections typically clear spontaneously. However, there are treatments for the diseases that are caused by HPV infection. Genital warts can resolve themselves or be removed by patient-applied or provider-administered therapy. Patient-applied therapy recommended by the CDC consists of podofilox 0.5% solution or gel, imiquimod 5% cream, or sinecatechins 15% ointment. Provider-administered therapy is cryotherapy with liquid nitrogen or cryoprobe applications ever 1-2 weeks, 10-25% podophyllin resin in a compound tincture of benzoin, 80-90% trichloroacetic acid (TCA) or bichloroacetic acid (BCA), or surgical removal by tangential scissor excision, tangential shave excision curettage, or electrosurgery. No evidence suggests that one treatment regimen is better than the other. Treatment against cervical lesions includes removal of precancerous lesions using cryotherapy and continuous preventative screening of cervical tissue.

A preventive strategy based on the development of vaccines against HPV is now widely available across the globe. A quadrivalent vaccine, Gardasil (Merck Co.) protects against 2 types of HPV that cause 75% of cervical cancer (HPV 16 &18) and the 2 types of HPV that cause 90% of genital warts (HPV 6 & 11). Gardasil can be used for both males and females ages 9-26. This vaccine is given in a series of 3 0.5 mL intramuscular injections at 0, 2, and 6 months. A bivalent vaccine, Cervarix (GlaxoSmithKline), protects against HPV 16 & 18 and is only approved for women ages 10-25. This vaccine is given in a series of 3 intramuscular 0.5 mL doses at 0, 1, and 6 months. Many countries have developed their own individual vaccine schedules. Both vaccines have been shown to be highly immunogenic and effective in prevention of incidence and persistent HPV infections that could lead to the development of precancerous lesions. It is recommended that vaccination begin at ages at which individuals have not yet become sexually active.

10. Herpes

Genital herpes is caused by herpes simplex viruses type 1 (HSV-1) or type 2 (HSV-2) with HSV-2 the primary genital STI. HSV-1 is acquired orally, usually in childhood, and typically

causes cold sores and sometimes keratitis. HSV-1 can also cause genital infection but recurrent episodes during infection with HSV-1 are much less frequent. HSV-1 and HSV-2 are typically transmitted during sexual contact by virus shed from herpes sores but virus can also be released intermittently between outbreaks from skin without apparent sores. Herpes virus enters through mucous membranes or breaks in the skin and replicates locally in mucosal epithelial cells. Between outbreaks the herpes virus ascends peripheral sensory neurons to the dorsal root ganglia and becomes latent.

10.1 Epidemiology

Herpes is the most common STI in the world and HSV-2 infection is the main cause of genital ulcers in developing countries. An estimated one sixth to one third of the world's population has genital herpes caused by HSV-2. HSV-2 prevalence is greater than 60% in sub-Saharan Africa and East Asia and between 25-40% in Latin America, Eastern Europe, South Asia, and South-east Asia. HSV-2 prevalence rates are less than 20% in North America and Western Europe and below 10% in North Africa, the Middle East, Japan, Australia and New Zealand. Most herpes infections are asymptomatic and herpes is usually spread by people who are unaware they have the disease. Symptomatic genital herpes infection is approximately twice as common in women as in men. Transmission from an infected male to a female partner is more likely than transmission from infected female to a male partner during vaginal intercourse. Rates of herpes infection are also higher in men who have sex with men and in HIV-positive individuals. Herpes seroprevalence rates are as high as 80% among HIV-positive populations in North America, Europe and Africa.

10.2 Clinical manifestations

Most individuals have no or only minimal symptoms from herpes infection and do not realize they are infected. Herpes appears 2-7 days after infection as small, pruritic and painful, usually multiple, grouped vesicles (blisters) with a red base on or around the genitals and rectum or on the buttocks or thighs. The vesicles will ulcerate to leave shallow lesions that heal in 2-4 weeks. During the initial episode additional groups of sores may appear. Fever, malaise, and bilateral inguinal lymphadenopathy that is firm and tender may also be present. Infection in women usually involves the vulva, vagina, and cervix. In men lesions usually appear on the glans penis, prepuce, or penile shaft. After resolution of the primary infection herpes enters a latent state. However, outbreaks will re-occur from weeks to months after the initial infection, particularly during periods of stress or illness, and typically 4 or 5 outbreaks occur within the first year. About one half of patients experience prodromal symptoms of tingling or pain at the eruption site 1-2 days prior to the appearance of lesions. Although herpes infection persists indefinitely outbreaks diminish in number and severity with time. The duration and intensity of outbreaks are usually more severe in persons with suppressed immune systems. Persons with immune deficiencies such as HIV-infected persons may have persistent, extensive, and severe mucocutaneous lesions involving large areas of perianal, scrotal, or penile skin. Complications of herpes infection include an aseptic meningitis in as many as 10% with primary infection, transverse myelitis, and perinatal transmission. Pregnant women experiencing primary genital herpes during birth can transmit a potentially fatal herpes infection to their infant and a caesarean delivery may be appropriate in these cases. The risk of transmission during birth is very low in women with recurrent disease. Infected neonates can experience disseminated disease with

organ failure, severe neurologic damage, ocular involvement, cutaneous and mucocutaneous sores, and even death.

10.3 Diagnosis

There are four types of testing employed in herpes diagnosis, DNA testing, antibody testing, antigen testing, and herpes culture. Nucleic acid amplification tests are very sensitive and can detect herpes DNA in samples from herpetic sores even when virus is in low copy number, such as in older lesions or in cerebrospinal fluid samples. It is the method of choice to detect HSV meningitis, encephalitis, and keratitis. HSV antibody tests are available to measure both IgM antibody, which can detect primary herpes infection after first several days of infection, and IgG antibody, which indicates prior HSV infection. The presence of HSV-2 antibody indicates anogenital infection but the presence of HSV-1 antibody does not distinguish genital infection from oral infection. Rapid antibody tests are available that detect antibodies to HSV-2 in blood from a finger stick within 10-15 minutes. The relatively quick turnaround and ease of this antibody tests makes it ideal for herpes screening for persons with undiagnosed disease as well as disease diagnosis. Fluorescently labeled antibody is used in antigen tests to detect markers expressed on herpes infected cells. Herpes culture is very specific but prone to false negative results, especially for recurrent infection, and requires several days to a week for results. Material from the base of the ulcer in fresh primary lesions is best for any herpes diagnosis as viral shedding decreases as lesions age and heal and in subsequent outbreaks. However, the requirement for tissue culture of host cells to grow herpes limits the utilization of this technique. Herpes can also be diagnosed by visual examination for the characteristic vesicles and sores although signs and symptoms of herpes can vary, making diagnosis problematic in some patients.

10.4 Treatment

There is no effective treatment for genital herpes but antiviral medications can lessen the duration and severity of outbreaks. Antiviral prophylaxis also reduces the chances of transmission from infected individuals to uninfected partners. Clinical episodes can be treated by acyclovir in a 7 day regimen for the initial episode or a 5 day regimen for subsequent episodes at 200 mg orally 5 times daily or 400 mg orally 3 times daily. Ideally treatment should begin within one day of the appearance of the herpes vesicles. Suppressive therapy uses acyclovir, 400 mg orally twice daily, continuously. Alternatively the acyclovir analogues valaciclovir and famciclovir can be used for treatment and prophylaxis although the dosages and regimens may vary. The acyclic nucleoside drugs are effective and well tolerated in most patients. Immunosuppressed individuals such as HIV-positive patients may respond poorly to treatment and require larger doses and longer treatment schedules or even parenteral drug administration.

11. Granuloma inguinale (Donovanosis)

Calymmatobacterium granulomatis is an intracellular Gram-negative facultative aerobic coccobacillus that is the causative agent of granuloma inguinale. Other designations for this disease include granuloma venereum and donovanosis, named for the discoverer of the infectious agent. A close phylogenetic relationship with *Klebsiella* spp. has led some to call for a reclassification of *Calymmatobacterium* into the genus *Klebsiella*. Some *C. granulomatis*

strains are capsulated. *Calymmatobacterium granulomatis* resides in the cytoplasm of mononuclear phagocytes or histiocytes in tissue.

11.1 Epidemiology

The incidence of granuloma inguinale has decreased in recent years but it still endemic in certain tropical and subtropical regions; south-east India, Indonesia, Papua New Guinea, South Africa, Guyana, Peru, Argentina, Brazil, and among aborigines of Central Australia. It is only occasionally reported in developed countries. Most infections occur in sexually active people 20-40 years of age and men are more than twice as likely to have disease. Granuloma inguinale is spread primarily thru vaginal or anal intercourse, infection via oral sex is rare. Non-sexual transmission via contact with infected material from lesions or by fecal contamination is possible.

11.2 Clinical manifestations

The infection begins approximately 10-50 days after exposure with the appearance of small, relatively painless, erythematous pustules or subcutaneous nodules. These will ulcerate to produce shallow and sharply demarcated lesions. Four types of lesions are described: ulcerogranulomatous ulcers, the most common, oozing lesions with a beefy red friable base that bleeds when touched, hypertropic ulcers with a raised irregular edge, sometimes completely dry, deep necrotic ulcers with an offensive smell from decaying tissue, and sclerotic ulcers with fibrous and scar tissue. In early stages the ulcers resemble chancroid, in later stages granuloma inguinale may resemble LGV. The lesions slowly expand destroying adjacent tissue. Anatomical areas most commonly infected in men include the sulcus, subprepucial region, and the anus. Women are most affected in the labia minor, fourchette, and occasionally in the cervix and upper genital tract. Extra-genital lesions occur in a minority of patients, these are secondary to the genital lesions. Oral lesions are the most frequent, loss of teeth indicates oral bone involvement, but lesions are possible on any surface. Very rarely disseminated donovanosis may occur, spreading to cause lesions in the liver, other organs, and bone, particularly tibia. Disseminated disease may be fatal as a diagnosis of donovanosis is rarely considered. Inguinal lymphadenopathy is generally absent. Untreated disease results in the destruction of genital tissue with scarring.

11.3 Diagnosis

Granuloma inguinale is diagnosed by clinical signs, particularly the presence of persistent spreading lesions, and the demonstration of intracellular Donovan bodies in the cytoplasm of mononuclear phagocytes or histiocytes present in scrapings or punch biopsies stained with Giemsa, silver, or Wright's stain. Specimens from just below the surface of the ulcer are most likely to yield positive results. Culture is difficult to perform as it requires growth of host cells and is not readily available.

11.4 Treatment

WHO guidelines recommend azithromycin, 1 g orally followed by 5500 mg daily or doxycycline, 100 mg orally twice daily but do not state the duration of therapy. Typically therapy is given for 3-6 weeks or until lesions are healed. Alternative regimens are erythromycin, 500 mg orally 4 times daily or tetracycline, 500 mg orally 4 times daily or trimethoprim 80 mg/sulfamethoxazole 400 mg, 2 tablets orally twice daily for a minimum

of 14 days. WHO recommends the addition of a parenteral aminoglycoside, such as gentamicin, for the therapy of HIV-positive patients. Treatment should be continued until complete healing is achieved. The intracellular residence of *C. granulomatis* makes it somewhat resistant to treatment.

12. STIs among travelers and immigrants from the tropics

Travel is known to be a major factor in the spread of STIs around the world, particularly in developing countries where STIs are endemic and very high rates are encountered in commercial sex workers. The rapid spread of antibiotic resistance around the world for a number of STIs and the spread of HIV infection are cases in point. It is difficult to assess the risk of acquiring STIs during travel in which sexual acts occur. Poverty and lack of legal enforcement certainly facilitate access to sexually compliant individuals in the developing world. In addition, risk-taking behavior increases when on vacation and often vacations involve higher risk activities than the traveler typically encounters at home. Studies have shown that engaging in sexual activity is the specific reason for travel, i.e. 'sex vacations', in some travelers. Reports of lower condom rate usage and higher rates of engagement in anonymous sex by travelers support a conclusion of increased risk. Travel clinics and physicians advising overseas travelers should counsel travelers about the risks and proper prophylactic regimens available. Travelers should also be strongly encouraged to be tested for STIs upon return if they have engaged in sexual activity. There are high rates of asymptomatic infection for many STIs and long incubation periods can also occur before there is an onset of symptoms. Immigrants and refugees pose a problem to the health care system in developed countries as well. STIs uncommon in the developed world such as chancroid, LGV, and donovanosis present a diagnostic challenge to the physician unfamiliar with these diseases. Incorrect diagnosis and subsequent incorrect treatment can delay resolution of the disease and increase the risk to the patient and sex partners, even permitting local mini-epidemics of new STIs. Health care providers should be aware of uncommon STIs present in their patient's country of origin when evaluating symptoms of genital infection in this population.

13. Prevention and control

Prompt diagnosis and treatment of infected individuals and public education of sexually active populations on proper prevention and prophylactic measures for STIs are the foundation of STI prevention and control. To be successful this approach should be supplemented with screening programs to identify individuals with subclinical infections as many, if not most, STIs are transmitted by individuals who do not know they are infected. However, the lack of adequate diagnostic capacity in most tropical settings severely compromises diagnosis and screening for STIs. This is the biggest barrier to addressing the STI epidemic in the developing world. Provision of a minimal diagnostic capacity, both equipment and trained individuals, at the initial point of contact with STI patients would yield enormous benefits. The development of affordable point-of care tests for STIs would also significantly advance efforts for controlling STIs in these countries. Public health education initiatives for STIs should encourage safe sex behavior and emphasize the advantages of prompt access to healthcare for suspected infections. Education is key to changing sexual behavior in high risk groups, especially adolescents and young adults who

are disproportionately afflicted by STIs. Some health care advisors have advocated a policy of treating all sexually active adolescents and adults in a village or locale as a means to controlling STIs, an approach similar to mass treatment with anti-helminthics utilized to control the endemic of intestinal worm disease. This approach may be the single most cost effective mechanism for managing the STI epidemic in developing countries and should be given careful consideration. Although perhaps not applicable for all STIs, certainly for highly prevalent STIs with drugs that are inexpensive, safe, efficacious, and well tolerated, such as metronidazole for treating trichomoniasis, this approach has much merit.

On an individual basis the only truly reliable protection is abstinence from sexual activity. People in long term monogamous relationships also have greatly reduced risk of STIs and HIV. Vaccines are available for the prevention of HPV infection and potential HIV vaccines are in clinical trials. Protective measures for sexually active individuals include reducing the number of sexual partners and the use of latex condoms and other barriers during sexual activity. Consistent and proper use of condoms has been shown to reduce the transmission of STIs and HIV. Male circumcision has also been shown to be significantly protective against transmission of HIV and STIs. STI treatment should include sexual partners of the index case whenever possible to prevent re-infection and to reduce disease transmission. Due to compliance issues and difficulties in following patients in many locales, directly observed single dose therapies are preferred for the treatment of STIs.

14. The future: Vaccines for STIs

Vaccination offers the ultimate tool for control of STIs; prevention before exposure. Safe and efficacious vaccines could eliminate the vast majority of STI-associated morbidity and mortality. Unfortunately this goal has only been attained relative to HPV infection (section 9.5) and prospects for additional STI vaccines in the immediate future are remote. In part, this is because the precise correlates of protective immunity have not been well-defined for these STIs. However, some progress has been made in the development of vaccines for genital herpes and *Chlamydia* infection. Three types of herpes vaccines have shown efficacy in animal models: (i) HSV-2 subunit vaccines combined with adjuvant; (ii) gene delivery vehicles, such as vaccinia virus, *Listeria* or *Salmonella typhimurium*, expressing HSV-2 proteins; and (iii) attenuated (replication-defective) HSV-2 viruses. Subunit vaccines based on herpes glycoproteins gD and gB have failed in two human clinical trials. Live attenuated viruses have not been tested in humans to date although they have shown the most promise in animal models. Recent progress in identification of T-cell epitopes mediating asymptomatic versus symptomatic disease manifestation should enhance future development of a herpes vaccine. *Chlamydia* candidate vaccines containing *Chlamydia* major outer membrane protein (MOMP) with HPV major capsid membrane protein L1, recombinant MOMP with cholera toxin, co-expressed *Chlamydia* Porin B and polymorphic membrane protein-D proteins in a *Vibrio cholerae* ghost delivery system, MOMP-based DNA vaccines, and live attenuated *Chlamydia* organisms have each shown efficacy in animal models but none have advanced to human trials.

Although the lack of progression to disease in some individuals infected with gonorrhea, syphilis, chancroid, and granuloma inguinale and the ability of immune responses to contain and clear infections in others in the absence of treatment indicates the theoretical feasibility of vaccination, progress on the development of a vaccine for these STIs has lagged. The syphilis spirochete, *Treponema pallidum*, has a unique molecular architecture and

the cell envelope consists of a dual membrane structure. The outer membrane is poorly immunogenic, lacking lipopolysaccharide and possessing few integral membrane proteins that could serve as surface antigenic targets for the host immune system. The strong antibody response observed in syphilis is principally generated by lipopolysaccharide and protein immunogens located in the inner membrane where they are inaccessible to this antibody response. To date, research on a syphilis vaccine has not progressed past the identification of these rare outer membrane proteins as candidate vaccine antigens. Development of a gonococcal vaccine has been hampered by the ability of *N. gonorrhoeae* to change surface antigens, especially Type IV pili, deficiencies in current animal models, and the lack of target capsular polysaccharides such as are present in *N. meningitidis*. Two candidate gonorrhea vaccines, utilizing killed whole gonococcal cells or pilus and pilus-associated proteins, have been tested in human clinical trials but neither produced protection. Recent work on gonococcal vaccines has been focused on the identification of potential B- and T-cell epitopes for candidate antigens. The relatively low incidence of chancroid and donovanosis in most developed countries is mirrored by limited research interest towards development of vaccines for *Haemophilus ducreyi* and *Calymmatobacterium granulomatis* infections and there is little in the way of published work or progress on vaccines for these organisms.

15. References

Barh, D., Misra, A. N., Kumar, A. & Azevedo V. (2010). A novel strategy of epitope design in *Neisseria gonorrhoeae*. *Bioinformation*, 5, 77-82, ISSN 0973-2063

Barry, P. M. & Klausner, J. D. (2009). The use of cephalosporins for gonorrhea: the impending problem of resistance. *Expert Opin. Pharmacother.*, 10, 555-577, ISSN 1465-6566

Bharadwaj, M., S. Hussain, V. Nasare, V. & Das, B. C. (2009). HPV & HPV vaccination: issues in developing countries. *Indian J. Med. Res.,* 130, 327-333, ISSN 0019-5359

Celum, C. (2010). Sexually transmitted Infections and HIV: epidemiology and interventions. *Top. HIV Med*., 18, 138-142, ISSN 1542-8826

Centers for Disease Control and Prevention. (2010). Sexually transmitted diseases treatment guidelines, 2010. *Morb. Mort. Wkly. Rep*., 59(RR-12), 1-110, ISSN 1057-5987

Corey, L. (2002). Challenges in genital herpes simplex virus management. *J. Infect. Dis*., 186 (Suppl 1), S29-S33, ISSN 0022-1899

Cox, D. L., Luthra, A., Dunham-Ems, S., Desrosiers, D. C., Salazar, J. C., Caimano, M. J. &Radolf, J. D. (2010). Surface immunolabeling and consensus computational framework to identify candidate rare outer membrane proteins of *Treponema pallidum*. *Infect. Immun*., 78, 5178-5194, ISSN 0019-9567

Cunningham, K. A. & Beagley, K. W. (2008). Male genital tract chlamydial infection: implications for pathology and infertility. *Biol. Reprod*., 79, 180-189, ISSN 0006-3363

Da Ras, C. T. & da Silva Schmitt, C. (2008). Global epidemiology of sexually transmitted diseases. *Asian J. Androl*., 10, 110-114, ISSN 1008-682X

Dasgupta, G., Chentoufi, A. A., Nesburn, A. B., Wechsler, S. L. & BenMohamed, L. (2009). New concepts in herpes simplex virus vaccine development: notes from the battlefield. *Expert Rev. Vaccines*, 8, 1023-1035, ISSN 1476-0584

Domantay-Apostol, G. P.; Handog, E. B. & Gabriel, M. T. G. (2008). Syphilis: the international challenge of the great imitator. *Dermatol. Clin*., 26; 191-202, ISSN 0733-8635

Eduardo, P.; Velho, N. F.; de Souza, E. M. & Belda, Jr., W. (2008). Donovanosis. *Brazil J. Infect. Dis*., 12, 521-525, ISSN 1413-8670

Edwards, J. L. & Butler, E. K. (2011). The pathobiology of *Neisseria gonorrhoeae* lower female genital tract infection. *Front. Microbiol*., 2, 102, ISSN 1664-302X

Grm, H. S., Bergant, M. & Banks L. (2009). Human papillomavirus infection, cancer and therapy. *Indian J. Med. Res.,* 130, 277-285, ISSN 0019-5359

Haggerty, C. L.; Gottlieb, S. M.; Taylor, B. D.; Low, N.; Xu, F. & Ness, R. B. (2010). Risk of sequelae after *Chlamydia trachomatis* genital infection in women. *J. Infect. Dis*., 201(S2), S134-S155, ISSN 0022-1899

Halford, W. P., Püschel, R., Gershberg, E., Wilber, A., Gershberg, S. & Rakowski B. (2011). A live-attenuated HSV-2 ICP0$^-$ virus elicits 10 to 100 times greater protection against genital herpes than a glycoprotein D subunit vaccine. *PLOS One*, 6, e17748, ISSN 1932-6203

Hilber, A. M., Francis, S. C., Chersich, M., Scott, P., Redmond, S., Bender, N., Miotti, P., Temmerman, M. & Low, N. (2010). Intravaginal practices, vaginal infections and HIV acquisition: systemic review and meta-analysis. *PLOS One*, 5, e9119, ISSN 1932-6203

Johnston, V. J. & Mabey, D. C. (2008). Global epidemiology and control of *Trichomonas vaginalis*. *Curr. Opin. Infect. Dis*., 21, 56-64, ISSN 0951-7375

Joint United Nations Programme on HIV/AIDS (UNAIDS). (2010). UNAIDS report on the global AIDS epidemic 2010. UNAIDS, ISBN 9789291738717, Geneva

Kamb, M. L.; Newman, L. M.; Riley, P. L.; Mark, J.; Hawkes, S. J.; Malik, T. & Broutet, N. (2010). A road map for the global elimination of congenital syphilis. *Obstet. Gynecol. Int*., 2010: 312798, 1-6, e-ISSN 1687-9597

Lewis, D. A. (2003). Chancroid: clinical manifestations, diagnosis, and management. *Sex. Transm. Infect*., 79, 68-71, ISSN 1472-3263

Looker K. J., Garnett, G. P. & Schmid, G. P. (2008). An estimate of the global prevalence and incidence of herpes simplex virus type 2 infection. *Bull. World Health Org*., 86, 805-812, ISSN 0042-9686

Low. N.; Broutet ,N.; Adu-Sarkodie, Y.; Barton, P.; Hossain, M. & Hawkes, S. (2006). Global control of sexually transmitted infections. *Lancet,* 368, 2001-2016, ISSN 0140-6736

McClelland, R. S., Sangare, L., Hassan, W. M. Lavreys, L., Mandaliya, K., Kiarie, J., Ndinya-Achola, J., Jaoko, W. & Baeten, J. M. (2007). Infection with *Trichomonas vaginalis* increases the risk of HIV-1 acquisition. *J. Infect. Dis*., 195, 698-702 ISSN 0022-1899

McGill, M. A., Edmondson, D. G., Carroll. J. A., Cook, R. G., Orkiszewski, R. S. & Norris, S. J. (2010). Characterization and serologic analysis of the *Treponema pallidum* proteome. *Infect. Immun*., 78, 2631-2643, ISSN 0019-9567

Memish, Z. A. & Osoba, A. O. (2006). International travel and sexually transmitted diseases. *Trav. Med. Infect. Dis*., 4, 86-93, ISSN 1477-8939

Mohammed, T. T. & Olumide, Y. M. (2008). Chancroid and human immunodeficiency virus infection – a review. *Int. J. Dermatol*., 47, 1-8, ISSN 0011-9059

Newman, L. M.; Moran, J. S. & Workowski, K. A. (2007). Update on the management of gonorrhea in adults in the United States. *Clin. Infect. Dis*., 44, S84-S101, ISSN 1058-4838

Nikolic, D. S. &Piguet V. (2010). Vaccines and microbicides preventing HIV-1, HSV-2, and HPV mucosal transmission. *J. Invest. Dermatol*., 130, 352-361, ISSN 0022-202X

Nusbaum, M. R., Wallace, R. R., Slatt, L. M. & Kondrad E. C. (2004). Sexually transmitted infections and increased risk of co-infection with human immunodeficiency virus. *J Am. Osteopath. Assoc*. 104, 527-35, ISSN 0098-6151

O'Farrell, N. (2002). Donovanosis. *Sex. Transm. Infect*., 78, 452-457, ISSN 1472-3263

Palefsky, J. M. (2010) Human papillomavirus-related disease in men: not just a women's issue. *J. Adolescent Health*, 46, S12-S19, ISSN 1054-139X

Schautteet, K., De Clercq, E. & Vanrompay, D. (2011). *Chlamydia trachomatis* vaccine research through the years. *Infect. Dis. Obstet. Gynecol*. 2011, 963513, ISSN 1098-0997

Ward, H. & Rönn, M. (2011). The contribution of STIs to the sexual transmission of HIV. *Curr. Opin. HIV AIDS*, 5, 305-310, ISSN 1746-630X

World Health Organization. (2001). *Global prevalence and incidence of selected curable sexually transmitted infections: Overview and estimates*, WHO Press, Geneva

World Health Organization. (2003). *Guidelines for the management of sexually transmitted infections*, WHO Press, ISBN 9241546263, Geneva,

World Health Organization. (2005). *Sexually transmitted and other reproductive tract infections*, WHO Press, ISBN 9241592656, Geneva,

World Health Organization. (2007a). *Global Strategy for the prevention and control of sexually transmitted infections: 2006-2015*, WHO Press, ISBN 9789241563475, Geneva

World Health Organization. (2007b). *Training modules for the syndromic management of sexually transmitted infections, 2nd edition*, WHO Press, ISBN 9241593407, Geneva

World Health Organization. (2011). *Global health sector strategy on HIV/AIDS 2011-2015*, WHO Press, ISBN 9789241501651, Geneva

Zhu, W., Chen, C.-J., Thomas, T. E., Anderson, J. E., Jerse, A. E. & Sparling P. F. (2011). Vaccines for gonorrhea: can we rise to the challenge. *Front. Microbiol*., 2, 124, ISSN 1664-302X

27

Re-Emergence of Malaria and Dengue in Europe

Rubén Bueno Marí and Ricardo Jiménez Peydró
Laboratorio de Entomología y Control de Plagas, Inst. Cavanilles de Biodiversidad y Biología Evolutiva, Universitat de València
Spain

1. Introduction

Currently, the emergence/reemergence of several vector-borne diseases in Europe is one of the most important threats for Public Health. In recent years, it is well known that global change have led to drastic modifications in the eco-epidemiology of various tropical and subtropical diseases. Global change can be defined as the impact of human activity on the fundamental mechanisms of biosphere functioning. Therefore, global change includes not only climate change, but also habitats transformation, water cycle modification, biodiversity loss, synanthropic incursion of alien species into new territories or introduction of new chemicals in nature. Consequently a holistic approach is a key factor to assessing the likelihood of vector-borne diseases transmission in Europe. Among these vectors, culicid mosquitoes are probably the most important because of its large vectorial capacity and its high degree of opportunism (Table 1).

Vector species	Distribution	Indigenous/exotic	Vectorial capacity
Ae. aegypti	Madeira (Portugal), The Netherlands	Exotic (recently imported)	Dengue (DEN), Yellow Fever (YF), Chikungunya (CHIK), West Nile (WN), Japanese encephalitis (JE), Saint-Louis encephalitis (SLE), La Crosse encephalitis (LACE), Murray valley encephalitis (MVE), Western equine encephalitis (WEE), Eastern equine encephalitis (EEE), Venezuelan equine encephalitis (VEE), Myxomatosis (MYX), Avian Malaria (AMAL), Dirofilariasis (DF)
Ae. albopictus	Mediterranean area, Central Europe	Exotic (first reported in Albania in 1979)	DEN, YF, CHIK, WN, JE, SLE, LACE, WEE, EEE, VEE, Jamestown Canyon (JC), Sindbis (SIN), Tahyna (TAH), DF

Vector species	Distribution	Indigenous/exotic	Vectorial capacity
Ae. vexans	All over Europe	Indigenous	WN, TAH, Tularaemia (TU), DF
Ae. vittatus	Spain, Portugal, France, Italy	Indigenous	DEN, YF, CHIK, AMAL
An. algeriensis	Mediterranean area, Eastern Europe, Central Europe, United Kingdom	Indigenous	Malaria (MAL)
An. claviger s.l.	All over Europe	Indigenous	MAL, WN, Batai (BAT), TAH, MYX, Anaplasmosis (ANA), Borreliosis (BO), TU, DF
An. maculipennis s.l.	All over Europe	Indigenous	MAL, WN, BAT, TAH, MYX, TU, DF
An. plumbeus	All over Europe	Indigenous	MAL, WN, DF
An. sergentii	Sicily (Italy)	Indigenous	MAL
An. superpictus	Southeastern Europe	Indigenous	MAL, DF
Cx. pipiens s.l.	All over Europe	Indigenous	WN, SIN, Usutu (USU), TAH, AMAL, DF
Oc. atropalpus	Italy, France, The Netherlands.	Exotic (first reported in Italy in 1996)	WN, JE, SLE, LACE, MVE, WEE, EEE
Oc. caspius	All over Europe	Indigenous	WN, TAH, MYX, TU, DF
Oc. japonicus	France, Belgium, Switzerland, Germany	Exotic (recently imported)	WN, JEV, SLE, LACE, EEE
Oc. triseriatus	Intercepted in a batch of used tyres imported from Louisiana (USA) to France in 2004	Exotic (not yet known as established)	DEN, YF, WN, SLE, LACE, WEE, EEE, VEE, JC

Table 1. Mosquito vectors in Europe with indication of distribution, indigenous or exotic status and vectorial capacity in each case.

2. Malaria

Malaria was a widespread disease in the whole of Europe until the second half of 20th century. The anthroponosis, often called "marsh fever" in the past, was particularly devastating between XVI and XIX centuries in Southern Europe due to the boom of irrigation techniques based on long flooding periods (e.g. rice fields). Several environmental modifications (mainly the drainage of swamps, moats, ditches and other stagnant waters), but particularly the availability of efficient synthetic antimalarial drugs and improved mosquito control activities including DDT spraying after World War II, have led to the disappearance of malaria from Europe (Bruce-Chwatt & de Zulueta, 1980). However, although *Anopheles* populations were significantly reduced by different control methods, in most cases, the vectors were not eradicated.

Today malaria annually affects 500 million people and threatens directly or indirectly 40% of world population (World Health Organization [WHO], 2007). However it is well known that these morbidity and mortality data show an asymmetric distribution, mainly depending on the economical, social and sanitary level of each country or region. The disease is endemic in much of Africa and several countries of Asia, Central America and South America. In Europe, the cycles of malaria transmission are relatively common in Georgia, Azerbaijan, Kyrgyzstan, Tajikistan, Uzbekistan and Turkey (WHO, 2010). This mosquito-borne parasitaemic disease is caused by protozoa of the genus *Plasmodium*. Although the simian parasite *Plasmodium knowlesi* (Knowles and Das. Gupta 1932) has been found recently as a cause of human malaria in Southeastern Asia (Luchavez et al., 2008), other four plasmodia species are the most recognized to infect humans in nature conditions: *Plasmodium falciparum* (Welch, 1897), *Plasmodium vivax* (Grassi & Feletti, 1890), *Plasmodium malariae* (Feletti & Grassi, 1889) and *Plasmodium ovale* (Stephens, 1922). About 90% of malaria mortality is caused by tropical strains of *P. falciparum* (most pathogenic species), which is also the species of *Plasmodium* most frequently imported to Europe (European Network on Imported Infectious Disease Surveillance [TropNetEurop], 2010). Furthermore, *P. vivax* shows the largest distribution range because it may also develop in temperate climates, being consequently the only species currently present in the cycles of transmission in Europe. Finally, *P. malariae* and *P. ovale* are characterized by its narrow distribution range and low parasitemia. Regarding to malaria vectors, there are about 40 *Anopheles* species with an important role in disease transmission (Kiszewski, 2004).

2.1 Malariogenic potential of Europe

The increasing of imported malaria cases in last decades, together with the high presence of anophelines in many Southern Europe regions (Romi et al., 1997; Ponçon et al., 2007; Bueno Marí & Jiménez Peydró, 2010a), has enabled the appearance of several autochthonous malaria cases, as recently has occurred in countries like Italy (Baldari et al., 1998), Greece (Kampen et al., 2002), France (Doudier et al., 2007) or Spain (Santa-Olalla Peralta et al., 2010). This situation forces us to investigate the possible reemergence of malaria in the current context of global change. One of the best methods to deep into the knowledge of possible malaria reemergence is the study of the malariogenic potential, which can be analyzed from the study of the receptivity, infectivity and vulnerability parameters (Romi et al., 2001; Bueno Marí & Jiménez Peydró, 2008).

2.1.1 Receptivity

Receptivity could be analyzed by the presence, density, and biological characteristics of vectors. At respect, the estimation of the Vectorial Capacity (VC) is postulated as a very useful tool to assess the receptivity of a determined territory in a concrete moment (Carnevale & Robert, 2009). The VC could be estimated by the MacDonald formula (MacDonald, 1957) according to the modifications proposed by Garrett-Jones (1964):

$$VC = ma2\ p^n\ /\ -\ln p$$

Where, m represents the relative vector density (number of vectors per man), *a* refers to human-biting frequency (number of human blood meals per vector and per day), *p* is the daily survival rate (life expectancy of the female mosquito) and *n* alludes to duration of the sporogonic cycle (length in days of the latent period of the parasite in the mosquito, i.e. extrinsic incubation cycle). It is important to note that *ma* is usually measured by collecting mosquitoes during an entire night using human bait. Consequently VC could be defined as the future daily sporozoite inoculation rate arising from a currently infective human case, on the assumption that all female mosquitoes biting that person become infected (Githeko, 2006). Of course VC changes from site to site, from vector to vector, and within and between transmission seasons.

2.1.1.1 Malaria receptivity in Southern Europe

Because of climatic conditions, the Southern Europe represents the territory of the Old Continent where disease cycles can be completed more likely. In terms of receptivity, of twenty species of *Anopheles* described in Europe twelve are confined in its distribution to Southern areas (Table 2). In the Iberian Peninsula rice cultivation was clearly associated with malaria endemicity until the beginning of the 20th century (Cambournac & Hill, 1938; Cambournac, 1939, Blázquez, 1974; Bueno Marí & Jiménez Peydró, 2010b). In these larval biotopes the species *Anopheles atroparvus* and, to a much lesser extent and only in the more arid areas, *Anopheles labranchiae* were supposed to be the major malaria vectors (Bruce-Chwatt & de Zulueta, 1977), although some other species, such as *Anopheles maculipennis* or *Anopheles claviger* may locally also have contributed to disease transmission (Bueno Marí, 2010). Currently *An. atroparvus* remains widespread in rice fields and other potential *Anopheles* breeding sites of Portugal and Spain (Capinha et al. 2009; Sainz-Elipe et al. 2010), since the most important western Mediterranean malaria vector *An. labranchiae* is considered dissapeared. *An. labranchiae* was found to be abundant in a restricted area of the contiguous Alicante and Murcia Provinces (South-eastern Spain) in 1946 (Clavero & Romeo Viamonte, 1948), but had disappeared by 1973 (Blázquez & de Zulueta, 1980) probably due to abandonment of rice cultivation in this area (Eritja et al., 2000). Recent surveys carried out in this area have revealed again the absence of *An. labranchiae* as well as high populations of the secondary vector *Anopheles algeriensis* also characterized by high domiciliation degrees (Bueno Marí, 2011). This was the only area where *An. labranchiae* has been able to establish itself in the Iberian Peninsula (Blázquez & de Zulueta, 1980). Though abundant along the African coastline between Ceuta and Tangiers, *An. labrachiae* has been unable to obtain a toe-hold in 15 km distant coastal plains of southern Spain, where rice fields support large populations of *An. atroparvus* (Ramsdale & Snow, 2000). It is important to note that the most important vector of the Iberian Peninsula *An. atroparvus* is suspected of being the vector of an autochthonous case of *Plamodium vivax* which recently occurred in Northeastern Spain (Santa-Olalla Peralta et al., 2010) and even also in other case of *Plamodium ovale* which happened in Central Spain, although airport malaria cannot be discarded in this last case due to the proximity of the patient's residence to two international airports (Cuadros et al., 2002).

Anopheles Species	European distribution	Malaria outbreaks
An. algeriensis	Brit, Ire, Fra, Cors, Spain, Bala, Port, Ger, Aust, Ital, Sard, Sic, Croa, Alb, Gree, Turk, Hung, Bulg, Moldv, Ukr, EurRus, Est	Argelia (non demonstrated vector in Europe)
An. atroparvus	Brit, Ire*, Swe, Den, Fra, Spain, Port, Belg, Neth, Ger, Aust, Czech, Slovk, Pol, Switz[a], Ital, Ser-Mon, Croa, Bosn, Slovn, Mace, Hung, Rom, Bulg, Moldv, Ukr, Bela, EurRus, Lith, Latv	Northern Europe, Central Europe, Eastern Europe, Mediterranean Europe
An. beklemishevi	Swe, Fin, EurRus	-
An. cinereus	Spain, Port	-
An. claviger	Brit, Ire, Nor, Swe, Den, Fra, Cors, Spain, Port, Belg, Neth, Lux, Ger, Aust, Czech, Slovk, Pol, Switz, Ital, Sic, Ser-Mon, Croa, Bosn, Slovn, Mace, Alb, Gree, Turk, Cypr, Hung, Rom, Bulg, Moldv, Ukr, Bela, EurRus, Lith, Latv, Est	Eastern Mediterranean countries, Central Asia
An. daciae [b]	Brit, Rom	-
An. hyrcanus	Fra, Cors, Spain, Ital, Sard, Sic, Ser-Mon, Croa, Mace, Alb, Gree, Turk, Hung, Rom, Bulg, Moldv, Ukr, EurRus	Asia (as An. hyrcanus s.l.)
An. labranchiae	Cors, Ital, Sard, Sic, Croa	France (Corsica), Italy (Peninsular Italy, Sardinia and Sicily), Southeastern Spain (disappeared since 1973)
An. maculipennis	Nor, Swe, Den, Fra, Cors, Spain, Port, Belg, Neth, Lux*, Ger, Aust, Czech, Slovk, Pol, Switz[a], Ital, Sic, Ser-Mon, Croa, Bosn, Slovn, Mace, Alb, Gree, Turk, Hung, Rom, Bulg, Moldv, Ukr, Bela, EurRus, Lith, Latv, Est	Coastal areas in the Balkans, Asia Minor, Northern Iran
An. marteri	Cors, Spain, Port, Ital, Sard, Sic, Alb, Gree, Turk, Bulg	-
An. melanoon [c]	Fra, Cors, Spain, Ital, Rom, EurRus	-
An. messeae	Brit, Ire[a], Nor, Swe, Den, Fra, Cors, Belg, Neth, Ger, Aust, Czech, Slovk, Pol, Switz[a], Ital, Ser-Mon, Croa, Bosn, Slovn, Mace, Alb, Gree, Hung, Rom, Bulg, Moldv, Ukr, Bela, EurRus, Lith, Latv, Est	Eastern Europe

An. multicolor	Spain	-
An. petragnani	Fra, Cors, Spain, Port, Ital, Sard, Sic	-
An. plumbeus	Brit, Ire, Swe, Den, Fra, Cors, Spain, Port, Belg, Neth, Lux, Ger, Aust, Czech, Slovk, Pol, Switz, Ital, Sic, Ser-Mon, Croa, Bosn, Slovn, Mace, Alb, Gree, Turk, Hung, Rom, Bulg, Ukr, Bela, EurRus, Lith, Est	England, Germany, Caucasus
An. pulcherrimus [d]	Turk	Middle East
An. sacharovi	Cors, Ser-Mon, Croa, Mace, Alb, Gree, Turk, Bulg, EurRus	Near East
An. subalpinus [c]	Fra, Cors, Port, Ser-Mon, Croa, Mace, Alb, Gree, Turk, Bulg, EurRus	Albania, Greece
An. sergentii	Sic	Mediterranean Africa
An. superpictus	Cors, Ital, Sic, Ser-Mon, Croa, Mace, Alb, Gree, Turk, Bulg, EurRus	Middle East

Note 1: Countries with anophelines records considered as doubtful or sporadic were not included. If it is thought that the species has been eradicated, the country is also not listed. *Note 2*: Brit (Britain), Ire (Ireland), Nor (Norway), Swe (Sweden), Den (Denmark), Fra (France), Cors (Corsica), Spain, Bala (Balearic Islands), Port (Portugal), Belg (Belgium), Neth (Netherlands), Lux (Luxemburg), Ger (Germany), Aust (Austria), Czech (Czech Republic), Slovk (Slovakia), Pol (Poland), Switz (Switzerland), Ital (Italy), Sard (Sardinia), Sic (Sicily), Malt (Malta), Ser-Mon (Serbia-Montenegro), Croa (Croatia), Bosn (Bosnia), Slovn (Slovenia), Mace (Macedonia), Alb (Albania), Gree (Greece), Turk (Turkey), Cypr (Cyprus), Hung (Hungary), Rom (Romania), Bulg (Bulgaria), Moldv (Moldavia), Ukr (Ukraine), Bela (Belarus), EurRus (Eropean Russia), Lith (Lithuania), Latv (Latvia), Est (Estonia).

[a] Records referred to *Anopheles maculipennis* s.l.

[b] Species recently described by molecular and morphological techniques.

[c] There is confusion with these two species.

[d] Present in Asiatic Turkey.

Table 2. *Anopheles* species with endemic presence in Europe and indication of historical data about its vectorial role (Ramsdale & Snow, 2000; Schaffner et al., 2001; Beck et al., 2003; Nicolescu et al., 2004; Linton et al., 2005; Becker et al., 2010; European Mosquito Taxonomists [MOTAX], 2010).

In France, the same two species mentioned above for the Iberian Peninsula, are also considered to be primary malaria vectors because of their abundance and their potential anthropophily: *An. atroparvus* in continental France and *An. labranchiae* in Corsica. In a former malaria-endemic area of Southern France, intensive samplings conducted recently in rice fields showed that *Anopheles hyrcanus* seems to be the only potential vector likely to play a role in malaria transmission in view of its abundance and anthropophily (Ponçon et al., 2007). Since 1994 several cases of vivax and falciparum malaria with no history of international travels, blood transfusion or injection drug use have been reported in Southern France (Delmont et al., 1994; Baixench et al., 1998; Doudier et al., 2007). In Corsica, where *An. labranchiae* still present in high densities in different regions (Toty et al., 2010), autochthonous *P. vivax* malaria transmission has been diagnosed, probably via the bite of a local *Anopheles* mosquito infected with *P. vivax* from a patient who had acquired infection in Madagascar (Armengaud et al., 2006). The second most important malaria vector of Corsica, *Anopheles sacharovi*, has not been detected in the island since 2002 (Toty et al., 2010).

Until the beginning of dichlorodiphenyltrichloroethane (DDT) application, the main malaria vectors in Italy were *An. superpictus* as well as two species of the *Anopheles maculipennis* complex: *An. labranchiae* and *An. sacharovi* (Hackett & Missiroli 1935). Despite *An. labranchiae* used to breed in various types of waters, such as marshes, streams, small pools or irrigation channels, the rice fields established in the 1970s currently represent its most important larval habitats in Central Italy (Bettini et al., 1978; Romi et al., 1992). Even in Western province of Grosseto *An. labranchiae* has replaced *Anopheles melanoon*, species that in 1970 represented for 100% of the anophelines fauna (Majori et al., 1970). Precisely in Grosseto region occurred the last autochthonous malaria case in Italy in August 1997 (Baldari et al., 1998). Nowadays of the anopheline species that have been vectors of malaria in Italy, only *An. labranchiae* and *An. superpictus* are still present in epidemiologically relevant densities (Romi et al., 1997). Moreover *An. atroparvus* is also present in Italy at low densities and *An. sacharovi* is currently considered disappeared, since last specimens of the vector were found 50 years ago (Sepulcri, 1963).

In Balkan countries (Bulgaria, Romania, Croatia, Serbia, Bosnia-Herzegovina, Montenegro and Albania, among others) the species *An. sacharovi* used to be the main malaria vector in coastal areas while *An. superpictus* and *An. maculipennis* were the primary vectors in inland areas due to the specific adaptations of their preimaginal stages (Hackett, 1937; Hadjinicolaou & Betzios, 1973; Bruce-Chwatt & de Zulueta, 1980). Larvae of *An. sacharovi* are tolerant against brackish water but not against salt water. On the other hand *An. superpictus* breeds in slowly flowing waters in hilly areas while *An. maculipennis* breeds in stagnant inland waters (Jetten & Takken, 1994). However, when sporadically *An. maculipennis* has colonized coastal areas of Balkans, Asia Minor and Northern Iran, it has also showed an important role in malaria transmission (Postiglione et al., 1973; Zaim, 1987; Manouchehri et al., 1992; Schaffner et al., 2001). Of the three most important vectors of Balkans, *An. superpictus* was never collected in Romania. Therefore in this country in addition to *An. sacharovi* and *An. maculipennis*, also *Anopheles messeae* and *An. atroparvus* have contributed to the endemism of malaria. Generally *An. messeae* has played a prominent role as a malaria vector in the Danube Valley and Delta, while *An. maculipennis* was mainly responsible for malaria transmission in the Romanian plains and *An. sacharovi* and *An. atroparvus* have been primary vectors at the Black Sea coast (Zotta, 1938; Zotta et al., 1940; Ciuca, 1966). All these issues represent the concept of "malaria stratification", which indicates a good relation between the distribution of the different anophelines species and the great "malaria geographic lines" (Nicolescu, 1996). Moreover a new species of the *An. maculipennis* complex, named *Anopheles daciae*, was recently first described in Romania (Nicolescu et al., 2004). It seems likely that *An. daciae* could be widespread in Eastern Europe and the Balkan States, and also could be responsible for malaria transmission in these regions that is currently attributed to *An. messeae*.

In order of relevance, *An. sacharovi*, *An. superpictus* and *An. maculipennis* were considered the main malaria vectors in Greece (Belios, 1955, 1978). During the recent years several autochthonous cases of *P. falciparum*, *P. malariae* and *P. vivax* have been diagnosed in Northern Greece (Kampen et al., 2002). At respect, it is important to note the proximity of this region to an unstable malaria country as Turkey. In Turkey malaria is still one of the most important vector-borne diseases in Turkey (Kasap et al., 2000; Alten et al., 2003), even remaining some endemic areas with hundreds of vivax cases yearly. The most important vectors in Turkey are *An. sacharovi* and *An. superpictus* (Kuhn et al., 2002), taking *An. maculipennis*, *An. claviger* and *Anopheles hyrcanus* a secondary role in malaria transmission.

If we analyze the VC of European anophelines we can extract several conclusions. In Spain the populations of *An. atroparvus* were deeply studied by several authors basically during the endemic period (Buen de, 1931, 1932; Buen de & Buen de, 1930, 1933; Torres Cañamares, 1934; Olavarria & Hill, 1935; Lozano Morales, 1946; Zulueta de, 1973; Blázquez, 1974). The estimation of VC shows that *An. atroparvus* was an important malaria vector in different wetlands of Spain mainly during summer months. The VC was especially high for *P. vivax* (in August VC=0.7–21.2) which has a shorter sporogonic cycle than *P. falciparum* (in August VC=0.2-5.3). In September VC values were lower for both *P. vivax* (VC=0.2–9.2) and *P. falciparum* (VC=0.04-2.3) and in October VC values were drastically reduced, but still relevant in the case of *P. vivax* (VC *P. vivax*=0.01-2.1 / VC *P. falciparum*=0.00007-0.02) (Bueno Marí & Jiménez Peydró, 2012). These results are similar to others derivates from different entomological researches carried out in Italy more recently. During August 1994 in Tuscany (Grosseto Province) were reported for *An. labranchiae* VC values ranging from 8.3-32.5 for *P. vivax* and 7.3-26 for *P. falciparum* (Romi et al., 1997). However VC was very low in early July, constituting no real risk for malaria transmission (<0.01 for both *P. vivax* and *P. falciparum*). Subsequently during 1998 in the same province but in areas where only natural anopheline breeding sites were reported, the VC of *An. labranchiae* from mid-July through the end of August ranged from 0.96-3.3 for *P. vivax* and 0.8-2.9 for *P. falciparum* (Romi, 1999). In other Mediterranean areas (North of Morocco), VC of *An. labranchiae* for *P. vivax* also showed high values during summer months (in July VC=17.2; in August=34; in September=18.3), while values from April to June were lower ranging from 0.5-3.7 (Faraj et al., 2008). On the other hand the average VC of *An. sacharovi* was found to be 0.22 (VC ranging from 0.63-0.014) in an endemic area of Southeastern Turkey (Tavşanoğlu & Çağlar, 2008). These last low VC values were probably related with very low percentages of human blood meals by anophelines.

Accordingly, although of course all these values of VC are purely theoretical, it is important to note that can be numerically shown that summer (from July to September, but especially in August) is an excellent season for malaria transmission, at least at receptivity level, in Southern Europe.

2.1.1.2 Malaria receptivity in Northern Europe

Endemic northern malaria reached to 68°N latitude in Europe during the 19th century, where the summer mean temperature only irregularly exceeded 16°C. It is important to note that precisely 16ºC is considered the lower limit needed for sporogony of *P. vivax* (Garnham, 1988). In Finland *Anopheles beklemishevi* has a northern distribution, while the other common species, *An. messeae*, is dominant in the southern part of the country (Gutsevich et al., 1974; Lokki et al., 1979; Kettle, 1995). Both species are known as an important malaria vectors (White, 1978). Despite other potential vectors, such as *An. claviger* and *An. maculipennis* have been observed (Utrio, 1979; Dahl, 1997), it is not possible to define certainly which mosquito species was most important for the malaria transmission in Finland. This is because temperature conditions of Finland, as well as in other northern countries, should have caused that malaria transmission have mainly occurred in indoor conditions due to transmission of sporozoites throughout the winter by semiactive hibernating mosquitoes (Huldén et al., 2005), since it is well known that in warm conditions the overwintering females of *Anopheles* can take several blood meals (Ekblom & Ströman, 1932; Encinas Grandes, 1982). Therefore, the best malaria vectors in Northern Europe will be those anthropophilic and endophagic anophelines which present hibernating females with

semiactive winter habits but not a complete diapause. In conclusion, northern malaria existed in a cold climate by means of summer dormancy of *P. vivax* hypnozoites in addition to the indoor feeding activity of overwintering *Anopheles* females previously mentioned.

In other Scandinavian countries such as Sweden or Denmark, besides the anophelines which has been mentioned above, there have been described other potential malaria vectors: *An. atroparvus* and *Anopheles plumbeus* (Ramsdale & Snow, 2000). Although *An. messeae* was probably the main vector during the malaria epidemics in Sweden, some authors proposed that *An. atroparvus* may have maintained malaria endemicity in certain coastal localities in the south of the country (Jaenson et al., 1986). Regarding to *An. plumbeus* there are several aspects that should be pointed to understand the increasing epidemiological importance of the species in Central Europe. *An. plumbeus* is the only hole breeding species of the genus *Anopheles* in Europe. Although it is a strictly dendrolimnic species, during dry periods females can also lay the eggs in small domestic and peridomestic containers, as well as other artificial breeding sites below the ground such as catch basins and septic tanks with water contaminated with organic waste (Bueno Marí & Jiménez Peydró, 2011). There are several reports in Europe about the presence of larvae in a biotope different from the tree cavity (Aitken, 1954; Senevet et al., 1955; Rioux, 1958; Tovornik, 1978; Bueno Marí & Jiménez Peydró, 2010a). Moreover, remarkable populations can also be found in urban situations, where the larvae develop in tree holes in gardens and parks, especially in Central Europe where *An. plumbeus* has increased in numbers during the last decades and can be a major nuisance species (Becker et al., 2010). This is a very important issue, because the continuous development of this species in urban environments could increase considerably the possibilities of interaction between malaria vectors and humans. In fact, *An. plumbeus* has been suspected to be responsible for two recorded cases of locally transmitted malaria in London, United Kingdom (Blacklock, 1921; Shute, 1954) and other two cases recently reported in Duisburg, Germany (Krüger et al., 2001). Of the five *Anopheles* species present in Britain only two, *An. atroparvus* and *An. plumbeus*, have been confirmed as malaria vectors in United Kingdom (James, 1917; Shute, 1954), while *An. messae* and *An. atroparvus* were the vectors involved in vivax epidemics occurred in Germany during the 20th Century (Kirchberg & Mamlok, 1946).

Therefore, it exists in Europe a latitudinal gradient in relation to the distribution of the species of the *An. maculipennis* complex. Without ignoring the possible participation of several species in malaria transmission cycles, the fact is that in Northern Europe (including European Russia) at 68ºN *An. beklemishevi* prevails as vector, being this species replaced by *An. messae* partially at 63ºN and fully about 59ºN. Around 56ºN *An. atroparvus* begins to acquire an important role in disease transmission and already in Mediterranean countries the situation of malaria receptivity is basically governed by *An. atroparvus*, *An. labranchiae* and *An. sacharovi* in Eastern, Central and Western Mediterranean respectively. As was previously pointed, this situation can be locally modified by the presence of other potential vectors widely distributed in Europe such as *An. claviger*, *An. hyrcanus*, *An. maculipennis* or *An. plumbeus*. Of course climate change could drastically modify not only the distribution of European anophelines, but also their phenology and overwintering patterns. However the changes in agricultural practices have a greater effect on the risk of malaria than an elevation in temperature of approximately 2°C (Becker, 2008), which is considered the average increasing temperature in Europe in next 50 years. Hence habitat modification is probably the factor with more influence in possible changes in malaria receptivity all over Europe.

2.1.2 Infectivity

Infectivity is defined as the degree of susceptibility of *Anopheles* mosquitoes to different *Plasmodium* species, i.e. refers to the possibilities that the sporogonic cycle of parasite could be completed within a concrete vector species. It is well known that mosquito populations of the same species but different geographic areas can differ drastically at infectivity level due to genetic reasons (Frizzi et al., 1975).

Infectivity tests carried out on European populations of species of the *An. maculipennis* complex showed that *An. atroparvus* can transmit Asian strains of *P. vivax* and African strains of *P. ovale* but is refractory to African strains of *P. falciparum* (James et al., 1932; Garnham et al., 1954; Ramsdale & Coluzzi, 1975; Teodorescu, 1983; Ribeiro et al., 1989). However, more recent studies have shown the ability of *An. atroparvus* to generate oocysts of *P. falciparum* (Marchant et al., 1998), but not to complete sporogony. Information about *An. labranchiae* is quite confusing due to the scanty and old infectivity tests conducted. Moreover laboratory studies have revealed that *An. labranchiae* can transmit *P. ovale* (Constantinescu & Negulici, 1967) but populations of the vector collected in Italy were refractory to African strains of *P. falciparum* (Ramsdale & Coluzzi, 1975; Zulueta de et al., 1975). Nevertheless recent researches with populations from Corsica have indicated that *P. falciparum* cycle can be successfully completed in *An. labranchiae* (Toty et al., 2010). Furthermore *An. labranchiae* has been involved in transmission of autochthonous vivax malaria cases and in Corsica (France), Greece and Italy (Sautet & Quilici, 1971; Zahar, 1987; Baldari et al., 1998) and even several outbreaks of *P. falciparum*, *P. malariae* and *P. vivax* in Morocco (Houel & Donadille, 1953). Under laboratory conditions, *An. sacharovi* has been demonstrated as an excellent vector of *P. vivax* (Kasap, 1990) and *An. messeae* was reported, not only as being the main vector of malaria over a large part of European Russia several decades ago (Detinova, 1953), but also the responsible of disease resurgence in Russia and Ukraine more recently (Nikolaeva, 1996). With regard to *An. maculipennis* it is known that in certain coastal areas in the Balkans, Asia Minor and Northern Iran (Postiglione et al., 1973; Zaim, 1987; Manouchehri et al., 1992), the species has participated actively in malaria transmission cycles. Due to its recent description, *An. daciae* yet must be tested on its susceptibility to *Plasmodium* species

Outside the species of the *An. maculipennis* complex is remarkable that European populations of *An. plumbeus* can produce sporozoites of tropical strains of *P. falciparum* (Marchant et al., 1998; Eling et al., 2003), as well as also Eurasiatic strains of *P. vivax* (Shute & Maryon, 1974). Even some authors suggest that *An. plumbeus* is capable of transmitting the four Plasmodium species (Shute & Maryon, 1969). However this hypothesis should be confirmed with modern molecular techniques. Respect to *An. algeriensis* and *An. claviger*, it is important to note that in natural populations it has been shown the presence of oocysts of *P. vivax* at intestinal level (Blacklock & Carter, 1920; Horsfall, 1972). In the case of *An. algeriensis*, even has been successfully tested the transmission of *P. falciparum* in laboratory conditions (Becker et al., 2010). *An. superpictus* can transmit *P. vivax* (Kasap, 1990) but its susceptibility to *P. falciparum* has not been tested, although this anopheline is probably sensitive, as it belongs to the subgenus *Cellia*, to which the principal African malaria vectors also belong. Another species of the subgenus *Cellia* poorly represented in Europe, such as *Anopheles multicolor* and *Anopheles sergentii*, have been also found parasitized by *P. vivax* and *P. falciparum* in natural conditions (Kenawy et al., 1990). Finally, there is no infectivity information about *An. marteri, An. cinereus* and *An. petragnani*. Anyway the epidemiological role of these species it seems secondary due to their zoophylic behaviour and rural distribution.

2.1.3 Vulnerability

Vulnerability is determined by the number of gametocyte carriers (malaria patients) during the suitable period for malaria transmission. If we analyze the data about imported malaria in Europe in recent years we can extract several conclusions. Malaria represents about 77% of tropical diseases imported in Europe (TropNetEurop, 2010). A total of 65.596 cases were reported in Europe between 2000 and 2009 (Table 3). However this number is clearly underestimated, since in last years the number of malaria reporting sites in Europe has increased significantly. Most of these cases are referred to immigrants (48.5%), and *P. falciparum* (81%) was the dominant species in analytic results. A high percentage of malaria cases in immigrants correspond to Visiting Friends and Relatives (VFR). This group of special epidemiological significance refers to those people who, once are established in their host countries, often travel to their origin countries to visit family or friends. Travels that these people can do to their origin countries exponentially increase the chances of disease contracting, since usually these areas are endemic regions and the stay within resident population and their customs is often long and intense (Gascón, 2006). Therefore this is an important collective to promote the need to take appropriate prophylactic measures during travels to endemic areas. Several studies have revealed that only 16% of VFR search for medical advice pre-travel, being malaria prophylaxis practically nonexistent in this collective (Leder et al., 2006). The European countries with higher number of imported malaria cases reported yearly are France and Germany, usually followed by other like Spain, Italy or Belgium. As it was shown before, malaria receptivity is remarkable in concrete regions of these countries.

	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	Total
Cases (sites reporting)	1120 (32)	3313 (38)	4555 (47)	5561 (44)	6536 (47)	7411 (50)	8544 (50)	8904 (52)	9509 (57)	10.143 (59)	65.596
P. falciparum	78.4%	70.0%	77.6%	82.4%	81.2%	81.6%	87.8%	82.8%	83.9%	84.0%	81%
P. vivax	11.5%	13.9%	11.7%	10.4%	11.2%	10.2%	7.5%	8.3%	8.2%	8.6%	10.1%
P. ovale	5.2%	5.3%	3.4%	3.1%	3.5%	4.4%	2.8%	4.3%	3.9%	3.1%	3.9%
P. malariae	2%	5.9%	4.3%	1.5%	1.5%	1.7%	1%	1.2%	1.5%	2.3%	2.3%
Unkn./Coinf.	2.9%	4.9%	3.1%	2.7%	2.4%	2.2%	0.9%	3.4%	2.7%	4.4%	2.7%
Imm./Refu.	30.5%	35.4%	44.8%	50.2%	54%	54.6%	52.8%	50.8%	55.2%	56.7%	48.5%
For. Vis.	11.8%	14.6%	7.5%	9.1%	7%	9.2%	10.3%	3.3%	5.1%	9.2%	8.7%
Eur. E.C.	53.8%	44.4%	38.9%	30.6%	30%	26.1%	26.2%	35.2%	34%	26.4%	34.5%
Eur. Exp.	3.9%	5.6%	8.8%	11.1%	9%	10.1%	10.7%	10.7%	5.7%	7.7%	8.3%

Note 1: Unkn./Coinf. (Plasmodium species unknown or coinfection of various species), Imm./Refu. (Immigrants/Refugees), For. Vis. (Foreign Visitors), Eur. E.C. (Europeans living in EC), Eur. Exp. (European Expatriates).

Table 3. Imported malaria in Europe between 2000-2009 (TropNetEurop, 2010).

The temporal distribution analysis of imported malaria cases indicates that high-risk months for disease transmission (between July and September) also coincides with the period of the most cases reported in Europe. Therefore most of cases occur during the epoch theoretically favorable for malaria transmission. In regard to the diagnostic delay, i.e. the average time between appearance of symptoms and malaria diagnosis (when therapy began), it shows disparate values according to each country. For example, in Eastern Spain the diagnostic delay of imported malaria was estimated in 13.7 days (Bueno Marí & Jiménez Peydró, 2012), while in other European countries like Sweden, France or Italy values are clearly lower, ranging from 3 to 8.2 days (Romi et al., 2001; Askling et al., 2005; Chalumeau et al., 2006). From an epidemiological point of view it is very important to reduce the diagnostic delay, because this is the period when malaria patients could be a source of infection for *Anopheles* females. Additionally, from an exclusively clinical perspective, delay to diagnosis leads of course to high parasitemia, which itself leads to severe forms of malaria.

3. Dengue fever and yellow fever

There are many similarities between dengue fever and yellow fever:

- Both are viruses of the genus Flavivirus (family Flaviviridae) and are strictly primatophilic, infecting only primates, including man.
- In their original habitat, both are zoonotic infections transmitted by forest mosquitoes.
- Their importance as human pathogens can be related with two forest mosquitoes characterized by high ecological plasticity that have become closely associated with the peridomestic environment.
- Both diseases have a history of transmission in temperate regions, including Europe, and share essentially the same selvatic and urban vectors.
- Transovarian transmission in female mosquitoes has been demonstrated for both viruses.
- The viruses and their urban vectors have a worldwide distribution due to transportation of goods and people.
- Both arboviruses are characterized by short incubation period and can provoke similar clinical symptoms, including hemorrhagic illness in humans, often with fatal consequences. However mortality rate is higher in yellow fever (20%) than in dengue (5%).

In the case of dengue fever its annual incidence has increased dramatically around the world in recent decades. It is estimated that over 2500 millions people who live in over 100 tropical and non-tropical countries, are currently at risk from dengue viruses globally. The rise in dengue incidence has been marked by geographic expansion of the virus and the vectors due to globalization, habitat modifications, lack of effective mosquito control programs and climate change. Although the major disease burden occurs in South East Asia, the Americas and the western Pacific, dengue was also a common disease in Europe in the past centuries. Large epidemics of dengue and yellow fever occurred in European ports of Spain, Portugal, France, Italy and even Wales and Ireland as the more northern countries of the continent (Eager, 1902; Monath, 2006). Last dengue epidemic in Europe, estimated at one million cases, occurred in Greece in 1927-28 (Papaevangelou & Halstead, 1977; Rosen, 1986).

Dengue is the most frequent tropical arboviruses imported in Europe and together with schistosomiasis both are considered, after malaria, the most important tropical diseases in

quantitative terms in Old continent. Of the hundreds of dengue imported cases reported yearly en Europe (Table 4), the vast majority are represented by tourists (about 84%). A difference to what happens with malaria, immigration (9%) seems to have comparatively little influence on dengue importation. This could be explained, of course by distinct perspectives and approaches of European tourists (e.g. travels to urbanized areas) and immigrants who come to Europe (e.g. Africa, where malaria is much prevalent than dengue, is the main origin from immigrants who arrive to Europe), but also by differences between incubation periods and existing prophylactic measures in both diseases. All dengue cases reported have shown the typical symptomatology of disease, including febrile symptoms in more than 90% of cases (TropNetEurop, 2010). However, it is important to note that the majority of imported dengue infections remain undiagnosed, with a ratio between symptomatic and asymptomatic travelers estimated in 1/3.3 (Cobelens et al., 2002). In general terms, it is estimated that about 80% of all dengue infections are asymptomatic (Farrar, 2008). This high asymptomatic, added to the fact that dengue is not a notifiable disease in much of European countries (Bueno Marí & Jiménez Peydró, 2010c; 2010d), allow us to consider that the knowledge of dengue virus circulation is very limited.

	2001	2002	2003	2004	2005	2006	2007	2008	2009	Total
Cases (sites reporting)	477 (37)	664 (47)	742 (46)	852 (48)	1023 (51)	1167 (50)	1273 (53)	1419 (57)	1553 (61)	9170
Imm./Refu.	10%	5.5%	8.2%	12.9%	6.8%	10.5%	9%	6.8%	11.3%	9%
For. Vis.	0.8%	0.5%	1%	0%	1.2%	4.8%	2.2%	0.8%	2.4%	1.5%
Eur. E.C.	86.7%	91.3%	79.6%	81.9%	87.7%	77.1%	81.3%	87.3%	83.9%	84.1%
Eur. Exp.	2.5%	2.7%	11.2%	5.2%	4.3%	7.6%	7.5%	5.1%	2.4%	5.4%

Note 1: Imm./Refu. (Immigrants/Refugees), For. Vis . (Foreign Visitors), Eur. E.C. (Europeans living in EC), Eur. Exp. (European Expatriates).

Table 4. Imported dengue in Europe between 2001-2009 (TropNetEurop, 2010).

Aedes aegypti is the primary urban vector of dengue and yellow fever basically because it exist a 'domesticated' form of the species that is rarely found more than 100 m from human habitation and feeds almost exclusively on human blood (Reiter, 2010). Both factors allow that *Ae. aegypti* will be considered as an excellent urban vector of viruses. Its distribution was traditionally limited by latitude between 45º N and 35º S according to the existence of January and July 10° C isotherms. Although records out of this latitude range are very rarely, it must be pointed that European northernmost collection of the species occurred in Brest (France) at 48º N (Christopher, 1960). Moreover recent studies have demonstrated that *Ae. aegypti* larvae can withstand temperatures of 2.5° C (Chang et al., 2007). In Eastern Europe it was also seen at its temperature limit at Odessa (Ukraina) at 46º N. (Korovitzkyi and Artemenko, 1933). Despite the species was relatively common in Mediterranean countries, it disappeared from the entire region in the mid-20th century, for reasons that currently are not clear but probably related with thermic tolerance and intensive mosquito

control campaigns with the employment of DDT. *Ae. aegypti* was common in the Iberian Peninsula mainly introduced from North Africa and was present in this Southern European region up to 1956 (Ribeiro & Ramos, 1999). Since the eradication of the species in Europe, its sporadic presence has been recognized in several countries, namely Britain, France, Italy, Malta, Croatia, Ukraine, Russia and Turkey (Snow & Ramsdale, 1999). However it must be pointed that the species has been reported in Madeira (Portugal) in 2005 (Margarita et al., 2006) and it seems that *Ae. aegypti* is now deeply established in this region because of continuous collections in later years (Almeida et al., 2007). This is the first report of the establishment of the species in Europe since mid-20th century. More recently *Ae. aegypti* has been also captured in The Netherlands (Scholte et al., 2010). In summary, we must pay some attention to surveillance and behavior of *Ae. aegypti* because globalization is provoking the arrival of the species to Europe and global warming could allow the definitive establishment of the species again in Southern areas.

On the other hand the situation is clearly divergent in regard to the secondary vector of dengue and yellow fever, *Aedes albopictus*, usually known as Asian tiger mosquito, due to its quick expansion in Europe in last years. There are several ecological factors that can help us to understand the different importance of *Ae. aegypti* and *Ae. albopictus* as primary and secondary vectors of human viruses respectively. Unlike patterns of oviposition and feeding exhibited by *Ae. aegypti*, Asian tiger mosquito is often abundant in the peridomestic environment, particularly in areas with plentiful vegetation, and feeds freely on humans and other animals. Consequently *Ae. albopictus* can also exist far from human habitation. Additionally *Ae. aegypti* has been globally dispersed from Africa by humans activities since several centuries ago while *Ae. albopictus* was firstly report out of its original Asiatic distribution range in 1979 in Albania (Adhami & Reiter, 1998). Current data indicate that *Ae. albopictus* has been detected much farer north than *Ae. aegypti* and one major difference between both species is that Asian tiger mosquito has the ability to adapt to cold temperatures by becoming dormant during the winter of temperate regions. The ability of *Ae. albopictus* to resist cold temperatures is partially related with its ability to synthesize a high amount of lipids, especially to produce larger amounts of yolk lipid in cold temperatures. At respect, it was demonstrated that larval lipogenesis of *Ae. albopictus* is much more efficient than that of *Ae. aegypti* (Briegel & Timmermann, 2001). Although *Ae. albopictus* occurs in both temperate and tropical areas, only temperate population, but not tropical ones, show a photoperiodic diapauses (Hawley, 1988). During the shortening daylight hours in late summer/early autumn, the reduced photoperiod stimulates the females of *Ae. albopictus* to produce eggs that enter facultative diapause (Estrada-Franco & Craig 1995). These eggs can resist hatching stimuli until the following spring and remain in a state of reduced morphogenesis as fully formed first instar larvae, exhibiting increased resistance to environmental extremes. Although the diapause occurs in the egg stage, only adults and pupae are known to be photoperiodically sensitive stages (Wang, 1966; Imai & Maeda, 1976; Mori et al, 1981).

Ae. albopictus has been found to be capable to transmit 26 viruses (Moore & Mitchell, 1997; Gratz, 2004; Paupy et al., 2009) and to be experimentally susceptible to several filariasis of veterinary interest (Cancrini et al., 1995; Nayar & Knight, 1999). Globalization has allowed the arrival of this species to Europe, mainly through the transport of eggs and larvae in used tires and gardening products (Reiter & Sprenger, 1987; Madon et al., 2002). The presence of Asian tiger mosquito has been confirmed in 16 European countries, but only in Southern ones the species is deeply established. Particularly interesting is the situation of Italy, where

the species was firstly detected in 1990 (Sabatini et al., 1990) and nowadays has colonized more than 2/3 parts of the territory, even having different areas of the country with mosquitoes densities in considerable epidemiological levels. Precisely these locally high densities have allowed the appearance of first cases of human viruses in Europe transmitted by *Ae. albopictus*. Specifically, in the province of Ravenna (Northeastern Italy) occurred an outbreak of Chikungunya virus in 2007. This virus is very similar to dengue and yellow fever (same vectors, bioecology and symptomatology), but much less pathogenic. Just in two and a half months, a total of 205 cases of Chikungunya were reported in two small towns of Ravenna where the infection of *Ae. albopictus* was also confirmed (Rezza et al., 2007). This outbreak of Chikungunya infection, outside a tropical country, was probably begun by a man from India, country that previous year had suffered an epidemic with more than 1 million cases (Ravi, 2006). The Indian man developed a febrile syndrome two days after his arrival in Italy and also had high titres of antibodies against Chikungunya. The phylogenetic analysis showed that the strain that caused Italian outbreak was similar to the strains detected on the Indian subcontinent (Yergolkar et al., 2006), showing in all cases a better adaption to *Ae. albopictus* than other variants. However most worrying scenario took place in 2010 with the re-appearance of first autochthonous cases of dengue in Europe transmitted by *Ae. albopictus*. In this year, two cases of autochthonous dengue fever were diagnosed in Nice (Southeast France) (La Ruche et al., 2010), region where *Ae. albopictus* is established at least since 2004 (Delaunay et al., 2007). Just days after two indigenous cases of Chikungunya in the districts of Alpes-Maritime and Var (also in Southeastern France) were detected through a routinely surveillance of dengue and Chikungunya (ECDC, 2010), which is yearly conducted since 2006 due to the establishment of *Ae. albopictus* in this region. In Greece, other Mediterranean country where *Ae. albopictus* is established at least since 2004 (Klobucar et al., 2006), two cases of indigenous dengue were diagnosed also in 2010 (Schmidt-Chanasit et al., 2010; Gjenero-Margan et al., 2011). The identification of these cases of dengue fever and Chikungunya occurred in 2010, which were in all cases well clustered in space and time, is strongly suggestive that autochthonous transmission of tropical viruses in Europe is ongoing.

According to these epidemiological perspectives it seems evident that there is a need to be able to predict the potential distribution and activity of *Ae. albopictus* in Europe to asses about possible re-emergence of dengue and other tropical arboviruses. At respect several Geographic Information Systems (GIS) have been developed in order to predict the number of weeks of activity of *Aedes albopictus* (ECDC, 2009). These GIS models have revealed that throughout much of Europe, more than 23 weeks are predicted to elapse between egg hatching in spring (in response to at least 11.25 hours of daylight and 10.5° C of mean temperature) and adult die-off in autumn (below critical temperature threshold of 9.5° C). Assuming that immature development takes about 2–4 weeks, this constitutes more than 20 weeks of adult activity in Central Europe and Southern United Kingdom, even increasing this activity to more than 40 weeks in southern areas (mainly Greece, Turkey and south of Iberian and Italic Peninsula), depending on availability of surface water for breeding. If these predictions would be fulfilled in Southern Europe, consequently could increase the speed of spread of the species, could also extend the episodes of medical and social alerts derivates from its feeding behavior in urban areas, and even could change the eco-epidemiology of viruses that *Ae. albopictus* can transmit.

It must be pointed that *Ae. albopictus* and *Ae. aegypti* are not the only aedine vectors with invasive behavior in Europe. Other exotic mosquitoes, such as *Ochlerotatus japonicus* and

Ochlerotatus atropalpus, have been also reported. *Oc. japonicus* is an Asian species and a competent vector of several arboviruses, including West Nile virus and Japanese encephalitis virus and is considered a significant public health risk (Sardelis & Turell, 2001; Sardelis et al., 2002a; 2002b; 2003). *Oc. japonicus* has been collected only in France, Belgium, Switzerland and Germany (Schaffther et al., 2003; 2009; Becker et al., 2011). On the other hand *Oc. atropalpus* is endemic to North America and has been observed in Italy, France and Netherlands (Romi et al., 1997; Adege-EID Méditerranée, 2006; Scholte et al., 2009). Although in the field, *Oc. atropalpus* has not been evidenced as an important vector of infectious diseases, under laboratory conditions, the species has been proven as a competent vector for West Nile virus, Japanese encephalitis virus, Saint-Louis encephalitis virus La Crosse encephalitis virus, among other arboviruses (King, 1960; Turell et al., 2001). Globalization, especially traffic of used tires, has led the arrival of *Oc. japonicus* and *Oc. atropalpus* to Europe. Out of these exotic vectors, we can not forget or ignore the presence of potential indigenous vectors of dengue and yellow fever in Europe. For example, *Aedes vittatus* is an important vector of yellow fever in different parts of Africa (Lewis, 1943; Satti & Haseeb, 1966) and also a potential vector of Chikungunya and four dengue serotypes (Mourya & Banerjee, 1987; Mavale et al., 1992). Although the species is deeply distributed in Mediterranean region (Spain, Portugal, France and Italy), the studies about its biology and phenology have been scanty in Europe. Anyway it seems unlikely that *Ae. vittatus* could start a cycle of virus transmission to humans because of its high degree of ruralism. Moreover *Ochlerotatus geniculatus* is a dendrolimnic species endemic to Europe that can efficiently transmit yellow fever, but this possibility has been evidenced only in laboratory conditions (Roubaud et al., 1937).

3.1 New challenges: The development of dengue vaccines

Although a vaccine based on live attenuated virus of the strain 17D is available for yelow fever since years, currently we haven´t any vaccine to be used with full warranty against dengue. However, the need for a dengue vaccine is clear. The most effective measures of an integrated mosquito control program (including changes in human habitation and behavior, the use of insecticides, and long-lasting modification of natural and man-made mosquito habitats) are difficult to implement and largely unsuccessful in most poverty-stricken settings, and consequently have not been carried out comprehensively enough to limit dengue's spread. While vector control is an integral part of any dengue prevention strategy, it is not enough on its own.

In recent years it has been obtained a better understanding of the disease and its etiopatogenicity, as well as of the necessary aspects to develop a vaccine that provides an effective and lasting protection against the virus. Dengue vaccine development is a very difficult task due to the possible participation of four related serotypes, since immunity to one serotype does not confer immunity to the remaining three. Complicating the scenario further is immune enhancement, which can result in severe dengue hemorrhagic fever or dengue shock syndrome in anyone who has been infected with one of the serotypes and subsequently becomes infected with another. Most of researchers agree that only effective solution is a tetravalent vaccine that simultaneously protects against all four serotypes. Regarding to this, it must be noted that tetravalent vaccines against dengue are currently in last phases of trials and is expected to be available for human population in the next following years.

4. Conclusions

Although malaria's receptivity is still high in different parts of Europe, we may conclude that the malariogenic potential of the Old Continent is low. Fortunately socio-economic and sanitary conditions of most European countries also support this assertion. While it is true that infectivity studies should be further promoted, percentages of imported malaria cases remain very low. However we must pay some attention to the increasing trend of malaria importation in last years, as well as also awareness among tourists and VFR's for to take corresponding prophylactic measures during their travels to endemic areas. Anyway, sporadic and local cases of autochthonous transmission mainly transmitted by *An. atroparvus, An. labranchiae, An. sacharovi* and/or *An. plumbeus*, can not been discarded in next years.

On the other hand, the answer to the question about if should be expected the re-emergence of dengue and other mosquito-borne tropical viruses in Europe in next years is indubitable: definitively yes. The arrival, establishment and expansion of dengue urbanite vectors due to global changes such as globalization, climate change and the lack of effective mosquito control programs, together with the increasing of imported cases in humans provokes that local and intense transmission of dengue could be a reality in next years in Southern Europe. To cope this possibility is necessary to enhance the entomological surveillance in potential areas of mosquitoes importation, such as airports or seaports, strength the monitoring of tropical viruses imported and awareness among citizens about their role in mosquito control and best prophylactic measures to take during the travels to tropical regions.

5. Acknowledgments

We wish to acknowledge that current work was partially funded by the Research Project CGL 2009-11364 (BOS), supported by the Ministry of Science and Innovation of Spain (Ministerio de Ciencia e Innovación del Gobierno de España).

6. References

Adege-EID Méditerranée. (2006). *Éléments entomologiques relatifs au risque d'apparition du virus Chikungunya en métropole* . Entente interdépartementale pour la démoustication du littoral (EID) Méditerranée, Montpellier, France.

Adhami, J.R. & Reiter, P. (1998). Introduction and establishment of *Aedes (Stegomyia) albopictus* Skuse (Diptera: Culicidae) in Albania. *Journal of American Mosquito Control Association*, Vol.14, No.3, (September 1998), pp. 340-343, ISSN 1046-3607.

Aitken, T.G.H. (1954). The Culicidae of Sardinia and Corsica (Diptera). *Bulletin of Entomological Research*, Vol.45, No.3, (September 1954), pp. 437-494, ISSN 0007-4853.

Almeida, A.P.; Gonçalves, Y.M.; Novo, M.T.; Sousa, C.A.; Melim, M. & Gracio AJ. (2007). Vector monitoring of *Aedes aegypti* in the Autonomous Region of Madeira, Portugal. *Euro Surveillance*, Vol.12, No.46, (November 2007), ISSN 1560-7917, Available online in: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=3311

Alten, B.; Çağlar, S.S.; Şimşek, F.M. & Kaynas, S. (2003). Effect of insecticide-treated bednets for malaria control in Southeast Anatolia - Turkey. *Journal of Vector Ecology*, No.28, Vol.1, (June 2003), pp. 97-107, ISSN 1081-1710. Date of submission:

Armengaud, A.; Legros, F.; Quatresous, I.; Barre, H.; Valayer, P.; Fanton, Y.; D' Ortenzio, E. & Schaffner, F. (2006). A case of autochthonous *Plasmodium vivax* malaria, Corsica, August 2006. *Euro Surveillance*, Vol.11, No.46, (November 2006), ISSN 1560-7917, Available online in: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=3081

Askling, H.H.; Ekdahl, K.; Janzon, R.; Henric Braconier, J.; Bronner, U.; Hellgren, U.; Rombo, L. & Tegnell, A. (2005). Travellers returning to Sweden with falciparum malaria: pre-travel advice, behaviour, chemoprophylaxis and diagnostic delay. *Scandinavian Journal of Infectious Diseases*, Vol.37, No.10, (October 2005), pp. 760-765, ISSN 0036-5548.

Baixench, M.T.; Suzzoni-Blatger, J.; Magnaval, J.F.; Lareng, M.B. & Larrouy, G. (1998). Two cases of inexplicable autochthonous malaria in Toulouse, France. *Medecine tropicale : revue du Corps de sante colonial*, Vol.58, No.1, (January 1998), pp. 62–64, ISSN 0025-682X.

Baldari, M.; Tamburro, A.; Sabatinelli, G.; Romi, R.; Severini, C., Cuccagna, P.; Fiorilli, G.; Allegri, M.P.; Buriani, C. & Toti, M. (1998). Introduced malaria in Maremma, Italy, decades after eradication. *The Lancet*, Vol.351, No.9111, (April 1998), pp. 1246-1248, ISSN 0140-6736.

Beck, M.; Galm, M.; Weitzel, T.; Fohlmeister, V.; Kaiser, A.; Arnold, A. & Becker, N. (2003). Preliminary studies on the mosquito fauna of Luxembourg. *European Mosquito Bulletin*, Vol.14, (January 2003), pp. 21-24. ISSN 1460-6127.

Becker, N. (2008). Influence of climate change on mosquito development and mosquito-borne diseases in Europe. Parasitology Research, Vol.103, No.1, (January 2008), 103:19–28, ISSN 0932-0113.

Becker, N.; Petric, D.; Zgomba, M.; Boase, C.; Madon, M.; Dahl, C. & Kaiser, A. (2010). *Mosquitoes and Their Control*. 2 nd ed. Springer, ISBN 978-3-540-92873-7, Berlin, Deutschland.

Becker, N.; Huber, K.; Pluskota, B. & Kaiser, A. (2011). Ochlerotatus japonicus japonicus – a newly established neozoan in Germany and a revised list of the German mosquito fauna. *European Mosquito Bulletin,* Vol.29 (April 2011), pp. 88-102, ISSN 1460-6127.

Belios, G.D. (1955). Recent course and current pattern of malaria in relation to its control in Greece. *Rivista di Malariologia*, Vol.34, pp. 1-24, ISSN 0370-565X.

Belios, G.D. (1978). From malaria control to eradication: problems and solutions. *Archeion Hygiene Athens*, Vol.27, pp. 54-59.

Bettini, S.; Gradoni, L.; Cocchi, M. & Tamburro, A. (1978). Rice culture and *Anopheles labranchiae* in Central Italy. WHO unpublished document, WHO/MAL 78.897, WHO/VBC series 78.686, Geneva, Switzerland.

Blacklock, B. (1921). Notes on a case of indigenous infection with *P. falciparum*. *Annals of Tropical Medicine and Parasitology*, Vol.15, pp. 59-72, ISSN 0003-4983.

Blacklock, B. & Carter, H.F. (1920). The experimental infection in England of *Anopheles plumbeus*, Stephens, and *Anopheles bifurcatus*, L., with *Plasmodium vivax*. *Annals of Tropical Medicine and Parasitology*, Vol.13, No.4, (March 1920), pp. 413-420, ISSN 0003-4983.

Blázquez, J. (1974). Investigación entomológica sobre anofelismo en el delta del Ebro. *Revista de Sanidad e Higiene Pública,* Vol.48, No.4 (April 1974), pp. 363-377, ISSN 0034-8899.

Blázquez, J. & Zulueta de, J. (1980). The disappearance of *Anopheles labranchiae* from Spain. *Parassitologia,* Vol.22, No.1-2, (January 1980), pp. 161-163, ISSN 0048-2951.

Briegel, H. & Timmermann, S.E. (2001). *Aedes albopictus* (Diptera: Culicidae): physiological aspects of development and reproduction, *Journal of Medical Entomology*, vol.38, No.4, (July 2001), pp. 566–571, ISSN 0022-2585.

Bruce-Chwatt, L.J. & Zulueta de, J. (1977). Malaria eradication in Portugal. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* Vol. 71, No.3, (March 1977), pp. 232-240, ISSN 0035-9203.

Bruce-Chwatt, L.J. & Zulueta de, J. (1980). *The Rise and Fall of Malaria in Europe*. Oxford University Press, ISBN 978-0198581680, New York, USA.

Buen de, E. (1931). Algunos estudios sobre biología del *Anopheles maculipennis* en lo que se refiere a la casa habitada por el hombre o animales. *Medicina de los Países Cálidos*, Vol.4, (September 1931), pp. 400-414.

Buen de, E. (1932). Algunos datos sobre la biología del *A. maculipennis* (*claviger*) en su fase de adulto. *Medicina de los Países Cálidos*, Vol.5, (November 1932), pp. 449-485.

Buen de, S. & De Buen, E. (1930). Notas sobre la biología del *A. maculipennis. Medicina de los Países Cálidos*, Vol.3, (September 1930), pp. 1-17.

Buen de, S. & Buen de, E. (1933). El *Anopheles maculipennis* y la casa; sus relaciones con la epidemiologia del paludismo en España. *Medicina de los Países Cálidos*, Vol.6, (July 1933), pp. 270-299.

Bueno Marí, R. (2011). El anofelismo en la Comunidad Valenciana: un ejemplo de estudio del potencial malariogénico de España. *Boletín de la Asociación española de Entomología*, Vol.35, No.1-2 (June 2011), pp. 47-83, ISSN 0210-8984.

Bueno Marí, R. (2010). *Bioecología, diversidad e interés epidemiológico de los culícidos mediterráneos (Diptera, Culicidae)*. Servei de Publicacions de la Universitat de València, ISBN 978-84-370-7987-5, Valencia, Spain.

Bueno Marí, R. & Jiménez Peydró, R. (2008). Malaria en España: aspectos entomológicos y perspectivas de futuro. *Revista Española de Salud Pública,* Vol.82, No.5, (September 2008), pp. 467-489, ISSN 1135-5727.

Bueno Marí, R & Jiménez Peydró, R. (2010a). New anopheline records from the Valencian Autonomous Region of Eastern Spain (Diptera: Culicidae: Anophelinae). *European Mosquito Bulletin,* Vol.28, (September 2010), pp. 148-156, ISSN 1460-6127.

Bueno Marí, R. & Jiménez Peydró, R. (2010b). Crónicas de arroz, mosquitos y paludismo en España: el caso de la provincia de Valencia (s. XVIII-XX). *Hispania* Vol.70, No.236, (September 2010), pp. 687-708, ISSN 0018-2141.

Bueno Marí, R. & Jiménez Peydró, R. (2010c). Situación actual en España y eco-epidemiología de las arbovirosis transmitidas por mosquitos culícidos (Diptera: Culicidae), Vol.84, No.3, (May 2010), pp. 255-269, ISSN 1135-5727.

Bueno Marí, R. & Jiménez Peydró, R. (2010d). ¿Pueden la malaria y el dengue reaparecer en España?, Vol.24, No.4, (July 2010), pp. 347-353, ISSN 0213-9111.

Bueno Marí, R & Jiménez Peydró, R. (2012). Study of the Malariogenic Potential of Eastern Spain. *Tropical Biomedicine,* in press.

Bueno Marí, R & Jiménez Peydró, R. (2011). *Anopheles plumbeus* Stephens, 1828: a neglected malaria vector in Europe. *Malaria Reports,* Available online in: http://www.pagepressjournals.org/index.php/malaria/article/view/malaria.2011.e2. ISSN 2039-4381.

Cambournac, F.J.C. (1939). A method for determining the larval *Anopheles* population and its distribution in rice fields. *Rivista di Malariologia* Vol.18, pp. 17-22, ISSN 0370-565X.

Cambournac, F.J.C. & Hill, R.B. (1938). The Biology of *Anopheles maculipennis* var. *atroparvu*s in Portugal. *Transactions of 3rd International Congress of Tropical Medicine & Malaria*. Vol.2, pp. 178-184.

Cancrini, G.; Pietrobelli, M.; Frangipane di Regalbono, A.F.; Tampieri, M.P. & della Torre, A. (1995). Development of *Dirofilaria* and *Setaria* nematodes in *Aedes albopictus*. *Parassitologia,* Vol. 37, No.2-3, (December 1995), pp. 141–145, ISSN: 0048-2951.

Capinha, C.; Gomes, E.; Reis, E.; Rocha, J.; Sousa, C.A.; Rosário, V.E..; Almeida, A.P. (2009). Present habitat suitability for *Anopheles atroparvus* (Diptera, Culicidae) and its coincidence with former malaria areas in mainland Portugal. *Geospatial Health* Vol.3, No.2, (May 2009), pp. 177-187, ISSN 1827-1987.

Carnevale, P. & Robert, V. (2009). *Les anophèles: Biologie, transmission du Plasmodium et lutte antivectorielle*, Bondy, IRD, ISBN 978-2-7099-1662-2, Montpellier, France.

Chalumeau, M.; Holvoet, L.; Chéron, G.; Minodier, P.; Foix-L'Hélias, L.; Ovetchkine, P.; Moulin, F.; Nouyrigat, V.; Bréart, G. & Gendrel, D. (2006). Delay in diagnosis of imported *Plasmodium falciparum* malaria in children. *European Journal of Clinical Microbiology & Infectious Diseases*, Vol.25, No.3, (March 2006), pp. 186-189, ISSN 0934-9723.

Chang, L.H.; Hsu, E.L.; Teng, H.J. & Ho C.M. (2007). Differential survival of *Aedes aegypti* and *Aedes albopictus* (Diptera: Culicidae) larvae exposed to low temperatures in Taiwan. *Journal of Medical Entomology*, vol.44, No.2, (March 2007), pp. 205-210, ISSN 0022-2585.

Christopher, S.R. (1960). *Aëdes aegypti (L.) the yellow fever mosquito, its life history, bionomics and structure*, Cambridge University Press, New York, United States of America..

Ciuca, M. (1966). *L'eradication de Paludisme en Roumanie*. Editions Medicales, Bucarest, Romania.

Clavero, G. & Romeo Viamonte, J.M. (1948). EI paludismo en las huertas de Murcia y Orihuela. Ensayos de aplicación de los insecticidas modernos, D.D.T. y 666, en la lucha antipalúdica. *Revista de Sanidad e Higiene Pública* Vol. 22, pp. 199-228, ISSN 0034-8899.

Cobelens, F.G.; Groen, J.; Osterhaus, A.D.; Leentvaar-Kuipers, A.; Wertheim-Van Dillen, P.M. & Kager, P.A. (2002). Incidence and risk factors of probable dengue virus infection among Dutch travellers to Asia. *Tropical Medicine and International Health*, Vol.7, No.4, (April 2002), pp. 331-338, ISSN 1360-2276.

Constantinescu, P. & Negulici, E. (1967). The experimental transmission of *Plasmodium malariae* to *Anopheles labranchiae atroparvus*. *Transactions of the Royal Society of Tropical Medicine and Hygiene,* Vol.61, No.2, pp. 182-188, ISSN 0035-9203.

Cuadros, J.; Calvente, M.J.; Benito, A.; Arévalo, J.; Calero, M.A.; Segura J. & Rubio, J.M. (2002). *Plasmodium ovale* Malaria acquired in Central Spain. *Emerging Infectious Diseases*, Vol.8, No.12, (December 2002), pp. 1506-1508, ISSN 1080-6040.

Dahl, C. (1997). *Diptera Culicidae, Mosquitoes*. In: *Aquatic Insects of northern Europe-A Taxonomic Handbook*. Vol.II, Nilsson, A.N. (Eds.), 163-186, Apollo Books, ISBN 8788757552, Stenstrup, Denmark.

Delaunay, P.; Mathieu, B.; Marty, P.; Fauran, P. & Schaffner, F. (2007). Historique de l' installation *d'Aedes albopictus* dans les Alpes-Maritimes (France) de 2002 à 2005. *Medecine tropicale*, Vol.67, No.3, (June 2007), pp. 310-311. ISSN 0025-682X.

Delmont, J.; Brouqui, P.; Poullin, P. & Bourgeade, A. (1994). Harbour-acquired *Plasmodium falciparum* malaria. *The Lancet*, Vol.344, No. 8918, (July 1994), pp. 330–331, ISSN 0140-6736.

Detinova, T.S. (1953). Age composition and epidemiological importance of the population of Anopheles maculipennis in the Province of Moscow. *Meditsinskaya Parazitologiya i Parazitarnie Bolezni*, Vol.22, pp. 486-495.

Doudier, B.; Bogreau, H.; De Vries, A.; Ponçon, N.; Stauffer, W.M. & Fontenille, D. (2007). Possible autochthonous malaria from Marseille to Minneapolis. *Emerging Infectious Diseases*, Vol.13, No.8, (August 2007), pp. 1236-1238, ISSN 1080-6059.

Eager JM. (1902). Yellow fever in France, Italy, Great Britain and Austria and bibliography of yellow fever in Europe. *Yellow Fever Institute Bulletin*, Vol.8, pp. 25-35.

European Center of Disease Control (ECDC). *EWRS Message: ID: 20100924FR0001*. 2010, Accessed 14th March 2011 Available online in: https://ewrs.ecdc.europa.eu/Pages/Secure/Messages/ViewMessage.aspx?id=20100924FR0001

European Center of Disease Control (ECDC). *Development of Aedes albopictus risk maps*. 2009, Accessed 14th March 2011 Available online in: http://ecdc.europa.eu/en/publications/Publications/0905_TER_Development_of_Aedes_Albopictus_Risk_Maps.pdf

Ekblom, T. & Ströman, R. (1932). Geographical and biological studies of *Anopheles maculipennis* in Sweden from an epidemiological point of view. *Kungliga Svenska Vetenskapsakademiens handling,* Vol.11, No.1, pp. 1-113.

Eling, W.; Van Gemert, G.J.; Akinpelu, O.; Curtis, J. & Curtis, C.F. (2003). Production of *Plasmodium falciparum* sporozoites by *Anopheles plumbeus*. *European Mosquito Bulletin*, Vol.15, (June 2003), pp. 12-13, ISSN 1460-6127.

Encinas Grandes, A. (1982). *Taxonomía y biología de los mosquitos del área salmantina (Diptera, Culicidae)*, Universidad de Salamanca, ISBN 8400050673, Salamanca, Spain.

Eritja, R.; Aranda, C.; Padrós, J.; Goula, M.; Lucientes, J.; Escosa, R.; Marquès, E. & Cáceres, F. (2000). An annotated checklist and bibliography of the mosquitoes of Spain (Diptera: Culicidae). *European Mosquito Bulletin*, Vol.8, (November 2008), pp. 10-18, ISSN 1460-6127.

Estrada-Franco, J.G. and Craig, G.B. (1995). *Biology, disease relationships, and control of Aedes albopictus*, Pan American Health Organization, ISBN 9275130426, Washington, United States of America.

European Mosquito Taxonomists (MOTAX). *Chart of European Mosquitoes*. 2010, Accessed 14th March 2011 Available online in: http://www.sove.org/motax/chart.htm

European Network on Imported Infectious Disease Surveillance (TropNetEurop). *Friend and observers Sentinel Surveillance Report.* 2010, Accessed 14th March 2011 Available online in: http://www.tropnet.net/reports_friends/reports_friends_index.html

Faraj, C.; Ouahabi, S.; Adlaoui, E.; Boccolini, D.; Romi, R. & El Aouad, R. (2008). Risque de réémergence du paludisme au Maroc étude de la capacité vectorielle d'*Anopheles*

labranchiae dans une zone rizicole au nord du pays. *Parasite*, Vol.15, No.4, (December 2008), pp. 605–610, ISSN 1252-607X.

Farrar, J. (2008). *Clinical features of dengue*. In: *Dengue*, Halstead, S.B. (Ed.), 171-191, Imperial College Press, ISBN: 978-1-84816-228-0, London, United Kingdom.

Frizzi, G.; Rinaldi, A. & Bianchi, L. (1975). Genetic studies on mechanisms influencing the susceptibility of Anopheline mosquitoes to plasmodial infections. *Mosquito News*, Vol.35, No.4, (December 1975), pp. 505-508, ISSN 0027-142X.

Garnham, P.C.C.; Bray, R.S.; Cooper, W.; Lainson, R.; Awad, F.I. & Williamson, J. (1954). Pre-erythrocytic stages of human malaria: *Plasmodium ovale*. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, Vol.49, No.1, (January 1954), pp. 158-167, ISSN 0035-9203.

Garnham, P.C.C. (1988). *Malaria parasites of man: life-cycles and morphology (excluding ultrastructure)*. In *Malaria: principles and practice of malariology*, Wernsdorfer W.H. & McGregor, I. (Ed), Vol. I, 61-96, Churchill Livingstone, ISBN 0443024170, Edinburgh, United Kingdom.

Garrett-Jones, C. (1964). Prognosis for Interruption of Malaria Transmission Through Assessment of the Mosquito's Vectorial Capacity. *Nature* Vol.204, (December 1964), pp. 1173-1175, ISSN 0028-0836.

Gascón, J. (2006). Paludismo importado por inmigrantes. *Anales del Sistema Sanitario de Navarra*, Vol.29, No.1, (January 2006), pp. 121-125, ISSN 1137-6627.

Githeko, A.K. (2006). *Entomological correlates of epidemiological impacts: how do we know it is working?* In: *Bridging Laboratory and Field Research for Genetic Control of Disease Vectors*, Knols, B.G.J., Louis, C. & Bogers, R.J. (Eds.), 215-219, Springer, ISBN 1-4020-3799-6, Dordrecht, Netherlands.

Gjenero-Margan, I.; Aleraj, B.; Krajcar, D.; Lesnikar, V.; Klobučar, A.; Pem-Novosel, I.; Kurečić-Filipović, S.; Komparak, S.; Martić, R.; Đuričić, S.; Betica-Radić, L.; Okmadžić, J.; Vilibić-Čavlek, T.; Babić-Erceg, A.; Turković, B.; Avšić-Županc, T.; Radić, I.; Ljubić, M.; Šarac, K.; Benić, N. & Mlinarić-Galinović, G. (2011). Autochthonous dengue fever in Croatia, August–September 2010. *Euro Surveillance*, Vol.16, No.9, (March 2011), ISSN 1560-7917, Accessed 14th March 2011, Available online in:
http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19805

Gratz, N.G. (2004). Critical review of the vector status of *Aedes albopictus*. *Medical and Veterinary Entomology*, Vol.18, No.3, (September 2004), pp. 215–227, ISSN 0269-283X.

Gutsevich, A.V.; Monchadskii, A.S. & Shtakelberg, A.A. (1974). *Fauna of the USSR. Diptera. Mosquitoes. Family Culicidae.* Vol.3, No.4., Keter Publishing House Jerusalem Ltd, ISBN 7065 1475, Jerusalem, Israel.

Hackett, L.W. (1937). *Malaria in Europe: An Ecological Study*. Oxford University Press, London, United Kingdom.

Hackett, L.W. & Missiroli, A. (1935). The varieties of *Anopheles maculipennis* and their relation to the distribution of malaria in Europe. *Rivista di Malariologia*, Vol.14, pp. 45-109, ISSN 0370-565X.

Hadjinicolaou, J. & Betzios, B. (1973). Resurgence of *Anopheles sacharovi* following malaria eradication. *Bulletin of the World Health Organization*, Vol.48, No.6, pp. 699-703, ISSN 0042-9686.

Hawley, W.A. (1988). The biology of *Aedes albopictus*. *Journal of the American Mosquito Control Association*, Vol.4, No.1, (March 1988), pp. 1–39, ISSN 1046-3607.

Horsfall, W.R. (1972). *Mosquitoes: their bionomics and relation to disease*. Hafner, New York, United States of America.

Huldén, L.; Huldén, L. & Heliövaara, K. (2005). Endemic malaria: an 'indoor' disease in northern Europe. Historical data analysed. *Malaria Journal*, Vol.4, No.4, (April 2005), ISSN 1475-2875, Accessed 14th March 2011 Available online in: http://www.malariajournal.com/content/4/1/19

Imai, C. & Maeda, O. (1976). Several factors effecting on hatching on *Aedes albopictus* eggs. *Japanese Journal of Sanitary Zoology*, Vol.27, pp. 363-372, ISSN 0424-7086.

Jaenson, T.G.T.; Lokki, J.; Saura, A. (1986). *Anopheles* (Diptera: Culicidae) and malaria in Northern Europe, with special reference to Sweden. *Journal of Medical Entomology*, Vol.23, No.1, (January 1986), pp. 68-75, ISSN 0022-2585.

James, S.P. (1917). Note recording the proof that *Anopheles maculipennis* is an efficient host of the benign tertian malaria parasite in England. *Journal of the Royal Army Medical Corps*, Vol.29, pp. 615, ISSN 0035-8665.

James, S.P.; Nicol, W.D. & Shute, P.G. (1932). *P. ovale* passage through mosquitoes and successful transmission by their bites. *Annals of Tropical Medicine and Parasitology*, Vol.26, pp. 139-145, ISSN 0003-4983.

Jetten, T.H. & Takken, W. (1994). *Anophelism Without Malaria in Europe: A review of the ecology and distribution of the genus Anopheles in Europe*. Wageningen Agricultural University Papers, Wageningen, The Netherlands, ISBN 906754373X.

Kampen, H.; Maltezos, E.; Pagonaki, M.; Hunfeld, K.P.; Maier W.A. & Seitz, H.M. (2002). Individual cases of autochthonous malaria in Evros Province, northern Greece: serological aspects. *Parasitology Research,* Vol.88, No.3, (March 2002), pp. 261-266, ISSN 0932-0113.

Kasap, H. (1990). Comparison of experimental infectivity and development of *Plasmodium vivax* in *Anopheles sacharovi* and *An. superpictus* in Turkey. *American Journal of Tropical Medicine and Hygiene*, Vol.42, No.2, (February 1990), pp. 111-117, ISSN 0002-9637.

Kasap, H.; Kasap, M.; Alptekin, D.; Lüleyap, U. & Herath, P.R.J. (2000). Insecticide resistance in *Anopheles sacharovi* Favre in southern Turkey. *Bulletin of the World Health Organization*, Vol.78, No.5, pp. 686-692, ISSN 0042-9686.

Kenawy, M.A.; Beier, J.C.; Asiago, C.M.: Said el S.E. & Roberts C.R. (1990). Interpretation of low-level Plasmodium infection rates determined by ELISA for anophelines (Diptera: Culicidae) from Egyptian oases. *Journal of Medical Entomology*, Vol.27, No.4, (July 1990), pp. 681-685, ISSN 0022-2585.

Kettle, D.S. (1995). *Medical and veterinary entomology*. 2nd edition. CAB International, ISBN: 0-851-98968-3, Cambridge, United Kingdom.

King, W.L.; Bradley, G.H.; Smith, C.N. & McDuffie, W.C. (1960). *A handbook of the mosquitoes of the southeastern United States*. *Handbook 173*. US Department of Agriculture, Washington, United States of America.

Kirchberg, E. & Mamlok, E. (1946). Malariabekämpfung in Berlin im Jahre 1946. *Ärztl Wochenschr*, Vol.1, pp. 119–122, ISSN 0365-6403.

Kiszewski, A.; Mellinger, A.; Spielman, A.; Malaney, P.; Sachs, S.E. & Sachs, J. (2004). Global Index. Representing the Stability of Malaria Transmission. *American Journal of*

Tropical Medicine and Hygiene, Vol.70, No.5, (May 2004), pp. 486-498, ISSN 0002-9637.

Klobucar, A.; Merdic, E.; Benic, N.; Baklaic, Z. & Krcmar, S. (2006). First record of *Aedes albopictus* in Croatia. *Journal of the American Mosquito Control Association,* Vol.22, No.1, (March 2006), pp. 147-148, ISSN 1046-3607.

Korovitzkyi, L.K. & Artemenko, V.D. (1933). Zur Biologie des *Aedes aegypti*. *Magasin de parasitologie de l'Institut zoologique de l'Acad´emie des Sciences de l'URSS*, Vol.2, pp. 400-406.

Krüger, A.; Rech, A.; Su, X.Z. & Tannich, E. (2001). Two cases of autochthonous *Plasmodium falciparum* malaria in Germany with evidence for local transmission by indigenous *Anopheles plumbeus*. *Tropical Medicine & International Health*, Vol.6, No.12, (December 2001), pp. 983-985, ISSN 1360-2276.

Kuhn, K.G.; Campbell-Lendrum, D.H. & Davies, C.R. (2002). A continental risk map for malaria mosquito (Diptera: Culicidae) vectors in Europe. *Journal of Medical Entomology*, Vol.39, No.4, (June 2002), pp. 621-630, ISSN 0022-2585.

Houel, G. & Donadille, F. (1953). Vingt ans de lutte antipaludique au Maroc. *Bulletin de l'Institut d'Hygiène du Maroc*, Vol.13, pp. 3–51.

La Ruche, G.; Souarès, Y.; Armengaud, A.; Peloux-Petiot, F.; Delaunay, P.; Desprès, P.; Lenglet, A.; Jourdain, F.; Leparc-Goffart, I.; Charlet, F.; Ollier, L.; Mantey, K.; Mollet, T.; Fournier, J.P.; Torrents, R.; Leitmeyer, K.; Hilairet, P.; Zeller, H.; Van Bortel, W.; Dejour-Salamanca, D.; Grandadam, M. & Gastellu-Etchegorry, M. (2010). First two autochthonous dengue virus infections in metropolitan France, September 2010. *Euro Surveillance*, Vol.15, No.39, (September 2010), ISSN 1560-7917, Accessed 14th March 2011, Available online in: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19676

Leder, K.; Tong, S.; Weld, L.; Kain, K.C.; Wilder-Smith, A.; von Sonnenburg, F.; Black, J.; Grown, G.V. & Torresi, J. (2006). Ilness in travelers visiting friends and relatives: a review of the GeoSentinel Surveillance Network. *Clinical Infectious Diseases*, Vol.43, No.9, (November 2006), pp. 1185-1193, ISSN 1058-4838.

Lewis, D.J. (1943). Mosquitoes in relation to yellow fever in the Nuba Mountains Anglo-Egyptian Sudan. *Annals of Tropical Medicine and Parasitology*, Vol37, No.1, pp. 65-76, ISSN 0003-4983.

Linton, Y.M.; Lee, A.S. & Curtis, C. (2005). Discovery of a third member of the Maculipennis Group in SW England. *European Mosquito Bulletin*, Vol.19, (April 2005), pp. 5-9, ISSN 1460-6127.

Lokki, J.; Saura, A.; Korvenkontio, P. & Ulmanen, I. (1979). Diagnosing adult *Anopheles* mosquitoes. *Aquilo Seriologica Zoologica*, Vol.20, pp. 5-12.

Lozano Morales, A. (1946). Contribución al estudio de la biología del *A. maculipennis var. atroparvus* en función del ambiente. *Revista de Sanidad e Higiene Pública*, Vol.20, pp. 239-250, ISSN 0034-8899.

Luchavez, J.; Espino, F.; Curameng, P.; Espina, R.; Bell, D.; Chiodini, P.; Nolder, D.; Sutherland, C.; Lee, K.S. & Singh, B. (2008). Human Infections with *Plasmodium knowlesi*, the Philippines. *Emerging Infectious Diseases* Vol.14, No.5, (May 2008), pp. 811-813, ISSN 1080-6040.

MacDonald, G. (1957). *The epidemiology and control of malaria*. Oxford University Press, London, United Kingdom.

Madon, M.B.; Mulla, M.S.; Shaw, M.W.; Kluh, S. & Hazelrigg, J.E. (2002). Introduction of *Aedes albopictus* (Skuse) in southern California and potential for its establishment. *Journal of Vector Ecology*, Vol.27, No.1, (June 2002), pp. 149-154, ISSN 1081-1710.

Majori, G.; Maroli, M.; Bettini, S. & Pierdominici, G. (1970). Osservazioni sull'anofelismo residuo nel Grossetano. *Rivista di Parassitologia*, Vol.31, No.2, pp. 147-154, ISSN 0035-6387.

Manouchehri, A.V.; Zaim, M. & Emadi, A.M. (1992). A review of malaria in Iran, 1975–90. *Journal of the American Mosquito Control Association,* Vol.8, No.4, (December 1992), pp. 381-385, ISSN 1046-3607.

Marchant, P.; Rling, W.; Van Gemert, G.J.; Leake, C.J. & Curtis, C.F. (1998). Could British mosquitoes transmit falciparum malaria? *Parasitology Today*, Vol.14, No.9, (September 1998), pp. 344-345, ISSN 0169-4758.

Margarita, Y., Santos Grácio, A.J.; Lencastre, I.; Silva, A.C.; Novo, T.; Sousa, C.; Almeida, A.P.G. & Biscoito, M.J. (2006). Mosquitos de Portugal: primeiro registo de *Aedes (Stegomia) aegypti* Linnaeus, 1762 (Diptera, Culicidae) na Ilha da Madeira. *Acta Parasitológica Portuguesa*, Vol. 13, No.1, pp. 59-61, ISSN 0872-5292.

Mavale, M.S.; Ilkal, M.A. & Dhanda, V. (1992). Experimental studies on the susceptibility of *Aedes vittatus* to dengue viruses. *Acta Virologica*, Vol.36, No.3, (August 1992), pp. 412–416, ISSN 0001-723X.

Monath, T.P. (2006). Yellow fever as an endemic/epidemic disease and priorities for vaccination. *Bulletin de la Societe de Pathologie Exotique*, Vol.99, No.5, (December 2006), pp. 341-347, ISSN 0037-9085.

Moore, C.G. & Mitchell, C.J. (1997). *Aedes albopictus* in the United States: ten-years presence and public health implications. *Emerging Infectious Diseases*, Vol.3,No.3, (July 1997), pp. 329–334, ISSN 1080-6059.

Mori, A.; Oda, T. & Wada, Y. (1981). Studies on the egg diapause and overwintering of *Aedes albopictus* in Nagasaki. *Tropical Medicine*, Vol.23, No.2, (June 1981), pp. 79-90, ISSN 0385-5643.

Mourya, D.T. & Banerjee, K. (1987). Experimental transmission of chikungunya virus by *Aedes vittatus* mosquitoes. *Indian Journal of Medical Research*, Vol.86, (August 1987), pp. 269–271, ISSN 0971-5916.

Nayar, J.K. & Knight, J.W. (1999). *Aedes albopictus* (Diptera: Culicidae): an experimental and natural host of *Dirofilaria immitis* (Filarioidea: Onchocercidae) in Florida, U.S.A. *Journal OF Medical Entomology*, Vol.36, No.4, (July 1999), pp. 441–448, ISSN 0022-2585.

Nikolaeva, N. (1996). Resurgence of malaria in the former Soviet Union (FSU). Society of Vector Ecology News, Vol.27, pp. 10–11.

Nicolescu, G. (1996). George Zotta (1886-1942). An early concept of malaria stratification. *Romanian Archives of Microbiology and Immunology*, Vol.55, No.2, (April 1996), pp. 173-79, ISSN 1222-3891.

Nicolescu, G.; Linton, Y.M.; Vladimirescu, A.; Howard, T.M. & Harbach, R.E. (2004). Mosquitoes of the *An. maculipennis* group (Diptera; Culicidae) in Romania, with the discovery and formal recognition of a new species based on molecular and morphological evidence. *Bulletin of Entomological Research*, Vol.94, No.6, (December 2004), pp. 525-535, ISSN 0007-4853.

Olavarria, J. & Hill, R.B. (1935). Algunos datos sobre las preferencias hemáticas de las *A. maculipennis*. *Medicina de los Países Cálidos*, Vol.8, pp. 169–173.

Papaevangelou, G. & Halstead, S.B. (1977). Infections with two dengue viruses in Greece in the 20th century. Did dengue hemorrhagic fever occur in the 1928 epidemic? *Journal of Tropical Medicine & Hygiene*, Vol.80, No.3, (March 1977), pp. 46-51, ISSN 0022-5304.

Paupy, C.; Delatte, H.; Bagny, L.; Corbel, V. & Fontenille, D. (2009). *Aedes albopictus*, an arbovirus vector: From the darkness to the light. *Microbes and Infection*, Vol.11, No.14-15, (December 2009), pp. 1177-1185, ISSN 1286-4579.

Ponçon, N.; Toty, C.; L'Ambert, G.; Le Goff, G.; Brengues, C.; Schaffner, F. & Fontenille, D. (2007). Biology and dynamics of potential malaria vectors in Southern France. *Malaria Journal*, Vol.6, No.18 (February 2007), ISSN 1475-2875, Accessed 14th March 2011, Available online in: http://www.malariajournal.com/content/6/1/18

Postiglione, M., Tabanli, B. & Ramsdale, C.D. (1973). The Anopheles of Turkey. *Rivista di Parassitologia*, Vol.34, No.2, pp. 127-159. ISSN 0035-6387.

Ramsdale, C.D. & Coluzzi, M. (1975). Studies on the infectivity of tropical African strains of *Plasmodium falciparum* to some southern European vectors of malaria. *Parassitologia*, Vol.17, No.1-3, (January-December 1975), pp. 39-48, ISSN 0048-2951.

Ramsdale, C. & Snow, K. (2000). Distribution of the genus *Anopheles* in Europe. *European Mosquito Bulletin*, Vol.7, (July 2000), pp. 1-26, ISSN 1460-6127.

Ravi, V. (2006). Re-emergence of Chikungunya virus in India. *Indian journal of medical microbiology*, Vol.24, No.2, (April 2006), pp. 83-84, ISSN 0255-0857.

Reiter, P. & Sprenger, D. (1987). The used tire trade: a mechanism for the worldwide dispersal of container breeding mosquitoes. *Journal of the American Mosquito Control Association*, Vol.3, No.3, (September 1987), pp. 494-501, ISSN 1046-3607.

Reiter. P. (2010). Yellow fever and dengue: a threat to Europe? *Euro Surveillance*, Vol.15, No.10, (March 2010), ISSN 1560-7917, Accessed 14th March 2011, Available online in: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19509

Rezza, G.; Nicoletti, L.; Angelini, Romi, R.; Finarelli A.C.; Panning, M.; Cordioli, P.; Fortuna, C.; Boros, S.; Magurano, F.; Silvi, G.; Angelini, P.; Dottori, M.; Ciufolini, M.G.; Majori, G.C. & Cassone, A.. (2007). Infection with chikungunya virus in Italy: an outbreak in a temperate region. *The Lancet*, Vol.370, No.9602, (December 2007), pp. 1840-1846, ISSN 0140-6736.

Ribeiro, H.; Batista, J.L.; Ramos, H.C.; Pires, C.A.; Champalimaud, J.L.; Costa, J.M.; Araújo, C.; Mansinho, K. & Pina, M.C. (1989). An attempt to infect *Anopheles atroparvus* from Portugal with African *Plasmodium falciparum*. *Revista Portuguesa de Doenças Infecciosas*, Vol.12, pp. 81-82, ISSN 1646-3633.

Ribeiro, H. & Ramos, H.C. (1999). Identification keys of the mosquitoes of Continental Portugal, Açores and Madeira. *European Mosquito Bulletin*, Vol.3, (January 1999), pp. 1-11, ISSN 1460-6127.

Rioux, J.A. (1958). *Les Culicidés du Midi méditerranéen. Encyclopédie Entomologique XXXV*. Paul Lechevalier, Paris, France.

Romi, R. (1999). *Anopheles labranchiae*, an important malaria vector in Italy, and other potential malaria vectors in Southern Europe. *European Mosquito Bulletin*, Vol.4, (June), pp. 8-10, ISSN 1460-6127.

Romi, R.; Severini, C.; Cocchi, M.; Tamburro, A.; Menichetti, D.; Pierdominici, G. & Majori, G. (1992). Anofelismo residuo in Italia: distribuzione nelle aree risicole delle provincie di Grosseto e Siena. *Annali Dell Istituto Superiore Sanita*, Vol.28, No.4, (December 1992), pp. 527-531, ISSN 0021-2571.

Romi, R.; Pierdominici, G.; Severini, C.; Tamburro, A.; Cocchi, M.; Menichetti, D.; Pili, E. & Marchi, A. (1997). Status of malaria vectors in Italy. *Journal of Medical Entomology*, Vol.34, No.3, (May 1997), pp. 263-271, ISSN 0022-2585.

Romi, R.; Sabatinelli, G.; Giannuzzi Savelli, L.; Raris, M.; Zago, M. & Malatesta, R. (1997). Identification of a North American mosquito species, *Aedes atropalpus* (Diptera: Culicidae), in Italy. *Journal of the American Mosquito Control Association*, Vol.13, No.3, (September 1997), pp. 245-246, ISSN 1046-3607.

Romi, R.; Sabatinelli, G. & Majori, G. (2001). Could malaria reappear in Italy? *Emerging Infectious Diseases*, Vol.7, No.6, (June 2001), pp. 915-919, ISSN 1080-6059.

Rosen, L. (1986). Dengue in Greece in 1927 and 1928 and the pathogenesis of dengue hemorrhagic fever: new data and a different conclusion. *American Journal of Tropical Medicine and Hygiene*, Vol.35, No.3, (May 1986), pp. 642-653, ISSN 0002-9637.

Roubaud, E.; Colas-Belcour, J. & Stefanopoulo, G. (1937). Transmission de fièvre jaune rar un moustique paléarctique répandu dans la region parisiennes, l'*Aedes geniculatus* Olivier. *Compte Rendus Hebdomadaires des Seances de l'Academie des Sciences*, Vol.202, pp. 182-183, ISSN 0567-655X.

Sabatini, A.; Raineri, V.; Trovato, G. & Coluzzi, M. (1990). Aedes albopictus in Italia e possible diffusione del la especie nell' area mediterranea. *Parassitologia*, Vol.32, No.3, (December 1990), pp. 301-304, ISSN 0048-2951.

Sainz-Elipe, S.; Latorre, J.M.; Escosa, R.; Masià, M.; Fuentes, M.V.; Mas-Coma, S.; Bargues, M.T. (2010). Malaria resurgence risk in southern Europe: climate assessment in an historically endemic area of rice fields at the Mediterranean shore of Spain. *Malaria Journal*, Vol.9, No.7, (July 2010), ISSN 1475-2875, Accessed 14th March 2011. Available online in: http://www.malariajournal.com/content/9/1/221

Santa-Olalla Peralta, P.; Vazquez-Torres, M.C.; Latorre-Fandós, E.; Mairal-Claver, P.; Cortina-Solano, P.; Puy-Azón, A.; Adiego Sancho, B.; Leitmeyer, K.; Lucientes-Curdi, J. & Sierra-Moros, M.J. (2010). First autochthonous malaria case due to *Plasmodium vivax* since eradication, Spain, October 2010. *Euro Surveillance*, Vol.15, No.41, (October 2010), ISSN 1560-7917, Accessed 14th March 2011. Available online in: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19684

Sardelis, M.R. & Turell, M.J. (2001). *Ochlerotatus j. japonicus* in Frederick County, Maryland: discovery, distribution, and vector competence for West Nile virus. *Journal of the American Mosquito Control Association, Vol.*17, No.2, (June 2011), pp. 137–141, ISSN 1046-3607.

Sardelis, M.R.; Dohm, D.J.; Pagac, B.; Andre, R.G. & Turell, M.J. (2002a). Experimental transmission of eastern equine encephalitis virus by *Ochlerotatus j. japonicus* (Diptera: Culicidae). *Journal of Medical Entomology*, Vol.39, No.3, (May 2002), pp. 480–484, ISSN 0022-2585.

Sardelis, M.R.; Turell, M.J. & Andre, R.G. (2002b). Laboratory transmission of La Crosse virus by *Ochlerotatus j. japonicus* (Diptera: Culicidae). *Journal of Medical Entomology*, Vol.39, No.4, (July 2002), pp. 635–639, ISSN 0022-2585.

Sardelis, M.R., Turell, MJ. & Andre, R.G. (2003). Experimental transmission of St. Louis encephalitis virus by *Ochlerotatus j. japonicus*. *Journal of the American Mosquito Control Association*, Vol.19, No.2, (June 2003), pp. 159–162, ISSN 1046-3607.

Satti, M.H. & Haseeb, M.A. (1966). An outbreak of yellow fever in the Southern Fung and Upper Nile province, Republic of the Sudan. *Journal of Tropical Medicine and Hygiene*, Vol.69, No.1, pp. 36-44, ISSN 0022-5304.

Sautet, J. & Quilici, R. (1971). A propos de quelques cas de paludisme autochtone contractés en France pendant l'été. *Presse Médicale,* Vol.79, pp. 524, ISSN 0755-4982.

Schaffner, F.; Angel, G.; Geoffroy, B.; Hervy, J.O. & Rhaeim, A. (2001). The mosquitoes of Europe / Les moustiques d' Europe [CD-ROM]. IRD Éditions and EID Méditerranée, Montpellier, France.

Schafther, F.; Chouin, S. & Guilloteau, J. (2003). First record of *Ochlerotatus (Finlaya) japonicus japonicas* (Theobald, 1901) in metropolitan France. *Journal of the American Mosquito Control Association*, Vol.19, No.1, (March 2003), pp. 1-5, ISSN 1046-3607.

Schaffner, F., Kaufmann, C. &Mathis, A. (2009). The invasive mosquito *Aedes japonicus* in Central Europe. *Medical and Veterinary Entomology*, Vol.23, No.4, (December 2009), pp. 448-451, ISSN 0269-283X.

Schmidt-Chanasit, J.; Haditsch, M.; Schöneberg, I.; Günther, S.; Stark, K. & Frank, C. (2010). Dengue virus infection in a traveller returning from Croatia to Germany. *Euro Surveillance*, Vol.15, No.40, (October 2010), ISSN 1560-7917, Accessed 14th March 2011. Available online in: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19677

Scholte, E.J.; Den Hartog, W.; Braks, M.; Reusken, C.; Dik, M. & Hessels, A. (2009). First report of a North American invasive mosquito species *Ochlerotatus atropalpus* (Coquillett) in the Netherlands, 2009. Euro Surveillance, Vol.14, No.45, (November 2009), ISSN 1560-7917, Accessed 14th March 2011. Available online in: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19400

Scholte, E.J.; Den Hartog, W.; Dik, M.; Schoelitsz, B.; Brooks, M.; Schaffner, F.; Foussadier, R.; Braks, M. & Beeuwkes, J. (2010). Introduction and control of three invasive mosquito species in the Netherlands, July-October 2010. *Euro Surveillance*, Vol.15, No.45, (November 2010) ISSN 1560-7917, Accessed 14th March 2011. Available online in: http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19710

Senevet, G.; Andarelli, L. & Adda, R. (1955). Presence of *Anopheles plumbeus* St. in Algerian shores. *Archives de l'Institut Pasteur d'Algérie*, Vol.33, No.2, (June 1955), pp. 138-139, ISSN 0020-2460.

Sepulcri, P. *La malaria nel Veneto*. (1963). Istituto Interprovinciale per la Lotta Antimalarica nelle Venezie, Venice, Italy

Shute, P.G. (1954). Indigenous *P. vivax* malaria in London believed to have been transmitted by *An. plumbeus. Monthly Bulletin of the Ministry of Health and the Public Health Laboratory Service*, Vol.13, pp. 48-51, ISSN: 0368- 881X.

Shute, P. & Maryon, M. (1969). Imported Malaria in the United Kingdom. *British Medical Journal*, Vol.28, (June 1969), pp. 781–785, ISSN 0959-8138.

Shute, P. & Maryon, M. (1974). Malaria in England past, present and future. *Journal of the Royal Society of Health*, Vol.94, (February 1974), No.1, pp. 23-29, ISSN 1466-4240.

Snow, K. & Ramsdale, C. (1999). Distribution chart for European mosquitoes. *European Mosquito Bulletin*, Vol.3, (January 1999), pp. 14-31, ISSN 1460-6127.

Tavşanoğlu, N. & Çağlar, S.S. (2008). The vectorial capacity of *Anopheles sacharovi* in the malaria endemic area of Şanlıurfa, Turkey. *European Mosquito Bulletin*, Vol. 26, (December 2008), pp. 18-23, ISSN 1460-6127.

Teodorescu C. (1983). Experimental infection of an indigenous strain of *Anopheles atroparvus* with imported species of *Plasmodium*. *Archives roumaines de pathologie experimentale et de microbiologie*, Vol.42, No.4, (October 1983), pp. 365-370, ISSN 0004-0037.

Torres Cañamares, F. (1934). Observaciones sobre los *A. maculipennis* y sus razas en Camporredondo (Jaén). *Medicina de los Países Cálidos,* Vol.7, (February 1934), pp. 53-72.

Toty, C.; Barré, H.; Le Goff, G.; Larget-Thiéry, I.; Rahola, N.; Couret, D. & Fontenille, D. (2010). Malaria risk in Corsica, former hot spot of malaria in France. *Malaria Journal*, Vol.9, No.8, (August 2010), ISSN: 1475-2875, Accessed 14th March 2011 Available online in: http://www.malariajournal.com/content/9/1/231

Tovornik, D. (1978). An atypical breeding place of the *Anopheles plumbeus* Stephens, 1828, in an unfinished house. *Biološki vestnik*, Vol.26, pp. 41-46, ISSN 0502-1969

Turell, M.J.; O'Guinn, M.L.; Dohm, D.J. & Jones, J.W. (2001). Vector competence of North American mosquitoes (Diptera: Culicidae) for West Nile virus. *Journal of Medical Entomology*, vol.38, No.2, (March 2001), pp. 130-134, ISSN 0022-2585.

Utrio, P. (1979). Geographic distribution of mosquitoes (Diptera, Culicidae) in eastern Fennoscandia. *Notulae Entomologicae*, Vol.59, pp. 105-123, ISSN 0029-4594.

Wang, K.C. (1966). Observations on the influence of photoperiod on egg diapause in *Aedes albopictus*. Acta Entomologica Sinica, Vol.15, pp. 75-77 ISSN 0970-3721

White, G.B. (1978). Systematic reappraisal of the *Anopheles maculipennis complex*. *Mosquito Systematics*, Vol.7, No.1, (January 1978), pp. 303-344, ISSN 0091-3669.

World Health Organization (WHO). *Malaria*. Fact sheet Nº 94, May 2007, Accessed 14th March 2011 Available online in: http://www.who.int/mediacentre/factsheets/fs094/en/print.html/

World Health Organization (WHO), Regional Office for Europe. (n.d.). *Centralized information system for infectious diseases (CISID) database. Malaria*., Accessed 14th February 2011, Available online in: http://data.euro.who.int/cisid/

Yergolkar, P.N; Tandale, B.V.; Arankalle, V.A.; Sathe, P.S.; Sudeep, A.B.; Gandhe, S.S.; Gokhle, M.D.; Jacob, G.P.; Hundekar, S.L. & Mishra, A.C. (2006). Chikungunya outbreaks caused by African genotype, India, *Emerging Infectious Diseases*, Vol.12, No.10, (October 2006), pp. 1580–1583, ISSN 1080-6040.

Zahar, A.R. (1987). *Vector bionomics in the epidemiology and control of malaria*. *Part II: The WHO European Region and the WHO Eastern Mediterranean Region*. VBC/88.5. World Health Organization, Geneva, Switzerland. Accessed 14th March 2011 Available online in: http://whqlibdoc.who.int/hq/1990/VBC_90.1_eng.pdf

Zaim, M. (1987). Malaria control in Iran: present and future. *Journal of the American Mosquito Control Association,* Vol.3, No.3, (September 1987), pp. 392-396, ISSN 1046-3607.

Zotta, G. (1938). Contribution à l'etude de la distribution des races d'*A. maculipennis* en Roumanie. *Archives Roumaines de Pathologie Experimentale et de Microbiologie*, Vol.11, pp. 209-246. ISSN 0004-0037.

Zotta, G.; Georgesco, M.; lonesco, V.; Lupasco, G.; Mardare, I. & Teodoresco, A.M. (1940). Nouvelle carte de la distribution des races d'*Anopheles maculipennis* en Roumanie. *Bulletin de la Section Scientifique de l'Académie Roumaine*, Vol.23, pp. 73-87.

Zulueta de, J.; Blazquez, J. & Maruto, J.F. (1973). Aspectos entomológicos sobre la receptividad del paludismo en la zona de Navalmoral de la Mata. *Revista de Sanidad e Higiene Pública*, Vol.47, No.10, (October 1973), pp. 853-870, ISSN 0034-8899.

Zulueta de J.; Ramsdale, C.D. & Coluzzi M. (1975). Receptivity to malaria in Europe. *Bulletin of the World Health Organization*, Vol. 52, No.1, pp. 109-11, ISSN 0042-9686.

28

Neonatal Thermoneutrality in a Tropical Climate

Hippolite O. Amadi
Imperial College London
United Kingdom

1. Introduction

Sub-Saharan African countries are notably among the nations with high neonatal mortality (NNMR) and morbidity rates (WHO, 2009). A number of issues have been previously raised in the literature in attempt to define some of the factors that contribute to these such as level of illiteracy among mothers and short supply of healthcare workers (Amadi et al., 2007). However, little has been said of the impact of environmental temperature regulation on the wellness and survival of neonates in this region. The sub-Saharan Africa is well-known for its harsh climatic conditions of high sun intensity and ambient temperatures, often in excess of 35°C, coupled with societal condition of abject poverty. Nursing environment of the neonate, especially pre-terms, is a crucial factor for the maintenance of appropriate body temperature for the physiological stability of the newborn. Classical management of neonatal thermoneutrality in this region of Africa has been dominated by procedures that were handed down from industrialised societies; these being fundamentally compliant to the peculiar climatic factors and social advantages of the countries of origin.

In the last decade, there has been concerted effort to scientifically investigate factors that may be subtly contributing to high neonatal mortality and morbidity in this region. These include meteorological, socio-cultural and technological factors that define the macro- and micro-environments immediate to the neonate. This knowledge is fundamental for the tweaking or outright replacement of the present morbidity-high techniques. This chapter will attempt to explore these factors and their consequences, and discuss the present interventions and techniques that are coincidentally yielding improved outcome in some neonatal centres in the region. The ideas expressed in this chapter were drawn from on-the-spot clinical practice experiences in a decade-on collaborative project that has involved up to 21 neonatal referral Centres across the entire geographical region of the West African state of Nigeria (Figure 1). Recent publications show that this region of African is currently far behind the United Nation's Millennium Development Goal (MDG) target on the survival of infants, and neonatal mortality rate is steadily making this worse (Federal Ministry of Health [FMOH], 2011). Neonatal survival might not necessarily improve by the flooding of the region with 'foreign-culture-biased' sophisticated incubator systems that are not so easy to handle by the users despite the high pricing of these that limit their procurement by the poor countries. There is a perceived socio-cultural dimension of the work place attitude that militates against effective practice of neonatal thermoregulation. This needed to be properly addressed perhaps by the use of affordable and manageable appropriate incubation technology that the people can easily identify with.

Ineffective thermoregulation leads to other complications and patients' poor response to treatment. Neonatal physiological stability enhanced by adequate thermoneutral control and humidification is an essential factor that enables the neonate to respond well to treatment thereby enabling effective management of associated tropical diseases. An adequate and hygienic incubation technique, appropriately designed for the peculiar tropical settings, will minimise neonatal cross-infection and also reduce disease transmission by the often freely roaming insects in and out of the incubators. Adverse climatic conditions and observed procedural inadequacies of incubator application often lead to overheating, compelling attendants to open up the incubator portholes and windows thereby compromising the microenvironment. The present work seeks to extend the incubator application to create procedures that ensures the minimisation of such compromises.

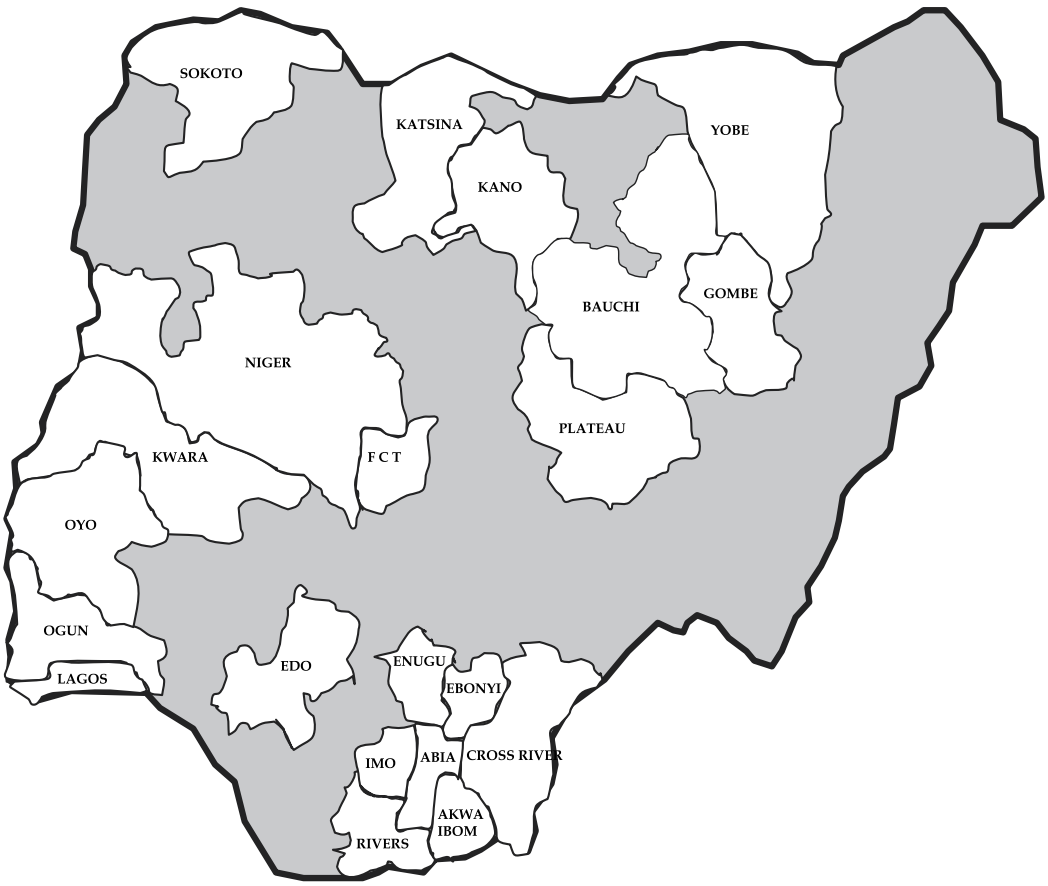


Fig. 1. Map of Nigeria showing all the states where the collaborating tertiary hospitals are located

In this chapter, four different environments that impact on the new-born baby would be examined. This knowledge is important to be able to effectively understand the thermal needs of the new-born during neonatal care, whether inside an incubator or an open cot. These are:

1. The prenatal environment or the mother's womb that provides for the nurturing of the foetus.
2. The micro-environment or the baby's compartment of an incubator or wrapping in an open cot.
3. The macro-environment or the outer room comprising the interior of the nursery building where the incubators and cots are situated for the nursing of the new-born.
4. The regional-environment or the outside surrounding of the nursery building directly being influenced by the regional climate.

2. Prenatal environment

The neonate's body system observes the law of "garbage in garbage out" in terms of what it does with the ambient temperature of its immediate environment. From clinical experience, it is very easy to observe that an extremely low birth weight neonate will quickly assume a body temperature equal to the ambient temperature of its immediate environment. This has been frequently observed at one of our collaborating neonatal centres in the very hot town of Nguru in Nigeria. In an overheated room, baby becomes hyperthermic and in a cold room, baby becomes hypothermic. Both extremes are devastating conditions, capable of claiming the baby's life and must be avoided. This is why the regulation of baby's environment must be done on patient-specific basis involving the exact extra warming or wrapping required to thermally stabilise such baby. Nature has made an adequate environmental provision for the prenatal period. Baby's immediate past housing, the womb, is a separately controlled environment independent of the foetus's body system and providing it with no worries for self-thermoneutral control. It is therefore essential to anticipate the possible fatal environmental shock that awaits a premature new-born as its organs are not yet functionally ready to completely support the baby outside the womb environment.

The body temperature of a healthy adult is physiologically stabilized about 37°C whether in a temperate climate of 1°C or tropical sub-Saharan African climate of 45°C. The adult expectant mother may be physiologically acclimatised to her 'freezing' or 'burning' weather respectively; however, the foetuses in both climates are accustomed to approximately the same thermal environment of the womb. Individual climatic adaptation for the baby only begins at birth. Therefore the design of a supportive environment for a premature baby must integrate the climatic peculiarities such foetus would be graduating into.

3. Neonatal micro-environment

It is an absolute necessity to provide an enabling environment immediately following premature birth. This calls for a sustainable artificial environment to allow the neonate enough time for its organs to fully mature to provide independent support to withstand the tougher climate of the outside world. This practice ameliorates the shock of a sudden change in environmental conditions that can become catastrophic for the premature new-born. What should such artificial environment possess then? A good design of controllable micro-environment for the baby might be achieved by applying constraints that are enriched with the good knowledge of the womb as well as the climatic and social factors of baby's place of birth. Incubator comes to mind in a clinical setting when neonatal microenvironment is mentioned. In a classical and basic sense, neonatal incubation might speak more of the

provision of a controlled warming for the new-born. It is understandable that provision of uncompromised all-day warming is essential for the neonate in a temperate regional climate of average room temperature of less than 10°C, for example. The baby's inability to maintain its own homeostasis meant that the extra 27°C or more required to attain a body temperature of 37°C must be provided and sustained artificially. The definition for this separate microenvironment for the new-born must however begin to modify when the regional climate changes to another of room temperatures in excess of 40°C. Such elevated ambient temperature in poor hygienic settings as the tropical culture would normally encourage fast breeding of disease transmitting insects even around hidden corners of the incubator. Therefore, appropriate incubation technique capable of effective neonatal care must recognise and integrate the climatic characteristics of the culture the neonate is graduating into. Neonatal incubator care, as is presently practiced in the high ambient sub-Saharan Africa is deficient of this fundamental factor. The race to lower neonatal mortality rate in Tropical Africa might hence not necessarily be dependent on increase of drug donations or over flooding the health facilities with sophisticated incubators. The race might however be won by going back to the fundamentals of neonatal thermoneutral care that are compliant to the conditions of the region. This is a natural 'preventive technique' that would lead to the minimisation of disease occurrences. An effective and climate sensitive neonatal care would ensure a healthy start in life for the promising infant.

Classical incubation techniques in this tropical African climate might not have been fully successful because these were designs developed and originally practiced in climates and cultures that were completely different. This creates opportunities for deficient practices that expose the delicate neonates to unhygienic conditions, insects and infections that soon make them morbid and eventually claim their lives. An unstable thermoneutral environment does not provide the thermal stability the neonate's body system requires to effectively respond to treatment. In response to this, an on-going research adopting new practice procedures in collaboration with 15 different neonatal centres across the region was initiated to address various adverse incubation factors. This has so far proffered solutions that coincidentally improved survival rates in the centres (Amadi et al., 2010). The aim of this work was to present the various thoughts, considerations and incubator care approaches that might have improved neonatal defence against exposure to tropical epidemics that could easily claim their lives.

4. Effect of poverty and illiteracy

A well-looked after pregnancy and professionally efficient antenatal care are essential factors that could promise a healthy baby. These factors would characteristically help to avoid the complications of preterm birth. Poverty and illiteracy create a clear distinction in neonatal mortality rate between developed nations such as the United Kingdom and any sub-Saharan African country such as Nigeria. This is evident from literature reports on neonatal and perinatal mortality rates from these regions (FMOH, 2011; Centre for Maternal and Child Enquiry, 2010). Societal abject poverty and high degree of illiteracy are factors that do not allow the expectant mothers to nurture healthy foetuses or seek professional antenatal care when these are available. It is arguable that most mothers would be happy to observe any rules that would guarantee them a healthy baby. Maternal poverty and illiteracy are outside the scope of the present work; however it sounds reasonable to mention that tackling poverty in sub-Saharan Africa as part of an assembly of measures would go a long way in lowering NNMR of the region.

4.1 Incubator availability

The odd effect of poverty shows up again during the management of the premature baby in terms of inadequacy in the supply of equipment for neonatal nursing. There is endemic insufficiency of functional incubators for the teaming population of the neonates requiring incubator care in a typical referral centre among those involved in the present study. The situation in the region was such that it was common to have only one centre where neonatal special care might be obtained in a catchment population of over 5 million, covering over 30,000 km^2 of land area. Journeys could take as much as 3 hours to reach the centres and often involving very poor access roads. Such a centre would normally have an average of 35 neonates on admission at any time, yet working with no more than two functional incubators and grossly under-staffed with qualified nurses and doctors. Sadly, up to three babies from different mothers were at times crammed into the same incubator in one of those horrible thermoneutral malpractices to be treated in later sections of this chapter. Our strategic plan to lower neonatal loses due to complications of thermal instability had to be carefully designed to discourage such wrong applications. Ibe (1993) indicated a culture of high admission deliveries for very low birth weight babies within the study region. This is compounded by a high number of referral cases of distressed tiny babies, often stretching the facilities beyond capacity as observed in our study centres. This therefore suggests that a hospital with average on-admission of over 30 patients should operate with a minimum of 20 well-maintained functional incubators, up to 3 radiant warmers, up to 10 phototherapy machines, up to 30 units of cot/incubator installable apnoea monitoring systems and enough attending nurses to guarantee a patient-to-nurse ratio of 5 during every shift. The provision of twenty functional incubators in one hospital alone is almost impossible when the poverty factors of these tropical countries come into play. Hence, an extra ordinary approach to this seemingly impossible situation had to be sought for.

4.2 Budget re-equipping of functional incubator

An earlier study in the tropical region of Africa by Ogunlesi et al (2008) indicated that high point-of-admission hypothermia and general thermal instability contribute a great deal to the high NNMR, stressing the need of incubators in adequate number to re-equip the centres. Many centres were discovered to have several units of broken-down, old and obsolete incubators stacked in equipment stores; others littered the walk ways of the hospital complexes, breeding insects and rodents, promoting environmental pollution and unhygienic facility. Many of these were kept back in the neonatal wards and being used as 'cages' for these babies in such unhygienic manner that could enhance infections and disease transmission (Figure 2). The horrific dirty sights that were revealed upon removal of the mattress trays were enough to wonder why any baby survived at all from the cages. Careful inspections would reveal that many of these still had reusable canopies and trolleys. Proposals for the purchase of adequate number of functional incubators during joint-task meetings with hospital managements would not go well. Lack of adequate funding for the hospitals meant that it was nearly impossible for any of them to acquire enough systems at the local prevailing costs of incubators. These were to be supplied to hospitals at costs higher than €20,000 per unit of incubator. This meant, for a Unit requiring up to 20 incubators, a huge budget of over €400,000 for the purchase of incubators alone in a poorly funded hospital with many other departments to run. The local repair of the broken-down incubators was not a feasible option as the spare parts of few of the current models could not be acquired due to poor supply chain. None of the companies that produced these

systems had any assembly plants or technical representatives to provide the needed technical support. Therefore sustainable solution was not envisaged through this approach. To acquire brand new systems, unlike the use of extended price-plan and after sale maintenance in developed countries, the hospitals in these poor nations would be required by the foreign companies to make outright payment of the full cost. High cost of logistics and unfavourable operating environments might have compelled these companies from any extended technical support to a country like Nigeria at the moment. They hence demanded full out right payment for the systems, leaving the country to bear the full liability of the carcasses when they broke down. An inventory carried out in a certain Teaching Hospital in Nigeria during this period revealed the presence of 45 carcasses of different models of dysfunctional baby incubators in their stores whilst they had no functional one to save tens of babies in its new-born Units.

Fig. 2. An incubator carcass showing the inside immediately below matrass tray (removed); system was in use as is when retrieved

At this junction it was clear that the idea of a new approach to this was inevitable. The old carcasses of incubators that littered the hospitals were again considered and fresh investigations revealed the high availability of these across all the 5 collaborating tertiary (university teaching) hospitals at the time. A research was hence initiated to evaluate and design a process that might apply to re-introduce these obsolete systems to active services in a neonatal ward without necessarily taking recourse to original manufacturers or spare parts. This was to consider the maintainability of the resulting system, carefully selecting options that would ensure availability of spare parts and simplifying the technology to make it easy for the local technicians to handle. Typically available carcasses to apply in these hospitals were of all sorts of brands, age and models, the state of some could be best described as 'horrible' (Figure 3). Carcasses were also literally recovered from hospital dump sites where they were abandoned awaiting evacuation. However, these constituted the closest solutions to the problem required to be solved. The wide variability of these carcasses resulted in the constraint requiring that a workable design must be easily adaptable to any 'model' and 'make' of an item of incubator carcass.

Fig. 3. Abandoned incubators for recycling, recovered from (A) rubbish dump (B) workshop (C) neonatal ward in use (D) scrap metal yard

5. Modelling Recycled Incubators

Recycled Incubator Technique (RIT) speaks of the successful application that most Nigerian tertiary hospitals have used to re-equip their Special Care Baby Units (SCBU) using their formally condemned or obsolete incubators. Many of these old systems were abandoned in the stores or used as ordinary cages (cots) in the Special Care Baby Units.

In the recycling technique that was designed in this study, the casings of the obsolete system were re-used but the functional assemblies of the power unit were completely re-engineered with customized generic digital components. RIT incubators currently make up 80% to 100% of the functional incubators in many Nigerian tertiary hospitals at the time of this report. These included University of Benin Teaching Hospital (UBTH) Benin-City, Lagos University Teachning Hospital (LUTH) Lagos, Jos University Teachnig Hospital (JUTH) Jos, Ebonyi State University Teaching Hospital (EBSUTH) Abakaliki, Aminu Kano Teachnig Hospital (AKTH) Kano, Federal Medical Centres (FMCs) at Nguru, Gombe, Owerri, EbuteMetta and Abeokuta. Others were University of Nigeria Teaching Hospital (UNTH) Enugu, University of Calabar Teaching Hospital (UCTH) Calabar, University of Ilorin Teaching Hospital (UITH) Ilorin and University of Abuja Teaching Hospital (UATH) Gwagwalada. An RIT system comparatively saves up to 75% of the cost of procuring and maintaining a modern state-of-the-art incubator whilst being functionally akin to these (Amadi et al, 2007). RIT has presently made it possible for some

of these hospitals to currently maintain up to 25 functional incubators whereas they could not simultaneously own up to 3 or none at all in the recent past. This has therefore been described by a Nigerian healthcare organisation as a significant contribution to national development in the Nigerian healthcare system (Committee of Chief Executives of Federal Tertiary Health Institution [CCEFTHI], 2007).

5.1 Original concept

The initial hypothesis proposed that the application of generic assemblies in the rebuilding of the functional mechanisms and electronics of an incubator would drastically reduce the unit cost of incubation in low income countries. To verify and implement this, modern manufacturing techniques based on standard generic assemblies was exploited in a careful design of interfaces for the linking of the generic assemblies. Using the internet market, individual mechanical, electrical and electronic assemblies that could apply to design the functional mechanism of an incubator were cost-competitively selected. These were assembled by design to yield desired outputs necessary for effective maintenance of the unique standard conditions required in an incubator's micro-environment.

5.2 Casing and trolley

System functionality was the primary focus of the RIT, however the overall peripheral finishing must be appealing necessitating a careful investigation of the abilities of self-employed local artesans. It was discovered that individual fabricators, welders and car painters around major cities in Nigeria were good enough in their arts such that with very close supervision these could do excellent jobs. Therefore the best of these artisans were identified and separately guided to renovate the old incubator casings and to finish these with the best possible standards.

5.3 Canopy and plastic components

There were identified artisans that worked on Perspex materials in the small and big cities. Some of these demonstrated good skills in the methods they used in their jobs. Various plastic components of different models of incubator amongst the carcasses were re-designed to be produced as spares for the replacement of the originals; the new designs being such as would be easy to fit into the crafting techniques of the local artesans. Most of the new designs therefore resulted in different shapes from those of the original incubator manufacturers. The changes in the RIT designs for these plastics/Perspex parts were necessary in order to simplify their production as required to ensure good finishing by a closely supervised artisan. The incubator hoods were assessed. Old age and handling could make some of these to become opaque after technical cleaning and must not be reused if not completely clear and transparent. Effort was hence made to reproduce the discarded hoods with locally available Perspex materials by applying simple procedures of working and reshaping of the materials.

5.4 Power unit modules

This is a detachable unit that houses the operational control elements in most incubator designs. A repairer intending to fix a broken-down incubator would normally detach and take this unit away in attempt to diagnose the source of a wrong system signal.

Unfortunately, many of these that were taken away from some of the hospitals by prospective repairers were returned without success as the spare parts were no longer available. Some repairers lost or did not even bother to return the removed power-units after their unsuccessful attempts. In RIT, the casing of the power module was normally reused. However, when these could not be traced to where they were sent to or by whoever removed them, a new simplified casing was designed and locally produced. In some cases, the high aesthetic finishing of the incubator was not altered by this major reconstruction. The Airshields model C100 systems at the Ebonyi State University Teaching Hospital (EBSUTH) Abakaliki and one of the C200 models at University of Benin Teaching Hospital (UBTH) were examples of incubators that were recycled with re-engineered power module casings (Figure 4).

Fig. 4. Recycled incubators, originally carcasses of (A) Airshields model C200 (B) ISIS Mediprema system

5.5 Operating system

Designing and developing a new assembly of an operating system to power the incubator was the most challenging step in the RIT procedure. The chain of electronic and digital communications that powered the intelligence system was generally achieved by interconnectivity of distinct units of circuitry. These were called assemblies and were generic constructs by different companies but selected and arranged by design in RIT systems (Figure 5). All used circuitry could be sourced for internationally through the internet. Specifications of input requirements and expected outputs of such units of assemblies enabled effective integration of these to achieve the ultimate incubator output during the design stage. Ability to design a functional system with this method required a good understanding of how the incubator worked and the different functions of the assemblies that powered it. A good knowledge of human anatomy and neonatal physiology were essentially applied to relate to the outputs of these assemblies to ensure the clinical suitability of the resulting system. These different assemblies or components were then purchased from their individual companies or marketers, appropriately reconfigured to design specifications and mounted in the power module casing. The various outputs, signals and actuations specified in the design were compatible with the applied transduction elements in the incubator; else design adjustments or matching transducers were sought for and applied.

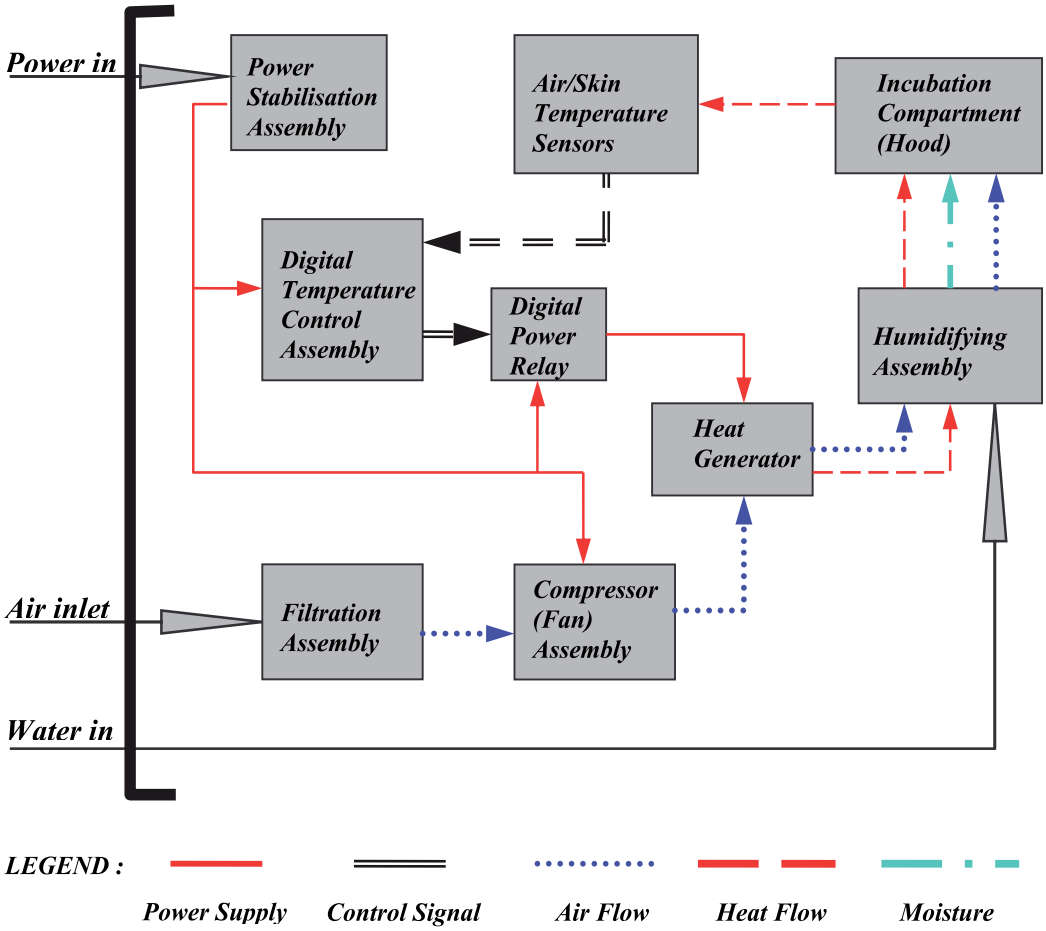


Fig. 5. RIT Assembly Block Diagram (Amadi et al, 2007)

6. Recycling own old incubators

The successful clinical trials of the initial RIT systems paved the way to the present in-depth study on neonatal thermoneutral control in this African tropical climate. The cost of producing an RIT system from a hospital's fleet of carcasses was demonstrated to be less than 20% of the cost of purchasing one state-of-the-art (STA) incubator (Amadi et al., 2007). This made the idea of incubator re-equipping of the SCBUs an attractive project to administrators of many of the tertiary hospitals that participated in the study. More hospital centres were later attracted to participate in Nigeria when the government approved the supply of STA Draeger Caleo system at a unit cost equivalent to the cost of recycling 7.5 old incubators. It was important to strategically tackle the provision of adequate number of functional micro-environments (housing) for the teaming population of the neonates within the limits of the available funding. It was well-understood that the STA systems have got the excellent finishing of modern technology and sophistication, however, it was necessary to consider the number of babies the same amount of money could house per unit time. RIT approach was hence the easy way of populating the SCBUs with many functional incubators

using the available meagre funding. This would hence create the platform to properly study thermoneutral application in a tropical busy centre. In order to keep the project focused and on course, some of the collaborating hospitals Managements were advised to adopt a 'slow but steady batch-by-batch' recycling approach. This approach allowed a hospital to put a time frame vision for the completion of the recycling of all available carcasses of incubators. The Lagos University Teaching Hospital, for example, had no functional incubators at the start of the project. Ten incubators were initially recycled; there after the Hospital Management recycled 5 more each year to achieve a fleet of 25 functional incubators in three years. This boosted patient flow and staff enthusiasm for work thereby enabling better investigations of the objects of this study in the Centre (Amadi et al., 2010).

The good dedication of most of the collaborators soon led to the successful recycling of all available old incubators in most of the participating hospitals. However, the drive to provide adequate number for the SCBUs meant that some of the hospitals had to take another step of procuring more incubators from the market. The confidence already reposed on RIT made it possible for the Hospitals Managements to use available funds to purchase affordable systems they could easily re-power with RIT component when these failed. This hence secured a sustainable fleet of functional incubators required for the study in each of the participating Centres. The Centres were also encouraged and assisted to add one or more transport incubators in the fleet. This helped to reduce the distress some of the neonates suffered during intra-hospital transports using inappropriate and crude techniques.

6.1 Incubator routine maintenance and functionality auditing

The high neonatal admission delivery in these hospitals coupled with high influx of referrals resulted in extensive demands on the incubators. Many of these functioned continuously for weeks and months without break except when they were to be cleaned for another waiting baby. There was then need to initiate another supportive programme to guarantee sustainability of the functional status of the incubators and freedom from frequent unexpected system failures. This was ensured by the introduction, in each Centre, of a routine maintenance culture. By this the Hospital Managements allowed all the RIT systems to undergo professional functionality auditing and thorough system servicing by qualified RIT in-country technicians once in every 6 months. Some employees of the hospital's engineering department were deployed to be trained to assist in the technical upkeep of the recycled systems. This programme ensured the replacement of any damaged or damaging part of the incubator before this could lead to the system being run to a stop.

7. Paediatrics incubation technique course

It was discovered that generations of medical students and staff had come and passed on from the Units without ever practicing with a really functional incubator. This created generations of staff who knew no better than how to crudely improvise cages to do the work of an incubator. This hence meant an absolute lack of the fundamental knowledge of how to operate and nurse babies in proper incubators. There was initial 'general' staff training on how to operate the newly recycled systems. This basically familiarised the systems to the nurses, clinical staff and the engineering technicians, demonstrating the various assemblies of the machine and how it worked. The course was basic enough to introduce the system with the assumption that the attending staffs were already trained in various aspects of

new-born management including incubator care. Over the succeeding months and across the peculiarities of the various participating hospitals in the region, there was continuous monitoring of how the nursing and clinical staff (care providers) attended to the patients with the incubators. Practice errors were thus identified; building up what was later to form the contents of a proper course work that would treat the fundamentals of incubator care in the tropical climate. This was to collate environmental, socio-cultural, human and technical factors as observed to design an elective course to demonstrate a customised approach to neonatal incubation within the climatic setting. This was to demonstrate to the attending care-providers how various wrong practices could have contributed to the mortalities in their Units. The second aspect of the course concentrated on educating participants on the 'dynamics of neonatal thermoneutral control' based on the best practice approach for achieving a steady body temperature for babies in incubators in the tropical climate. This two-graded elective course coined 'Paediatrics Incubation Techniques' thus had level 1 as 'fundamentals of neonatal incubator care' and level 2 as 'dynamics of neonatal thermoneutral control'. The general aim of level 1 was to apply theories and on-the-spot practical demonstrations to explain the basic physics of incubation and how the incubator achieved this. This also demonstrated how familiar wrong practices could have prevented the incubator from properly achieving its aims thereby delimiting the overall neonatal care quality. Level 2 was a short course that taught participants how to interactively set and re-set the incubator set-point to avoid the common confusions encountered when using the incubator to thermally stabilise the baby. The contents of these courses are briefly set out below.

7.1 Fundamentals of neonatal incubator care

The course was started with an introductory segment that was aimed at making participants to understand the general make-up of the incubator. This presented its features and mode of operation in a way as to simplify any complexities that would make care-providers see this as a mystery machine. This emphasised that Newborn babies were precious and generally delicate while the premature, among them, were much more delicate to nurse, especially during the first few days of their arrival. The premature baby, during the first few days of its arrival, needed above every other thing, controlled and regulated warmth that could provide it with a comfortable environment in its struggle for survival. The Incubator, in doing this job, becomes the obvious best friend of the premature baby during this period. A mishandling of the Incubator would mean everything DANGER to the precious premature baby inside it. Therefore a carefree attitude towards an incubator on duty could expose the child to suffocation, electric shock and many other dangers that could claim its life. Hence it was necessary that every nurse and clinician was adequately informed and trained before he/she could effectively nurse a baby with it. This emphasized that the incubator was precious and delicate just like its best friend and hence must be handled with care.

The course module explained some differences in designs of infant incubators, describing these as diverse and versatile, pointing out probable constraints necessitating the design variations. Although there is a generally acceptable basic programme of operation of an incubator, different manufacturers enhanced their designs over others with automation technology that improved their values. Modern designs incorporate microprocessors that aid automatic operation of the machine, enabling it to do much more than what older analogue and hybrid systems could do. The microprocessors fostered advanced artificial intelligent systems that regulated the humidity of the incubation chamber or neonate's

compartment. Some apply affixed probes to independently monitor baby's temperature, breathing, heartbeat rates and weight-gains.

The module explained why some infant incubators were designed as "Transporters" or "Rescue" Units; discussing the kinds of "Power Sources" that keep the system functional whilst on transit. The power source could be assemblies incorporating a lead-acid accumulator (car battery) or rechargeable uninterrupted power supply (UPS) units. Transporter designs are used for ambulatory services, i.e. to move premature babies, for example, from the labour room to the neonatal intensive care unit. During such transport operation, the machine power source would be switched to battery or UPS. In cases where the baby arrives in a distant hospital or maternity, the transport incubator could be inter-phased with the car or ambulance electrical systems for operation throughout the drive or flight. More sophisticated transport incubators are designed with integral neonatal ventilators for life support and to minimize the possibilities of successful apnoea attack. Transporter units are designed to also make use of the conventional electric mains supply during normal operation in the ward. Diagrams and photos of different models of transport incubators were used to buttress on the diversity of transporter designs where none was physically available in the hospital.

The ability of the incubator to make artificial (programmed) decisions was explained. This facility is installed in incubators at different capacity levels depending on the taste and design constraints of the manufacturer. The intelligence is mostly installed take care of

a. Incubator over-heating through 'air' temperature sensors or baby over-heat through 'skin' probes on baby.
b. Electric Current leakages which can cause electric shock and hence harmful to both the attendance and the premature baby inside it.
c. Humidity control for the comfort of the baby. Most of the earlier designs incorporated manually operated humidity controls.

Other primary allowable design capabilities, either automatically operated through its artificial intelligence or manually were explianed. These included Weighing features for checking baby's weight gains; Oxygen supplies through inbuilt oxygen concentrators or direct feed through independent oxygen cylinders or supply plants. The need and necessity for incorporation of cooling facility in tropical incubator designs to aid heat extraction for climatically overheated incubators was also explained.

Participants were also taken aback in history to trace the origin of modern neonatal incubation. This segment of the course endeavoured to show the progress in the development of the ideas and technologies that led to the design and production of the modern systems that could be seen today. The contributions of early players were discussed, including: the early Egyptian applications, the 1588 Giovanni Battista della Porta's idea, the 1609 Cornelius Drabbel's 'Athenor incubator' with thermostat, the 1770 John Champion's London design and patent of 1846. Other pioneering works examined were those of 1837 work of Dr Crede of Leipzig, Odile Martin's 'Couveuse' at Paris Maternity Hospital and the remarkable 1896 Earl's Court exhibition in London [Drebbel, 2011; Neonatalogy on the web, 1897].

The course isolated different basic assembly modules of an infant incubator and practically demonstrated these features to intimate students on the operational links of these to achieve regulated warmth for the baby. This includes: (a) Power source/input assembly (b) Electromechanical/Electronic compartment (c) Compressor/fan assembly (d) Thermal generation assembly (e) Humidity assembly (f) Incubation chamber/Neonate's

compartment (g) Thermostatic assembly and (h) External communication assembly. Cartoon illustrations were applied in some instances to ensure fair understanding of the ideas behind these various assemblies (Figure 6).

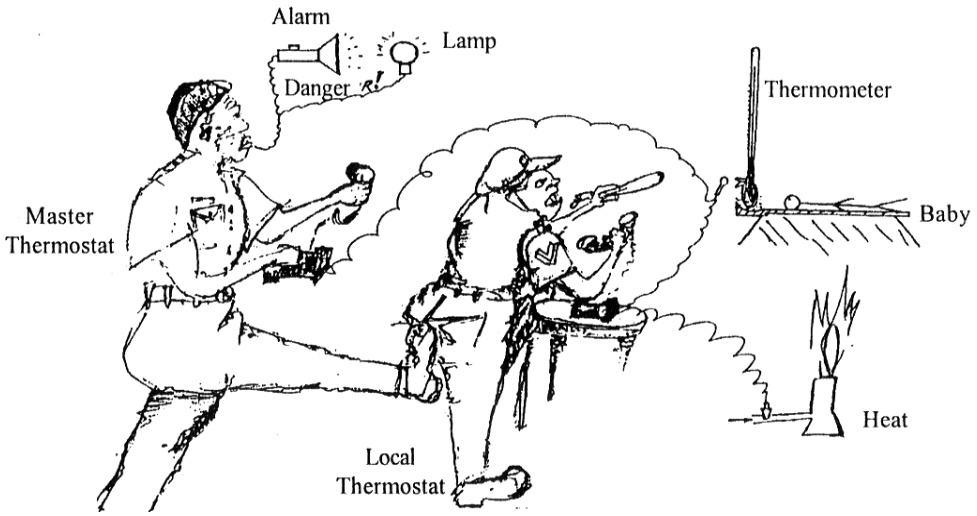


Fig. 6. Cartooned illustration of thermostatic operation

[This assembly is the thermal policeman of the incubator. It controls and gives the thermal generators instructions on when to supply heat and when to stop. It always checks up the temperature of the incubation chamber and compares it with the operator's input (set point). The thermostat's 'ON and OFF' or 'REDUCE and INCREASE' commands to the thermal generators help it to keep a check and guard against an under- or over -shoot of incubation temperature. It does this by what is known as "feedback control mechanism"]

7.1.1 Operational safety

This was a special segment of the course taught in relation to the neonate, the care-provider, the immediate nursery ward and the incubator itself. Necessary steps for the care of incubator were examined so that participants would appreciate the conducts that were required for their systems to remain functional and hygienic. Some of these were:

a. Preventive Maintenance Culture (PMC): This was an important routine that every newborn intensive care manager was encouraged to imbibe. The PMC is a practice of keeping the incubators and all other neonatal equipment in the neonatal intensive unit under a regular and routine technical check by qualified service personnel to ensure safety and avoid frequent breakdowns. Such breakdowns can occur at odd times especially when the services of the machine are most needed by a neonate. The PMC helps to put the systems in regular six or four monthly technical check-ups and performance auditing.
b. Cleaning: There was need for a regular cleaning and dusting of the machine outer casing everyday so long as the machine was in use. A thorough 'in and out' cleaning is very important before the machine is put into use after a long time of being parked. Various tested locally available disinfectants were recommended for use during such cleaning exercise. The cleaning of the incubators was discouraged from being grouped among the relegated menial jobs given to recruited 'ordinary' people to do. These often

low-paid casual workers were recruited to mop floors, clean windows and dust chairs. These also went ahead to handle the incubators, often with neonates inside, the same way as they cleaned chairs using same filthy and smelly rags. What a perfect way of introducing bugs that reduce neonatal survival and also cause damages to the incubator! The course stressed the need for hygiene around the neonates and the incubators and to keep off such infection vendors as outside visitors, improperly masked staff and mothers. Participants were encouraged to use their improved understanding of the neonatal microenvironment during the course to ensure effective cleaning standards that would be professionally acceptable.

c. Mains Input: There was always insufficient power sockets on the walls of nursery wards to take up all electrical appliances required to be on at the same time. This was an observed failure common to all participating neonatal centers without an exception. This deficiency in the nursery electrification often causes care-providers to multiply power ports by the use of 'cabled extension'. Unfortunately, this indiscriminately run all over the place, staying in the way for movement and potentially posing the hazard of a staff tripping over them. This might result in injury to the staff or the baby such may possibly be carrying. The use of extensions causes care-providers to innocently overload these cables and the host power sockets causing electrical sparks and shocks, endangering lives and damaging equipment. This was therefore labeled a hazardous and unprofessional practice that must be discouraged. New wiring designs were proposed and implemented in each Centre to correct this deficiency.
d. Humidity Reservoir: The care of the humidity reservoir was a segment that highlighted on the effective management of the incubator humidifiers. This explained how the reservoir might be kept from becoming nursery for infection-causing micro plants and organisms. Hence, humidifier tank must be drained of water whenever the machine was discharged of a neonate and kept dry if not in use. The tank should however be refilled with distilled water whenever the machine was to be used again. Water could be boiled and stored in a clean plastic container for use as an alternative to the use of distilled water if unavailable.
e. Thermometer: The operator must closely monitor the temperature of the incubator with the thermometer provided to probe the microenvironment. This helps to notice when the temperature might be indicative of unfavorable condition inside. This is important especially for cases when there is a thermostatic assembly failure.
f. Voltage Stabilizer: Use of single incubator-specific automatic voltage regulator (AVR) sets was introduced and encouraged to be applied. Erratic, poor quality and incessant power failures in these tropical countries often exposed the incubators to power surges and under voltage supplies that crippled their effective function (Figure 7). The functional inadequacies of this phenomenon were demonstrated diagrammatically to enable self-appreciation of the problem by the trainees so as to have personal drives to protect the systems during use.
g. Earthed Lines: Trainees were encouraged to carefully inspect incubators being delivered for use before acceptance. It was important to reject incubators with obvious electrical hazards such as un-fused systems and the absence of the 'earthing line' pin (E) on the plug head, whether the system was a new product from a supplier or repaired system from the maintenance workshop. Cartooned illustrations were used to demonstrate how these related to the general building earthing and how these served as safety devices to protect baby and care-providers from 'static' and 'mains' electric shocks (Figure 8).

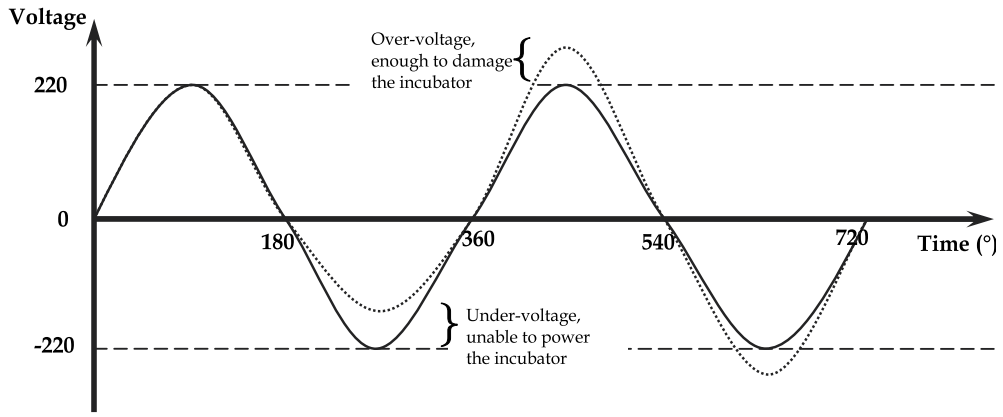


Fig. 7. Exaggerated sinusoids illustrating unsteady power supply

[There may be need for a voltage stabilizer set to run an incubator in these countries, especially for modern microprocessor based systems that might not operate with deficient power supplies. However, it must be noted that at adverse, erratic power supplies as has been witnessed, the stabilizer also stood in danger of being blown up together with the incubator it was supposed to be protecting. At this condition, if need be, it was necessary to power down the incubator.]

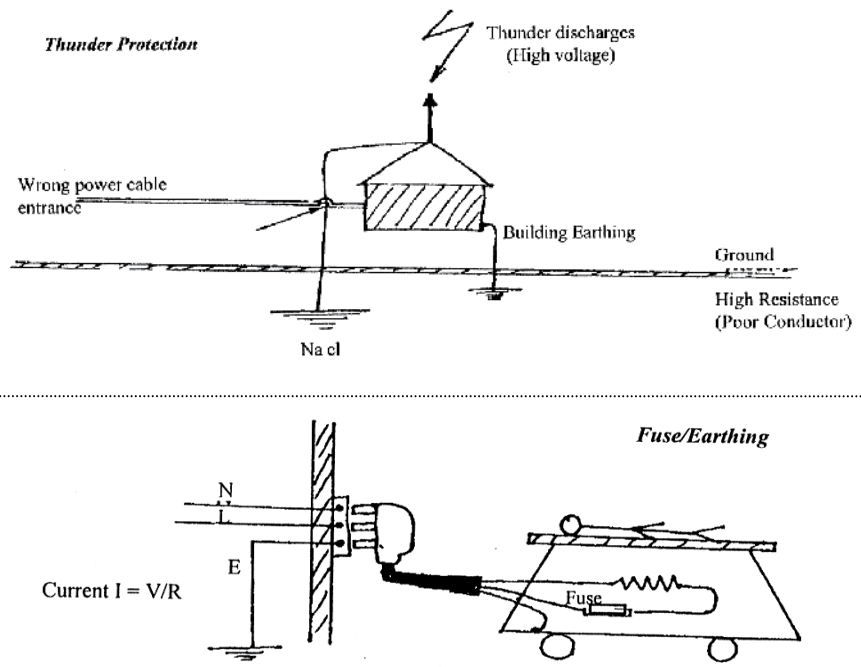


Fig. 8. Cartooned illustration of earthing line

[There must be a regular check of the effectiveness of the electrical earth conductor of the building that is housing the incubators. Improper 'earth' conduction or failure of the electrical earth line is an electrical hazard and can cause such accidents as electric shock to the baby or attending clinician. Poorly installed earth conductor such as shown in the diagram can cause interference of thunder discharges with the power supply to the machines. This can lead to damages to the incubators and other systems. Inexperienced contractors as has been noted can wrongly pass the copper conductor of thunder discharges directly over an electric cable that supplies the building. The interference that resulted from this in one occasion destroyed all connected appliances in the nursery.]

h. Socket Pin: Burnt wall sockets and plug head were observed being used to power incubators and appliances in the nursery irrespective of their conditions. The dangers of this practice were communicated and the continuous use of incubators with broken or partially burnt plug heads was discouraged. Hence, every broken power plug, burnt socket or wrong fuse must be changed before these were used to power the incubators. Figure 9 shows a couple of dangerous plug head/socket applications captured during usage.

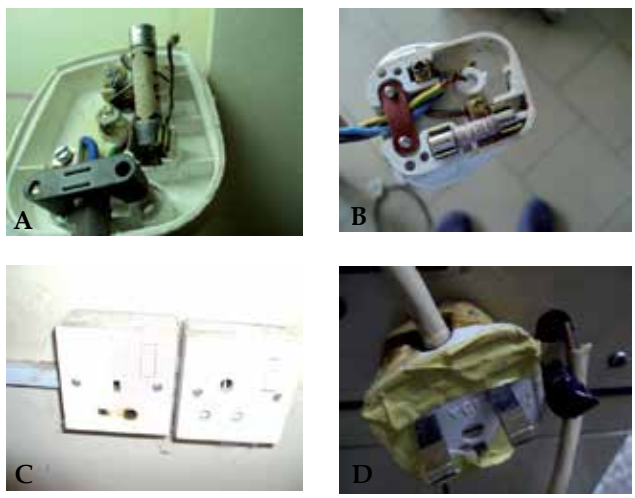


Fig. 9. Unsafe socket and plug heads (A) plug head with hair wire instead of properly rated fuse (B,D) broken plug head with missing earth-pins (C) burnt socket

i. Operator: Unit Heads were encouraged to ensure that untrained operators or nurses should not be allowed to man the machines until these were properly lectured on how to use them to provider effective care for the neonate. This pointed out the dangers of wrongly operating an incubator and how this might turn around to destroy the baby rather than saving it.

7.1.2 Incubator overheat

It was a common event across all centres to observe practices that led to an incubator overheating beyond their set point values. Such situations often resulted in neonatal hyperthermia for the babies inside the incubators. It was therefore necessary to carefully study how the identified conditions and practices resulted in the malfunctioning of the

incubators. A segment of the course module was dedicated to this, emphasising possible remedies as peculiarly applied to each Centre. The identified causes were of two types, namely externally induced warming and locally induced warming. External warming of the incubators happened as a result of wrong positioning of the system within the nursery building or close to other heat generating gadgets in the same rooms. The consequence of external sources of incubator warming is not necessarily a new discovery as this has already been commented in the literature (Lyon, 2004). However there was no practical evidence of a working knowledge of this in any of the Centres. Related errors were therefore identified, studied and included in the course module to educate users on how their habits were contributing to the situation. Few of these are briefly explained below.

a. Incubator access doors: Wrong usage of the incubator access doors and portholes was the primary cause of locally induced extra warming of the incubator. Attendants were often observed to carry out long procedures on the neonate right inside the incubators with all portholes or access doors left wide open whilst the incubator was still on (Figure 10). Such procedure might start at a time when incubator had already attained and maintained the set-point value. Opening of the portholes for a long period of time compromises the integrity of the microenvironment by a sudden drop in temperature within the chamber. This happened as a result of the nursery cooler air that uncontrollably rushed into the incubator chambers.

Fig. 10. A long clinical procedure being carried out inside a functioning incubator

The incubator air probe senses the drop in the chamber temperature and sends signals to the controller. This makes the thermal generators to increase heat output in attempt to counter the losses through the open portholes. This condition could cause the thermal generators to maintain an unnecessary 100% heat output for the period of the procedure without this being noticed because the extra heat was being fed to the wider nursery. However, soon

after the procedure has been completed and the portholes and access doors closed, the extra heat already generated within the thermal compartment is then concentrated in the recovered microenvironment overshooting the set-point value. The incubator intelligence would sense this and automatically withdraw heat generation. However it would take a long time for the accumulated chamber temperature to fall, within which this effect could instigate neonatal hyperthermia. Following from other studies, the literature has commented that the use of open bed is more convenient than the closed incubator during special procedures such as endotracheal intubation or arterial catheterisation (Tunell, 2004). Procedures requiring long period of time was advised to rather be carried out on a resuscitaire or 'work-bench' under a radiant warmer as this also allows the clinician enough space to work. Alternatively, the incubator should be switched off if such procedures must necessarily be carried out inside the incubator. This overheating effect was also practically demonstrated during normal working periods when such errors occurred and this helped to accelerate a change in this attitude of workers within the Centres. This practice was also identified to be unhygienic as many of the incubators were observed to contain various leftover items of work materials such as syringes, needles, wrist bands, cotton wool, plaster, sample bottles etc (Figure 11). A currency note and caps of water bottles were also items recovered right inside the incubators in some cases.

Fig. 11. Items of clinical and other materials trapped inside humidifier/heater chambers of three different incubators on active service.

b. Radiant warmer: Literature has previously pointed out external heat sources that could contribute to incubator overheat (Lyon, 2004). All through the observatory period of the present work, Centres never paid attention to incubator positioning

relative to open radiant warmers. This hence contributed a great deal to the re-occurring cases of incubator overheat and neonatal hyperthermia for systems located very close to warmers. Causes to this were mistaken by attendants to be local to the incubators until these were practically demonstrated during the series of trainings to show how the incubators displayed 0% local heat output whilst the chamber temperature was steadily rising. The practice of incubator positioning in these Centres were hence modified, avoiding incubators to be located next or very close to the location of open radiant warmers.

c. Phototherapy lamps: Neonatal jaundice is a widespread disease simultaneously treated whilst baby is being incubated. Therefore it was common to observe various kinds of phototherapy machines set over incubator canopies. Many of these often locally-produced systems operated on conventional household fluorescent lighting tubes. These deliver very high intensity of heat across the canopy to the baby, uncontrollably overwarming the microenvironment. The operating heights of these systems were often not adjustable or professionally fixed to avoid the consequent induction of incubator overheat and neonatal hyperthermia. It was hence demonstrated that the use of scientifically recommended phototherapy light tubes would ensure effective outcome without compromising baby's thermal stability. Constructions to adjust the operating heights of the local systems were recommended and carried out, especially when lack of spare parts or poor financing was the cause of resorting to the make-shift systems.

d. Nursery windows: There seemed to be no guiding rule for positioning an incubator in the nursery as some of these were placed directly behind windows. High heat intensity of sunlight gained easy direct access into the nursery through the glassy windows. This takes over the warming of the microenvironment through the transparent canopy. This uncontrollable external heating was often the cause of incubator overheats and hyperthermia during the day times. Incubators were therefore discouraged from being placed against glassy windows as much as possible within the nursery. Alternatively, appropriate window blinds that were able to minimise the solar radiation were fixed; these could be chosen to be drawn when necessary.

e. Nursery walls: Incubators were recommended to be placed no less than 45 centimetres from the nursery walls. This minimised the adverse effect of heat radiation from the overheated walls by the sun. The structural building pattern for new-born wards did not have any special consideration to minimise radiant heat storage. The observed poor designs easily made the walls work like a capacitor, being recharged by the sun from the outside during the day's heat. This retained the heat which was later discharged into the nursery, uncontrollably warming the incubators and babies in cots. This 'capacitance effect' was identified to be responsible for the common cases of periodic feverish attack on most babies in the ward. Though not yet reported in any medical literature, this experience is prevalent in all the Centres that participated in this study. This was observed to begin in many of the Centres at different times during the 'pm' periods of the day. This uncontrollable 'evening fever syndrome' would often confuse care-providers as they struggled to narrow the feverish event to any particular pathological or clinical cause. Most Centres were also observed to resort to using water to sponge babies to lower their temperatures. Others opened all incubator portholes and doors in attempt to lower temperatures thereby exposing neonates to the open environment and all kinds of air-borne infection vendors.

7.1.3 Further common errors

There were other commonly identified errors that might have contributed negative impact on the general wellness of the neonates, impoverishing practice outcomes. These were studied as applied to the local setting, trying out and proffering some coincidental effective solutions. Some of these were:

a. Humidification: Adequate humidification of the microenvironment has been said to enhance effective neonatal incubation (Silverman et al., 1958, 1963). Humidifier assembly is therefore a basic feature of a standard infant incubator. In most incubator designs this is located beneath the mattress tray at the covered lower aspect of the machine. Humidifier fill-ports from which this could be refilled or drained of water are usually visibly located in front or at the sides of the incubator for easy access. The often harsh climatic weather brings about a very low atmospheric Relative Humidity (RH), as low as 20% in the northern parts of Nigeria. It could therefore be a serious clinical challenge to keep baby adequately hydrated during resuscitation and neonatal nursing if this facility was not properly applied. The practice of running an incubator with dry humidifiers whilst baby was inside was a common failure observed in all the Centres at the beginning of this project. Quick staff interviews revealed that some attendants were not aware of any humidification facilities in incubator designs. Many of those aware of this confessed never bothered or remembered to add water to operate this. Many practical incidents occurred in Centres that perfectly assisted as good traditional events to convey the reasons behind these inevitable scientific procedures.

On one occasion during a whole day consultation in one of the collaborating centres, a certain incubator was running at a set point of 35.5°C. It was on a sunny dry 'harmattan' period, described as a West African season of hot, dry and dusty trade wind that normally blows from the Sahara during the month of November, carrying large amounts of dusts out over the Atlantic Ocean (Wikipedia, 2011; Britannica Online, 2011). The afternoon of the said day was about 35.2°C outside air temperature, nursery room temperature and RH of 34°C and 22% respectively. About half an hour later the same incubator with the same 5-day old baby was observed to have been reset to 37°C. The set-point of this was again, another 1hour 45 minutes later seen to have been increased to 40°C. The system was a 'dial-knob control', mercury meniscus-guide thermometer, Narco Isolette Airshields incubator model C86 at the SCBU of UPTH Port-Harcourt. Upon the observer's request, baby's skin temperature at that instant was measured to be an extreme hypothermic 33.2°C. The baby was said to have been on a steady temperature decline over several hours and throughout the period the incubator set-point was being raised. There was the presence of obvious clinical confusion as the attending clinician seemed to have eliminated some possible reasons for this situation and expressed fears of losing the baby. This true situation recalled similar events that had been observed in other Centres across the country. The incubator humidifier was at this point checked and discovered to be completely dry. As this called for emergency, available 'clean' water was called for and introduced, incubator set-point arbitrarily lowered to 37°C and humidification set at 'maximum' mark. This was also advised for the rest of the incubators in the ward as these were also being operated without functioning humidifier. To the amazement of the clinicians and nurses on duty who got involved in the frenzied situation, baby's condition dramatically reversed. Baby started to improve, gaining skin temperature after only 10 minutes and reaching 36.2°C in 35 minutes. This obvious wrong practice culture at UPTH at the time was to change forever as the science behind the drama of this event was explained to the wider audience of the Unit's care-providers.

Etiology: The science of molecular equilibrium in an open environment expects molecular migration from an area of higher concentration to a lower one. This general law was also expected of a microenvironment with very low RH as in this real life example. As baby was incubated 'naked', i.e. without clothes or wrapping, the microenvironment was a continuum with the porous-skinned neonate. This meant that baby, having more water than the immediate surroundings, was dehydrating by losing water to a thirsty atmosphere as the microenvironment sought to reach its saturation. Unfortunately, this instigated another general law of basic physics that 'evaporation causes cooling' and manifested in the dropping of baby's skin temperature. The subsequent practice response of the increased incubator heating made things worse because the microenvironment became dryer and hungrier for more water thereby exacerbating the baby's condition. Hence, this practice was never going to improve baby's condition under the present circumstances. Introduction of water in the humidifier chambers quickly saturated the microenvironment's atmosphere, reversing the concentration gradient of water molecules in the continuum in favour of the baby. As baby gained moisture and headed for saturation, evaporation immediately seized and neonatal cooling stopped. Hence, baby began to regain thermal stability as it retained the moderately supplied warming. It was possible for other neonatal complications to result in baby's loss of temperature as this; however, practice experience in this climatic region showed that elimination of possible causes should start with a check on humidification.

A near opposite of this occurred during heavy torrential rainy season that was also common to this climate, around the months of June. This would leave pockets of surface puddles scattered all over the area due to poor drainage systems. The atmospheric humidity of nearby neonatal nurseries had been measured to reach full saturation affecting the functioning of certain models of incubator such as the Vickers models 59, 79 and 77. The humidity control mechanism of such systems did not allow full stoppage of moisture supply to the microenvironment, especially when the humidifier contained maximum water. The reluctance of the wider nursery atmosphere to accept escaping moisture from the incubator soon led to saturation and condensation within the inside walls of the canopy. The resulting misty covering, often referred to as 'steaming' by the care-providers, blinds the see-through canopy, confusing the less experienced workers. The direct effect of such over-humidification on the wellness of the neonate had not been fully studied within the present project, however, literature points to a possible neonatal discomfort and a poor overall outcome (de Carvalho et al., 2011). A coincidental practice remedy to this was to fully minimise the setting of the humidity control followed by a possible drastic reduction of the humidifier water level.

b. Incubator overcrowding: This term refers to the wrong practice of putting more than one baby into a single functioning incubator, a common method initially observed in all the collaborating SCBUs (Figure 12). There are a lot of imaginable consequences of this practice on the general outcome.

i. This can easily lead to neonatal cross infection among the inmate babies and can potentially cause the loss of all of them to the same infection outbreak.

ii. This makes it absolutely difficult to regulate the incubator to suit all babies at the same time. Neonatal thermoneutrality is supposed to be a patient-specific application because thermal responses to the same environment can rapidly differ among neonates. This practice hence has the potential of saving one baby whilst adversely chocking the rest to death. Therefore this must be avoided where possible, even for a possible lower-risk carrying cohorts of a multiple-birth.

iii. There is a potential risk of mistaken administration of the wrong medication to the wrong patient by the often over worked nurses on duty.
iv. This increases the dangers of possible fall through less secured incubator portholes as reported by Health Devices (2010).

Fig. 12. Two babies sharing a single incubator.

This wrong approach was reported to be due to inadequacy of functional systems to independently support all needy babies, mostly blamed on poverty and poor funding. The seemingly moral reason of giving equal share to all needy babies as argued by some care-providers must be seriously weighed against the above consequences and for the sake of clinical hygiene.

7.2 Dynamics of neonatal thermoneutral control

This was the level 2 aspect of the 'Paediatrics Incubation Technique' course. The content of this aspect was drawn from lots of observed unscientific manner the incubators were operated during neonatal nursing. There was absolute lack of knowledge or any algorithm on how to re-regulate the incubator set-points based on the state of the neonate to achieve a physiological thermal equilibrium for the baby. Modern incubation techniques rely on algorithms that have been discussed in the literature stemming from the knowledge of 'central or core temperature' (t_c) and 'peripheral temperature' (t_p) of the neonate (Lyon and Oxyley, 2001). This technique requires the probing of the baby's skin temperature at two separate spots, notably the t_c from baby's back, in-between the scapulae and the t_p from the sole of baby's feet. This technique primarily measures a differential blood temperature (t_d) based on blood stream closest to the cardiac exit (chest level) and farthest travelled stream (foot level); $t_d = f(t_c, t_p)$. Instantaneous values of t_c and t_p are applied to proposed equations and situations to obtain the appropriate marginal values for upward or downward resetting of the set-point (Lyon and Oxyley, 2001). The proposed equations and resetting algorithms are theoretically sensible and supposed to be practically helpful for application in any setting of clinical practice. However, there were observed difficulties in its clinical usage in

the present situation as most of the clinicians and nurses would require a standby calculator to work out the values of t_d each time the incubator was to be reset. This soon became more frequent than to be tolerated by the often too busy few staff on shift. This difficulty was made worse by the high volume of neonates that were usually on admission during the shifts. In probable recognition of this difficulty, modern STA incubators such as the Draeger Caleo system are designed with a similar algorithm inbuilt in them. Therefore it is possible to permanently affix the temperature probes on the designated portions of baby's skin for the incubator to automatically and appropriately reset the incubator when baby's condition changed. Unfortunately, over 97% of the incubators in use in the studied tropical region were of older generation of incubator systems, requiring manual application of this modern algorithm by the attendants. On-the-spot monitoring and study of how attendants operated the few STA systems was carried out. The findings however raised new concerns for the consequences of inaccurate application of the temperature probes.

7.2.1 Dangers of 'skin mode' control

The presence of some skin-mode servo-controlled modern incubators and open warmers should have been a welcomed advantage for the few Centres that had them. However, a sound knowledge of the working principles of such advanced systems was extremely crucial for their services to produce effective automatic neonatal thermoneutral control. Unfortunately, our initial practice observations in these Centres showed insufficient understanding of these as has been expressed in the literature (Perlstein et al., 1997; Dollberg et al., 1993; De La Fuente et al., 2006). Users knew little about the possibilities of true skin temperature attenuation as the thermistor probes placement was always improperly done. These reported their previous experience and fears when baby's temperatures were noticed to soar whilst incubator displayed a desired 36.5°C. These could not effectively interpret the reason and hence resorted to manual control via 'air' mode. It was reported that some instances produced serious consequences of neonatal hyperthermia before baby's ordeal could be discovered. Tunell (2004) pointed out the complexities of the 'servo control' technique and suggested the use of manual regulation during the first days after birth. The important steps and assiduous care required to ensure that such automatic machines did not pose any threat to the life of the babies might have been stressed in the working manuals or by a trained company representative. However lack of the presence of quality professionals from these companies does not allow this. Users are often left at the mercy of common market traders, with no professional understanding, who act as middlemen or vendors for the big companies in the developed countries.

7.2.2 Handy approach

Sophistications and cutting-edge technologies are good, especially in developed countries where expectations compare to the scientifically advanced culture. However, as relates to West Africans with a different culture of poverty, illiteracy and underdevelopment, how do we communicate this sophistication in a sustainable manner? The primary goal of any standard was to save the highest possible number of needy neonates within the limits of poor funding and manpower. A culturally compatible approach would therefore be (1) clinically functional (2) relatively non capital intensive (3) highly simplified, locally-sustainable technology; operational techniques must be (1) easy-to-remember (2) simple for quick mental evaluation of control parameters (3) based on simple but functional

algorithms. These factors guided the development of the 'handy' approach currently being used in all the collaborating Centres. This was a simplified operational algorithm for achieving thermal stability in neonates. A recent follow-up study reported that over 80% of applying nurses believed that the usage of the technique was a boost to their practice enthusiasm [Amadi et al., 2010].

Principles: The handy approach might not be the best technique ever used but this was definitely better than the unscientific 'trial and error' methods observed at the inception of this project. This was developed by a long study that paid very close attention to the worst case scenarios such as the weakness of an 'extreme preterm baby' in an 'extremely harsh weather' of Nguru town, Nigeria. Nguru is a north-eastern ancient city of Nigeria notable with up to 47°C ambient temperatures during certain periods of the year. One of Nigeria's Federal Medical Centres (FMCnguru) was located in this grossly under-developed town. During the periods of these experiments approved by the FMCnguru's ethical committee, informed consents were obtained from mothers that were happy to permit the extended neonatal observations required for the study. The allowable standards for neonatal body temperature in most Nigerian neonatal centres including FMCnguru was a lower-upper limits of 36.5°C-37.4°C, measured from the axilla. Baby's thermal reaction to prevailing room temperatures were noted and compared to how volunteer healthy adults responded to the same harsh weather. Baby's skin temperatures behaved differently with those of the adults as these were observed to be always equal to the room temperature even when this increased to 43°C or decreased to 34°C whilst the adults maintained a constant range of 36.2°C-37°C all the times. This pattern was not exactly the same with higher birth weight and older postnatal age babies. Although such babies were all the same observed to be hyper- or hypothermic during these periods, their body temperatures were slightly lower or higher than room temperatures respectively. The local responsorial procedure at FMCnguru during high ambient heats was to sponge babies with water to minimise hyperthermia. Based on these findings, it was assumed that the neonate was very likely going to become hypothermic or hyperthermic depending on the relative overheating or under-heating of its host incubator. A neonate's thermal equilibrium set-point was therefore defined as the incubator air-mode set point that thermally stabilised a neonate to a body temperature of 36.5°C-37.4°C; incubator being appropriately humidified. The mid-point of this range was set at 36.9°C and used as a target for neonatal stabilisation. Therefore a general guiding principle for restabilising a deviating neonate was to increase or decrease incubator set-point value by an amount equal to baby's deviation from 36.9°C. This also demanded a compulsory recheck of baby's situation at intervals of no more than 30 minutes until baby re-stabilised. This was to allow enough time for the incubator to achieve the new set-point and for the baby to fully respond to the new changes, incorporating a possible neonatal cyclic temperature changes described by De La Fuente et al (2006). A disease process such as infection would be suspected and investigation initiated if baby's situation failed to respond positively to these changes after the 2nd cycle. It must however be first established that hyperthermia was not due to any domineering external warming of the incubator as described in section 7.1.2 of this chapter.

In quick easy-to-remember steps, the handy approach sets a good stage for the admission and systematic management of a neonate in an incubator thus:

a. Feeder Unit alerts SCBU: At the inception of this project the SCBU of most centres had poor or no existent system of pre-admission communication with feeder departments

such as the labour-ward/theatre for the in-born babies and any available reception unit for referral neonates. Therefore there was always a routine of frenzy and chaotic emergencies at the sudden appearance of an unexpected baby with a group of panicking adults. The confusion often created distracted the normal work flow of attendance on the nursery inmates. A standard of 'feeder unit alert' was hence established mandating a pre-admission alert with expected arrival time (EAT). A fair knowledge of the EAT for a prospective inmate allowed the SCBU management to make adequate preparations and properly assign respective duties to the attending staff in good time for the arriving baby.

b. Designation and readiness of the expected incubator: Following feeder unit alert, preparations and provisional designation of duties would start. The expectant incubator was the incubator chosen to host the expected baby upon arrival and after all the admission protocols and possible initial clinical routines has been completed on a resuscitation table. The steps for preparing the expectant incubator was: (1) Cleaning with a standardised disinfectant solution of a combination of antiseptic fluids and water, normally referred to as 'carbolisation'. The hood and matrass tray with all the interior of the canopy were thoroughly disinfected during this procedure. (2) Fresh cover spread was laid on the matrass and access portholes requiring replaceable covers were covered with fresh sterile blinds. (3) The humidifier that should have been completely drained of water after the last use was then re-filled with the appropriate incubation water. This was distilled water or in the alternative 'boiled and cooled' water. Reservation of the alternative incubator water was practiced provided this was done in a plastic container as the use of metal containers could generate rust and contaminate the water. (4) The incubator was then switched on. (5) Oxygen in-line supply was connected and tested for function. The supply was then turned off and kept on standby. (6) A provisional set-point for the incubator was fixed at the lower limit of the neonatal clinical range, i.e. 36.5°C. It was necessary to keep the provisional set-point closer to normal body temperature as the baby's point-of-admission temperature was yet unknown, whether this was going to be within acceptable range or not. (7) The incubator was then allowed to run to achieve this set point in good time before baby's EAT.

c. Admission, resuscitation and stabilisation: Upon baby's arrival, all the normal protocols were carried out by the admitting clinician and baby handed over for neonatal nursing.

d. Start of incubation: Initial thermal stabilisation started as soon as baby was introduced into the incubator. Baby's entrance temperature was checked and noted. Attendants would ensure that baby was securely place on the matrass and all the access doors and porthole covers were securely latched. Opening of all canopy access windows to work on the baby were kept at minimum. Baby's temperature was re-checked no later than 30 minutes after incubation began to confirm a possible re-adjustment of the incubator set-point, to search for the thermal equilibrium set-point.

e. Subsequent thermal re-stabilisation: This followed the procedure described earlier in this section to find a new equilibrium set-point whenever baby's temperature deviated from the allowable range. Figure (13) shows the guiding flowchart for this dynamics.

8. Externally influenced deficiencies

Gradual elimination of the various identified and rectified SCBU errors has steadily improved practice in the collaborating hospitals. However, there are other highly influential

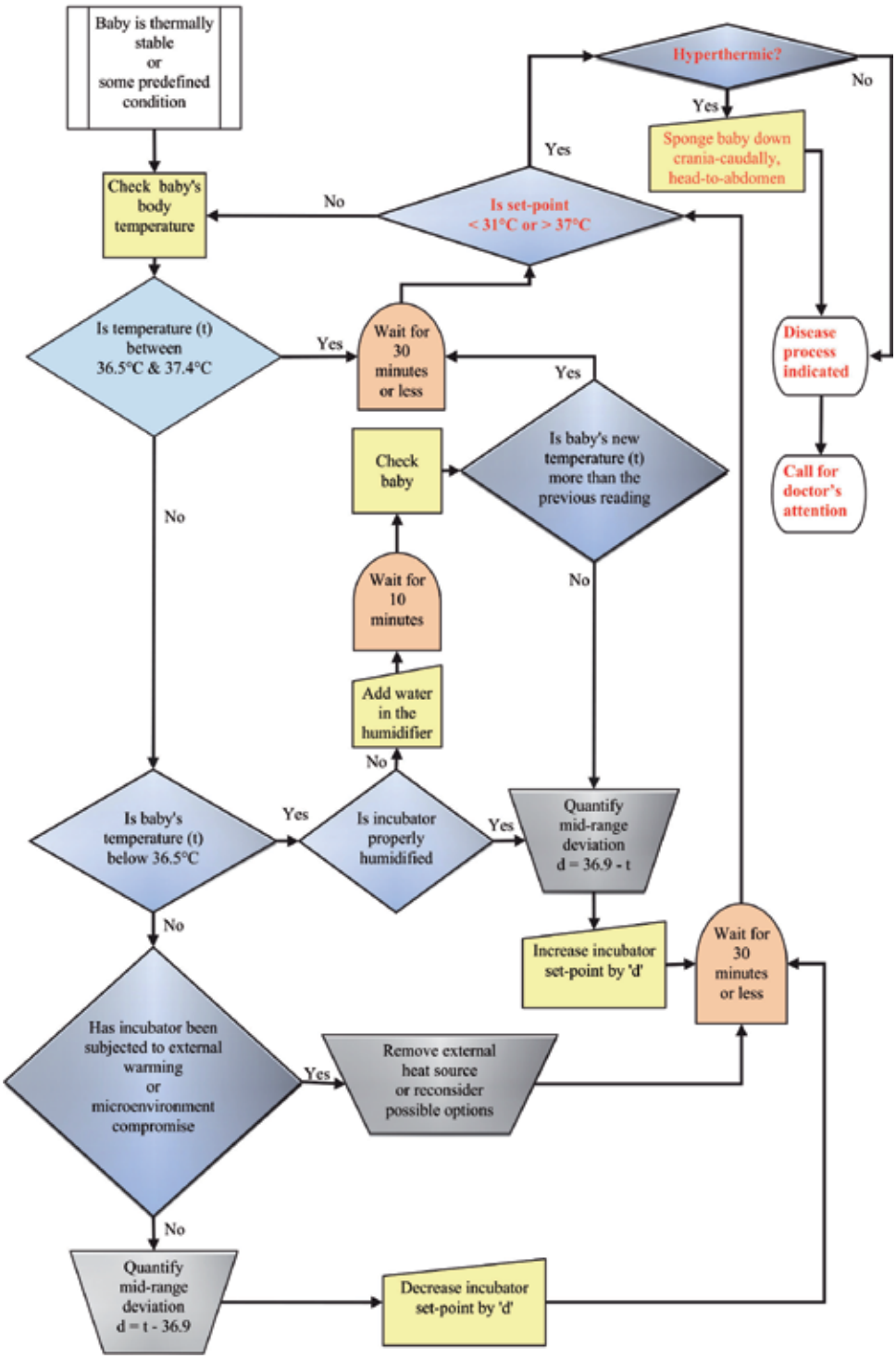


Fig. 13. Thermoneutral control flowchart

external factors that are possibly contributing to lower practice outcome. This category of problems might be beyond the ability of immediate SCBU to correct, hence required the cooperation of the higher institutional management to resolve.

8.1 Epileptic power supply

Inside a functioning incubator and yet wrapped! One would expect that a well-documented and known practice of nursing neonates naked inside a functional incubator would not need to be overemphasised anywhere in the world (Lyon, 2004). However this was initially observed to be one of the wrong incubator applications in the collaborating Centres. Some of the users showed evidence of this knowledge but could not stop because they needed to protect baby from cold stress that sets in upon power failure. However, there were no clear reasons given for this practice during the periods when the system functioned. Uninformed and indiscriminate electric power outages that last for several hours are common to West African countries. Unfortunately, very sensitive units as the SCBU of hospitals suffer from this problem at which point the incubator suddenly fails exposing baby to danger of cold stress and hypothermia. The use of 'standby' generators is widespread but this still does not effectively cover up this deficiency in most Centres. There were also reports of incubator damages due to power surges from malfunctioning generators. Full-sine-wave power inverting technology was therefore considered an option to investigate. A full-sine-wave power inverter system with a cascade of sealed batteries function to convert the DC power of the batteries to AC power required to operate the incubators upon conventional mains power failure. This operates with an automatic power change over system that allows it to stay 'on' to recharge the batteries when mains AC supply is available and switches incubators to draw inverted battery power when mains supply fails. A 5KVA system installed with up to 8 pieces of '12 volts 200 amp-hour' batteries was found to be able to continuously support up to 11 incubators simultaneously for up to 10 hours. This was enough to provide uninterrupted power supply to the most critical neonates in the few Units that could afford to implement this, hence minimising the effect of operating power deficiencies.

8.2 Inadequate nursing staff

The sudden increase in the number of patients seeking to be admitted for neonatal care as reported by Amadi et al (2010) meant that more hands were needed to cope with the present volume of work in each participating SCBU. The Units were hence faced with the lack of adequate manpower and challenges to retain the already experienced ones on employment. External issues of government policies on employment and local administration of the Nursing Department in these hospitals were contributing factors to the challenges. The direct effect of this to the present project was the resulting inability to effectively offer adequate care using all the developed techniques and procedures in this project. Some of these challenges as enumerated below are currently being tackled through engaging the various hospital managements to demonstrate the importance of discriminate staffing of the new-born Units.

1. Maintaining a sizable number of SCBU nursing staff as compared to adult or young-adult wards. Most of the SCBUs are currently having up to 40 inmates on admission at the same time with as few as 3 nursing staff to look after them during some rota shifts. Our current study shows that the quality of attention offered to these babies becomes

clinically unacceptable at more than a patient-to-nurse ratio of 5. This mark is frequently being exceeded, hence calling for urgent review on nurses' deployment to the Units. This has also led to some preventable loses to apnoea as many attacks were not detected early enough to commence resuscitation. It therefore became important to propose and implement the provision of integral digital apnoea monitors on all incubators and cots. These raised audible alarms during attacks, enabling the few nurses on duty to be aware of the points of emergency even whilst they were busy with other babies.

2. Frequent shovelling of experienced nurses has militated against more excellent results from the Centres. It was a common routine in 80% (12/15) of the hospitals to re-shovel senior nurses among all the departments including the neonatal wards. This was entirely governed by the Nursing Department as a measure to allow nurses acquire experiences of how things worked in various departmental wards and happened as frequent as every two years or less. There might be good intentions for this; however, our findings from the present study showed that this was producing a serious counterproductive effect on the SCBU target. Nurses needed to stay in the Units for up to 18 months to fully understudy the new systems and procedures being implemented to positively alter the neonatal mortality as these ideas were completely new to most of them. During this period they would have attended the level 1 and perhaps level 2 of the Paediatric Incubation Technique courses. This has hence frequently created occasions when all three or four nurses on duty were completely untrained newcomers to the new procedures, hence slowing down the progress of the Unit. Minimizing the unequal shovelling of well-experienced and trained neonatal care-givers with inexperienced ones has therefore become a major issue to settle in all the hospitals. A proposal was drawn and negotiated with the various Nursing Administrative Departments of the hospitals to implement a 'neonatal 70-30' agenda whereby their normal shovelling exercise must ensure that 70% of SCBU qualified nurses were specialised or have at least 15 months experience and certified on the course levels 1 and 2. This excluded the numerous yet-to-qualify and short-staying nursing students that must work under full supervision of at least one experienced nurse on duty. This is presently working well and yielding good results in 6 of our 15 collaborating centres. Quantified in terms of incubation hours denied due to system breakdown, and comparing one calendar quarter before and after full implementation of the agenda, this has on the average saved 81% (10,886.4 hours) of the total incubation time lost to system breakdown before implementation. It is evident from these 6 Centres that frequency of system breakdown due to mishandling has dropped, thereby reducing maintenance costs and providing for more babies to save.
3. Compulsory theoretical course (requiring a pass in an end of course test) was initiated and currently being implemented by some of the Nursing Administrative Departments as a prerequisite for posting a new nurse to the SCBU. In the new guideline, resident doctors that were specialising on new-born care were advised to complete the 2 levels of the elective course.

9. Conquering the climate

The conclusions being drawn from the entire project suggest that culture and climate were major forces to conquer in order to realise the MDG target on neonatal mortality. Our on-

going studies at the University of Ilorin Teaching Hospital and the Federal Medical Nguru have identified a number of parameters that could be altered to reduce the negative impact of climate on new-born morbidity. We studied all the nursery buildings at our disposal, these being all distinctively different from each other in design, structure and relative location. The impact of high sunlight intensity as a source of uncontrollable external warming of the incubator was used to identify the parameters that were aiding or preventing the harsh climate. Incubators functioned well, adequately maintaining their set points, when these had absolute control of the warming of their microenvironments. This occurred during cooler periods of the day or the night when the nursery ambient temperature dropped well below 30°C. However this often changed during the day when the macro-environment of the inside of the nursery became excessively hot due to radiation from the sun. We therefore hypothesised that minimising the outside influence of climatic heat on the nursery would enhance effective thermoneutral control and achieve better success rates. It is understood that the use of air-conditioners could artificially cool the nursery wards to counter room warming during the day. This was tried but not considered a sustainable solution as the high frequency of breakdowns without immediate repair or replacement often sent the Unit back to the same ugly situation. Again, this was also noticed to present threats of hypothermia on the other full-term babies in cots as these shared the open nurseries with the incubator babies. It therefore became necessary to find enabling parameters that could be altered to attain the best naturally cooled condition in the nursery. Parameters were preliminarily identified by comparing nursery warming in any two Centres that have direct opposite circumstance. Parameters currently being studied in details were:

1. Siting of the nursery building within hospital complex. Nurseries that were cited as the eastern-most building among the rest in the hospital complex and without any other immediate building east to this seemed to be hotter than those elsewhere cited.
2. Locating nursery within building structure. Nursery apartments that were located on the topmost floor of a multi-storey building seemed hotter than those located on the ground floor.
3. Structural design of nursery outside wall. Nursery designs that provided the main ward at the middle of other flanking rooms, stores or side labs seemed cooler than designs where the wall of the main ward was directly next to the outside. This suggests that some kind of wall lagging designs might provide the needed natural cooling for the macro-environment.
4. Floor to roof height of nursery. Nurseries with higher roofs from the floor level seemed cooler than the shorter ones.
5. Nursery floor level, nursery window height and nursery window blinding material were also identified to seem to create some differences and hence also being studied.

10. Conclusion

This project has been an individual coordinating effort in a drive to lower neonatal mortality rate, restore nursing enthusiasm and patient- carer's confidence in the tropical region of West African state of Nigeria. The project originally set out to find alternative solution to the provision of functional incubators to re-equip the referral hospitals in the country. This began in each of the participated hospital at a time when most of these had no functional incubator. The development and application of the idea of Recycled Incubator Technique (RIT) helped to realise the initial objective as this has made it possible for some of the

hospitals to move from a condition of having no functional incubator to having 15 or more within a short period of time. However, the restoration of proper usage of the incubator to nurse babies exposed the knowledge deficiency of care-providers in incubator application. It was evident that immediate cultural setting and the quality of care with the provided incubator were capable of promoting the spread of diseases among the neonates. Hence, this project extended to the study and proposition of corrective procedures that were easily applicable to the people.

The implementation of the various ideas developed in this study has brought cultural dimension to tweak already established practice facts. This was another way of using the local language to communicate the medicine of neonatal thermocontrol in this tropical region. The methods were easily acceptable and adaptable and seemed to have led to improved outcome among all participating Centres.

Overall, the entire project has achieved significant success across the landscape of Nigeria among all the applying hospitals as published by Amadi et al., 2010. This study was unable to explicitly isolate successes due to the provision of incubators as an initial project and the duo of training courses and modified thermoneutral algorithm as an extended application. These were applied simultaneously. It is commonly acknowledged among hospital administrators in Nigeria that the advent of RIT and the subsequent thermocontrol procedures represented a significant contribution to Nigeria's improving neonatal healthcare delivery (CCEFTHI, 2007). Further investigations are still continuing on how the climate is impacting and militating against overall outcome. We hope to fully define this and proffer solutions on how to ameliorate this and boost survival rate in the region.

11. Acknowledgement

The support of all the Chief Medical Directors of the hospitals that have so far collaborated is hereby acknowledged. Some of these were outstanding in their cooperation at various segments of this project. These were Akin Osibogun, Bello Kawuwa, Uriah Etawo, Dan Iya, Sulyman Kuranga, Abulhameed Dutse and Eugene Okpere. Other individuals whose supportive roles are worthy of mention are Jonathan Azubuike, Olugbenga Mokuolu and Peter Onyeri.

12. References

Amadi, H.O.; Azubuike, J.C.; Etawo, U.S.; Offiong, U.R.; Ezeaka, C.; Eyinade, O.; Adimora, G.N.; Osibogun, A.A.; Ibeziako, N.; Iroha, E.O.; Dutse, A.I.; Chukwu, C.O.; Okpere, E.E.; Kawuwa, M.B.; El-Nafaty, A.U.; Kuranga, S.A. & Mokuolu, O.A. (2010). The impact of recycled neonatal incubators in Nigeria: a 6-year follow-up study. *Int J Paeditr*. Vol.2010, Article ID 269293, 7 pages doi:10.1155/2010/269293

Amadi, H.O.; Mokuolu, O.; Adimora, G.N.; Pam, S.D.; Etawo, U.S.; Ohadugha, C.O. & Adesiyun, O.O. (2007). Digitally recycled incubators: better economic alternatives to modern systems in low-income countries. *Annals Tropical Paeditr*, Vol.27, No.3, (September 2007), pp. 207-214, ISSN 0272-4936

CCEFTHI. (July 2007). Communiqué issued at the end of the 58th quarterly meeting of the Committee of Chief Executives of Federal Tertiary Hospitals, University of Benin Teaching Hospital Nigeria, 16.02.2010, Availbale from ccefth.org/document/communique_53rd_meeting.pdf

Centre for Maternal And Child Enquiry. (2010). Overview of perinatal and neonatal mortality in the UK. In: *Perinatal mortality 2008: United Kingdom,* J. Dorling (Ed.), 9-15, CMACE, ISBN 978-0-9558055-3-0, London, United Kingdom

de Carvalho, M.; Torrao, C.T. & Moreira, M.E. (2011). Mist and water condensation inside incubators reduce the efficacy of phototherapy. *Arch Dis Child Fetal Neonatal Ed*, Vol.96, No.2, (March 2011), pp.F138-F140

De La Fuente, L.; Campbell, D.E.; Rios, A.; Grieg, A.; Graff, M. & Brion, L.P. (2006). Frequency analysis of air and skin temperature in neonates in servo-controlled incubators. *Journal of Perinatology*, Vol.26, No.5, (May 2006), pp. 301-305

Dollberg, S.; Xi, Y. & Donnelly, M.M. (1993). A non-invasive transcutaneous alternative to rectal thermometry for continuous measurement of core temperature in the piglet. *Pediatr Res*, Vol.34, pp. 512

Drebbel. (June 2011). Incubator, In: *Cornelis Drebbel's inventions and instruments: references and descriptions*, pp. 3, 18.06.2011, Available from http://www.drebbel.net/Drebbels%20Instrumenten.pdf

Encyclopedia Brittanica. (June 2011). Harmattan wind. In: *Brittanica Online Encyclopedia*, 18.06.2011, Available from http://www.britannica.com/EBchecked/topic/255457/harmattan

Federal Ministry of Health. (2011). Saving newborn lives in Nigeria: Newborn health in the context of the Integrated Maternal, Newborn and Child Health Strategy. *Federal Ministry of Health Abuja, Save the Children, Jhpiego*, 2nd edition, 24.06.2011, Available from http://resourcecentre.savethechildren.se/content/library/documents/nigeria-newborn-health-report-context-integrated-maternal-newborn-and-chilHealth

Devices. (2010). Hazard report-Infants may fall through insecure ports on incubators. *Health devices*, Vol.39, No.1, pp. 25-26

Ibe, B.C. (1993). Low Birth Weight and Structural Adjustment Programme in Nigeria. *Journal of Tropical Paediatrics,* Vol. 39, pp. 312-313

Lyon, A. (2004). Applied physiology: temperature control in the new born infant. *Current Paediatr*, Vol.14, pp. 137– 144

Lyon, A.J. & Oxley, C. (2001). Heat Balance, a computer program to determine optimum incubator air temperature and humidity: a comparison against nurse settings for infants less than 29 weeks gestation. *Early Hum Dev*, Vol.62, pp. 33–41

Neonatology on the web. (1897). The use of incubator for infants. *The Lancet*, pp. 1490-1491, Available from http://www.neonatology.org/classics/lancet.incubators.html

Ogunlesi, T.A.; Dedeke, O.I.; Adekanmbi, F.A.; Fetuga, B.M. & Okeniyi, A.J. (2008). Neonatal resuscitation – knowledge and practice of nurses in western Nigeria. *South African Journal of Child Health*, Vol.2, pp. 23-25

Perlstein, P.H. (1997). Physical environment. In: *Neonatal–Perinatal Medicine: Diseases of the Fetus and the Infant,* A. Fanaroff & R. Martin, (Eds.), 481–501, Mosby, Philadelphia, PA

Silverman, W.A.; Agate, F.J. & Fertig, J.W. (1963). A sequential trial of the nonthermal effect of atmospheric humidity on survival of human infants of low birth weight. *Pediatr*, Vol.31, pp. 710–724

Silverman, W.A.; Fertig, J.W. & Berger, A.P. (1958). The influence of the thermal environment upon survival of newly born preterm infants. *Pediatr*, Vol.22, pp. 876–885

Tunell, R. (2004). Prevention of neonatal cold injury in preterm infants. *Acta Paediatr*, Vol.93, pp. 308–310, ISSN 0803-5253

WHO African Region. (September 2009). Child and adolescent health. *World Health Organisation,* 07.09.2009, Availabe from http://www.who.int/countries/nga/areas/cah/en/index.html

Wikipedia. (June 2011). Harmattan. In: *Wikipedia, the free encyclopedia*, 18.06.2011, Available from http://en.wikipedia.org/wiki/Harmattan

Associations Between Nutritional Indicators Using Geoadditive Latent Variable Models with Application to Child Malnutrition in Nigeria

Khaled Khatab
Institute of Occupational and Social Medicine, Medical School, RWTH Aachen University
Germany

1. Introduction

Childhood undernutrition is amongst the most serious health issues facing developing countries. It is an intrinsic indicator of well-being, but it is also associated with morbidity, mortality, impaired childhood development, and reduced labor productivity (Svedberg 1996; UNICEF 1998; Sen 1999)

To assess nutritional status, the 2003 DHS obtained measurements of height and weight for all children below five years of age.(Survey 2003) Researchers distinguish between three types of malnutrition: wasting or insufficient weight for height indicating acute malnutrition; stunting or insufficient height for age indicating chronic malnutrition; and underweight or insufficient weight for age which could be a result of both stunting and wasting.

These three anthropometric variables are measured through z-scores for wasting, stunting and underweight, defined by

$$Z_i = \frac{AI_i - MAI}{\sigma}, \tag{1}$$

where AI refers to the individual anthropometric indicator (e.g. height at a certain age), MAI refers to the median of a reference population, and σ refers to the standard deviation of the reference population. Each of the indicators measures somewhat different aspects of nutritional status. Note that higher values of a z-score indicate better nutrition and vice versa. Therefore, a decrease of z-scores indicates an increase in malnutrition. This has to be taken into account when interpreting the results. The reference standard typically used for the calculation is the NCHS-CDC Growth Standard that has been recommended for international use by WHO. (WHO 1999) The reference population are children from the USA. More precisely, the children, up to the age of 24 months are from white parents with a high socio-economic status, while children older than 24 months are from a representive sample of all US children. The selection of the reference populations can affect the results, for example a higher z-score can be caused by the change of the reference population.

Latent variable model: Previous analyses are often based on Demographic and Health Surveys (DHS) as a well-established data sources with reliable information on childhood

undernutrition, and they rely on statistical inference with various forms of regression models. Because of methodological restraints, it is difficult to detect nonlinear covariate effects adequately, for example, age, and it is impossible to recover small-scale, district-specific spatial effects with common linear regression or correlation analysis. Recent research has therefore applied geoadditive regression models (Fahrmeir L 2001; Fahrmeir 2004) .They have been used in regression studies of risk factors for acute or chronic undernutrition (e.g., Kandala et al., 2001; Adebayo 2003; Khatab, 2007) and for morbidity.(Kandala 2001; Adebayo 2003; Kandala, Magadi et al. 2006; Kandala, et al. 2007; Khatab 2007) These models can account for nonlinear covariate effects and geographical variation while simultaneously controlling for other important risk factors.

However, in all these studies regression analyses are carried out separately for certain types of undernutrition such as stunting, wasting or underweight, neglecting possible association among these response variables and without aiming at the detection of common latent risk factors. Because of common and overlapping risk factors, separate analyses may fail to give a comprehensive picture of the epidemiology for the malnutrition and the joint effects of childhood malnutrition at population level.

To asses the association between the the nutritional indicators, we applied the recently developed latent variable model. This model gives us the opportunity to study the association or interrelationship between the three types of malnutrition as indicators for nutritional status. The factor loadings describe the association between these indicators and their impact on the nutritional status of a child. Latent variable model permits modeling of covariate effects on the latent variables through a flexible geoadditive predictor

The objective of this study is to determine the associations between nutritional indicators among Nigerian children under 5 and also to examine the impact of socioeconomic and public health factors on the nutritional status.

Nutritional status is known to have various risk factors including geographical locations as a proxy of socioeconomic and environmental factors that affect the disease prevalence and incidence.

Spatial heterogeneity in these factors influences the nutritional status pattern. Consequently, efforts to reduce the burden of childhood undernutrition should include investigations into the influence of the associations between the different measurements of the malnutrition status of children and their distribution among the locations on child health.

Two approaches of latent variable models (joint model) analysis of malnutrition have emerged: the measurement model which accommodates and describes the effect of the latent variables and a set of observed covariates (e.g. child's sex, mother's educational attainment, working status, etc) on the nutritional indicators such as stunting, wasting and underweight.

The structural model is linking a set of observed covariates which have indirect effects (such as child and mother's age, etc), with the latent variables.

In the latent variables overall specific risks are estimated having adjusted for covariates, and in addition, the correlation of risk between measurements of the malnutrition can be quantified.

In this study, we considered the latent variable model to jointly analyse childhood stunting, wasting and underweight, with the objective of highlighting spatial patterns of these indicators.

To build a regression model for undernutrition, we first have to define a distribution for the response variable. In this application, it is reasonable to assume that z-score is Gaussian distributed; thus in principle, could be applied.
The analysis started by employing a separate geoadditive Gaussian model to continuous response variables for wasting, stunting and underweight. The author then applied geoadditive latent variable models, based on these separate analyses results, which were reported in Khatab, 2007, where the three undernutrition variables were taken as indicators for the nutritional status of a child.
All computations have been carried out with R Programs using the MCMC package; see(Raach 2005; Khatab 2007)

2. Data & methods

DHS collects information on household living conditions such as housing characteristics, on childhood morbidity, malnutrition and child health from mothers in reproductive ages (15-49). There were 6029 children's records in the 2003 survey of Nigeria. Each record consists of information on childhood malnutrition and diseases and the list of covariates that could affect the health and nutritional status of children. In the following, we provide some more information about the nutritional indicators, which were used as response variables and information about the covariates considered in this study.
Stunting. Stunting is an indicator of linear growth retardation relatively uncommon in the first few months of life. However it becomes more common as children get older. Children with *height-for-age* z-scores below minus two standard deviations from the median of the reference population are considered short for their age or stunted.
Wasting. Wasting indicates body mass in relation to body length. Children whose *weight-for-height*'s z-scores are below minus two standard deviations (z-scores $< -2SD$) from the median of the reference population are considered wasted (i.e. too thin for their height) which implies that they are acutely undernourished otherwise they are not wasted.
Underweight. Underweight is a composite index of stunting and wasting. This means children may be underweight if they are either stunted or wasted, or both. In a similar manner to the two previous anthropometric incidences, children may be underweight when their z-score is below minus two standard deviations and they are severely or moderately so if their z-score is lower than two standard deviations. The included variables in Table 1 were considered in the analysis to study child nutritional status.

3. Statistical analysis

In the following, we focus on geoadditive Gaussian models for continuous response variables to analyze the effects of metrical, categorical, and spatial covariates on stunting, wasting and underweight response variables in latent variable analyses. Furthermore, we use "nutritional status" as the indicator in the analysis of the latent variable models as mentioned.

3.1 Geoadditive gaussian model

In this analysis, we apply a noval approach by exploring regional patterns of childhood malnutrition and possible nonlinear effects of the factor within latent model framework using geoadditive Bayesian gaussian model for continuous response variable.The model

Factor	N(%)	Coding effect
Place of residence		
Urban	2237(33.58%)	1
Rural	4424(66.42%)	-1.ref
Child's sex		
Male	3487(52.35%)	1
Female	3174(47.65%)	-1.ref
Working		
Yes	1209(18.15%)	1
No	5452(81.85%)	-1.ref
Mother's Education		
No,		
Incomp.prim,		
Comp.prim,		
Incomp.sec	4194(62.97%)	1
Compl.sec,		
Higher	2467(37.04%)	-1.ref
Pregnancy's treatment		
Yes	697(10.46%)	1
No	5964(89.54%)	-1.ref
Drinking water		
Controlled	5374(80.68%)	1
Not controlled	1287(19.32%)	-1.ref
Missing	1%	
Had radio		
Yes	5374(80.68%)	1
No	1559(19.32%)	-1.ref
Has electricity		
Yes	6203(93.12%)	1
No	458(6.88%)	-1.ref
Toilet facility		
Own flush toile facility	1768(28%)	1
Other and no toilet facility	4511(71.8%)	-1.ref
Missing	1%	
Antenatal visit		
Yes	4181(63%)	1
No	2342(35%)	-1.ref
Missing	2%	

Table 1. Factors analyzed in malnutrition study

used for this investigation has been described else where.(Raach 2005; Khatab 2007) . Basicaly in the early stage of this study we used the geoadditive Bayesian gaussian model for the separate analysis. In this model we replace the strictly linear predictor

$$\eta_{ij}^{lin} = x_{ij}'\beta_j + w_{ij}'\gamma_j \quad j = 1,..,3, \tag{2}$$

With geoadditive predicator, to have geoadditive model

$$\eta_{ij}^{geo} = \beta_{0j} + f_1(Chage_i) + f_2(BMI_i) + f_3(Mageb_i) + f_{spat_i}(s) + w_{ij}'\gamma_j \tag{3}$$

where w includes the categorical covariates in effect coding. The function f_1, f_2 and f_3 are non-linear smooth effects of the metrical covariates (body mass index, child, and mother's age) which are modelled by Bayesian P-splines, and f_{spat} is the effect of the spatial covariate $s_i \in 1;...;S$ labeling the districts in Nigeria. Regression models with predictors are referred to as geoadditive models. However, in this work we have used g**eoadditive latent variable models** to overcome the drawbacks of separate analysis.

3.2 A bayesian geoadditive LVM (latent varaible models)

A latent variable model with covariates consists of two main approaches: the measurment model for continuous response with covaraites influencing the indicators directly (direct effects); and the structural model explaining the modificatio of the latent variables by covariates (indirect effects) (Fharmeir and Raach, 2007; Khatab, 2007)

3.2.1 Mesurment model

$$y_{ij} = \lambda_0 + a_j'w_i + \lambda_j\upsilon_i + \varepsilon_{ij},\ i = 1,..,n,\ j = 1,..,p, \tag{4}$$

Where υ_i represents the nutritional status with independent and identically distributed Gaussian errors $\varepsilon_{ij} \sim N(0,\sigma^2)$. In this model, υ_i is the unobservable value of υ for individual i, λ_j is the "factor loading", and $\lambda_j\upsilon_i$ is the effect of υ_i. In addition, w_i are the direct effects which affect the observed variables directly and a_j is the vector of regression coefficients. The restriction to $\sigma_\upsilon = var(\upsilon) = 1$ is necessary for identifability reasons . (Fahrmeir L 2007; Khatab and Fahrmeir 2009).
Continuous variables are observed directly, hence the underlying variable is obsolete.

3.2.2 General geoadditive structural model

$$\upsilon_i = u_i'\alpha + f_1(x_{i1}) + ... + f(x_{iq}) + f_{geo}(s_i) + \delta_i, \tag{5}$$

with independent and identically distributed Gaussian errors $\delta_i \sim N(0,1)$. The restriction to $\sigma_\upsilon = \text{var}(\upsilon) = 1$ is necessary for identifiability reasons.
RESULTS. We applied a geoadditive latent variable model, using the three types of undernutrition as indicators of latent nutritional status. The decision which covariates should be used in the measurement model, and which should be used in the structural equation, is based on the same criteria that was used in (Khatab 2007; Khatab and Fahrmeir 2009).

Our interest is in analyzing the three types of undernutrition of children using latent variable models, and in investigating how they can be established as indicators of the latent variable "undernutrition status". Based on the previous separate analyses (Khatab, 2007), we are able to determine which factors can have direct effects and which can have indirect effects on the indicators.

In order to choose the covariates used in the measurement model (which have direct effects on the disease indicators); or in the case of the structural model, those have indirect effects via their common impact on the latent variable "nutritional status," we used the following criteria: if the effects of covariates turned out to be significantly different (in terms of confidence intervals) for the three diseases, we decided to keep them in the measurement model, otherwise covariates were included in the geoadditive predictor of the structural equation for the latent variable (Khatab and Fahrmeir 2009).

We started by using the easiest model possible, a classic factor analysis for continuous indicators. The predictor of the structural equation of the model yields LMV0:

$$\eta = 0 \tag{6}$$

Estimates of factor loadings are depicted in Table 2. The estimated mean factor loadings show that indicator 2 (*weight-for-age*) has the highest factor loading. That means the most effect on the z-scores is on underweight for age and is followed by the indicator of stunting.

The classic factor analysis model has been extended by introducing direct and indirect parametric covariates, which modified the latent construct.

Parameter	Mean	Std	2.5%	97.5%
Factor Loadings				
1. stunting λ_{11}	1.244	0.02	1.206	1.28
2. underweight λ_{21}	1.36	0.08	1.353	1.38
3. wasting λ_{31}	0.770	0.015	0.739	0.801

Table 2. Results of Model LVM0 of Z-scores indicators with $\eta = 0$.

The next model was selected based on the previous separate analyses (reported in Khatab, 2007). This leads to the latent variable model.

In the fundamental analysis (LVM1), the vector a_j comprises the covariates urban, antenatal visits, educational level of mothers, access to flush toilet, and availability of electricity, with direct effects on y_j ; and u_i' comprises the remaining categorical covariates sex, work, treatment during pregnancy and access to controlled water and radio, having common effects on the latent variable υ . However, the results of model LVM1 (not reported here) have been extended or changed to model LVM2 by including some covariates that have direct effects on the parametric direct covariates in LVM2. The results of model LVM2 (Table 3) shows that most of the parametric direct covariates are significant and remained quite stable when including these covariates in the direct parametric effects. It demonstrates that the female children whose mothers are educated, had treatment during their pregnancy, had access to controlled water, had access to radio and working currently have higher Z-score of *weight-for-age* and are better nourished. However, males whose mothers are currently working are associated with a higher level of *(weight-for-height)*(at 97%). Although working status has a slight effect on the indicator of stunting, it is associated with

other indicators. According to the covariate of radio, it has mostly a non-significant effect. Moreover, the results of LVM2 indicate a negative effect of the education on the indicator 2.

Parameter	Mean	Std	2.5%	10%	90%	97.5%
Factor Loadings						
stunting λ_{11}	1.041**	0.021	1.00	1.02	1.079	1.095
underweight λ_{21}	1.191**	0.007	1.178	1.187	1.208	1.210
Wasting λ_{31}	0.673**	0.017	0.644	0.656	0.703	0.714
Parametric indirect Effects						
urban	-0.057	0.049	-0.153	-0.119	0.011	0.044
anvis	0.054	0.065	-0.058	-0.013	0.153	0.198
toilet	0.142**	0.059	0.017	0.060	0.212	0.250
elect	0.0683*	0.056	-0.026	0.010	0.151	0.186
Parametric Direct Effects						
male (a_{11})	–0.238**	0.0518	-0.321	-0.285	-0.153	-0.119
work (a_{12})	0.09	0.055	-0.042	-0.007	0.134	0.168
trepr (a_{13})	0.155*	0.069	-0.004	0.041	0.226	0.274
water (a_{14})	0.083**	0.035	0.0148	0.0384	0.127	0.153
educ (a_{15})	0.216**	0.039	0.143	0.167	0.265	0.291
radio (a_{16})	0.062	0.0300	-0.029	-0.0095	0.0711	0.093
male (a_{21})	–0.064**	0.0138	-0.082	-0.067	-0.032	-0.030
work (a_{22})	0.109**	0.0176	0.051	0.056	0.085	0.107
trepr (a_{23})	0.072**	0.023	0.024	0.026	0.085	0.117
water (a_{24})	0.048**	0.007	0.039	0.043	0.057	0.065
educ (a_{25})	0.067**	0.013	0.0507	0.058	0.074	0.076
radio (a_{26})	0.047**	0.0056	0.004	0.005	0.020	0.039
male (a_{31})	0.051*	0.042	-0.015	0.010	0.119	0.148
work (a_{32})	0.096*	0.0453	-0.006	0.021	0.135	0.163
trepr (a_{33})	-0.056	0.056	-0.182	-0.141	0.005	0.045
water (a_{34})	0.001	0.028	-0.054	-0.036	0.037	0.056
educ (a_{35})	–0.076**	0.032	-0.135	-0.115	-0.035	-0.015
radio (a_{36})	0.0018	0.0248	-0.068	-0.050	0.013	0.032
Smoothing Parameters						
Chage	0.035**	0.028	0.008	0.01	0.065	0.107
BMI	0.004**	0.0056	0.0006	0.001	0.010	0.018
Mageb	0.003**	0.0045	0.0004	0.0006	0.007	0.015
reg	0.121**	0.045	0.055	0.071	0.175	0.227

Table 3. Results of LVM2, including direct and indirect effects. (**: Statistically significant at 2.5% and 10%)

The reason for this is that in the analysis of latent models, we used three indicators (which were assumed to have high level of correlations among each other) instead of one indicator, which was used by the separate analysis.

It is observed that the indicators have a higher correlation which can affect the results, so we have made a further analysis excluding the indicator of wasting (*weight-for-hight*) to examine the effects of various factors on the other indicators (underweight and stunting), and results are compared (LVM3) with analysis when all three indicators (LVM2) are present.
The results of LVM3 (Table 4) indicate that the antenatal visits and the availability of electricity are associated positively with nutritional status. With regard to the direct covariates, the females and the education level of mothers have a positive significant effect on the indicator of stunting. While, only the work status is associated positively with the indicator of underweight. The factor loadings estimates show that the *weight-for-height* is seen to be more serious in Nigeria (its higher factor loading of 1.14).

Parameter	Mean	Std	2.5%	97.5%
Factor Loadongs				
stunting λ_{11}	1.147*	0.028	1.097	1.203
underweight λ_{21}	0.987*	0.0274	0.934	1.040
Parametric Indirect Effects				
urban	0.0357	0.060	-0.357	0.152
anvis	0.346*	0.075	0.205	0.492
toilet	0.156	0.082	-0.013	0.313
elect	0.153*	0.058	0.033	0.269
Parametric Direct Effects				
male (a_{11})	–0.242*	0.059	-0.357	-0.1372
work (a_{12})	0.087	0.064	-0.028	0.211
trepr (a_{13})	0.124	0.083	-0.044	0.290
water (a_{14})	0.065	0.086	-0.1033	0.241
educ (a_{15})	0.184*	0.067	0.055	0.330
radio (a_{16})	0.019	0.0365	-0.049	0.088
male (a_{21})	-0.057	0.045	-0.150	0.026
work (a_{22})	0.118*	0.053	0.0155	0.224
trepr (a_{23})	0.022	0.060	-0.090	0.137
water (a_{24})	0.0079	0.069	-0.124	0.139
educ (a_{25})	0.046	0.0529	-0.051	0.154
radio (a_{26})	0.028	0.029	-0.027	0.089
Smoothing Parameters				
Chage	0.016*	0.018	0.064	0.143
BMI	0.004*	0.011	0.075	0.319
Mageb	0.135*	0.085	0.0003	0.009
reg	0.159*	0.054	0.081	0.291
Chage	0.016*	0.018	0.064	0.143

Table 4. Estimates of factor loadings of the LVM3 with only two indicators in Niegria.

Figure 1 shows the non-linear effect of the child's age to be associated with a malnutrition status in Nigeria for LVM1 and LVM2, respectively. It shows that the rates of malnutrition of children increase sharply from about 5 to around 20 months of age. The rates of malnutrition are at low level between 20 and 30 months of age, then rise again through the

remainder of the third year. This pattern highlights the first two years of life as the most nutritionally vulnerable for children in Nigeria.

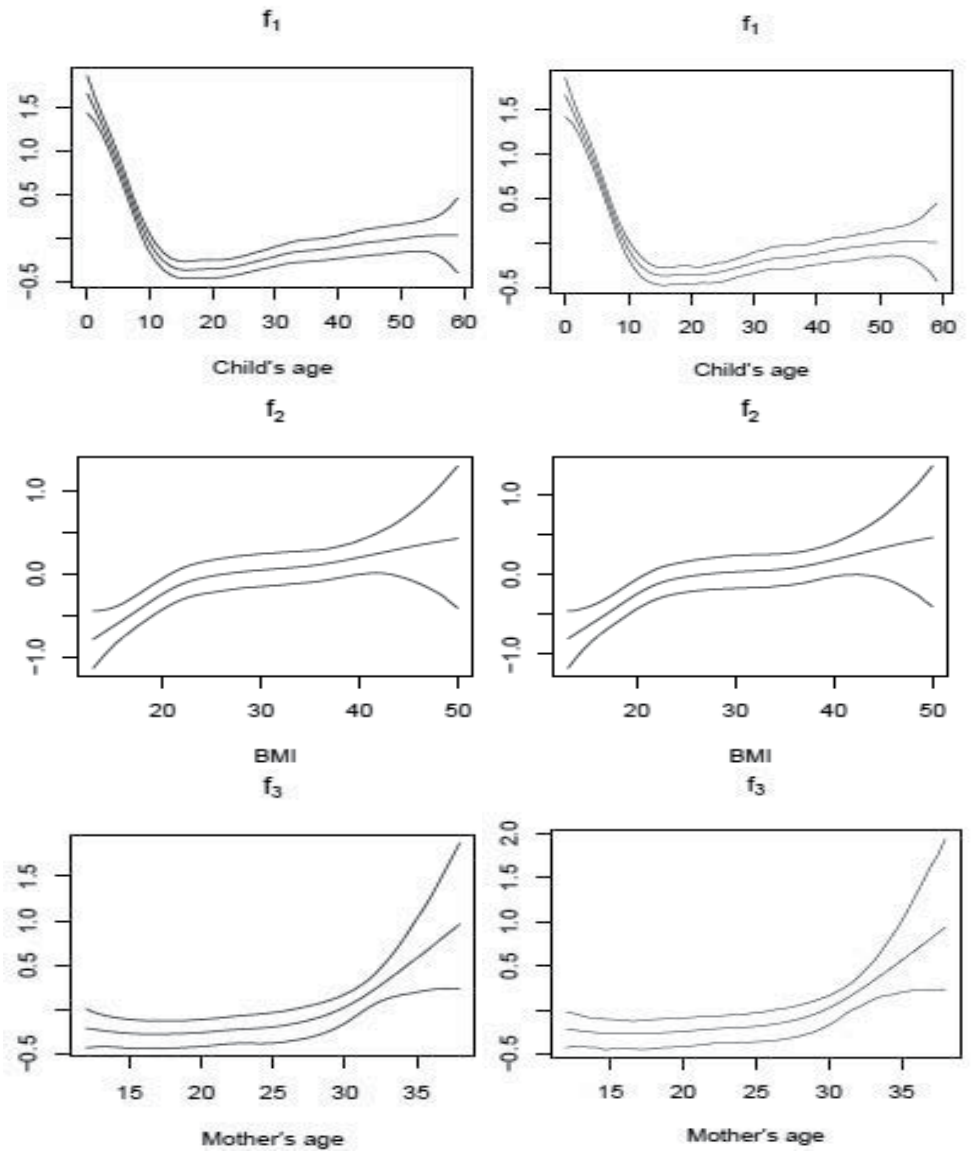


Fig. 1. Nonlinear effects from top to bottom: child's age, mother's BMI and mother's age at birth for LVM1 (left) and LVM2 (right) of "malnutrition status" of children for Nigeria, using latent varaible model for continuous responses

The nonlinear effect of the BMI of the mother shows that obesity of the mother probably poses less of a risk for the child's nutritional status, due to the fact that a very low BMI suggested acute undernutrition of the mother. The Z-score is highest (and thus stunting lowest) at a BMI of around 30-40 months.

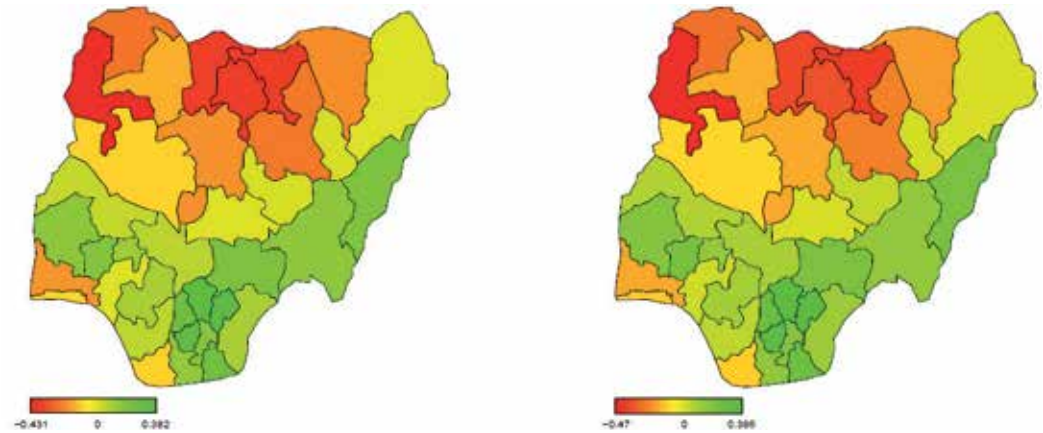


Fig. 2. Posterior mean for leatent varaiable model for LVM1 (left panel) and LVM2 (right panel) on malnutrion status for Nigeria

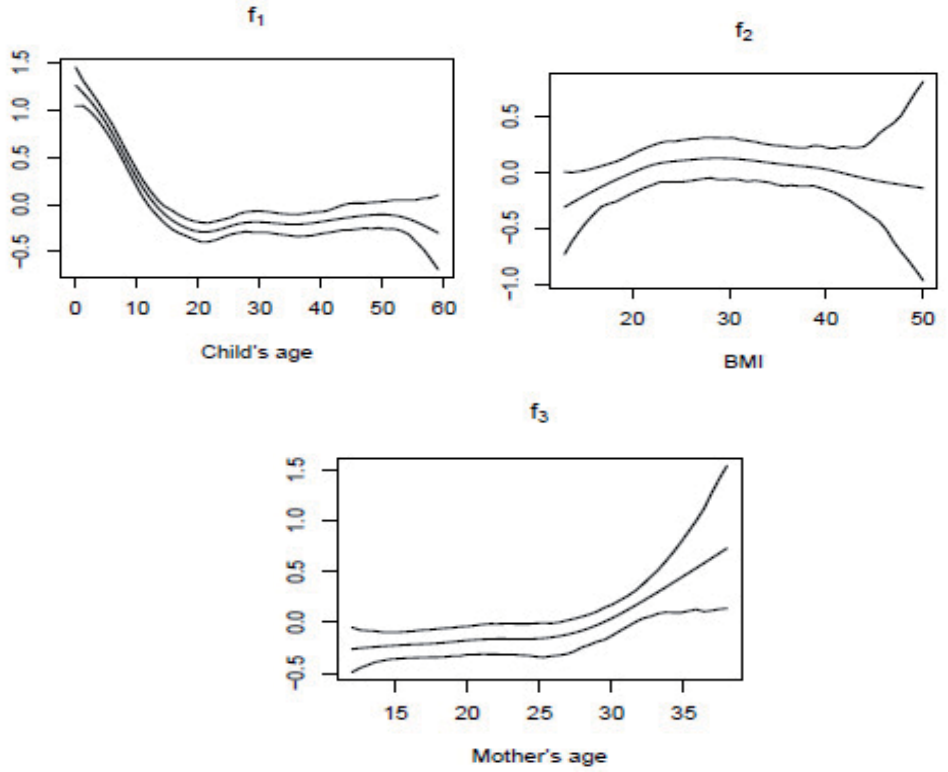


Fig. 3. Nonlinear effects from top to bottom: child's age, mother's BMI and mother's age at birth using only two indicators of latent varaible "malnutrition status" of children for Nigeria, using latent varaible model for continuous responses.

The effect of the mother's age seems to be slight on the Z-scores of children up till about the age of 25 months; thereafter, there is a strong effect shown.

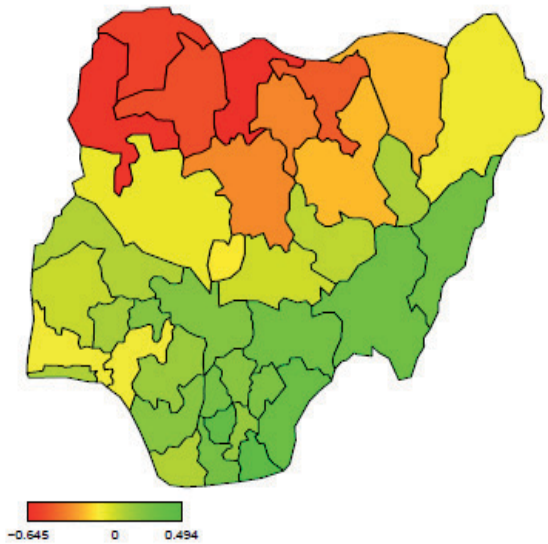


Fig. 4. Posterior mean for Latent varaible model, using only two idicators of latent varaible "Malnutrion status" for Nigeria

Fig. 5. Map of Nigeria showing the different states

In addition, the patterns of the nonlinear effect in LVM3 (Figure 3) are similar to the patterns of LVM1 and LVM2. The same is true with regard to the spatial effects of LVM3 (Figure 4). Figure 2 shows that the districts in the southeastern through the southern part of the country are associated with better nutrition of children in Nigeria.

4. Discussion

The results of estimating the geoadditive latent variable models with continuous response variables are indicated and suggest the following:

4.1 Child's sex

The likelihood of being stunted and underweight was lower for girls than for boys; a finding consistent with .(Klasen 1996; Lavy, Strauss et al. 1996; Svedberg 1996; Gibson 2001; Kandala 2001; Borooah 2002; S.B 2003); on the other hand, Gibson (2001) did not find any significant gender difference between the *height-for-age* and the *weight-for-age* in Papua, New Guinea.

4.2 Malnutrition among residences

Although, rural living was expected to have many problems, such as, poor health, use of unprotected water supplies, lack of charcoal as fuel, lack of milk consumption, and lack of personal hygiene (which assumed to be the risk factors of nutritional status), the results indicate that the place of residence is not associated with significant effects on wasting, underweight, and for stunting. This is consistent with some studies, but not with others: Adebayo (2003) found that where the mother lives (rural/urban) has no statistical significance for child's *weight-for-height*, and a similar impact of where the mother lives, as in *height-for-age*, is observed in *weight-for-age*, though Kandala found that urban areas have a statistical significance for a child's *height-for-age* in Tanzania and Malawi (Lavy, Strauss et al. 1996; Gibson 2001; Borooah 2002)

4.3 Mother's education

Maternal education, which is related to household wealth, is a determinant of good child-care knowledge and practices. The education attainment of mothers is mostly significant in the analysis of LVM.

The results with two indicators are quite similar to the results with three indicators with regard to this variable. This result supports the suggestion that an educated mother assumes the responsibility of taking a sick child to receive health care. Further, the time that mothers spend discussing their child's illness with a doctor is almost directly proportional to their level of education: consequently, illiterate women (with sick children) get much less out of visiting a doctor than literate women do. These findings are consistent with many studies in the context of developing countries (Africa Nutrition chartbooks 1996, Borooah 2002), which reported that maternal education has a strong and significant effect on stunting. They found that at primary levels of education,, effects on stunting are small or negligible, and they increase only at secondary or higher levels.(Chartbooks 1996; Borooah 2002).

4.4 Working mothers

Work has a non significant effect on the malnutrition status of children in Nigeria. The results are consistent with some previous studies and not consistent with others. Some

studies reported that when mothers are working, the household income is increased and the access to better food will be increased, as well as the access to a quality level of medical care. On the other hand, when mothers are employed outside the home, the duration of full breastfeeding is shortened and necessitates supplementary feeding. This is usually preformed by illiterate care-takers, which might affect the health of children negatively.

4.5 Drinking water

A household's source of drinking water has been shown to be associated with the nutritional status of a child in Nigeria (*weight-for-age*) in separate analysis (Khatab, 2007), and it seems to be mostly significant in the results of LVM. In other words, the source of water is associated with the nutritional status of a child through its impact on the risk of childhood diseases such as diarrhea, and is affected indirectly as a measure of wealth and availability of water.

4.6 Access to toilet

The type of toilet used by a household is an indicator of household wealth and a determinant of environmental sanitation. This means that poor households, which are mostly located in rural areas, are less likely to have sanitary toilet facilities. Consequently, this results in an increased risk of childhood diseases, which contributes to malnutrition.
The results indicate that in households where a flush toilet exits, stunting and underweight (separate analysis) are significantly lower and the nutritional status of children (analysis with LVM) is better.

4.7 Availability of electricity and radio in household

Despite access to electricity and radio, which facilitates the acquisition of nutritional information allowing more successful allocation of resources to produce child health (Kandala, 2001), only the availability of electricity was significant and had a positive effect on reducing stunting, and underweight with separate analysis, and it seems to be significant on the LVM "nutritional status". This may be because mothers allocate their leisure time to radio or television, but it doesn't help improve the level of nutrition of their children. At the same time, it reduces the length of time spent engaging in their children's affairs.(Kandala 2001)

4.8 Antenatal visits

The variables that deal with access to health care, such as children of mothers who obtained clinical visits during pregnancy and had vaccines and treatment, have a positive and significant effect on malnutrition status. Therefore, health service investments are more effective in reducing stunting, wasting and underweight among indigenous communities. Our results indicate that children of mothers who had clinical visits and got medical care during pregnancy are less likely to be stunted and to be underweight than their counterparts in Nigeria. The results with two indicators also indicate that the *anvis* has a positive effect.

4.9 Child's age

In the analysis, it was discovered that the situation among children who are stunted is quite similar; however, the deterioration in nutritional status is set between 5-20 months of age.

Similarly, deterioration in child's *weight-for-height* sets during the first 4-5 months of age, as reported in much of the literature, is due to supplementation. However, it reaches its minimum level between ages 13 and 15 months, then rises again and reaches its minimum level between 16 and18 months of age; which is earlier than the case of stunting. A sudden pick-up effect is noticeable from age 18 months until about 45 months, where it attains its maximum level.

An improvement commenced after age 20 or 25 months and rose gradually until age 50 months. Previous studies assumed that it is an average effect of low *height-for-age* and *weight-for-height* during this period of life (Adebayo, 2003)

The level of wasting suggests that insufficient food intake may be an important factor in the rise of malnutrition in both countries. In addition, the implication of this finding is that wasting is not clearly noticeable in the first four months of life. As soon as a child is fed with other supplementation such as liquids or other forms of diet, which due to the unhygienic source of preparation of such supplementation, may facilitate infections and diseases such as diarrhoea, then acute malnutrition may set in.

4.10 Mother's BMI

A mother's nutritional status affects her ability to successfully carry, deliver, and care for her children and is of great concern in its own right. The BMI pattern shows linear trends with positive slopes. Malnutrition in women is assessed using BMI. When the BMI of non-pregnant women falls below the suggested cut-off point, which is around 18:5$kg/m2$, malnutrition is indicated. Women who are malnourished (thinness or obesity) may have complications during childbirth and may deliver a child who can be wasted, stunted or underweight. The results indicate that there is an association between the thinness of the mother and the nutritional status of the child.

4.11 Mother's age at birth

The results show that the influence of mothers who are younger than 20 years is higher on the nutritional status of children.

A possible cause for this is childbirth among very young girls, whose bodies are not physically ready to endure the processes of childbirth. The problem is compounded by the fact that some African countries have poor obstetric care. Furthermore, these mothers could not reach health facilities, or, when they do, it is too late. Effective ways must be devised to delay age at first marriage and first birth. These two factors will almost certainly determine the number of children she will have in her lifetime. While early age at first birth has health implications, it also has economic implications.

In addition, one study obtained in Nigeria reported that younger mothers (teenagers) are less likely in comparison to older mothers to breastfeed their children after birth, which means that the age of the mother at birth of a child influences whether the child will receive colostrum or not, which might affect the nutritional status of children (Adebayo and Fahrmeir 2005).

Moreover, previous studies which were obtained in some developing countries have shown that some African countries do not allow girls back to go back school after they give birth. As a consequence, a girl who drops out of school will continue the cycle of poverty(Alderman H 1997; Wasao; 1999).

4.12 Malnutrition in region

As reported in the 2003 NDHS, the trend in the nutritional status of Nigerian children has worsened with regard to stunting and wasting (from 36% in 1990 to 46% in 1999 for stunting and 11% in 1990 to 12% in 1999 for wasting). The results, based on our analysis, indicate that mostly districts in the northeast and southeast and northwest are more likely to be associated with *nutritional problems*, providing a more complete picture of the situation. The result also revealed striking regional variations, with the northeast, south and southeast in much worse situations in terms of stunting and underweight than the northwest and southwest For more information about the different states in Nigeria, see figure 5 . On the other hand, the children who live in the northwest part of the country are more likely to be wasted than their counterparts in other parts of the country. These regional and zonal disparities may reflect the contribution of other factors, such as socio-cultural conditions and morbidity of children, in determining the nutritional status of children under the age of five. The high prevalence of stunting observed in the 2003 NDHS survey is in the context of large-scale deepening poverty and household food insecurity. Severe rural poverty appears to be found in the southwest of Nigeria, in the north-center, and in the extreme northeast.
These results are consistent with some previous studies which discuss the relation between poverty and malnutrition as persistent problems in Nigeria.

4.13 Summary

The results showed that the place of residence, mother's working, type of toilet and availability of electricity and radio in households have negligible effects on the undernutrition of children.
We find that the analysis identify the association of child's age, mother's age at birth and mother's BMI as effecting undernutrition. It was found that children are at a high risk during the first 15-20 months of life and that the risk rises again between ages 25-50 months. The effect of BMI on the child's nutritional status is approximately linear with positive slope, which means that there is an association between the thinness condition of mothers and nutritional status. According to the mother's age at birth, it shows that younger mothers are less likely to affect their children's nutritional status positively.
It is found that children living in some provinces in the southeast regions and some regions in the southern part of the country are associated with undernutrition.

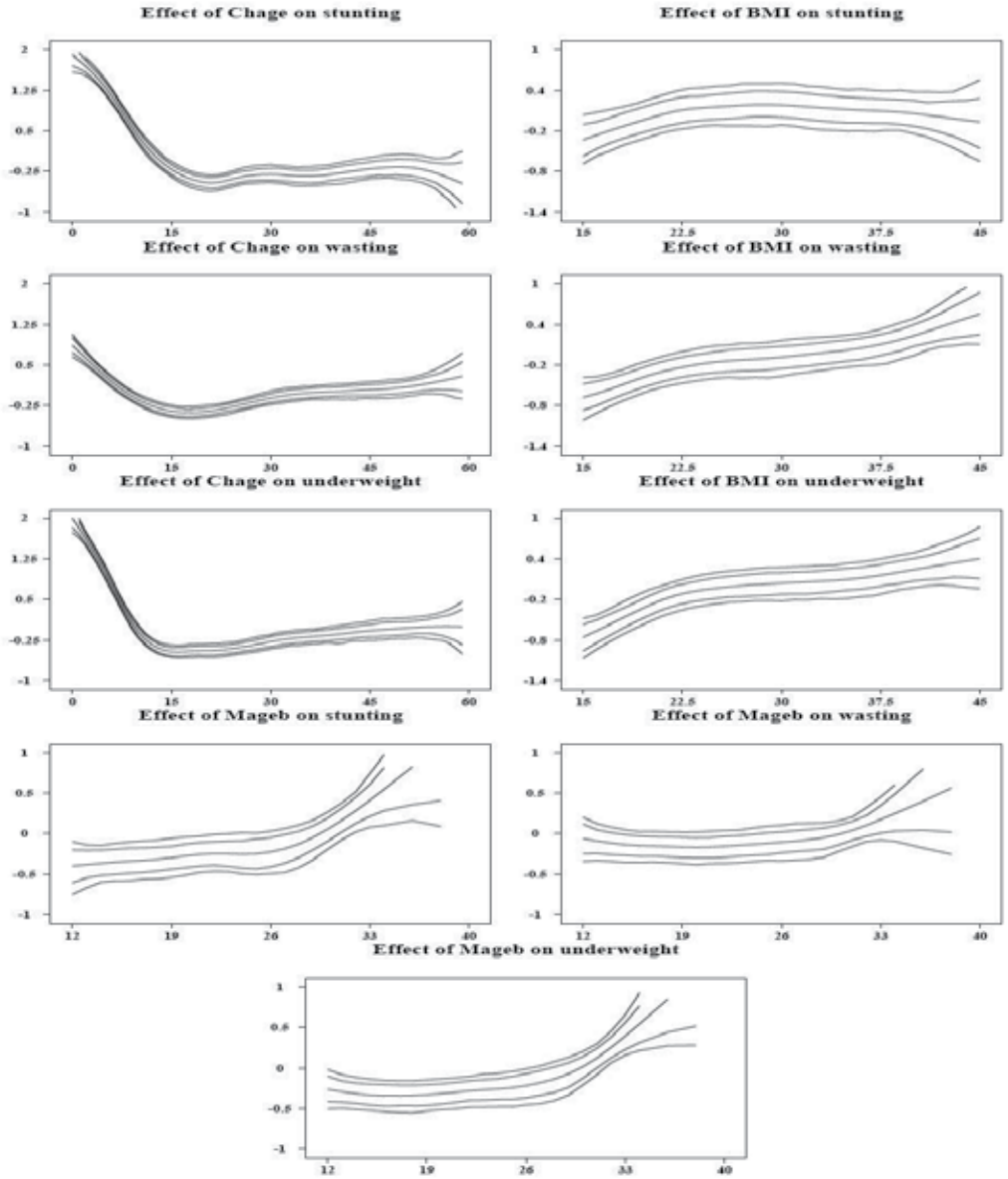
4.14 Policy implications

Affected areas should improve socioeconomic conditions. Because, if living standards are improved, there will be better health care and a reduction in infant and child diseases, child malnutrition and child mortality.
The policymakers need to give more attention to some areas which have high rates of poverty, such as the southern part of Nigeria. These areas are more likely to have a higher proportion of undernutrition compared to other areas, due to poor health facilities and complications during childbirth or even carelessness and misdiagnosis during hospital care. Therefore, the most important issues to address in these areas are health care, proper food, and raising the educational level of parents.

5. Appendix

As presented in this paper, LVMs are based on the results of the separate models which were presented in Khatab, 2007. At that stage, we have used separate geoadditive probit

models with the binary target variables for diarrhea, cough, and fever using covariate information from the 2003NDHS. The computations for the separate models were carried out using BayesX program (Brezger A, Kneib T, Lang S, 2005). We are showing here the results of Model 3, which was selected from a long hierarchal analysis based on its DIC (the value of deviance information criterion).



Source:Khatab, 2007

Fig. A1. Nonlinear effects of child's age, BMI, and Mother's age at birth using separate Bayesian Gaussian Model children for Nigeria using Bayesian latent variable model for continuous responses.

Variable	Mean	S.dv	10%	median	90%
const	–1.133*	0.154	-1.33	-1.133	-0.94
male	–0.117*	0.030	-0.156	-0.117	-0.077
urban	0.032	0.039	-0.020	0.032	0.083
work	0.027	0.033	-0.016	0.025	0.070
trepr	0.075*	0.039	0.026	0.074	0.128
anvis	0.147*	0.039	0.095	0.147	0.199
radio	0.017	0.037	-0.030	0.017	0.063
elect	0.131*	0.039	0.077	0.129	0.180
water	0.044	0.044	-0.008	0.043	0.106
educ	-0.543	0.943	-1.766	-0.509	0.606
toilet	0.078*	0.048	0.013	0.078	0.140

Table A1. Fixed effects of separate model using Bayesian geoadditive model on Stuniting

Variable	Mean	S.dv	10%	median	90%
const	–0.710*	0.121	-0.863	-0.718	-0.551
male	–0.032*	0.022	-0.061	-0.033	-0.004
urban	-0.022	0.030	-0.059	-0.023	0.016
work	0.044*	0.026	0.009	0.043	0.077
trepr	0.014	0.031	-0.027	0.014	0.053
anvis	0.079*	0.030	0.040	0.080	0.116
radio	0.035*	0.028	0.0007	0.034	0.072
elect	0.065*	0.029	0.024	0.067	0.101
water	0.046*	0.033	0.001	0.047	0.089
educ	0.063*	0.038	0.013	0.064	0.111
toilet	0.105*	0.044	0.051	0.106	0.159

Table A2. Fixed effects of separate model using Bayesian geoadditive model on underweight

Variable	Mean	S.dv	10%	median	90%
const	–0.041*	0.127	-0.214	-0.032	0.116
male	0.026	0.024	-0.005	0.025	0.058
urban	-0.051	0.030	-0.111	-0.050	0.006
work	0.049*	0.027	0.011	0.050	0.083
trepr	-0.046	0.034	-0.116	-0.045	0.022
anvis	-0.038	0.030	-0.076	-0.039	0.0004
radio	0.018	0.032	-0.023	0.020	0.060
elect	-0.019	0.031	-0.060	-0.019	0.020
water	0.028	0.036	-0.016	0.026	0.075
educ	0.030	0.041	-0.022	0.030	0.0847
toilet	0.0037	0.048	-0.055	0.0006	0.068

Table A3. Fixed effects of separate model using Bayesian geoadditive model on wasting

6. References

[http://hdr.undp.org/docs/events/Berlin/Background_paper.pdf] webcite, U. N. U. S.-S. A. t. h. c. o. t. b.-a.-u. s.

Adebayo, S. B. and L. Fahrmeir (2005). "Analysing child mortality in Nigeria with geoadditive discrete-time survival models." *Stat Med* 24(5): 709-28.

Alderman H, J. B., V Lavy and R Menon (1997). "Child Nutrition, Child health and School Enrollment:A longitudinal Analysis." *The World Bank* Policy research Working (Papwe.1700).

Borooah, V. (2002). "The Role of Maternal Literacy in Reducing the Risk of Child Malnutrition in India." *ICER - International Centre for Economic Research*.

Brezger A, K. T., Lang S (2005). "BayesX - Software for Bayesian Inference based on Markov Chain Monte Carlo simulation Techniques." *Available at: http://www.stat.uni-uenchen.de/lang/BayesX* 1.4.

Chartbooks, A. N. (1996). "Nutrition of Infants and young Children in Mali. Macro international Inc." *Calverton, Maryland, U.S.A*.

Fahrmeir L, a. L. S. (2001). "Bayesian Inference for Generalized Additive Mixed Models Based on Markov Random Field Priors." *Applied Statistics* 50: 201-220.

Fahrmeir, L., Kneib, T.and Lang, S (2004). "Penalized structured additive regression of space-time data:a Bayesian perspective." *Statistica Sinica* 14: 731-761.

Fahrmeir L , R. A. (2007). "A Bayesian semiparametric latent variable model for mixed responses " *Psychometrika* 72: 327– 346.

Gibson, J. (2001). "Literacy and Intra-household externalities." *World Development* 29(155-66).

Kandala, N. B., C. Ji, et al. (2007). "Spatial analysis of risk factors for childhood morbidity in Nigeria." *Am J Trop Med Hyg* 77(4): 770-9.

Kandala, N. B., Lang, S., Klasen, S. and Fahrmeir, L (2001). "Semiparametric Analysis of the Socio-Demographic Determinants of Undernutrition in Two African Countries." *Research in Official Statistics* 4(1): 81-100.

Kandala, N. B., M. A. Magadi, et al. (2006). "An investigation of district spatial variations of childhood diarrhoea and fever morbidity in Malawi." *Soc Sci Med* 62(5): 1138-52.

Khatab, K. (2007). "Analysis of childhood diseases and malnutrition in developing countries of Africa." *Dr.Hut Verlag -Munich, Germany*.

Khatab, K. and L. Fahrmeir (2009). "Analysis of childhood morbidity with geoadditive probit and latent variable model: a case study for Egypt." *Am J Trop Med Hyg* 81(1): 116-28.

Klasen, S. (1996). "Nutrition, Health, and Mortality in Sub-Saharan Africa: Is there a Gender Bias?" *Journal of Development Studies* 32: 913-932.

Lavy, V., J. Strauss, et al. (1996). "Quality of health care, survival and health outcomes in Ghana." *J Health Econ* 15(3): 333-57.

Pelletier DL, F. E. (1995). "The effects of malnutrition on child mortality in developing countries." *Bulletin of the World Health Organization* 73: 443-448.

Pelletier, D. L., E. A. Frongillo, Jr., et al. (1995). "The effects of malnutrition on child mortality in developing countries." *Bull World Health Organ* 73(4): 443-8.

Pelletier, D. L. a. E. A. F. (2003). "Changes in Child Survival Are Strongly
Associated with Changes in Malnutrition in Developing Countries." *Journal of Nutrition* 133: 107-119.

Raach, A. W. (2005). "A Bayesian semiparametric latent variable model for binary, ordinal and continuous response." *Dissertation, available from edoc.ub.uni-muenchen.de*.

S.B, A. (2003). "Semiparametric Bayesian Regression for Multivariate Responses." *Ph.D Thesis, Hieronymus Verlag, Munich, Germany*.

Sen, A. (1999). "Development as Freedom." *Oxford: Oxford University Press*.

Smith LC, H. L. (2000). "Explaining child malnutrition in developing countries: a cross country analysis. International Food Policy Research Institute, Food Consumption and Nutrition Division discussion paper." 60.

Smith LC, O. A., Jensen HH (2000). "The geography and causes of food insecurity in developing countries." *Agricultural Economics* 22: 199-215.

Survey, D. a. H. (2003). "Demographic and Health Survey for Nigeria." *DHS*.

Svedberg, P. (1996). "Gender Bias in Sub-Saharan Africa: Reply and Further Evidence." *Journal of Development Studies* 32: 933-943.

UNICEF (1998). "The State of the World's Children." *New York: UNICEF*.

UNICEF (2008). "The State of the world children." *UNICEF*.

Vella, V., A. Tomkins, et al. (1992). "Determinants of child nutrition and mortality in north-west Uganda." *Bull World Health Organ* 70(5): 637-43.

Wasao., T.-R. C. a. S. (1999). "Factors Affecting Children's Nutritional and Health Status in Kenya: A District-Level Analysis." *Paper presented at the Population Association Of America annual meeting in New York Marriot Marquis Hotel on March*: 25-27.

WHO (1999). "Infant and Young Child Growth: The WHO Multicentre Growth Reference Study.Executive Board:Implementation of Resolu- tions and Decisions " *EB105/Inf.doc/1.Geneva:WHO.*

Edited by Alfonso J. Rodriguez-Morales

Tropical Medicine has emerged and remained as an important discipline for the study of diseases endemic in the tropic, particularly those of infectious etiology. Emergence and reemergence of many tropical pathologies have recently aroused the interest of many fields of the study of tropical medicine, even including new infectious agents. Then evidence-based information in the field and regular updates are necessary. Current Topics in Tropical Medicine presents an updated information on multiple diseases and conditions of interest in the field. It Includes pathologies caused by bacteria, viruses and parasites, protozoans and helminths, as well as tropical non-infectious conditions. Many of them are considering not only epidemiological aspects, but also diagnostic, therapeutical, preventive, social, genetic, bioinformatic and molecular ones. With participation of authors from various countries, many from proper endemic areas, this book has a wide geographical perspective. Finally, all of these characteristics, make an excellent update on many aspects of tropical medicine in the world.

ISBN 978-953-51-4322-2

Photo by Montest / iStock

IntechOpen