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Ticks and Tick-Borne Pathogens

Edited by Muhammad Abubakar and Piyumali K. Perera

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Meet the editors

Dr. Muhammad Abubakar, a scientist from the National Veterinary Laboratory, Islamabad, Pakistan, has over 15 years of experience in various areas of veterinary sciences. His expertise on transboundary animal diseases (TADs) at national and international levels is well known. He has established various diagnostic laboratories for diagnosis of TADs. He has been involved in organisation and conduct of various training programs for field and laboratory staff. He has published numerous research papers, review articles, and book chapters on different areas of veterinary sciences. He has also worked in academia in teaching and research supervision roles at graduate and undergraduate levels. He is currently supervising two journals in the area of veterinary sciences (Research journal for Veterinary Practitioners and Veterinary Sciences; Research and Reviews) as an editor-in-chief.

Dr. Piyumali K. Perera received her BSc (Hons) in Zoology from the University of Peradeniya, Sri Lanka (2011) and her PhD in Parasitology from the Faculty of Veterinary and Agricultural Sciences, the University of Melbourne, Australia (2015). She received an International Postgraduate Research Scholarship and Australian Postgraduate Award to conduct her doctoral research on theileriosis, a major tick-borne disease in Australia. For her outstanding performance during her PhD, she received several prestigious awards/prizes including World Association for the Advancement of Veterinary Parasitology - Louisiana State University Travel Award (2015), VW officer prize in Veterinary Science (2014), and Dairy Farmers of Victoria Postgraduate Scholarship (2013). She is currently serving as a Lecturer in Zoology, teaching Parasitology to undergraduates at the Faculty of Science, University of Peradeniya. Her current research interests are in the fields of ticks and tick-borne diseases, parasitic zoonoses, and human gut microbiome. She has published many research articles in high impact journals in the field including PloS one, Infection, Genetics and Evolution, Ticks and Tick-borne Diseases, Journal of Clinical Microbiology, Parasites & Vectors and Veterinary Parasitology.

Contents

Preface

This book is intended for students in parasitology, biologists, parasitologists involved in molecular diagnostics of tick-borne diseases, practicing veterinarians, and for others who may require information on ticks and tick-borne diseases.

Ticks transmit a range of pathogens including viruses, bacteria, and protists to vertebrate hosts, including humans and domestic and wild animals. Tick-borne diseases of humans and domestic animals can substantially affect their health and livestock production, respectively. Hence, it is of paramount importance to investigate these ticks and tick-borne diseases, particularly the emerging infections harboured by ticks. When we consider a particular tick-borne disease, it is critical to have a thorough understanding of the vectors involved, biology of vectors, pathogenesis, hosts affected, diagnostic methods, treatment, and control.

In this book, we have put together a collection of chapters focused on different aspects of ticks and tick-borne diseases mainly to provide the reader with novel information in the field, but not the basic generalised information provided by many textbooks. This book includes topics such as high-throughput technologies in diagnosis, discovery of novel tick vaccines, identification of new pathogens transmitted by ticks, and new epidemiological information of certain well-known ticks and tick-borne diseases. Our target audience is the parasitologists, parasitology students, and veterinarians who have a basic knowledge of the details of taxonomy and classification of these ticks and tick-borne diseases, life cycles, diagnosis, treatment, and control. Therefore, the novel findings have been discussed in detail, while basic information is either deliberately omitted or mentioned briefly in the introduction of each of these chapters.

These chapters were authored by parasitologists from all over the world, giving an insight to the reader about the significant ticks and tick-borne diseases prevalent in those particular geographical regions with the local expert's point of view. Each of the chapters have separate reference lists, making it easier for the reader to find additional reading material related to their topic of interest.

I would like to express my gratitude to all the contributors of this book including the authors of the accepted chapters. My special thanks to the Author Service Manager, Ms. Anita Condic, and other staff of IntechOpen publishing for their kind support

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Section 1

Tick Borne Infections

Chapter 1

Introductory Chapter: Ticks and Tick-Borne Pathogens

Muhammad Abubakar, Piyumali K. Perera, Abdullah Iqbal and Shumaila Manzoor

1. Introduction

Ticks are obligate ectoparasites that feed on the blood of their hosts. Ticks belong to the phylum Arthropoda, class Arachnida, subclass Acari, order Parasitiformes, and suborder Ixodida [1, 2]. There are three families of ticks classified as Ixodidae (hard ticks), Argasidae (soft ticks), and Nuttalliellidae (limited to Tanzania and South Africa) [3, 4]. More than 900 species of ticks have been classified in the world. Ticks not only cause physical damage to their hosts by sucking blood and injuring skin, but many of these tick species also have the ability to transmit pathogens to their host. The population of ticks in any region depends upon various factors such as climate, the presence of predators, and competitor species [5].

According to an estimate, every year, ticks and tick-borne pathogens cause the US $13.9–18.7 billion loss. Annually, tick infestations result in a loss of almost 3 billion hides of cattle [6]. Ticks transfer pathogens from their gut to host bloodstream by their saliva [7]. Ticks transmit a range of pathogens including viruses, bacteria, and protists to vertebrate hosts, including humans, domestic, and wild animals. These pathogens cause many viral diseases (e.g., Crimean-Congo hemorrhagic fever, West Nile fever, Omsk hemorrhagic fever, and Colorado tick fever), bacterial diseases (Lyme disease, Q fever, borreliosis, and relapsing fever), fungal diseases (dermatophilosis), protozoal diseases (theileriosis and babesiosis), and rickettsial diseases (anaplasmosis, ehrlichiosis, Brazilian spotted fever, and Rocky Mountain spotted fever) [7–11]. Tick-borne diseases (TBDs) of domestic animals (e.g., cattle, sheep, and goats) can substantially affect livestock production, food supply, and economy of many regions worldwide. TBDs cause production losses mainly as a consequence of infertility, abortions, reduced weight gain, decreased milk production, lower quality of milk, and mortality. In addition, costs associated with control and preventive measures, such as dipping with acaricides, vaccination, chemotherapy, veterinary services, and monitoring, also contribute considerably to economic losses (Brown, 1997). In addition, most of these pathogens are very serious zoonotic threats due to the worldwide distribution of ticks and lack of vaccine availability against these viruses and other pathogens [12].

Tick-borne pathogens are not the only problem due to tick infestation. When ticks feed on their host, they draw blood and cause damage to the skin. Injury of skin and subcutaneous tissues leads to edema, pruritus, erythema, scaling, and ulceration [13]. Excoriation can result in secondary bacterial infections. Along with these physical damages, ticks affect the productivity of animals by disturbing their normal behavior [14].

2. Tick-borne viruses

Tick-borne viruses (TBV) are specifically named as tiboviruses, and all of them belong to a group of arboviruses [15]. These viruses require ticks and vertebrate host to complete their life cycle. Combined evolution of ticks and tiboviruses results in the development of such a life cycle that totally matches the feeding cycle of ticks. These viruses belong to nine families of viruses. Among nine tiboviruses families, eight are RNA families (Flaviviridae, Reoviridae, Rhabdoviridae, Orthomyxoviridae, Nyamiviridae, Phenuiviridae, Nairoviridae, and Peribunyaviridae) and one DNA family (Asfarviridae) [13, 16].

To date, almost 19 diseases of livestock and 16 diseases of humans have been reported by TBV [17, 18]. Flaviviridae viruses are most common tiboviruses that include tick-borne encephalitis virus, West Nile virus, louping ill virus, Powassan virus, and Kyasanur Forest disease virus that are transmitted by *Dermacentor reticulatus*, *Ornithodoros moubata*, *Ixodes ricinus*, *Ixodes scapularis*, and *Haemaphysalis punctata*, respectively [19, 20]. West Nile virus is endemic in many African and European countries [21]. African swine fever virus is also a tick-borne virus that belongs to family Asfarviridae and transmitted by *Ornithodoros porcinus*. African swine fever disease is a very serious threat for pigs due to its high mortality rate [22]. Thogoto virus of family Orthomyxoviridae is transmitted by tick species such as *Rhipicephalus appendiculatus*, *Boophilus microplus*, *Hyalomma dromedarii*, *Rhipicephalus evertsi*, and *Amblyomma variegatum* [23].

Two major tick-borne viruses of Nyamiviridae are Nyamanini virus and Midway nyavirus. Reoviruses include tiboviruses such as Colorado tick fever virus, Great Island virus, and Chobar Gorge virus. Colorado tick fever virus is prevalent in the United States and Canada. This virus is transmitted to mammals by a tick *Dermacentor andersoni*. Fever, meningitis, rash, and conjunctivitis are typical clinical signs of Colorado tick fever [9].

Rhabdoviridae includes tick-borne viruses such as Isfahan vesiculovirus, Connecticut virus, and Barur ledantevirus [13]. Nairoviridae contains two major tick-borne viruses; those are Crimean-Congo hemorrhagic fever virus and Nairobi sheep disease virus. Crimean-Congo hemorrhagic fever outbreaks have been reported from many African, Asian, and European countries in the last two decades. This virus is mainly transmitted by *Hyalomma marginatum*, *H. lusitanicum*, *H. truncatum*, *Rhipicephalus bursa*, and *Dermacentor marginatus* [24].

3. Tick-borne bacteria

Tick-borne bacterial (TBB) diseases not only affect the productivity of animals but also have zoonotic importance. Lyme disease is one of the major tick-borne bacterial diseases that is caused by *Borrelia burgdorferi* [25]. These bacteria are transmitted to mammal host by *I. ricinus*, *I. hexagonus*, *I. pacificus*, *I. scapularis*, and *I. persulcatus*. Lyme disease is rapidly spreading in Europe. It is estimated that about 10% of the total population of ticks are positive for *B. burgdorferi* in Europe, and annually more than 85,000 human cases of Lyme are reported from the European countries [26]. Lyme disease also affects domestic animals. Clinical signs and symptoms of Lyme disease in animals include lethargy, anorexia, lameness, and urinary disorder [25].

Another TBB is *Francisella tularensis* that causes tularemia. Ticks of species *I. ricinus*, *D. andersoni*, *D. variabilis*, *D. marginatus*, and *A. americanum* act as biological vectors for *Francisella tularensis*. These bacteria can cause disease in humans, rodents, rabbits, and rarely sheep [27]. Q fever is also a tick-borne zoonotic bacterial disease that is

caused by *Coxiella burnetii*. Ticks of species *Haemaphysalis bispinosa* and *I. holocyclus* can also act as reservoir hosts and biological vectors [28].

4. Tick-borne Rickettsiae

Tick-borne Rickettsiae (TBR) can spread to new geographic areas and susceptible population by ticks. Anaplasmosis is an eminent tick-borne rickettsial disease of cattle that is caused by *Anaplasma marginale*. This is transmitted by *Rhipicephalus microplus*. The mortality rate of anaplasmosis in cattle varies from 30 to 50%. Another tick-borne rickettsia is *Rickettsia rickettsii* that causes spotted fever [29]. In the USA, *R. rickettsii* causes Rocky Mountain spotted fever, and in Brazil, it causes Brazilian spotted fever. Rocky Mountain spotted fever spreads mainly by ticks of species *Amblyomma americanum*, *A. cajennense*, *D. andersoni*, *D. variabilis*, and *R. sanguineus sensu lato* [30]. Medically significant vectors of Brazilian spotted fever include *A. aureolatum* and *A. cajennense*. The mortality rate of Rocky Mountain spotted fever in the USA and Brazil has been reported 10% and 30–40%, respectively. African tick bite fever is another tick-borne rickettsial disease that is caused by *Rickettsia africae*. Major vectors of *Rickettsia africae* are ticks of *A. variegatum* and *A. hebraeum* species [31].

Heartwater or cowdriosis is another tick-borne rickettsial disease that is caused by *Ehrlichia ruminantium*. *E. ruminantium* is mainly transmitted by *Amblyomma variegatum*, *A. pomposum*, and *A. hebraeum* [32]. This disease is limited to Africa and South Africa. Cowdriosis is a serious threat to ruminants in sub-Saharan Africa, where up to 90% mortality rate has been reported [33]. In dogs, *Ehrlichia canis* causes Ehrlichiosis. *E. canis* has been reported from the many Asian, European, and American countries and transmitted from one dog to another dog by *Rhipicephalus sanguineus sensu lato*. Clinical signs of this disease include high fever, lethargy, anemia, and nose bleeding [34].

5. Tick-borne fungi

Ticks are also involved in the transmission of a fungal pathogen, *Dermatophilus congolensis*, to mammals. *Dermatophilus congolensis* causes a skin disease Dermatophilosis [35]. This pathogenic fungus is transmitted by a tick vector *A. variegatum*. Dermatophilosis causes exudative dermatitis in sheep and cattle which leads to significant economic loss due to the devaluation of hide quality [36].

6. Tick-borne protozoa

Ticks can transmit many blood protozoan parasites to their vertebrate hosts. Among these, two main groups of TBDs are of importance to the livestock: theileriosis (i.e., tropical theileriosis and East Coast fever (ECf)) and babesiosis, posing major health and management problems to cattle and small ruminants, mainly in tropical and subtropical regions worldwide. The most pathogenic species are *T. annulata* and *T. parva*, the causative agents of tropical or Mediterranean theileriosis and ECf, respectively. Other species, such as *Theileria mutans*, *Theileria taurotragi*, and members of the *T. orientalis* complex, are usually considered to cause asymptomatic infections in livestock. *Theileria parva* is transmitted to cattle by *Rhipicephalus appendiculatus* (East Africa) or *R. zambeziensis* (South Africa) and causes ECf [35]. Tropical theileriosis is caused by *Theileria annulata* and

transmitted by *Hyalomma* spp. It is characterized by lymph nodes swelling, high fever, and dyspnea [37].

Babesiosis is another tick-borne protozoal disease. Bovine babesiosis is caused by *Babesia bovis* and *B. bigemina*. These protozoans are transmitted by *Rhipicephalus microplus* and *R. annulatus*. In bovines, animals having babesiosis show clinical signs including hemoglobinuria, jaundice, rapid breathing, and high fever [38]. In canines, *Babesia canis* causes piroplasmosis. *B. canis* is transmitted to dogs by *R. sanguineus* and *D. reticulatus*. In humans, *B. microti* and *B. divergens* are responsible for babesiosis [39].

7. Conclusion

Ticks prevalent in the dairy or poultry industries lead to economic losses either by direct damage to hide and stress to animals or indirectly by pathogens that they transmit to animals and humans. Prevalence of ticks and tick-borne pathogens is influenced by environmental factors and quarantine measures. Tick control at any level can prevent the outbreak of diseases caused by tick-borne pathogens.

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Chapter 2

Oriental Theileriosis

Jerald Yam, Daniel R. Bogema and Cheryl Jenkins

Abstract

Theileria orientalis, the causative agent of oriental theileriosis, is an apicomplexan haemoparasite and is one of several tick-borne *Theileria* spp. infecting cattle. Unlike the highly pathogenic transforming *Theileria* species (*T. annulata* and *T. parva*) which induce uncontrolled lymphocytic proliferation, *T. orientalis* is a non-transforming strain exerting its major pathogenic effects via erythrocyte destruction. Clinical symptoms associated with oriental theileriosis are largely consequences of the underlying anaemia. Because of its non-transforming nature, *T. orientalis* was previously considered a benign parasite, however, in the recent years, clinical outbreaks of *T. orientalis* have been increasingly observed throughout Asia and Australasia. Recent rapid spread of clinical theileriosis has been linked to a pathogenic genotype of the parasite, genotype Ikeda (Type 2). The geographic distribution of clinical outbreaks correlates to the range of the major vector tick, *Haemaphysalis longicornis*, although other vectors and modes of transmission are possible. This review includes discussion of *T. orientalis* epidemiology, transmission, pathogenesis, treatment and control and provides an update on the taxonomy of this organism which is still under debate.

Keywords: *Theileria orientalis*, cattle, taxonomy, epidemiology, transmission, control

1. Introduction

T. orientalis has been reported to cause mortality in up to 5% of infected cattle. Clinical outbreaks commonly occur when naïve cattle are introduced into endemic herds, when animals undergo stress through transportation or are immunosuppressed. Pregnant heifers and calves are particularly susceptible to infection, with late term abortions also commonly reported. The parasite is globally spread but countries impacted by clinical theileriosis include Australia, New Zealand, Japan, Korea, China and Vietnam [1–3]. Oriental theileriosis represents a major economic burden to cattle production. In Australia in 2010 the economic impact of the parasite was estimated at $20 million AUD per annum. However, the costs associated with disease are likely to have increased substantially since that time with the subsequent spread of bovine theileriosis into new areas of the country. In New Zealand, although the total economic impact has not been well established, clinical outbreaks were estimated to cost up to $NZ 1 million on a single large dairy farm [4]. Recently, clinical outbreaks of theileriosis were documented for the first time in dairy cattle undergoing transport stress during importation to Vietnam from Australia [3], highlighting the potential importance of this disease in the live cattle trade. In countries like Japan and China where multiple tick species have been identified as potential disease vectors, economic impacts have been significant [5, 6].

The lack of preventive measures or suitable vaccines complicates the management of *T. orientalis*. Currently, there are limited therapeutic options available for

treatment of oriental theileriosis and no vaccines available for this disease globally. Vaccine and/or therapeutic development has been identified as a research priority for bovine theileriosis; however as in malaria studies, an understanding of the taxonomy and genetic variability within parasite populations is essential to ensure vaccine and therapeutic efficacy.

2. Taxonomy of *T. orientalis*

2.1 Taxonomic history of *T. orientalis*

Historically, the taxonomy of *T. orientalis* (formerly referred to as the *Theileria orientalis/sergenti/buffeli* group) has been a subject of some confusion, due to similarity in strain morphology, variability of host animals and transmission vectors, occurrence of mixed infections, parasite genetic diversity and the difficulty in extracting pure isolates for studies, especially in benign infections where parasitaemia is low [7]. Originally, these parasites were classified based on geographic origin [8, 9]. Further attempts to classify this group of parasites led to suggestions that the group should be classified into one species [1, 8–11]. More recently, variations in the major piroplasm surface protein (MPSP) gene have been used to classify members of the *T. orientalis* group, separating it into 11 genotypes [1].

Members of the *Theileria orientalis* group were first identified in Australian cattle in 1910 and the organism classified as *T. mutans* [12] due to the morphological similarity to the previously described African species [13]. Some years later, Wenyon [14] made the first description of a similar blood parasite from sheep and named it *Babesia sergenti*. The morphological drawings of *B. sergenti* [9] corresponded to *Theileria* spp. morphology and it was later found that the parasite he described was indeed a theilerial parasite of sheep [15, 16]. However, in the intervening years, a new parasite of cattle in Eastern Siberia was described and *T. sergenti* [17]. The sheep parasite thus has precedence with respect to the name *T. sergenti,* rendering this name invalid for the cattle parasite; nonetheless the name *T. sergenti* had been used widely for this organism in the literature. Following the initial description of "*T. sergenti*" in Siberian cattle, a similar cattle haemoparasite was found in the same area and the authors named it *T. orientalis* [18].

Serological and morphological studies [19] later revealed that the *T. mutans* isolate identified in Australia [12] was the same species as "*T. sergenti*" [17] and not the African *T. mutans* described by [8]. Authors [15] suggested that the Australian isolate was either *T. orientalis* [18] or *T. buffeli* [20]. Serological and morphological studies conducted on *Theileria* stocks from Australia, Britain, Iran, Japan, USA and a higher pathogenicity stock from Korea concluded that the nomenclature of Australian *Theileria* should be *T. orientalis* [8]. But, a few authors still propose that the name *T. buffeli* should be designated due to the transmission of parasite from buffalo to cattle and the fact that isolates characterised at that point of time were all infective for buffalo [11, 21, 22]. Studies in Japan suggested *T. orientalis* and *T. buffeli* to be separated from *T. sergenti* and be classified as a different group due the serological and transmissibility differences [9, 23, 24]. Regardless of these findings, it was concluded that designation of the name *T. sergenti* should not be used for any blood parasite of ruminants with the exception of sheep [15, 16, 22].

2.2 Taxonomic classification using molecular techniques

As serological and morphological techniques were not suitably discriminatory for distinguishing isolates from the *Theileria orientalis/sergenti/buffeli* group,

molecular techniques became more prevalent. The use of the MPSP and 18S rRNA genes further clarified the relationships within this taxonomic group. Early PCR analysis of the MPSP gene revealed four major genotypes, Ikeda, Chitose and Buffeli and Thai type [25, 26]. The Buffeli genotype type was also separated into sub-genotypes B1 and B2 due to variability observed between these isolates [25]. Genotyping of the V4 variable region with the 18S rRNA gene which was previously shown to enable classification of *Theileria* spp. [27] revealed seven genotypes (Genotypes A to G) [28]. Subsequent examinations of *Theileria orientalis/sergenti/buffeli* group taxonomy utilised MPSP sequences due to greater observed sequence variation, producing stronger branch support in phylogenetic analyses [29, 30]. By 2010, eight MPSP genotypes (1–8) were classified including the unclassified genotype from Brisbane, Australia (*T. buffeli* Warwick) [29–31]. MPSP genotype 6 found in cattle and yak was reclassified and the taxonomic name *Theileria sinensis* was suggested to reflect divergence from the other members of the *Theileria orientalis/sergenti/buffeli* group [6, 32]. Three new genotypes from sheep, water buffalo and cattle were further identified [33] in Vietnam (N1, N2 and N3 respectively) bringing to current number of classified MPSP genotypes to 11 (Types 1–8 and N1–N3). Retrospective analysis of the genotypes previously identified with the 18S rRNA gene [28] against the current MPSP genotyping scheme shows that genotype A corresponds to Chitose while genotypes B and E correspond to Ikeda. 18S rRNA Genotypes C and D correspond to the Buffeli and Type 6 MPSP genotypes respectively. Further analysis revealed the 18S rRNA genotypes F and G identical to *Theileria cervi* a species found in elk. Buffeli sub-genotypes B1 and B2 identified in [25] correspond to the MPSP buffeli genotype and Type 4 respectively.

Molecular examinations have considerably clarified the taxonomy of *T. orientalis*. Asian isolates previously referred to as *T. sergenti* were found to be a mix of MPSP genotypes that were also commonly found in *T. buffeli* and *T. orientalis* isolates. Both Types 1 (Chitose) and 3 (Buffeli) were commonly found in both Australia and East Asia, with Type 3 spread globally. Hence more recent studies have begun to refer to this group by the common name *T. orientalis* [1, 34, 35].

Great efforts have been made by researchers to genetically characterise *T. orientalis*. However, current genetic characterisation methods utilise relatively few molecular markers. It has been well established that the primary mechanism driving genetic diversity in apicomplexans is through the sexual recombination; in the case of *Theileria* parasites, this occurs within the tick vector. Recombination has been relatively poorly studied in *T. orientalis*, however it has been suggested that recombination between MPSP genotypes is unlikely due to the low sequence identities between types [33] and high sequence identities within each clade [1, 31, 36, 37].

2.3 Current taxonomic state of *T. orientalis*

Genetic diversity within and between the MPSP genotypes should be further investigated as it has the potential to resolve the controversy surrounding the taxonomic classification of *T. orientalis,* elucidate virulence factors driving differential pathogenicity, and has implications for vaccine design. A complete genome of *T. orientalis* (Ikeda) has now been sequenced and annotated and is available for further research [34], and whole genome sequencing of large numbers of isolates is now feasible. A recent study which presented draft genomes of Australian isolates of Ikeda, Chitose and Buffeli genotypes confirmed the MPSP phylogenies indicating that the apathogenic Chitose and Buffeli genotypes are more closely related to each other than to the pathogenic Ikeda genotype [37]. That study further suggested that *T. orientalis* may indeed encompass multiple species and subspecies. The average nucleotide identity (ANI) between the Ikeda genome

and those of the Chitose and Buffeli genotypes (82%) was comparable to that of *T. annulata* and *T. parva* (80%). While sequencing of additional representatives of these genotypes is desirable, the evidence from the ANIs combined with the differential pathogenicity of these genotypes suggests that *T. orientalis* Ikeda is a separate species to *T. orientalis* Chitose and *T. orientalis* Buffeli. Moreover, the ANI between *T. orientalis* Chitose and *T. orientalis* Buffeli (86%) was comparable to that of the murine *Plasmodium* spp. suggesting that there may be further species or subspecies-level diversity within *T. orientalis* genotypes. [37]. Whole genome sequencing of additional *T. orientalis* genotypes is warranted to determine whether a new species designation should be applied to *T. orientalis* Ikeda and whether this may extend to include the phylogenetically related Type 7 which has also been associated with clinical disease [38]. Additional genome-wide studies will also enable researchers to formulate vaccine strategies by characterising possible vaccine targets and allow genetic diversity investigations within parasite populations [1]. Current efforts to understand the recombination mechanisms of other species of *Theileria* that lead to genetic diversity and taxonomic uncertainties [39–41] have been fruitful and it warrants researchers to conduct further investigations to answer the taxonomic questions surrounding *T. orientalis*.

3. Epidemiology

T. orientalis is a cosmopolitan parasite of cattle that also affects buffaloes and yaks [8]. *T. orientalis* infections have been globally reported in Australia [42–45], New Zealand [46], Southeast Asia [3, 33, 47–50] East Asia [6, 29–31, 36], South Asia [38, 51–53], Middle East [54–56], Africa [57–59], Europe [8, 60–65] and the Americas [1, 10, 66]. The distribution of *Theileria* species is dependent on the availability and competence of suitable tick vectors [7]. The principle vector of *T. orientalis*, *H. longicornis,* can be found in most of the countries where disease outbreaks have been reported (**Table 1**). In countries where distribution of *H. longicornis* is sparse or where the species is not known to occur, other *Haemaphysalis* spp. or other genera of ixodid ticks (**Table 1**) have been identified to be capable of transmitting the parasite, although the comparative competency of these species is unclear. The significance of these ticks as vectors of *T. orientalis* warrants further investigation.

3.1 Clinical disease outbreaks: Japan, Australia, New Zealand

In Japan, *T. orientalis* sourced from grazing cattle in Hokkaido was reported to cause 0.1% and approximately 2.5% of mortality and morbidity respectively [83]. In 2009, PCR analysis of the MPSP and p23 gene of *T. orientalis* revealed the presence of at least four genotypes (1, 2, 4 and 5) [29]. Further analysis [68] revealed Type 3 (Chitose) to be present in Japan and with earlier studies the authors suggested a total of seven genotypes (1–3, 4, 5, 7 and 8) to be present [69, 84]. Studies conducted over a number of years implicated *T. orientalis* Ikeda (Type 2) as being linked to clinical disease [42, 83, 85].

In recent years, outbreaks of oriental theileriosis have been increasingly observed in a number of different countries and are usually identified as being associated with MPSP genotype Ikeda (Type 2) [29, 45, 46, 68]. Australia and New Zealand recently experienced major disease incursions linked to *T. orientalis* Ikeda despite other genotypes of the parasite being present in these countries for many years.

T. orientalis was first observed in Australian herds in 1910, and the introduction was linked to the importation of *T. orientalis* infected *H. longicornis* ticks on

Country	*T. orientalis* MPSP genotypes	Host species	Vectors	References
Australia	1, 2, 3, 5	Cattle	*H. longicornis*	[1, 42, 44]
New Zealand	1, 2, 3, 5	Cattle	*H. longicornis*	[46, 67]
Japan	1, 2, 3, 4, 5, 7, 8	Cattle	*H. longicornis*, *H. mageshimaensis*, *H. douglasi*, *I. persulcatus*, *I. ovatus*	[5, 29, 34, 36, 68–70]
Korea	1, 2, 3, 4, 5, 8	Cattle	*H. longicornis*	[30, 71–74]
Taiwan	3	Cattle	*H. longicornis*	[31]
Vietnam	1, 2, 3, 5, 7, N3	Cattle	*Rhipicephalus microplus*	[3, 33, 49]
	5, N1, N2	Water buffalo	Unspecified	
	N1	Sheep	Unspecified	
Indonesia	7	Cattle	Unspecified	[48]
Thailand	1, 3, 5, 6, 7, N3	Cattle	Unspecified	[10, 47, 75]
	1, 3, 4, 5, 7, N2, N3	Water buffalo	Unspecified	
Cambodia	1, 3	Cattle	Unspecified	[49]
Myanmar	1, 3, 4, 5, 7, N3	Cattle	*R. microplus*, *Haemaphysalis* spp.	[50]
Philippines	Unspecified, but possible Type 1 and/or Type 3	Cattle	Unspecified	[76, 77]
India	1, 3, 7	Cattle	*H. bispinosa, R. microplus*	[38, 52]
	N2	Water buffalo	*R. microplus*	[53]
Sri Lanka	1, 3, 5, 7	Cattle	Unspecified	[1, 51]
	N1, N2	Water buffalo	Unspecified	
China	1, 2, 3, 5, 6, 8	Cattle	*H. longicornis, H. qinghaiensis*	[6, 32, 78–80]
	3	Water buffalo	*H. longicornis*	
	6	Yak	*H. qinghaiensis*	
Mongolia	1, 3, 5, 7, N3	Cattle	*Dermacentor nuttalli*	[36]
Russia	1	Cattle	*H. longicornis*	[60]
Egypt	1, 2	Cattle	Unspecified	[56]
	2	Water buffalo	Unspecified	
Kenya	3, 5	Cattle	Unspecified	[58]
United Kingdom	3	Cattle	*H. punctata*	[1, 8]
Italy	1, 3	Cattle	*R. bursa*	[62]
Hungary	Unspecified, PCR of 18S rRNA was done to identify presence of *T. orientalis*	Cattle	*H. punctata*	[65]
Portugal	Unspecified, RLB assay was done to identify presence of *T. orientalis*	Cattle	*H. punctata*	[64, 81]

Country	*T. orientalis* MPSP genotypes	Host species	Vectors	References
Spain	Unspecified, RLB assay was done to identify presence of *T. orientalis*	Cattle	*Haemaphysalis* spp.	[63]
Greece	Unspecified, IFAT—Indirect fluorescent antibody test for *T. orientalis* antigens	Cattle	*H. punctata*	[61]
Brazil	1, 2, 3, 4, 5, 7, N2 ,N3	Cattle	*R. microplus*	[1]
	Unspecified	Water buffalo	*R. microplus*	[66]
USA	6	Cattle	Unspecified	[1, 10]
Ethiopia	1, 2, 3, 5	Cattle	Unspecified, but *T. orientalis* DNA found in *Amblyomma* and *Rhipicephalus* species	[59]
Iran	Unspecified	Cattle	*H. punctata*, *H. longicornis*	[55, 82]
Turkey	1, 3	Cattle	*Hyalomma excavatum*, *R. annulatus*	[54, 62]
Central Africa	Unspecified	Cattle	*A. variegatum*	[57]

Majority of the unspecified vectors were suggested to be Haemaphysalis spp. MPSP genotypes Type 1 = Chitose, Type 2 = Ikeda, Type 3 = Buffeli. The other eight genotypes (4–8 and N1–N3) have yet to be named.

Table 1.
The global distribution of T. orientalis MPSP genotypes reported in four different host species and the possible transmission vectors.

cattle from Japan [86, 87]. Surveys of cattle in New South Wales (NSW, Australia) performed in the mid-20th century revealed the presence of *T. orientalis* in 60% of examined blood smears [42, 86] and later studies found herd and individual animal seroprevalence of 75% and 41% respectively in endemic parts of Queensland [42, 87]. The parasite was considered to be relatively benign as it caused only mild anaemia [42]. Prior to 2006, reports of clinical theileriosis in Australia were rare and experimental transmission studies were unable to establish clinical infection in test animals, suggesting that Australian strains of *T. orientalis* were of the benign Buffeli genotype [8, 22, 42, 88]. Samples from cattle imported into Japan from Australia were shown to be positive for the Chitose genotype by MPSP restriction fragment length polymorphism (RFLP), showing evidence that Chitose was present in Australia prior to 1998 [31]. However, since 2006, there was a large increase in clinical *T. orientalis* outbreaks in coastal and highlands regions of NSW [44, 89] and other parts of Australia such as Queensland [43], Victoria [90, 91], Western Australia [92] and South Australia [93, 94] (**Figure 1A**). Most clinical theileriosis outbreaks were linked to the movement of periparturient cattle from inland areas to the coast and the introduction of naïve cattle into endemic areas and/or introduction of infected cattle to *T. orientalis* non-endemic areas [2, 42, 89]. Large scale surveillance efforts identified the Ikeda genotype as the sole infecting type or as a mixed infection with other genotypes in all herds examined [43–45, 90].

T. orientalis was first reported in New Zealand in 1982 [95] with suggestions that the parasite could have been introduced through the importation of cattle from Britain or Australia where the parasite was prevalent. Prior to 2012, the Ikeda genotype was not associated with clinical theileriosis in New Zealand. Since then outbreaks of *T. orientalis* of the Ikeda genotype have been reported in beef and dairy

Figure 1.
Map of Australia (A) and New Zealand (B) showing the extent of spread of theileriosis during the recent disease incursions in each respective country. The areas in which T. orientalis Ikeda is enzootic closely mirrors the distribution of the vector tick H. longicornis.

cattle herds in multiple regions of the North Island [96, 97]. In 2012, genotyping tests conducted on affected cattle herds of *T. orientalis* outbreaks further revealed three other genotypes present, Chitose, Buffeli and Type 5 [97]. Of the four genotypes, Ikeda was identified to be more pathogenic than Chitose and Buffeli in New Zealand [67]. Prevalence and spatial distribution studies showed *T. orientalis* Ikeda to predominantly occur in the Northland (33 out of 35 herds; 94%) and Auckland and Waikato regions (63 out of 191 herds; 33%) where the transmission vector, *H. longicornis* is known to occur [96, 98] (**Figure 1B**). Only 2 out of 204 (1%) herds tested positive for *T. orientalis* Ikeda in the South Island of New Zealand where the distribution of *H. longicornis* is sparse and less common [96, 98].

3.2 Global distribution of *T. orientalis*

The geographic distribution of *T. orientalis* MPSP genotypes was previously reviewed by [1]. Since then, new clinical cases have been reported in Ethiopia [59] where *T. orientalis* was not known to occur, Type 5 was identified in cattle in Kenya [58], Type 2 Ikeda was recently identified in Vietnam via cattle imported from Australia [3], and studies in Kerala, India, revealed for the first time that MPSP genotype N2 to cause clinical theileriosis in Asian water buffaloes [53]. The majority of molecular distribution studies are based on the genetic characterisation of the *T. orientalis* MPSP gene. Some studies utilise other molecular markers such as the ITS 1, ITS 2, COX III and 18S rRNA genes to identify or characterise the parasite [46, 66, 99]. Studies based on molecular markers other than MPSP could not accurately classify MPSP genotypes, therefore, the identity of the MPSP genotypes found in some studies remain unclear [1].

As described above, most studies have implicated *T. orientalis* Ikeda (Type 2) in oriental theileriosis outbreaks [29, 43, 46, 68]. However, some studies have suggested MPSP genotypes Chitose (Type 1) [46, 74] and 7 [38] to be associated with clinical disease. The clinical relevance of these genotypes cannot be confirmed as COX III and 18S rRNA genes were used to characterise the samples instead of the MPSP gene in one study [46] or the possibility of mixed infections with Ikeda genotype was not investigated [1, 38, 74]. Nonetheless, Type 7 is phylogenetically related to the Ikeda genotype [1], and may indeed represent a pathogenic genotype

and should be the subject of further study. MPSP genotype N2 seems to be predominant among water buffalo populations although it has also been reported in cattle in Brazil. Type N2 was identified to cause fatal oriental theileriosis in Asian water buffaloes [53] but its virulence against cattle and other animals is unclear. Further distribution studies are required in order to determine host specificity of type N2. Cross-infection profiles between host animals in different countries may vary. For example, in India, Types 1, 3 and 7 are found in cattle and only type N2 is found in water buffaloes. But, in Thailand, Types 1, 3, 5, 7 and N3 can be found in both cattle and water buffaloes [47, 75]. This suggests that the tick vectors of a specific region may display host specificity limiting transmission to the preferred host or the tick vectors may have different preference for different genotypes. Previously, studies on *T. parva* have demonstrated that different tick populations have different preference for particular genotypes [39]. Whether this holds true for *T. orientalis* remains unclear, and warrants further investigation.

3.3 Vectors of *T. orientalis*

Although, the ixodid tick, *Haemaphysalis longicornis*, is considered to be the principal vector of *T. orientalis* [5, 67, 89, 94], the parasite has been detected in other arthropods such as mosquitoes [100] and lice [94, 101, 102]. Several studies have also revealed several possible tick vectors other than *H. longicornis* (**Table 1**). Prior to the recent Australian *T. orientalis* outbreak, *H. bancrofti* and *H. humerosa* [103–106] were found to be more competent and efficient vectors compared to *H. longicornis* under experimental conditions, although it is noted that the *H. humerosa* used in these studies were latterly believed to be *H. bremneri* [106, 107]. These studies employed the 'Warwick stock' of *T. orientalis* which is of the Buffeli genotype. Interestingly, the extent of spread of clinical theileriosis in Australia (**Figure 1A**) caused by *T. orientalis* Ikeda corresponds very well to the known range of *H. longicornis* rather than to that of *H. bremneri*, *H. bancrofti* or indeed, *H. humerosa* Furthermore, studies on a range of tick species collected from the Gippsland region of Victoria, within the theileriosis endemic zone, only detected the presence of *T. orientalis* in *H. longicornis* [94]. Similarly, in New Zealand, disease is only detected within the known range of *H. longicornis* (**Figure 1B**) and indeed, *H. longicornis* is the only *Haemaphysalis* tick present in that country [98]. In parts of Australia, *T. orientalis* Buffeli and Chitose are known to occur outside the areas in which disease in enzootic and outside the known range of *H. longicornis.* Together, these findings suggest that different ticks transmit different genotypes of *T. orientalis* with different efficiencies or that the tick species displays variable selection for the different genotypes. In *T. parva*, particular genotypes have been shown to be favoured when passaged through different tick clones, suggesting that these genotypes are selected for in tick vectors [39]. Also in China, *T. sinensis* is limited to the surrounding regions of the Tibetan plateau [108] as the vector *H. qinghaiensis* is limited to this region [6]. Indeed, recent genome sequencing studies revealed that the Ikeda, Chitose and Buffeli genotypes are sufficiently divergent to be considered different species or subspecies [37] and therefore may be adapted to different tick hosts. Vector competency for the different genotypes aside from *T. orientalis* Buffeli [87, 104, 105] have not yet been investigated in detail.

Currently, information on tick species transmitting disease is somewhat confounded because the vector competency for the different genotypes has not been thoroughly investigated. In Japan, *H. megaspinosa*, *H. douglasi*, *I. persulcatus* and *I. ovatus* have been identified as other potential vectors of *T. orientalis* [5]. Additionally, these four ticks were found to preferentially transmit the pathogenic

T. orientalis Ikeda [42]. In Europe, *H. punctata* seems to be the predominant tick vector to transmit *T. orientalis* [8, 61, 64, 65, 81], but in other geographical locations such as East Asia [5, 6], Australia [94] and New Zealand [98], *H. longicornis* is identified as the predominant tick vector. Although only limited molecular surveys have been undertaken in Europe, *T. orientalis* Ikeda has not been identified in this region. The specific relationship between type Ikeda and *H. longicornis,* in Japan [5], China [6], Australia [94] and New Zealand [98] where the Ikeda genotype is limited to *H. longicornis* distribution, combined with the absence or sparse distribution of *H. longicornis* in Europe [109], suggests that the Ikeda genotype may have a specific relationship with *H. longicornis*.

The epidemiology of *T. orientalis* is important as it enables researchers to understand distribution patterns and set up appropriate biosecurity measures. It is clear that there are gaps in the current knowledge of *T. orientalis* transmission and distribution. Further research is essential to identify potential tick vectors that may preferentially transmit certain MPSP genotypes of *T. orientalis*. Molecular characterisation and investigations of the MPSP genotypes coupled with whole genome studies could provide insights on the pathogenicity and genetic diversity, therefore enabling the implementation of efficient control strategies against this emerging disease agent.

4. Lifecycle and transmission

Evidence suggests that *H. longicornis* is a major vector of *T. orientalis*. This species is a three host tick meaning that each life stage of the tick will feed on a different host before each moult. *H. longicornis* parasitises cattle and other domestic ruminants [98, 110] and it undergoes obligate parthenogenesis to reproduce, as the adult female is able to lay fertile eggs in the absence of a male [111]. The three host lifecycle of *H. longicornis* has four life stages, an egg, larvae, nymphal and adult stage. Eggs hatch 30–90 days after being laid. The hatched larvae begin questing for its blood meal by climbing vertically on blades of grass to seek a host. *H. longicornis* have enhanced survivability as they are not specific in feeding even though they have a preference for cattle. Each engorgement occurs for 3–4 days before the tick falls to the ground and moults to the next stage.

In the tick vector, the *Theileria* lifecycle begins with blood engorgement on a mammalian host during which infected erythrocytes containing piroplasms are ingested by the tick. These piroplasms differentiate into gametocytes in the midgut of the tick and undergo a brief sexual stage to form zygotes that enter the gut epithelial cells. Motile kinetes are developed by meiotic division within the gut epithelial cells. Following meiosis, the parasite escapes into the haemolymph during the tick moulting phase and migrates to the salivary glands where sporogony occurs. *Theileria* kinetes invade salivary cells, develop into sporoblasts, and then into infectious sporozoites which are injected into the mammalian host when the moulted tick feeds again [112]. Sporozoites are inoculated into the mammalian host through the hypostome of the feeding tick. In *T. parva* and *T. annulata*, sporozoites invade the mammalian host leukocytes to develop multinucleate syncytial schizonts. At this point *Theileria* spp. can be separated into two evolutionary groups based on their ability to transform host leukocytes leading to clonal expansion of infected lymphoid cells [113]. Unlike *T. parva* and *T. annulata, T. orientalis* does not transform the invaded leukocytes. The schizonts undergo merogony to develop merozoites and rupture the leukocytes to invade the erythrocytes and form piroplasms [114]. When the tick feeds on the infected mammalian host, the *T. orientalis* lifecycle is completed.

Transmission of *T. orientalis* in the tick is transstadial, as the parasite can be transmitted from one instar to the next. Ticks that ingest erythrocytes infected with piroplasms transmit the parasite when they moult to the next instar [115]. Transovarial transmission, parasite transmission from adult female to the next generation of eggs, has yet to be scientifically demonstrated [103] by any transmission studies although some researchers have speculated that *Theileria* might involve transovarial transmission in ticks [116, 117].

Interestingly, *T. orientalis* infection dynamics varies depending on the genotype transmitted. A study on *T. orientalis* temporal dynamics in 10 animals revealed that Ikeda was detected first when naïve animals are exposed to herds infected with a mix of Ikeda, Chitose and Buffeli genotypes [35]. Thus the Ikeda genotype possesses a shorter pre-patent period than the other two genotypes, which may be due to a faster growth rate, out-competition of the other genotypes, or perhaps more efficient transmission by the tick vector [35]. Similar observations were made in temporal monitoring of mixed Ikeda and Chitose infections in experimentally infected cattle in Japan [25, 118].

Transplacental parasite transfer from pregnant cattle to offspring through the placenta has been confirmed through molecular and serological methods for a range of *Theileria* spp. This mode of transmission has been demonstrated in species such as *T. annulata* [119] *T. equi* [120, 121], and *T. lestoquardi* [122]. Transplacental transmission also occurs in *T. orientalis* infection [71, 123, 124]. Early studies [123] used blood film examination to demonstrate that transplacental transmission occurs in calves but at a low rate of 5% (5/100 calves that are 1–2 days old). The authors also determined the parasitaemia of newborn calves and post-grazing calves to be similar and suggested the low levels of parasitaemia in newborn calves to be ineffective in producing immunity against *T. orientalis* [123]. In contrast, 100% of the calves (n = 5) from experimentally infected dams were demonstrated to be *T. orientalis* positive and infected dams sometimes aborted the calves (two out of five dams) at approximately 6–7 months of gestation [71]. However, the dams in the study had an extremely high tick burden of approximately 200 ticks which had been artificially fed on cows with high parasitaemia [71]. In contrast, another recent study in New Zealand [125] did not detect transplacental transmission despite using sensitive molecular techniques. Recently, an Australian study [124] used molecular methods to confirm transplacental transmission of *T. orientalis* in field-affected cattle, but at low rate of approximately 2% (2/98 calves) similar to the study of [123]. In that study, abortion did not appear to correlate with transplacental transmission of *T. orientalis*, instead the authors posited that, abortion may occur due to hypoxia in the foetal calves due to maternal anaemia, placental insufficiency, or other factors related to maternal pathology [123].

In addition to ticks, *T. orientalis* can also transmit mechanically through the inoculation of infected blood [8, 101] or via other biting arthropods such as the sucking louse (*Linognathus vituli*) [102, 126] and potentially the horse flies (*Tabanus trigeminus*) and stable flies (*Stomoxys calcitrans*). These biting arthropods have been hypothesised to be able to mechanically transmit *T. orientalis* through the proboscis of the biting flies or regurgitation of blood into the animal host [101, 102, 126]. In Australia, *Theileria* DNA was not detected in March flies (*Dasybasis* sp.) collected in outbreak regions in Gippsland, Victoria [94]; however, *T. orientalis* was detected in mosquitoes collected from the same area. In addition, a xenosurveillance study in the United Kingdom has revealed *T. orientalis* in 16 out of 105 (15.2%) blood meals in mosquitoes [100]. The risk of transmission by mechanical vectors is likely to be dependent on the parasitaemia of the infected blood being transferred by these biting arthropods [101].

Mechanical transmission through routine husbandry practices is another potential method of *T. orientalis* transmission. A recent Australian study showed that *T. orientalis* could be mechanically transmitted with volumes as low as 0.1 mL of blood and persist for at least 5 months in the infected bovine after blood inoculation [101]. Thus, injuries sustained during yarding and transport of cattle, or routine husbandry procedures such as vaccination, blood transfusion, castration or ear notching performed where contaminated instruments are re-used can result in iatrogenic transfer of *T. orientalis* infection. Aside from blood transmission, there is potential for mechanical transfer of the parasite via the oral route. Dam to calf transfer of the apicomplexan *Neospora caninum* has been suggested to occur via the colostrum with pathogen entry via the oral mucosa. Recent findings that *T. orientalis* is present in colostrum raise the possibility that a similar mode of transfer may be possible by this species in calves, although this is yet to be confirmed [101].

Although there is now clear evidence from a number of studies that *T. orientalis* can be transmitted mechanically, including by haematophagous arthropods, this mode of transmission would not be expected to maintain the parasite life cycle. Mechanical transfer bypasses the sexual stage of the lifecycle where genetic recombination occurs. The direct transfer of haploid stage piroplasms from one host to another may result in reduced genetic diversity, a feature of apicomplexans which facilitates immune evasion [127–129]. Thus, mechanical transmission of *T. orientalis* may allow the organism to persist in the herd when tick numbers are low, but passage through the tick is likely to be important for the overall survivability of the parasite [101].

Although different forms of *T. orientalis* transmission have been identified, more research is required in order to increase awareness and formulate efficient control and preventive strategies to reduce disease incidence and stress on livestock.

5. Pathogenesis

Unlike *T. parva* and *T. annulata* that transform host leukocytes leading to fatal lymphoproliferation [130–132], the major pathogenic effect caused by *T. orientalis* is through the destruction of host erythrocytes and subsequent anaemia. Schizonts can be detected transiently in the lymph nodes, spleen and liver of infected cattle approximately 10 days post-inoculation with sporozoites [132]. However, schizonts in *T. orientalis* are rarely associated with major pathogenic effects as the schizont-infected cells are not commonly found in the peripheral blood [132]. Piroplasms can be detected in the host erythrocytes approximately 10 days post-inoculation and anaemia develops approximately 10 days later following detection of piroplasms when parasite load and serological response peaks [133]. Host animals sometimes also experience transient pyrexia and reduction in white blood cell count as anaemia develops [132, 134]. Animals that have been immunologically exposed to *T. orientalis* have lower parasitaemias and recover from infections earlier and with less morbidity. However, the haemoparasites can persist in the host, potentially until death, and can cause relapse through the resumption of piroplasm proliferation when animals face stress from pregnancy, lactation or rapid changes of environmental or rearing conditions [3, 132].

The pathogenic effects of anaemia consequent to infection although not well established [135]; have been studied extensively. Splenic capture of erythrocytes is likely the primary cause of anaemia rather direct lysis of erythrocytes by the pathogen [133]. In malaria infection, splenic clearance of both infected and uninfected erythrocytes is known to occur and may be the consequence of activation of splenic

macrophages or altered red pulp resulting in an increase in mechanical erythrocyte retention [136]. Yagi et al. [137] demonstrated that survival of both infected and uninfected erythrocytes decreased in *T. orientalis* infected calves and suggested that denaturation of blood plasma may play a role in this reduced survivability as reported for other protozoan infections [138–140]. Studies of *T. annulata* have demonstrated that anaemia might be an immune-mediated process as indicated by the presence of a haemagglutinin [141]. However, in *T. orientalis* infection the destruction of erythrocytes can occur in the absence of immunoglobulin or the involvement of complement [142]. Oxygen radicals released from the lysed erythrocytes may also play a role in pathogenesis as observed for *Plasmodium* infections [140]. Indeed, [143] demonstrated the development of anaemia in association with elevated levels of methemoglobin, a product of haemoglobin oxidation. Oxidative damage of erythrocytes occurs when superoxide radicals are released simultaneously to the increased levels of methemoglobin which may result in their removal from circulation by the reticuloendothelial (mononuclear phagocyte) system [132, 143].

As described in detail in Section 3, the pathogenicity of *T. orientalis* is genotype-dependent unlike the transforming theilerias *T. parva* and *T. annulata* [1]. However this may reflect the fact that the *T. orientalis* genotypes display species-level divergence [37] and pathogenicity of *T. orientalis* Ikeda may be driven by as-yet unidentified virulence factors.

6. Clinical disease, infection dynamics and the immune response

In the early stages of clinical oriental theileriosis, signs of muscle weakness, ataxia, and abortion are observed in infected animals. A variety of clinical findings such as the lack of appetite, pyrexia, elevated heart rate, abnormal breathing, pale mucous membranes and jaundice have been reported [89]. Aggression in clinically affected animals has occasionally been observed and may be caused by the alteration of mentation as a result of cerebral hypoxia [89]. All of these symptoms are a result of the anaemia in the host animal. Identification of anaemia can be achieved by measuring haematocrit (packed cell volume), which in severely infected cattle can be as low as 8% [144]. In *T. annulata* infections, bovine cerebral theileriosis associated with aggression was identified as a result of lymphocytic proliferation and blood vessel inflammation [145].

In *T. orientalis* both clinical and subclinical infections are known to frequently occur as a combination of genotypes [29, 42, 44, 146]. In Japan and Australia, *T. orientalis* Ikeda occurs with Chitose genotypes at high frequency with or without the presence of benign genotypes [45, 90, 147] and surveys from the Eastern coast of Australia have revealed genotypes Buffeli and Chitose occur in most subclinical infections [43]. The Ikeda genotype has been linked with higher parasite load and is evident in 100% of the samples that are clinically infected with Ikeda only or a mixture of Ikeda and Chitose [146]. In the clinically mixed infections, semiquantitative data revealed Ikeda to be the dominant genotype (58%) [146]. Within the genotype Chitose, there are two subtypes, Chitose A and Chitose B [35, 146]. In clinical samples from Australia, Chitose A was been noted to commonly occur with Ikeda at a high frequency (approximately 95% of cases examined) and is often detected at high parasite loads, while Chitose B occurs with Ikeda at a lower frequency [35]. Whether Chitose A is contributing to pathogenesis remains unclear. Although, the genotype Chitose was suggested to be able to solely establish a clinical infection in New Zealand cattle [46], the cytochrome oxidase III and 18S rRNA genes rather than the MPSP gene were used to characterise the samples, therefore the genotype of the parasite involved in that study remains unconfirmed. Nonetheless, if the

cattle were naïve to Chitose genotype it is possible that this may have led to clinical disease. Regardless, the Ikeda genotype has been associated with recent clinical outbreaks in New Zealand [67, 96, 97]. Another Korean study [74] also suggested Chitose to independently establish clinical infection in cattle, but mixed infections were not accounted for in the study.

Higher susceptibility to clinical theileriosis is observed in association with cattle movements; especially where naïve cattle are newly introduced to an endemic area, and/or infected animals are introduced to a non-endemic area with competent vectors [43]. Naïve cattle become rapidly infected in the presence of infected vector ticks, with time to patency (as determined by qPCR) as early as 11 days post-introduction to an infected herd [35]. Overall parasite load peaks around 40 days post-introduction with the onset of anaemia occurring 8–10 days later, although drops in haematocrit commence at the onset of the patent period. Interestingly, in mixed infection with Ikeda and Chitose genotypes (with or without Buffeli genotype), the Ikeda genotype is detected first and also peaks first. Declines in Ikeda genotype are then followed by an increase in the Chitose suggesting a genotype switching mechanism which may be driven by the host immune response [35, 148].

Additional factors may drive disease susceptibility in cattle such as breed or the age of the animal. In Japan, beef cattle of the Wagyu breed have been reported as being less susceptible to clinical infections [149]. Although potentially a factor in disease susceptibility, the effect of age has not been well-studied. Some cases occurring in regions where adult cattle had previously been exposed *T. orientalis* reported calves at 6 to 14 weeks of age to have high mortality and severe morbidity [150, 151] which coincides with high parasitaemias which are consistently observed in calves from *Theileria*-endemic areas [124]. While MPSP antibodies are sometimes detectable in the colostrum of dams and appear to be transferred to calves [101], any passive immunity appears to be short lived, with antibodies undetectable in calves by 4 weeks of age [124]. Lack of protection from maternal antibodies likely explains the high infection intensities and clinical disease observed in calves.

In adult cattle, seroconversion to the MPSP occurs approximately 14 days after patency and humoral responses to this protein persist for at least 11 weeks post-infection [133]. However, a study of 256 *T. orientalis*-infected animals showed that humoral responses to the MPSP are much more frequently observed in animals experiencing clinical anaemia (89%) versus those with subclinical infections (45%). It is unsurprising therefore that seroconversion to the MPSP is also strongly correlated with both parasite load and the Ikeda genotype [133]. Another study demonstrated that humoral responses to experimental infection with *T. orientalis* (via mechanical transfer) are variable and only established after persistent infection [152]. The role of humoral immunity in protecting against *T. orientalis* infection in adult cattle is unclear. Cell-mediated rather than humoral immunity is generally considered more important in responding to intracellular pathogens; however once established, humoral immunity may assist in preventing the pathogen from gaining cell entry, as for *Babesia bovis* [153]. Further work is required to determine whether animals that have experienced clinical theileriosis are immune to disease recrudescence and whether immunity against one genotype confers protection against another.

Studies of the transforming theilerias, *T. parva* and *T. annulata*, have shown that cattle that recover from infection are able to establish immunity against homologous strains but succumb to heterologous strains suggesting that immune responses are highly specific for particular parasite epitopes [154, 155]. Immunity is mediated via cytotoxic T lymphocytes (CTL) which target parasitized lymphocytes but allow parasitized erythrocytes to persist [129]. Thus the immune pathways important in protection against non-transforming theilerias such as *T. orientalis* may be more akin to those of *Babesia* species [133].

7. Diagnosis

Oriental theileriosis can be diagnosed by various methods such as microscopy, serology, molecular techniques and xenodiagnosis. Bovine erythrocytes are anucleate, therefore those infected with piroplasms can be visualised under a light microscope using DNA stains (such as Giemsa or Diff-Quik) [156]. In carrier-state animals, erythrocyte infections are commonly observed in the low parasitaemia range of 0.02–0.03% [85, 157]. Parasitaemia in clinically affected animals suffering severe anaemia and other related clinical signs may range from >1–30% [46, 89]. Light microscopy is a quick and inexpensive method for the initial differential diagnosis of possible clinical theileriosis [89]. It has been used to describe many of the first species of *Theileria* after Koch's [158] initial description of *T. parva* [159]. However, the technique is limited as a diagnostic tool as it is considerably less sensitive than PCR and does not enable the differentiation of morphologically similar piroplasms [160, 161]. The differentiation between similar piroplasms is important to distinguish the clinically important species such as *T. parva; T. annulata* and *T. orientalis* from other less clinically significant species such as *T. taurotragi* and *T. mutans.* Light microscopy is unable to differentiate between pathogenic and apathogenic genotypes of *T. orientalis*. Furthermore, light microscopy lacks the sensitivity to adequately detect clinically-benign carrier animals [45, 159].

While a number of serological tests exist for the detection of *T. orientalis* [29, 87, 133, 152, 162], these assays are currently not genotype-specific and in some cases also cross-react with other *Theileria* species [152, 162]. Serological tests are of a similar sensitivity to blood smear examination and are most reliable when the animals are clinically affected, but are unsuitable for testing newly infected animals that have not yet seroconverted [133]. Currently, serological methods do not offer any advantage over molecular methods for determining whether animals have been exposed to *T. orientalis* since this organism establishes lifelong infections and can be detected in the blood well beyond the initial infection period.

PCR is currently the gold standard for sensitive detection of *T. orientalis* [133]. PCR can detect infection in cattle up to 2 weeks before the infected erythrocytes can be observed under a light microscope [29]. Conventional PCR methods have high sensitivity and have been validated for diagnostic use [43–45, 69]. However, conventional PCR assays are laborious to perform and do not provide information on parasite load making it impossible to distinguish between clinically infected animals and subclinical carriers. To address these problems, a number of real time semi-quantitative and quantitative PCRs have been developed for the detection of *T. orientalis* [146, 163–166]. The majority of these assays have been designed to specifically detect the pathogenic Ikeda genotype [93, 146, 163, 164, 166] and in some cases several genotype specific assays have been multiplexed [146, 163, 166]. Genotype discrimination has been most successfully achieved using assays targeting the MPSP gene [146, 163] while some other molecular markers have been shown to be insufficiently discriminatory [167].

The high prevalence of subclinical carrier animals infected with clinically-relevant genotypes [43] makes accurate quantification critical to correct diagnosis, particularly in the presence of confounding factors. In order to address this, a TaqMan probe-based assay targeting the *T. orientalis* MPSP was used to establish clinical thresholds for disease to facilitate diagnosis [146]. Using this assay, animals with *T. orientalis* gene copy numbers above 300,000 are highly likely to display clinical signs; while those with gene copy numbers below 15,000 are considered subclinical carriers. Cattle with gene copy numbers between 15,000 and 300,000 are frequently clinically affected but may also be recovering from disease or in-contact with clinically affected cohorts [146].

8. Treatment and control of *T. orientalis*

8.1 Chemotherapy

The increase in oriental theileriosis outbreaks in recent years highlights the need for effective treatment and control measures for this disease. Chemotherapy remains an important strategy in combating protozoan diseases [168]. Chemotherapeutics such as imidocarb, oxytetracyclines and halofuginone have been used to treat oriental theileriosis [2]. In Australia, imidocarb and oxytetracyclines are some of the registered chemicals which in some studies, appeared to have a positive response on cattle with low parasitaemia, but a poor response in severely infected cattle [169]. Menoctone, a hydroxynaphthoquinone compound was discovered to have anti-theilerial properties [170] and two active analogues, parvaquone [171] and buparvaquone [172] were developed shortly thereafter; which treated *Theileria* infections in cattle with high efficacy [173]. Total elimination of *T. orientalis* infection was achieved in splenectomised calves by a chemical mixture of primaquine and buparvaquone, or primaquine with halofuginone [174]. In Japan, buparvaquone was demonstrated to be effective enough to be used as a single chemical treatment [175]. A single intramuscular injection dose of 2.5 mg/kg buparvaquone was sufficient to treat the Buffeli, Chitose and Ikeda genotypes [2, 176]. In contrast, imidocarb was identified to have little effect on *T. orientalis* infection [177]. Prior to 2010, buparvaquone resistance in *T. annulata* has never been documented [173]. However, in *P. falciparum* and *Toxoplasma gondii*, resistance against atovaquone, a hydroxynaphthoquinone compound, was well documented to be caused by the mutation of the mitochondrial cytochrome b gene [178, 179]. The mode of action of buparvaquone in *T. orientalis* is not well established, but a study in coccidian parasites suggests an effect on the generation of energy [180]. While buparvaquone treats *Theileria* infections with great efficacy when used in the early stages of disease, resistance observed in apicomplexan infections are a growing concern and is a problem with chemotherapeutic agents in general.

An Australian study [169] showed that treatment with buparvaquone leads to the retention of residues in cattle tissue. The tissue residues were present up to 147 days post treatment with buparvaquone and as such this chemotherapeutic has long withholding periods and has not been approved for use at all in Australia. Previously in Japan, chemicals such as pamaquine and primaquine phosphate were commonly used treat *T. orientalis* infections but due to declining efficacy, its usage was discontinued [177]. This declining efficacy further revealed the inefficacy of primaquine phosphate to eliminate *T. orientalis* alone [174]. It requires a combination of chemicals as discussed above to successfully eliminate *T. orientalis* infection. As such, chemotherapy options have been limited due to the variations of drug efficacy. The development and identification of chemical compounds suitable for the treatment of *T. orientalis* is important, however, drug discovery is both time consuming and expensive. There are other important preventive measures worthy of investigating such as the control of competent vectors and management of animals that can facilitate the reduction of *T. orientalis* outbreaks.

8.2 Vector control and animal management

Vector control is important to reduce the rapid spread of *T. orientalis* outbreaks. Restriction of grazing cattle movements may assist in reducing exposure to infected *H. longicornis* ticks. Control of this vector can also be achieved by using acaricides such as multi-seasonal pour-on flumethrin [83]. This method has been successfully

demonstrated in Japan to reduce *T. orientalis* infection [83], but it is not permitted for use in Australia due to the possibility of unacceptable residues [181]. Currently, the acceptable methods of tick control in Australia are the application of synthetic pyrethroids in the form of short-acting dips and sprays that can contain amitraz, cypermethrin with chlorfenvinphos and deltamethrin with ethion [2, 181]. The usage of these cheap and common acaricides although economic (A$0.50–A$1.50 per head), might lead to resistance in the tick vectors, which in the long run will incur higher cost due to the requirement for more expensive macrocyclic lactones (approximately A$600 per treatment for a 100-cow herd) for tick control [182]. *H. longicornis* as described above is a three-host tick; therefore it is a challenge for these acaricides to be effective at controlling this species due to a limited host attachment period.

An alternate method to control *T. orientalis* transmission by ticks would be the development of a vaccine that targets exposed antigens of the tick [132]. Currently, there is a commercialised vaccine against *B. microplus* [183] and similar attempts have been made by utilising tick saliva proteins (p29, p34 and p35) against *H. longicornis* to produce a vaccine [184, 185]. The immunised animals when exposed to ticks, display interference that reduce tick growth and increase mortality of the ticks.

Besides controlling the tick vectors, proper management of animals can also reduce *T. orientalis* infection or re-infection. Infected animals are susceptible to relapses when faced with stress factors as discussed above. Supportive therapy such as blood transfusion can be performed to improve the anaemic conditions in affected animals; however, these therapies are time-consuming and expensive and may only be practical to treat valuable stud animals. Animal movement should be kept to a minimum to prevent elevated blood pressure which can cause the animal to collapse [186]. Intravenous fluids and nutritional supplements may also benefit affected animals [2] and intramuscular injection with iron dextran over the course of 3 days can aid recovery of infected animals [187]. The treatment and control of *T. orientalis* is multi-faceted and it requires all of the different elements discussed in order to be effective.

8.3 Vaccine development

Vaccination is viewed as the preferred method of control for oriental theileriosis. Unfortunately no vaccines currently exist for this disease; however, live vaccines for *T. parva* and *T. annulata* have been successfully used to treat East Coast fever and tropical theileriosis for over 40 years. Vaccination with highly passaged macroschizont-infected cell lines is possible for *T. annulata* due to the stimulation of immunity with low doses of attenuated cells which do not induce clinical disease. In contrast, for *T. parva*, the doses required to stimulate an immune response also induce clinical disease, therefore vaccination against *T. parva* involves simultaneous vaccination with sporozoites (homogenised ticks) and treatment with long-acting formulation of oxytetracycline to suppress disease. Because vaccination with a single strain of *T. parva* leaves animals susceptible to heterologous challenge, immunisation involves a mixture of three isolates which provides broad protection against disease [188].

The vaccination strategy employed for *T. annulata* is not directly transferrable to *T. orientalis* due to the non-transforming nature of this species and a lack of cultivation methods for this organism. The "infect and treat" method used for *T. parva* has potential promise for control of *T. orientalis* but is currently somewhat limited by a lack of suitable chemotherapeutic agents for parasite suppression.

Vaccination against tick fever, caused by the closely related piroplasmids, *Babesia bovis* and *B. bigemina,* also employs live attenuated organisms and is administered

to calves between 3 and 9 months of age when they are less susceptible to disease. This vaccination strategy has not been attempted with *T. orientalis* but unlike for tick fever, calves are highly susceptible to oriental theileriosis [150, 151]. Nonetheless, live vaccination is still considered one of the most promising approaches for control of oriental theileriosis. It has been suggested that vaccination with benign genotypes of *T. orientalis* may provide cross protection against the pathogenic genotypes [189]; however recent genome studies suggesting that the differences between genotypes are at the subspecies or species level make this more doubtful [37]. Furthermore, despite a relatively high seroprevalence *T. orientalis* in Australia (due to the presence of benign strains), extensive outbreaks caused by *T. orientalis* Ikeda occurred across the entire range of the vector tick. Combined with data showing that infections with *T. orientalis* are usually of mixed genotype [45, 90, 146, 190], there is little evidence to suggest that vaccination with *T. orientalis* Buffeli, Chitose or other genotypes would provide cross protection against *T. orientalis* Ikeda.

Development of a subunit vaccine is another possible avenue for control of oriental theileriosis. Early studies showed that passive immunisation of calves with an anti-MPSP monoclonal provided protection against development of disease upon challenge [191]. Therefore the MPSP was selected for use in subunit vaccine formulations consisting of recombinant baculovirus-expressed MPSP or synthetic MPSP peptides (containing KEK motifs) mixed with Freund's adjuvant or encapsulated in mannan-coated liposomes. Following immunisation with these vaccine formulations, calves were splenectomised and challenged with Ikeda or Chitose sporozoite stocks. Animals immunised with high dose peptide or recombinant MPSP had reduced parasitaemias relative to control calves and were protected from clinical signs of oriental theileriosis [190]. Despite these promising preliminary results, a subunit vaccine for *T. orientalis* has not been pursued further. Subunit vaccines are generally considered problematic when working with apicomplexans due to genetic diversity among strains. Indeed, in their subunit vaccine trial Onuma et al. observed homologous rather than heterologous protection between *T. orientalis* MPSP genotypes [190]. Furthermore, antigens such as the MPSP which are immunogenic are also under immune pressure, resulting in genetic drift. These issues may be overcome by using multiple antigens in the subunit vaccine formulation or targeting antigens which are not normally immunogenic. A greater understanding of how the bovine immune system responds to *T. orientalis* is required before further work on vaccine development can meaningfully proceed. Despite the hurdles in developing a vaccine for *T. orientalis*, it remains a worthy goal given the ongoing burden that this disease imposes on cattle production throughout Asia and Australasia.

9. Conclusion

T. orientalis is an apicomplexan parasite of economic significance around the world to both beef and dairy industries. This review has highlighted several knowledge gaps surrounding oriental theileriosis from taxonomic uncertainties, vector preferences and treatment and control measures. Development of effective therapeutics or prophylactic measures such as vaccines remains a priority due to recent spread of oriental theileriosis into new areas across the Asia Pacific region. Advancements in whole genome sequencing technologies promise to provide new insights into the *T. orientalis* taxonomy, genetic diversity and the underlying mechanisms of pathogenesis, all of which underpin successful development and implementation of efficient control strategies against this emerging parasite.

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Chapter 3

Handling the Microbial Complexity Associated to Ticks

Alejandro Cabezas-Cruz, Thomas Pollet, Agustín Estrada-Peña, Eleonore Allain, Sarah I. Bonnet and Sara Moutailler

Abstract

Ticks and the pathogens they transmit constitute a growing burden for human and animal health worldwide. In the last years, high-throughput detection and sequencing technologies (HTT) have revealed that individual ticks carry a high diversity of microorganisms, including pathogenic and non-pathogenic bacteria. Despite several studies have contributed to the availability of a catalog of microorganisms associated to different tick species, major limitations and challenges remain ahead HTT studies to acquire further insights on the microbial complexity associated to ticks. Currently, using next generation sequencing (NGS), bacteria genera (or higher taxonomic levels) can be recorded; however, species identification remains problematic which in turn affects pathogen detection using NGS. Microfluidic PCR, a high-throughput detection technology, can detect up to 96 different pathogen species, and its combination with NGS might render interesting insights into pathogen-microbiota co-occurrence patterns. Microfluidic PCR, however, is also limited because detection of pathogen strains has not been implemented, and therefore, putative associations among bacterial genotypes are currently unknown. Combining NGS and microfluidic PCR data may prove challenging. Here, we review the impact of some HTT applied to tick microbiology research and propose network analysis as an integrative data analysis benchmark to unravel the structure and significance of microbial communities associated to ticks in different ecosystems.

Keywords: high-throughput technologies, network analysis, ticks, tick-borne pathogens, microbiota

1. Introduction

Ticks are hematophagous ectoparasites of vertebrates that derive nutrition through blood feeding and are efficient vectors of major pathogens. Feeding habits and the process of blood digestion in ticks greatly differ from that in hematophagous insects (e.g. mosquitoes) and may influence pathogen acquisition and transmission. In ticks, digestion is a slow intracellular process [1, 2]. Argasidae, or "soft ticks," feed quickly and several times during their lifetime (approximately 40–60 minutes per feeding in most species). In adult soft ticks, full digestion only proceeds once mating occurs. In contrast to soft ticks, Ixodidae, or "hard ticks," feed for longer periods of time. Adult virgin females of Ixodidae Metastriate ticks attach to the host and take only a small quantity of blood before mating [3]. Mating

induces females to fast feeding, increasing their weight approximately 100 times within few days [3]. Thus, feeding times in female hard ticks can last from few days to weeks depending on the stage and the availability of males. After hatching from the eggs, the three following developmental stages (i.e. larvae, nymphs and adults) of Prostriate *Ixodes* ticks feed on different hosts. Potentially, while feeding on a host, each of these stages can transmit and acquire new pathogens [4]. Once acquired, most, if not all, tick-borne pathogens (TBPs) are transmitted transstadially (i.e. the ability of a microorganism to pass from one to the next developmental stage of the vector), and thus, ticks are 'hubs' in pathogen's circulation cycles [5]. In consequence, a considerable proportion of ticks are found to be coinfected in field surveys [6–9]. The above characteristics, among others, enable ticks to transmit a great variety of pathogens, including bacteria, viruses, protozoa and helminths, which constitute a growing burden for human and animal health worldwide [4, 10]. Among arthropod vectors, ticks transmit the most diverse array of disease agents [11].

Despite tick biology favors the acquisition and transmission of a great diversity of pathogens, most studies on TBPs prevalence in ticks focused in single infections. This was probably influenced by technical limitations to detect multiple pathogens and, possibly, by the fact that initial discoveries on the role of ticks as vectors linked "one-pathogen" to "one-tick-species." After the first demonstration of pathogen transmission by ticks, when Smith and Kilbourne [12] demonstrated that *Rhipicephalus annulatus* transmit *Babesia bigemina*, several studies established the role of ticks as vectors of several pathogens including *Borrelia duttonii* transmitted by *Ornithodoros mouhata* [13]; *Rickettsia rickettsii* transmitted by *Dermacentor andersoni* [14]; *Rickettsia conorii* transmitted by *R. sanguineus* [15]; and later, in the 1980s, *B. burgdorferi* s.l. responsible for Lyme borreliosis and transmitted by *Ixodes* spp. [16, 17]. These initial discoveries may have influenced the conception of a "single-pathogen" epidemiology. Thus, until recently, our experimental and theoretical models of pathogen transmission by ticks were limited because they frequently included single pathogen species [5]. Discoveries made using novel technologies [18], however, changed our current understanding of TBPs epidemiology: from the "single-pathogen" view, we are now at the bridge of unraveling the impact of "multiple-pathogen" in TBPs epidemiology. Coinfections, when multiple pathogen species coexist within an individual, are very common in ticks [9, 19, 20] and influence pathogen acquisition [21], transmission [19] as well as host infection risk [22]. When pathogens share a reservoir, they can interact directly via pathogen-pathogen interactions [23] and indirectly via host immune-mediation or they can also compete for host resources [24]. Within-host interactions are so strong that the dynamics of one pathogen, within a host and within a host population, cannot be understood without knowledge of other co-occurring pathogens [22, 25].

Pathogen coinfection in ticks can be studied by standard PCR using primers that detect known pathogens suspected to occur in a given tick species of a particular geographic region. This approach is the most frequently used; however, it is strongly biased and makes pathogen detection to be strongly influenced by particular research interests [5]. This may be the reason why one of the most studied coinfection is that between two of the most prominent TBPs, *Anaplasma phagocytophilum*, an intracellular bacterium that causes human granulocytic anaplasmosis (HGA), and *B. burgdorferi* s.l., an extracellular bacterium that produces Lyme borreliosis [6, 8, 21, 26, 27]. The approaches based on high-throughput technologies provided novel combinations of pathogen coinfections in ticks [9] with potential impact on vector competence. For example, Moutailler and colleagues [9] found 31 different pathogen confections in *Ixodes ricinus* ticks (see below and

Table A1). The most important realization of the recent research, however, is that most of the tick-associated microorganisms are not pathogens. Likely mirroring the revolution in microbiota research in model organisms [28–30], less than 10 years ago, tick researchers started applying next-generation sequencing (NGS) to explore the composition of tick microbiota [31]. The results showed a higher diversity of bacteria genera associated to ticks [32] compared to model organisms like *Drosophila melanogaster* [28]. This was surprising because while ticks have a restricted diet, *Drosophila* feed on a variety of decaying matter which could be the source of a complex microbiota. Possibly, allowing a high bacterial diversity is part of the evolutionary strategy of ticks to cope with their complex life cycle and metabolic deficiencies.

A major challenge of high-throughput data is data analysis, and therefore, integrative analytical tools are needed to improve our current understanding of tick-pathogen-microbiota interactions. Network analysis, a branch of graph theory, is a mathematical tool for the analysis of complex systems composed of many components which may interact with each other. Network analysis has been used to unravel complex microbial communities such as those present in soil [33], water [34] and human [35, 36] and tick microbiota [37]. This chapter focuses on the impact of high-throughput technologies in the current understanding of the microbial complexity associated to ticks. In addition, we propose to combine high-throughput data with network analysis to gain new insights into the structure of microbial communities associated to ticks and their impact on pathogen circulation. Throughout this review, we will use the term "microbiota" as "the microbial taxa associated with a given host" and "microbiome" as "the catalog of these microbes and their genes." A distinction can be established between these terms, while the microbiome includes information about the microbiota composition, the latest term does not necessarily includes information about gene composition.

2. New technologies and the microbial universe of ticks

2.1 Microfluidic PCR

2.1.1 General background on the technology

Frequently, studies on TBPs prevalence in ticks focused mainly on bacteria and parasites and only few species or genera are targeted in each study. Detection assays (e.g. PCR, nested PCR or real-time PCR) are designed to detect a restricted number of pathogens that are known or suspected to be transmitted by particular tick species collected at a particular location. In addition to the "*research interest*" bias, using standard PCR methods, only few microliters of total DNA are available per sample, which limits the number of pathogens that can be tested in each sample and confirmation by sequencing becomes difficult. Ideally, to better understand the epidemiology of TBPs, researchers should be able to detect in each sample (i.e. individual ticks or tick pools) most of the pathogens that ticks could potentially transmit, regardless of the tick species or the location. For this purpose, Michelet and collaborators [18] have developed a new high-throughput tool to detect a high number of TBPs in a high number of samples by real-time PCR in a single experiment [18]. Briefly, they developed a chip (BioMark™ dynamic arrays, Fluidigm Corporation) targeting TBPs (bacteria and parasites) of worldwide distribution. The designed epidemiologic arrays may detect simultaneously 48 pathogens in 48 samples (or potentially 96 pathogens in 96 samples) corresponding to 2304 real-time microfluidic PCRs (or potentially 9216 real-time microfluidic PCRs). Specific

primers and TaqMan probes were designed for each pathogen, and their specificity was tested *in silico* using Blast.

A brief workflow of the microfluidic PCR is provided **Figure 1**. Firstly, ticks are homogenized in cell culture medium (i.e. D-MEM) completed with 10% of fetal calf serum to preserve viral particles and separated into three aliquots: one dedicated to total DNA extraction, one to total RNA extraction and one conserved at −80°C for back-up. Secondly, RNAs are reverse transcribed into cDNA using random primers (only 1 µL of RNA is used per reaction), and then cDNA and DNA are preamplified with a pool of primers/probe targeting TBPs to increase the signal of TBPs relative to the signal of tick RNA/DNAs. Remarkably, only 1.25 µL per sample are needed to test all the pathogens simultaneously. Two different chips were run in the BioMark™ dynamic array system: one to detect RNA viruses using the preamplified cDNAs and the other to detect DNAs from bacteria/parasites using the preamplified DNAs. In the chip, samples and primers/probes are added into the right and left wells, respectively. Pressure and oil allow the distribution of each sample and primers/probe sets into the microfluidic PCR chambers in the middle of the chip. Each sample will be mixed with all the primers/probes sets and each primers/probe set will be mixed with all samples, allowing 2304

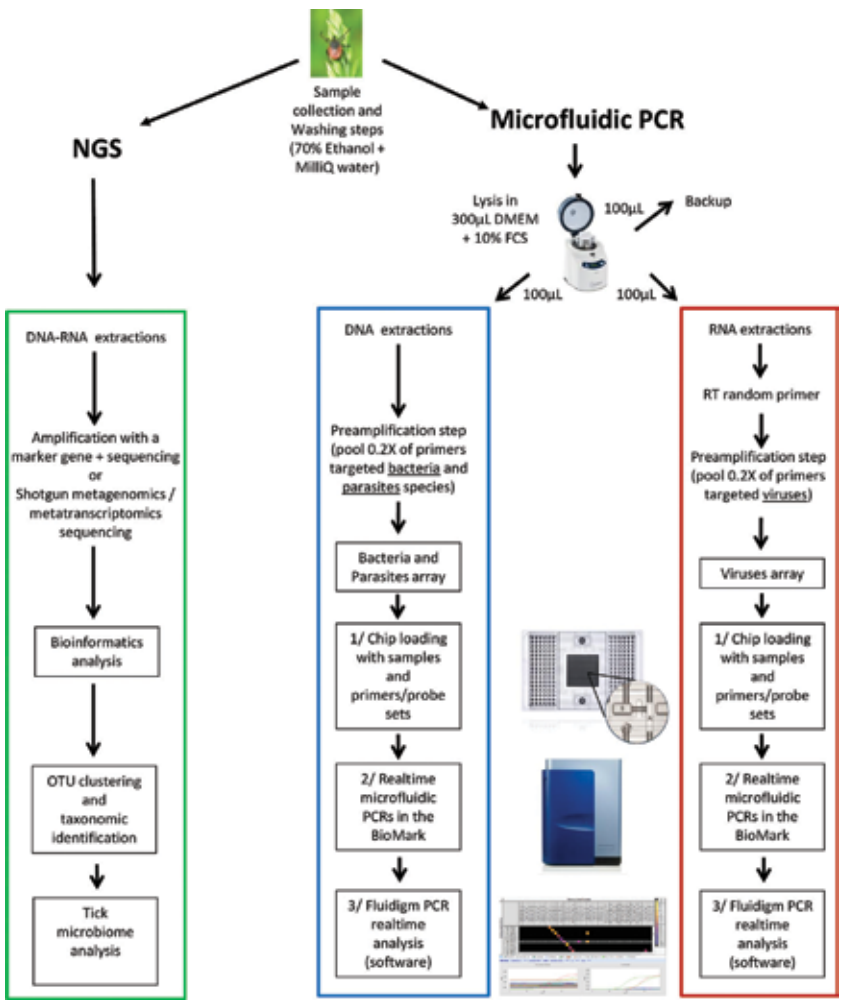

Figure 1.
General workflows of high-throughput screening of ticks using the real-time microfluidic PCR system and NGS.

individual real-time PCRs at a final volume of six nanoliters per reaction. For further details, we refer the reader to [18].

2.1.2 Tick-borne pathogen coinfections revealed by microfluidic PCR

The first application of microfluidic PCR targeted 37 pathogens including *Francisella tularensis*, *Coxiella burnetii*, *Candidatus* Neoehrlichia mikurensis, five species of *Anaplasma*, three species of *Ehrlichia*, eight species of *Borrelia* (seven from the Lyme borreliosis group and one, *B. miyamotoi*, from the relapsing fever group), two species of *Bartonella*, four species of *Rickettsia*, ten species of *Babesia* and two species of *Theileria* [18]. To confirm the morphological characterization of the tick species analyzed and to control the quality of DNA extraction, primers specific to five species of ticks, including three species of *Ixodes* and two species of *Dermacentor*, were tested. Sensitivity of primers and probes was tested on a dilution range of reference DNAs of the targeted pathogens on a Lightcycler 480 real-time PCR system. Then, the specificity was tested on the BioMark™ dynamic array system. The resulting chip was further evaluated on field samples corresponding to 47 pools of 25 *I. ricinus* nymphs each collected in two sites per country in France, The Netherlands and Denmark, 7050 samples in total. Several pathogens were successfully detected, and the prevalence of *A. phagocytophilum*, *Ca*. N. mikurensis, *Rickettsia helvetica*, *Bartonella henselae*, five different genospecies of *B. burgdorferi* s. l., *B. miyamotoi*, *B. divergens* and *B. venatorum* was determined [18]. Positive samples were validated by PCR amplification and sequencing of selected gene fragments [18]. Notably, this study revealed for the first time the presence of five pathogens previously unreported in Denmark. This work highlighted the potential of unbiased pathogen detection. A similar tool targeting 22 tick-borne viruses (TBVs) has also been developed and evaluated on European ticks (unpublished data). These fast and low-cost tools allow comprehensive testing of TBPs and can be customized to fit regional demands or to accommodate new or emerging pathogens. Indeed, specific sets of primers/probe are continuously designed by our team. These tools represent a major improvement for surveillance and future epidemiological studies.

This new high-throughput technology has been used mainly during epidemiological studies of TBPs in specific countries with different tick species screened as *I. ricinus* in Ireland [38] and Denmark [39], *Ornithodoros* spp. in France [40], *Rhipicephalus microplus* in Galápagos Islands [41] and TBVs in *Hyalomma* spp. ticks collected on migratory birds in Sweden [42]. Remarkably, this allowed the detection of expected pathogens (i.e. *Borrelia* species in *I. ricinus*), rare (i.e. *Bartonella* species in *I. ricinus* and *Borrelia* from the relapsing fever group in *Ornithodoros* spp.), or unexpected pathogens (i.e. Alkurma virus in *Hyalomma* spp.) in different regions.

Moreover, these high-throughput screenings of TBPs in individual ticks have highlighted the co-occurrence of several pathogens in one tick, known as tick coinfections. Before the use of this novel technique, tick coinfections were evaluated by classical PCR, nested PCR or real-time PCR, and related publications focused in few pathogens, less than 10 different genera screened per publication [43–59]. After the year 2016, two publications have demonstrated the presence of up to five and four different pathogen species in *I. ricinus* female ticks collected in France and Romania, respectively, using this high-throughput system [9, 20]. The advantages of microfluidic PCR over classical PCR detection methods (i.e. qualitative PCR, nested PCR, or real-time PCR) can be summarized: (i) small amount of sample is needed for detection of tens of microorganisms, (ii) convenient and easy to implement when thousands of samples are to be tested and (iii) price per sample run is lower. Tick coinfections among bacteria, parasites and/or viruses described in the literature in the last 4 years are listed in **Table A1**. Not surprisingly, the most

commonly found coinfections are those between *Borrelia* spp. and *A. phagocytophilum* or *Rickettsia* spp. as well as between different species of *Borrelia* included in the Lyme borreliosis group. Nevertheless, this result could reflect the reality or could be a bias resulting from the high quantity of research projects focusing on the above bacteria.

2.1.3 Challenges and perspectives

Unfortunately, only few publications are available regarding coinfection by bacteria and parasites or bacteria and viruses or parasites and viruses in ticks [49, 50, 52, 54, 60]. To solve this gap of information regarding inter-taxa coinfections, a system to detect simultaneously bacteria, parasites and viruses will be, without any doubt, an improvement of available tools. Nevertheless, even if this high-throughput system allows a rapid detection of numerous pathogens present in a high number of samples, confirmation of doubtful results or presence of unexpected pathogens should be confirmed by classical or nested PCR. Knowing the fact that for each pathogen different genotypes/strains could exist, this confirmation step could allow us to sequence different genes per pathogen leading to a better characterization of the epidemiological history of TBPs present in the targeted region/ecosystem.

High-throughput identification of pathogen strains would be also a significant improvement to current microfluidic PCR protocols. Genetic diversity of bacteria species resulting in novel strains can be associated to changes in pathogenicity, virulence and host specificity. A classic example of this is that different strains of the bacterium *Escherichia coli* can provide health benefits or produce deadly diseases. In particular, *E. coli* strain Nissle 1917 is used as a probiotic [61] and *E. coli* strain O157:H7 has been responsible for a number of deadly food-borne pathogen outbreaks [62]. It has been reported that multiple strains of *A. phagocytophilum* circulate in Europe, with minimal overlap in their reservoir associations [63]. One of these strains is a generalist infecting a wide range of mammalian species, including livestock and other domestic animals [64–66]. A second strain appears to specialize almost exclusively on roe deer [63]. Both of these strains are transmitted by *I. ricinus* and both could affect humans. A third strain has a host range restricted to rodents and is circulated by *I. trianguliceps* [64]. Targeting different *A. phagocytophilum* strains in a high-throughput system may allow studying not only tick vector specificity of this bacterium but also coinfections among and between strains of *A. phagocytophilum* and other pathogens. Thus, systematic detection of pathogen strains using high-throughput approaches would provide a more comprehensive view of TBPs diversity and may inform on host specificity and the emergence of novel TBPs. By including primers/probe sets targeting pathogen strain-specific markers, current microfluidic PCR protocols can be updated for strains detection and identification.

An additional challenge to high-throughput detection is how to detect novel strains or species. The emergence of novel pathogens is a dynamic process. For example, a novel species of *Ehrlichia*, *E. minasensis* [67], evolved from variable strains of the pathogen *E. canis* [68], and it was associated to new invertebrate and vertebrate hosts. While the common tick vector for *E. canis* is *R. sanguineus* s.l. [69], *E. minasensis* was isolated from *R. microplus* hemolymph [70], and while *E. canis* is mainly pathogenic for dogs [71], *E. minasensis* was found to be pathogenic for cattle [67, 72]. An alternative for the detection of novel pathogen strains or novel pathogens closely related to recognized pathogen species is the amplification and sequencing of genetic markers followed by phylogenetic analysis to assess strain diversity in samples positive to given pathogens. Emergence of novel strains is frequently associated with

genetic variability in surface proteins which can be used as genetic markers to assess strain diversity [68, 71].

Finally, high-throughput quantification of TBPs in tick organs could be a useful approach to assess some components of tick vector competence, for example, vector colonization by pathogens. It is known that the simple detection of pathogen DNA in a tick does not demonstrate the vector competence of this tick species for this pathogen. Vector competence depends effectively on genetic factors determining the ability of a vector to transmit a pathogen and has to be demonstrated under controlled conditions [10]. A typical TBP colonizes tick midgut and migrates to salivary glands to be transmitted with tick saliva to the host. The detection and quantification of the pathogen in different organs including midgut and salivary glands could be a step forward from pathogen detection to tick vector competence assessment. As an example, Berggoetz et al. [73] detected different pathogens (i.e. *Babesia*,*Theileria*, *Anaplasma* and *Ehrlichia*) with variable prevalence in the salivary glands of four tick species (*Rhipicephalus evertsi evertsi*, *Rhipicephalus decoloratus*, *Amblyomma hebraeum* and *Hyalomma rufipes*) collected in ruminants. In addition to describe new vector-pathogen combinations, this approach using tick organs allowed to detect *Theileria bicornis*,*Theileria* sp. (giraffe), *Theileria* sp. (Kudu) and *Babesia* sp. (sable) for the first time in ticks and more precisely in salivary glands suggesting vector competence of the studied tick species. As another example, Budachetri et al. [74] detected *Rickettsia parkeri*, known to cause human rickettsiosis, in the midgut, salivary glands and the saliva of questing ticks *Amblyomma maculatum*. Detection and quantification of TBPs in tick organs can provide new insights into the distribution of pathogens within ticks in different ecological settings. High pathogen levels relative to negative controls and in salivary glands relative to midgut may inform on pathogen replication in tick tissues and thus vector colonization by pathogens. The BioMark™ dynamic array system offers the possibility to achieve this by using a specific chip dedicated to digital PCR. This technology has been used to quantify viruses in food and/or in different organs of mice, and it can be adapted to TBPs detection and quantification in different tick organs [9, 75].

2.2 Next-generation sequencing

2.2.1 General background on the technology

During the past decade, NGS technologies have provided new insights into microbial community dynamics and ecology. These tools allow high-throughput analysis of complex and diverse microbial communities in multiple ecosystems such as soils and aquatic systems or in the microbiota of host organisms such as plant, animals and humans. With the development of these new sequencing approaches, it has definitively become faster and more economical to comprehensively evaluate the complexity of microbial species and strains in various ecosystems. Three main sequencing strategies are commonly used to study microbial communities: (i) marker gene approaches (i.e. SSU rRNA genes) with amplicon sequencing to identify microbiota composition (the 16S rRNA gene being the most used), (ii) shotgun metagenomics to characterize the functional potential of the microbiome and (iii) shotgun metatranscriptomics to determine actively expressed genes [76]. For further details on these different sequencing approaches, the reader is referred to [77, 78].

2.2.2 Tick microbial communities revealed by NGS

While ticks are known to be one of the main vectors of various pathogenic agents [4, 9, 10, 20, 73, 79, 80], it is now recognized that TBPs in ticks coexist with

microorganisms considered non-pathogenic for humans. Studies using NGS have shown that specific TBPs are frequently found together with other pathogens, symbionts and commensals [81]. This tick microbial complex, recently named "pathobiome" [82, 83], is influenced by the environment, and the interactions between its different components might influence pathogen acquisition by ticks and transmission to the host. In this context, the identification and characterization of tick microbiota has become essential to understand tick-pathogen interactions [84, 85]. While at the beginning of the twenty-first century, some studies started to characterize microbial communities associated to ticks using fingerprinting approaches (e.g. [86, 87]), the development of NGS technologies allowed higher resolution in the identification of tick microbiota bacteria and revealed an

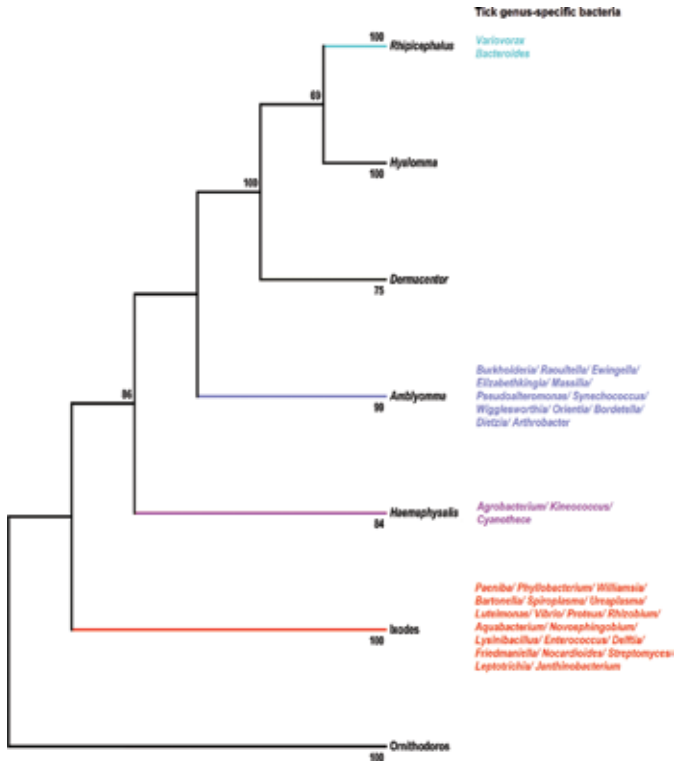

Figure 2.
Bacteria genera found across tick genera. The figure is a cladogram displaying the phylogenetic relation among major tick genera. Information on bacteria genera specific to each tick genus was collected from published data available in Table A2. The cladogram is based on a maximum parsimony phylogenetic tree of subolesin nucleotide sequences that were aligned using MAFFT followed by codon alignment. The final alignment contained 576 total sites of which 329 were gap-free. Bootstrap values (500 replicates) are shown next to the branches. Branches were collapsed at the genus level. Sequences were collected from GenBank and transcriptome projects, and accession numbers are as follow: Ixodes scapularis (AY652654), I. persulcatus (KM888876), I. ricinus (JX193817), I. ariadnae (KM455971), I. hexagonus (JX193818), Rhipicephalus evertsi (JX193846), R. appendiculatus (DQ159967), R. microplus (EU301808), R. sanguineus (JX193845), R. haemaphysaloides (KP677498), R. annulatus (JX193844), R. decoloratus (JX193843), R. zambeziensis (GFPF01005851), R. bursa (GFZJ01017781), R. pulchellus (GACK01006228), Dermacentor silvarum (JX856138), D. sinicus (KM115649), D. marginatus (KU973622), D. variabilis (AY652657), D. reticulatus (JX193847), Amblyomma variegatum (JX193824), A. hebraeum (EU262598), A. cajennense (JX193823), A. americanum (JX193819), A. maculatum (JX193825), A. aureolatum (GFAC01005925), A. triste (GBBM01002796), A. sculptum (GFAA01000261), Hyalomma anatolicum (KT981976), H. rufipes (JX193849, H. marginatum (DQ159971), H. excavatum (GEFH01000904), Haemaphysalis longicornis (EU289292), Hae. elliptica (JX193850), Hae. qinghaiensis (EU326281), Hae. flava (KJ829652), Hae. punctata (DQ159972), Ornithodoros moubata (JX193852), O. savignyi (JX193851), O. turicata (GDIE01114362), O. erraticus (HM622148), and O. rostratus (GCJJ01005500).

unexpected microbial diversity in these arthropods [88–90]. The general workflow commonly used to study tick microbiota using NGS is presented in **Figure 1**.

Since the first study using NGS to describe the bacterial diversity in the cattle tick *R. microplus* [91], different NGS technologies have been applied to identify the microbiota of various tick species. In consequence, the microbiota of several tick species of the genera *Ixodes*, *Dermacentor*, *Haemaphysalis*, *Rhipicephalus* and *Amblyomma* has been studied, and its composition in different locations was reported. A review of studies using these tools and describing tick microbial community composition at the genera level is presented in **Table A2**. Focusing on metagenomics approaches, both Illumina MiSeq and 454 pyrosequencing represented the most used sequencing techniques, even though the Illumina chemistry is now the most used due to the higher number of sequences generated by this approach. Most of our knowledge about tick microbial diversity and composition comes from sequencing the 16S rRNA gene based on DNA extracts (**Table A2**). Interestingly, the diversity of genus-specific microorganisms detected in ticks varies among the main tick genera (**Figure 2**). While a large number of bacterial genera are exclusively associated with *Ixodes*, not a single bacteria genus was found yet to be exclusively associated to *Dermacentor* (**Figure 2**). Whether this is related with the fact that more studies are available on *Ixodes* spp. (i.e. [17]) than on *Dermacentor* (i.e. [8]), microbiota is unknown; however, this finding warrants further research. Not only *Ixodes* has the highest number of genus-specific microorganisms (**Figure 2**), but it can also accommodate most of the bacteria found in other tick genera (**Figure 3**). Despite clear differences in the microbial communities of different tick genera (**Figures 2** and **3**), several bacteria genera were shared by all tick genera including *Rickettsia*, *Pseudomonas*, *Acinetobacter*, *Coxiella and Flavobacterium*. These findings should be approached under the hypothesis that these bacteria have a deep influence on the physiological processes of the tick or they would be not tightly associated to such diverging tick genera [81].

2.2.3 Challenges and perspectives

NGS methods have improved increasing in sequencing depth (i.e. a higher number of sequences obtained per sample) and thus a better estimation of the microbial diversity. However, the read length of the most widely used sequencing

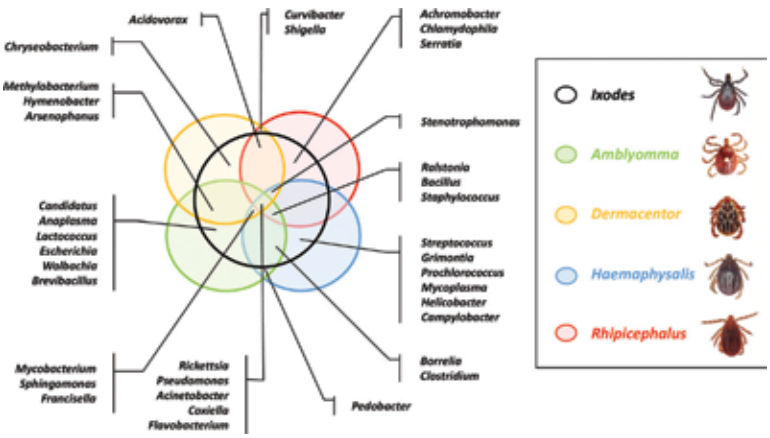

Figure 3.
Bacteria genera shared by major tick genera. Information on bacteria genera shared by more than one tick genera was collected from published data available in Table A2. For figure display reasons, the bacteria genera shared by Ixodes, Rhipicephalus and Amblyomma are not shown. These three tick genera share bacteria of the genera Corynebacterium and Propionibacterium.

platforms today is very short (few hundreds base pairs) and requires the researchers to choose a region of the 16S rRNA gene to sequence. For NGS purposes, the 16S rRNA gene is divided into nine regions (i.e. V1–V9). Most of the previous studies that used the 454 pyrosequencing approach amplified the V1–V3 region (**Table A2**). Studies that used the MiSeq approach mainly amplify the second part of the 16S rRNA gene with the V3–V4/V3–V5 or V5–V6 regions (**Table A2**). In this context, many bacteria genera may share the same amplified region, and the taxonomic resolution of profiling is inherently limited with incomplete information on tick microbial composition at the species level. There is a need for a simple 16S rRNA gene-based profiling approach that avoid the short read length to provide a much larger coverage of the gene to obtain higher taxonomic resolution in tick microbiota identification. The limitation of 16S rRNA gene sequencing (DNA-based) for microbial community analyses is the inability to differentiate between active and non-active cells. In comparison, 16S rRNA sequencing (RNA-based) can target metabolically active cells which produce rRNA. It is thus essential to include RNA and metatranscriptomic approaches to characterize the tick microbiota [92–94]. In addition, limitations linked to the 16S rRNA gene sequencing include polymerase chain reaction (PCR) bias, resulting, as previously mentioned, in low taxonomic resolution (typically genus-level) and limited functional insight into the microorganisms. These limitations hamper our ability to investigate how the non-pathogenic members of the tick microbiota interact with the pathogens and influence their presence and transmission. One way to avoid these biases is to use whole genome sequencing (WGS) to sequence thousands of genes from hundreds of microorganisms in a given sample. By gaining access and annotating the whole genome, it would become possible to reconstruct the putative metabolism of individual microbial species and gain insight into their potential role in tick-borne pathogens and diseases.

Using NGS techniques, many studies described tick microbial community composition and diversity and reported lists of microorganisms associated to several tick species. However, as underlined by Shade [95], diversity and composition without context provide limited insights into the mechanisms underpinning community patterns. Measurement of microbial diversity should be the starting point for further inquiry of ecological mechanisms rather than the "answer" to community outcomes [95]. Studying microbial communities associated to ticks needs thus contextual data, and it appears crucial to know the dynamics in space and time of these communities and the influence of environmental factors on their dynamics. In addition to factors associated with tick biology, the composition of tick microbial communities can be highly variable due to environmental factors such as biogeography, temperature, light-dark cycles, hygrometry, and vegetation [87–89, 96, 97]. Future studies on tick microbiota will have to consider these different variables and define more deeply their role in the dynamics of microbial communities associated to ticks. Biotic interactions are also important drivers of diversity, and the nature and strength of interactions can result in complex multimember interactions. Considering the pathobiome concept, one additional challenge for the understanding and control of tick-borne diseases is to increase the measurements of microbial diversity and calls for identifying potential associations/interactions between pathogens and other tick microbes. Finally, after identifying the tick microbiota including symbionts, it becomes crucial to determine the relationships between ticks and these bacteria. Ticks are strict hematophagous arthropods, and this specific diet is limited in B vitamins. Duron et al. [98] have recently demonstrated that the exploit of this unbalanced diet is possible because an intracellular bacterial symbiont of the genus *Francisella* supplies missing nutrients and that this nutritional symbiont is essential for tick development and survival to adulthood.

Similar studies have to be carried out in the future to better understand the complex roles of these symbionts in tick ecology.

3. Network analysis

3.1 General background on network analysis methodology

Networks are formed by components, known as nodes, and the relationships between these components are named links (**Figure 4**). The network may be undirected (there is not directionality in the link) or directed (there is directionality in the link). In microbial networks, each node represents a species and each link, representing co-occurring bacteria, resulting in undirected networks. Directed networks would be those resulting from, for example, parasites "on" vectors or microbes "in" a reservoir. The complete set of records can be then weighted according to the number of times one node is linked to another node (**Figure 4**). Several indices can be used to measure network properties from which the relationships among the co-occurring bacteria are derived. The degree centrality (DC, i.e. number of links connecting a given node to other nodes) is the most basic measure of a network and is calculated after weighting the total number of records containing this interaction. The DC provides an estimation of the strength of the association but does not evaluate the importance of each node in the context of the network. The node betweenness centrality (NBC) indicates how often a node is found on the shortest path between two nodes in the network [99, 100]. The implicit meaning of the NBC in microbial networks is the importance of a node in the flow of other components of the network and is considered a basic index defining the relative importance of a node in an ecological network. The PageRank

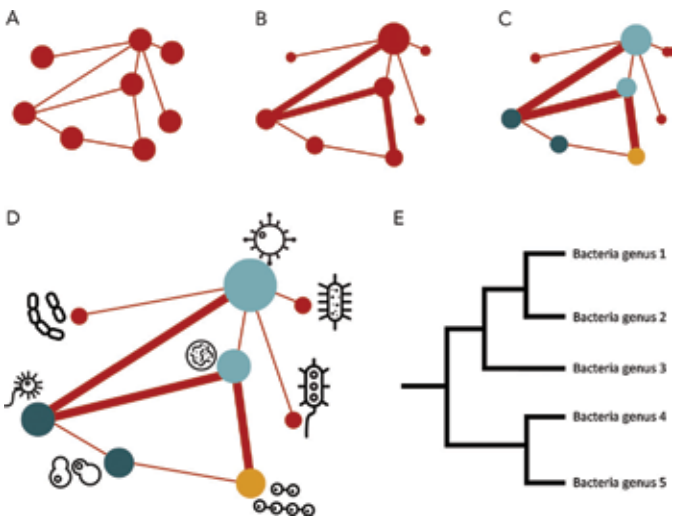

Figure 4.
A schematic explanation of the construction of networks for co-occurring bacteria in the microbiome. (A) A network is composed of nodes (circles) and links (lines). Each pair of bacteria that co-occur is connected by a link. The absence of a link means that a given pair of bacteria was not found to co-occur in any carrier. (B) The relative importance of each bacterial taxa and the importance of the links between co-occurring bacteria can be measured with indexes of centrality. In the schematic representation, larger circles mean higher centrality and wider links mean frequently detected co-occurring bacteria. Then, clustering algorithms (C) can detect communities of co-occurring bacteria (randomly colored in the figure). Once the complete network is built (D), results can be translated to a phylogenetic tree of the detected taxa to obtain important indexes of phylogenetic diversity and tracking the phylogenetic signal of the quantitative traits of the network (E).

(PR) is an index of centrality that assigns a universal rank to nodes based on the importance of the other nodes to which it is linked. Therefore, the NBC and PR are complementary measures for capturing the importance of each node in the linkage of other nodes throughout the network. These three indexes capture the ecological relationships between the interacting partners.

Real-world networks have been shown to separate into logical clusters in which nodes are tightly connected to each other but only loosely connected to nodes outside of their module [101]. They thus represent sets of organisms that interact more among them than with the others. This modularity separates the complete network into compartments that can be observed as naturally segregated niches in which a subset of taxa has a statistically higher affinity among them than with other species in the network.

3.2 Network analysis to disentangle the microbial complexity associated with ticks

The important value of the tick microbiota is the ecological interpretation of the associations or co-occurrence rates of the microorganisms detected in a collection of ticks. Whether these ticks were collected in different ecosystems, or associated to different hosts, or surveyed at different time intervals, the most important purpose is capturing the ecological meaning of these associations among the detected bacteria. Therefore, it is necessary to determine the relationships among the microorganisms, identify 'dominant' taxa in the microbiota and to study how they interact.

It is logical to assume that microorganisms that co-occur in the network are those that "overlap in the habitat" provided by the carrier of a given microbiota. This high co-occurrence likely ensures cohesiveness and persistence of the network improving the circulation of the microorganisms. Most important, a phylogenetic tree of the detected bacteria can be built, and the indexes of centrality can be tracked over the branches of the resulting tree (**Figure 4**). This is commonly known as "tracking the phylogenetic signal of quantitative traits" [102]. A common empirical observation for organisms is that continuous traits (i.e. morphological features, or the occupancy of ranges of the variables shaping its environmental niche) of closely related species in a phylogeny are often similar, meaning that these traits are under selection pressure. The link between phylogeny and continuous trait values is commonly referred in the literature as the phylogenetic signal. Therefore, it is possible to test the phylogenetic signal of the network indexes, which are actually quantitative traits, over the branches of the tree. Several indexes and dedicated computer packages are available to measure the phylogenetic signal [102]. Tracking these indexes on the phylogenetic tree explains the relative importance of the taxa of the microbiota and how it is organized in a population of ticks. The phylogenetic distance of the microorganisms detected in ticks can be calculated. This could be used to evaluate the phylogenetic diversity carried by ticks according to the habitat, the season of the year or the environmental conditions driving the tick phenology and survival. It is necessary to stress that an index of phylogenetic distance, together with the centrality indexes of the realized network, provides ecological or possibly physiological information of the microbiota composition. This cannot be achieved by listing bacterial taxa.

Most of the guidelines expressed above have been addressed in a recent study on the microbiota of *Ixodes ricinus* ticks and one of its main hosts, the vole *Myodes glareolus* [37]. In this study, NGS was combined with network analysis to measure the impact of the ecosystem in the composition of tick and vole microbiota. One of the main conclusions of the study is that the similarity of the microbiota between ticks and hosts is low, with a clear impact on the type of ecosystem in which ticks

were collected on the resulting microbiota. These findings could be a consequence of the different range of hosts available for the tick in two different ecosystems. Regardless of the causes of these findings, the study demonstrated that the tick microbiota seems to be optimized for the co-occurrence of bacteria with low phylogenetic similarities. This could be interpreted in two ways: (i) the high phylogenetic diversity of bacteria in ticks evolved to decrease the competition for the 'tick niche' of closely related taxa, since it is expected that largely divergent taxa would have very different requirements in the tick and (ii) the microbiome is organized to provide the tick with a large number of bacterial metabolic routes that benefit the physiological processes of the tick; therefore, a high diversity of taxa in a tick would ensure a high diversity of these 'physiological complementarity' supporting the physiology of the tick in many different ways. The lack of empirical data in this field warrants further research, either from field studies or from laboratory controlled studies.

The current impossibility to obtain germ-free ticks is a gap in this field of study. Colonization of ticks with single species of bacteria could help to understand the contribution of individual bacteria to tick physiology. However, accumulating evidence demonstrated that most of these bacteria are fundamental for tick physiological processes and survival in the environment. Therefore, the information about the ecological and physiological relationships between the tick and the microbiome must be obtained from field surveys and subjected to big data analysis as proposed before. We firmly believe that the next step forward in the field of tick microbiome must be a change of paradigm from 'taxonomical listing' to the functional characterization of tick microbiome in the environment. Classic statistics can be of little help in such task.

4. Conclusions

High-throughput technologies have improved our current understanding of the microbial complexity associated to ticks. These technologies allowed us to move from the "one-tick-one-pathogen" paradigm to the "one-tick-many-microorganisms" paradigm. This new concept can be summarized: ticks are associated with complex microbial communities, including pathogenic and non-pathogenic microorganisms, which interact between them and with the vector and are together under the influence of the environment. Future developments may be related with the characterization of tick microbiome at the species level and with inclusion of strain diversity analysis in high-throughput pathogen detection. Finally, high-throughput data analysis could benefit from tools assessing the relevance and contribution of individual nodes of the microbial network. Network analysis can be used to calculate co-occurrence patterns and centrality indexes that may assist in the identification of highly important members of tick microbiota.

Acknowledgements

The authors thank the members of their laboratories for fruitful discussions.

Conflict of interest

The authors declare that they have no competing interests.

A. Appendix

Tick species	Tick stage	Microorganism detected			% of co-infection	Technique/s of detection and targeted genes	Feeding status of ticks, Engorged (E) and non-engorged (NE)	Country	Reference
		Bacteria	Parasites	Viruses					
Ixodes ricinus	Nymphs/ adults	*Borrelia burgdorferi s.l.* + *Rickettsia spp.* (*R. helvetica* mainly)	NT	NT	7.3	Realtime PCR (5S and 23S rRNA genes of Intergenic Spacer region)	NE	Germany	[44]
		B. burgdorferi s.l. + *Anaplasma phagocytophilum*	NT	NT	0.3				
		B. burgdorferi s.l. + *Rickettsia spp.* (*R. helvetica mainly*) + *A. phagocytophilum*	NT	NT	0.1				
	Adults	—	*Babesia microti* + *Toxoplasma gondii*	NT	42	Nested PCR (conservative regions of the flagelline gene)	E	Poland	[45]
		—	*B. microti* + *T. gondii*	NT	32		NE		
	Adults	*B. burgdorferi s.l.* + *Rickettsia spp.*	NT	NT	12.7	Realtime PCR (5S and 23S rRNA genes of Intergenic Spacer region)	NE	Germany	[58]
	Nymphs	*B. burgdorferi s.l.* + *Rickettsia spp.*	NT	NT	12.7				
	Nymphs/ adults	*Different genospecies of B. burgdorferi s.l. (detail not provided)*	NT	NT	3.6				
	Nymphs	*B. afzelii* + *Ca. N. mikurensis*	NT	NT	3.3	Realtime PCR (16S rRNA and *hbb* gene)	NE	Norway	[59]
	Nymphs/ adults	*Different genospecies of B. burgdorferi s.l. (detail not provided)*	NT	NT	2.1	Realtime PCR (*fla* gene fragment)	NE	Poland	[46]
	Nymphs/ adults	*B. burgdorferi s.l.* + SFG *Rickettsia*	NT	NT	3.7	Realtime PCR (*opsA* and flagelin genes)	NE	The Netherlands	[57]
	Adults	*R. helvetica* + *A.phagocytophilum*	NT	NT	0.4	Realtime Microfluidic PCR (16S rRNA encoding *rrs* genes)	NE	France	[9]
		R. helvetica + *B. afzelii*	NT	NT	0.4				
		R. helvetica + *B. garinii*	NT	NT	0.4				
		R. helvetica + *B. valaisiana*	NT	NT	0.7				

Tick species	Tick stage	Microorganism detected			% of co-infection	Technique/s of detection and targeted genes	Feeding status of ticks, Engorged (E) and non-engorged (NE)	Country	Reference
		Bacteria	Parasites	Viruses					
		R. helvetica + *Bartonella henselae*	NT	NT	3.0				
		B. burgdorferi + *B. valaisiana*	NT	NT	0.4				
		B. garinii + *B. afzelii*	NT	NT	0.7				
		B. garinii + *B. burgdorferi*	NT	NT	0.4				
		B. garinii + *B. henselae*	NT	NT	0.4				
		B. miyamotoi + *B. henselae*	NT	NT	0.4				
		Candidatus Neoehrlichia mikurensis + *B. miyamotoi*	NT	NT	0.7				
		An. phagocytophilum+ *B. henselae*	NT	NT	0.4				
		B. spielmanii + *B. henselae*	NT	NT	0.4				
		R. helvetica + *B. afzelii* + *B. garinii*	NT	NT	0.7				
		R. helvetica + *B. valaisiana* + *B. burgdorferii*	NT	NT	0.4				
		R. helvetica + *An. phagocytophilum* + *B. afzelii*	NT	NT	0.4				
		B. garinii + *B. afzelii* + *B. spielmani*	NT	NT	0.4				
		B. garinii + *B. afzelii* + *B. valaisiana*	NT	NT	0.7				
		B. garinii + *B. burgdorferi* + *B. valaisiana*	NT	NT	0.7				
		B. garinii + *B. burgdorferi* + *B. henselae*	NT	NT	0.4				
		B. garinii + *B. afzelii* + *B. henselae*	NT	NT	1.1				
		B. garinii + *B. miyamotoi* + *B. henselae*	NT	NT	0.4				
		B. garinii + *R. helvetica* + *B. henselae*	NT	NT	0.4				
		B. garinii + *B. afzelii*	*B. divergens*	NT	0.4				
		B. garinii + *B. afzelii* + *Ca. N. mikurensis*	NT	NT	0.7				
		R. helvetica + *B. afzelii* + *B. garinii* + *A. phagocytophilum*	NT	NT	0.4				

Tick species	Tick stage	Microorganism detected			% of co-infection	Technique/s of detection and targeted genes	Feeding status of ticks, Engorged (E) and non-engorged (NE)	Country	Reference
		Bacteria	Parasites	Viruses					
		B. afzelii + *B. garinii* + *B. burgdorferi* + *B. henselae*	NT	NT	0.4				
		B. afzelii + *B. garinii* + *B. burgdorferi* + *R. helvetica*	NT	NT	0.4				
		B. afzelii + *B. garinii* + *B. burgdorferi* + *B. spielmanii*	NT	NT	0.7				
		R. helvetica + *B. afzelii* + *B. garinii* + *B. valaisiana* + *B. burgdorferi*	NT	NT	0.4				
		B. henselae + *B. afzelii* + *B. garinii* + *B. spielmanii* + *B. burgdorferi*	NT	NT	0.4				
	Nymphs/ adults	*Ca.* N. mikurensis + *A. phagocytophilum*	NT	NT	0.1	Realtime PCR	NE	Slovakia	[48]
	Nymphs/ adults	*B. miyamotoi* + *B. burgdorferi s.l.*	NT	NT	0.29	Realtime PCR (*glpQ* gene and 5S-23S rDNA IGS)	NE	Slovakia	[51]
		B. miyamotoi + *B. afzelii*	NT	NT	0.12				
	Larvae	*B. burgdorferi s.l.* + *R. helvetica*	NT	NT	4.5	Realtime PCR (*flaB* and *opsA* genes)	E	The Netherlands	[103]
		R. helvetica + *Ca.* N. mikurensis	NT	NT	0.7				
		A. phagocytophilum + *R. helvetica*	NT	NT	0.7				
	Nymphs	*B. burgdorferi s.l.* + *R. helvetica*	NT	NT	9.6				
		B.burgdorferi s.l. + *Ca.* N. mikurensis	NT	NT	3.5				
		B. burgdorferi s.l. + *A. phagocytophilum*	NT	NT	3.5				
		R. helvetica + *Ca.* N. mikurensis	NT	NT	1.9				
		A. phagocytophilum + *R. helvetica*	NT	NT	1.5				
		B. burgdorferi s.l. + *B. miyamotoi*	NT	NT	0.2				
		B. miyamotoi + *Ca.* N. mikurensis	NT	NT	0.2				
		B. burgdorferi + *R. helvetica*+ *Ca.* N. mikurensis	NT	NT	1.3				
		B. burgdorferi s.l. + *R. helvetica* + *A. phagocytophilum*	NT	NT	0.6				

Tick species	Tick stage	Microorganism detected			% of co-infection	Technique/s of detection and targeted genes	Feeding status of ticks, Engorged (E) and non-engorged (NE)	Country	Reference
		Bacteria	Parasites	Viruses					
	Nymphs/ adults	*B. garinii* + *B. afzelii*	NT	NT	4.3	Realtime Microfluidic PCR + PCR [*gltA (Bartonella-Rickettsia* spp.), 23S rRNA-*rpoB-fla-ospA-glpQ (Borrelia* spp.), *groEL (Candidatus* Neoehrlichia mikurensis)]	NE	Romania	[20]
	Nymphs/ adults	*B. garinii* + *B. lusitaniae*	NT	NT	3.0				
	Nymphs	*B. garinii* + *B. spielmanii*	NT	NT	0.7				
	Nymphs	*B. afzelii* + *B. bissettii*	NT	NT	0.2				
	Nymphs	*B. afzelii* + *B. lusitaniae*	NT	NT	0.2				
	Nymphs	*B. garinii* + *B. valaisiana*	NT	NT	0.2				
	Nymphs	*B. garinii* + *B. afzelii* + *B. valaisiana*	NT	NT	0.9				
	Nymphs	*B. garinii* + *B. afzelii* + *B. lusitaniae*	NT	NT	0.2				
	Adults	*B. garinii* + *B. afzelii* + *B. spielmanii*	NT	NT	1.3				
	Adults	*B. garinii* + *B. valaisiana* + *B. lusitaniae*	NT	NT	1.3				
	Nymphs	*B. garinii* + *R. monacensis*	NT	NT	0.4				
	Nymphs/ adults	*B. valaisiana* + *Bartonella spp.*	NT	NT	0.4				
	Nymphs	*B.afzelii* + *Ca.* N. mikurensis	NT	NT	0.4				
	Nymphs	*B. valaisiana* + *Ca.* N. mikurensis	NT	NT	0.4				
	Adults	*B. valaisiana* + *R. monacensis*	NT	NT	1.3				
	Nymphs	*B. valaisiana* + *Rickettsia spp.*	NT	NT	0.2				
	Nymphs	*B. afzelii* + *Rickettsia spp.*	NT	NT	0.2				
	Nymphs	*B. miyamotoi* + *Ca. N. mikurensis*	NT	NT	0.2				
	Nymphs	*B. miyamotoi* + *Bartonella spp.*	NT	NT	0.2				
	Nymphs	*B. garinii* + *Bartonella spp.*	NT	NT	0.2				
	Nymphs	*B. spielmanii* + *A. phagocytophilum*	NT	NT	0.2				
	Nymphs	*B. garinii* + *Rickettsia spp.*	NT	NT	0.2				

Tick species	Tick stage	Microorganism detected			% of co-infection	Technique/s of detection and targeted genes	Feeding status of ticks, Engorged (E) and non-engorged (NE)	Country	Reference
		Bacteria	Parasites	Viruses					
	Nymphs	*B. garinii* + *Ca.* N. mikurensis	NT	NT	0.2				
	Nymphs	*B. afzelii* + *R. helvetica*	NT	NT	0.2				
	Nymphs	*Borrelia spp.* + *Ca.* N. mikurensis	NT	NT	0.2				
	Nymphs	*Borrelia spp.* + *Bartonella spp.*	NT	NT	0.2				
	Nymphs/ adults	*B. garinii* + *B. afzelii* + *Rickettsia spp.*	NT	NT	0.6				
	Nymphs/ adults	*B. garinii* + *B. lusitaniae* + *Rickettsia spp.*	NT	NT	0.4				
	Nymphs	*B. garinii* + *B. afzelii* + *R. monacensis*	NT	NT	0.4				
	Nymphs	*B. valaisiana* + *B. spielmanii* + *R. monacensis*	NT	NT	0.2				
	Nymphs	*B. garinii* + *B. valaisiana* + *R. helvetica*	NT	NT	0.2				
	Nymphs	*B. garinii* + *B. afzelii* + *A. phagocytophilum*	NT	NT	0.2				
	Nymphs	*B. garinii* + *B. afzelii* + *Ca. N. mikurensis*	NT	NT	0.2				
	Nymphs	*B. garinii* + *B. valaisiana* + *Ca. N. mikurensis*	NT	NT	0.2				
	Nymphs	*B. garinii* + *R. helvetica* + *Bartonella spp.*	NT	NT	0.2				
	Nymphs	*B. valaisiana* + *R. monacensis* + *Ca.* N. mikurensis	NT	NT	0.2				
	Nymphs	*B. valaisiana* + *Rickettsia spp.* + *Ca.* N. mikurensis	NT	NT	0.2				
	Nymphs	*Borrelia spp.* + *R. monacensis* + *Ca.* N. mikurensis	NT	NT	0.2				
	Nymphs	*B. garinii* + *B. afzelii* + *B. lusitaniae* + *Ca.* N. mikurensis	NT	NT	0.2				
	Nymphs	*B. burgdorferi s.l.* + *B. miyamotoi*	NT	NT	0.4	Realtime PCR (*glpQ* gene)	NE	The Netherlands	[55]

Tick species	Tick stage	Microorganism detected			% of co-infection	Technique/s of detection and targeted genes	Feeding status of ticks, Engorged (E) and non-engorged (NE)	Country	Reference
		Bacteria	Parasites	Viruses					
I. frontalis	Adults	*B. burgdorferi s.l.* + *A. phagocytophilum*	NT	NT	18.2	Realtime PCR (*flaB* and *ospA*)		The Netherlands	[103]
	Larvae	*B. valaisiana* + *B. turdi*	NT	NT	2.5	Nested PCR (*flaB*, 5S and 23S rRNA IGS)	E	Spain	[56]
I. holocyclus and I. tasmani	Adults	—	*Trypanosoma . irwini, T.gilletti, T. copemani and T. vegrandis*	NT	27,3 and 12,2	NGS (18SrRNA)	E	Australia	[53]
I. scapularis	Nymphs/ adults	*B. burgdorferi s.l.* + *A. phagocytophilum*	NT	NT	1.8	Realtime PCR [23S (*Borrelia*), tubulin (*Babesia*), *msp2* (*Anaplasma*)]	E	USA	[49]
		B. burgdorferi s.l.	*B. microti*	NT	1				
		A. phagocytophilum	*B. microti*	NT	0.4				
		B. burgdorferi s.l. + *A. phagocytophilum*	*B. microti*	NT	0.3				
I. persulcatus	Nymphs/ adults	*B. garinii* + *SFG Rickettsia*	NT	NT	16.2	Nested PCR	NE	China	[47]
		B. burgdorferi + *A. phagocytophilum*	NT	NT	4.9				
		SFG *Rickettsia* + *A. phagocytophilum*	NT	NT	2.9				
		B. burgdorferi + *A. phagocytophilum* + SFG *Rickettsia*	NT	NT	2.5				
	Adults	*B. burgdorferi s.l.*	NT	TBEV	1.6	Realtime PCR (*gltA* and *ompA*)	E	Russia	[52]
		B. burgdorferi s.l. + *Ehrlichia chaffeensis*	NT	NT	1.6				
		B. burgdorferi s.l. + *A. phagocytophilum*	NT	NT	1.6				
Dermacentor marginatus	Adults	*R. raoultii*	NT	TBEV	4.2				

Tick species	Tick stage	Microorganism detected			% of co-infection	Technique/s of detection and targeted genes	Feeding status of ticks, Engorged (E) and non-engorged (NE)	Country	Reference
		Bacteria	Parasites	Viruses					
D. reticulatus	Adults	*A. phagocytophilum*	NT	TBEV	0.32	PCR [*gltA* (*Rickettsia* spp.), *fla* (*B. burgdoferi* s. l.), B1 fragment (*T. gondii*), 18S rRNA gene (*Babesia* spp.)]	NE	Poland	[54]
		R. raoultii	NT	TBEV	4.26				
		B. burgdorferi s.l.	NT	TBEV	0.16				
		A. phagocytophilum + *R. raoultii*	NT	NT	0.63				
		R. raoultii + *B. burgdorferi s. l.*	NT	NT	1.1				
		R. raoultii	*Babesia spp.*	NT	0.47				
		R. raoultii	*Toxoplasma gondii*	NT	0.95				
			Babesia spp. + *Toxoplasma gondii*	NT	0.16				
			Toxoplasma gondii	TBEV	0.45				
Haemaphysalis longicornis	Nymphs/ adults	*A. capra*	NT	SFTSV		PCR (*Pmy* gene)	NE	China	[50]
Rhipicephalus sanguineus	Adults	*E. canis*	*H. canis* + *L. infantum chagasi*	NT	28.6	PCR [16S rRNA (*Anaplasma*), 18S rRNA (*Babesia*), 16S rRNA (*Ehrlichia*), 18S rRNA (*Hepatozoon*), kinetoplast DNA (*Leishmania*)]	E	Brazil	[43]

NT, not tested; TBEV, tick-borne encephalitis virus; SFTSV, severe fever with thrombocytopenia syndrome virus.

Table A1.
Coinfections reported in the literature in the last 4 years.

Tick species	Tick stage	Bacteria detected	Technique of detection	Country	References
I. ricinus	Adults	*Rickettsiella*, *Rickettsia*, *Midichloria*, *Paenibacillus*, *Borrelia*, *Lactococcus*, *Ralstonia*	Ion torrent [16S [V1–V2])	Australia	[104]
	Nymphs	*Borrelia*, *Escherichia*, *Rickettsia*, *Candidatus Neoehrlichia*, *Wolbachia*, *Methylobacterium*, *Mycobacterium*, *Phyllobacterium*, *Sphingomonas*, *Hymenobacter*, *Pseudomonas*, *Williamsia*	454 pyrosequencing [16S (V6)]	Italy	[88]
	Adults	*Borrelia*, *Escherichia*, *Rickettsia*, *Candidatus Neoehrlichia*, *Methylobacterium*, *Mycobacterium*, *Phyllobacterium*, *Sphingomonas*, *Hymenobacter*, *Pseudomonas*, *Williamsia*			
	Nymphs	*Anaplasma*, *Coxiella*, *Ehrlichia*, *Borrelia*, *Rickettsia*, *Bartonella*, *Francisella*	Hiseq (bacteria)	France	[92]
	Adults				
	Adults	*Borrelia*, *Ehrlichia*, *Ca midichloria*, *Spiroplasma*, *Anaplasma*, *NeoEhrlichia*	RNA seq (bacteria)	Czech Republic	[94]
	Adults	*Borrelia*, *Lactobacillus*, *Streptococcus*, *Ureaplasma*, *Grimontia*, *Bacillus*, *Luteimonas*, *Vibrio*, *Rickettsia*	454 pyrosequencing (Bacteria and Archaea)	Japan	[32]
I. persulcatus	Adults	*Proteus*, *Acinetobacter*, *Rickettsia*, *Pseudomonas*	MiSeq [16S (V4)]	China	[50]
	Adults	*Rickettsia*, *Spiroplasma*, *Coxiella*	454 pyrosequencing [16S (V1–V3)]	Japan	[106]
	Adults	*Pseudomonas*, *Sphingomonas*, *Acidovorax*	MiSeq [16S (V3–V5)]	Russia	[107]
	Adults	*Pseudomonas*, *Enterobacter*, *Serratia*, *Stenotrophomonas*, *Achromobacter*	Hiseq (bacteria)	China	[93]
	Adults	*Chlamydophila*, *Ureaplasma*, *Streptococcus*, *Helicobacter*, *Campylobacter*, *Prochlorococcus*, *Borrelia*, *Mycoplasma*, *Clostridium*	454 pyrosequencing (Bacteria and Archaea)	Japan	[32]
I. scapularis	Adults	*Rickettsia*, *Brevibacillus*	454 pyrosequencing [16S (V1–V3)]	America	[108]
	Adults	*Rickettsia*, *Francisella*	454 pyrosequencing [16S (V1–V3)]	America	[109]
	Nymphs	*Rickettsia*, *Sphingomonas*, *Rhizobium*	MiSeq [16S (V3–V4)]	America	[90]

Tick species	Tick stage	Bacteria detected	Technique of detection	Country	References
	Adults	*Rickettsia*, *Wolbachia*, *Sphingomonas*, *Methylobacterium*, *Pseudomonas*			
	Nymphs	*Rickettsia*, *Acidovorax*, *Novosphingobium*, *Aquabacterium*	454 pyrosequencing [16S (V2)]	America	[84]
	Adults				
	Nymphs	*Acinetobacter*, *Rickettsia*, *Lysinibacillus*, *Corynebacterium*, *Staphylococcus*, *Enterococcus*, *Delftia*	MiSeq [16S (V4)]	America	[85]
	Adults				
I. affinis	Adults	*Rickettsia (>70%)*, *Methylobacterium*, *Borrelia*	MiSeq-454 pyrosequencing [16S (V1–V3; V4)]	America	[97]
I. holocyclus	Adults	*Wolbachia*, *Sphingobacterium*, *Hymenobacter*, *Friedmaniella*, *Nocardioides*, *Streptomyces*, *Paenibacillus*, *Clostridium*	Ion Torrent [16S [V1–V2])	Australia	[104]
	Nymphs	*Propionibacterium*, *Corynebacterium*, *Staphylococcus*, *Streptococcus*, *Ca. Midichloria*, *Ralstonia*	MiSeq [16S (V1–V2)]		[105]
	Adults	*Propionibacterium*, *Mycobacterium*, *Corynebacterium*, *Streptococcus*, *Ca. Midichloria*, *Ralstonia*			
I. ovatus	Adults	*Spiroplasma*, *Coxiella*, *Ehrlichia*, *Rickettsia*, *Leptotrichia*	454 pyrosequencing [16S (V1–V3)]	Japan	[106]
	Adults	*Rickettsia*, *Ureaplasma*, *Mycoplasma*, *Clostridium*, *Ehrlichia*, *Helicobacter*, *Francisella*, *Borrelia*	454 pyrosequencing (Bacteria and Archaea)	Japan	[32]
I. pacificus	Nymphs	*Rickettsia*, *Methylobacterium*, *Flavobacterium*, *Sphingomonas*	MiSeq (16S)	America	[110]
	Adults	*Rickettsia*, *Methylobacterium*			
I. pavlovskyi	Adults	*Acinetobacter*, *Rickettsia*, *Chryseobacterium*, *Escherichia*, *Janthinobacterium*	MiSeq [16S (V3–V5)]	Russia	[107]
Amblyoma americanum	Nymphs	*Rickettsia*, *Coxiella*, *Borrelia*, *Wolbachia*, *Midichloria*, *Ehrlichia*, *Pseudomonas*	454 pyrosequencing [16S (V1–V3)]	America	[111]
	Adults	*Rickettsia*, *Coxiella*, *Borrelia*, *Wolbachia*, *Midichloria*, *Ehrlichia*, *Pseudomonas*			
	Nymphs	*Rickettsia*, *Coxiella*			[112]

Tick species	Tick stage	Bacteria detected	Technique of detection	Country	References
	Adults	*Rickettsia*, *Midichloria*, *Coxiella*, *Ehrlichia*, *Sphingomonas*			
	Adults	*Coxiella*, *Brevibacterium*, *Rickettsia*, *Staphylococcus*	MiSeq [16S(V3–V4)]		[113]
	Adults	*Hymenobacter*, *Flavobacterium*, *Rickettsia*, *Methylobacterium*, *Ehrlichia*, *Burkholderia*, *Anaplasma*	MiSeq [16S(V1–V4)]		[114]
	Adults	*Coxiella*, *Rickettsia*, *Arsenophonus*, *Pseudomonas*, *Acinetobacter*	?? [16S (V1–V9)]		[96]
A. longirostre; A. nodosum, *A. maculatum*, *H. juxtakochi*	Adults	*Lactococcus*, *Raoultella*, *Wolbachia*, *Francisella*, *Propionibacterium*, *Ewingella*, *Elizabethkingia*, *Rickettsia*, *Massilia*, *Methylobacterium.*	454 pyrosequencing [16S (V1–V3)]	America	[115]
A. maculatum	Adults	*Francisella*, *Propionibacterium*, *Rickettsia*, *Pseudomonas*, *Corynebacterium*, *Escherichia*,	454 pyrosequencing [16S (V1–V3)]	America	[74]
A. testudinarium	Nymphs Adults	*Pseudoalteromonas*, *Rickettsia*, *Synechococcus*, *Wigglesworthia*, *Clostridium*, *Orientia*, *Bordetella*, *Bacillus*	454 pyrosequencing (Bacteria and Archaea)	Japan	[32]
A. triguttatum	Adults	*Francisella*, *Rickettsia*, *Flavobacterium*, *Pedobacter*, *Ralstonia*, *Mycobacterium*	MiSeq [16S (V1–V2)]	Australia	[105]
A. tuberculatum	Adults	*Rickettsia*, *Francisella*, *Dietzia*, *Arthrobacter*, *Acinetobacter*	454 pyrosequencing [16S (V1–V3)]	America	[116]
D. andersoni	Adults	*Francisella*, *Rickettsia*, *Arsenophonus*	Pacific Bioscience (PacBio, Menlo Park, USA) [16S (V1-V9)]	America	[117]
	Adults	*Arsenophonus*, *Acinetobacter*, *Francisella*, *Rickettsia*	454 pyrosequencing [16S (V4)]	America	[118]
D. marginatus	Adults	*Flavobacterium*, *Rickettsia*, *Curvibacter*, *Acidovorax*, *Shigella*	454 pyrosequencing [16S (V1–V3)]	Turkey	[119]
D. occidentalis	Adults	*Rickettsia*, *Francisella*, *Sphingomonas*, *Methylobacterium Hymenobacter*	MiSeq [16S (V4)]	America	[120]
D. reticulatus	Adults	*Francisella*, *Rickettsia*, *Acinetobacter*, *Acidovoraxi Chryseobacterium*	MiSeq [16S (V3–V5)]	Russia	[107]

Tick species	Tick stage	Bacteria detected	Technique of detection	Country	References
D. silvarum	Adults	*Pseudomonas*, *Coxiella*, *Rickettsia*, *Acinetobacter*	454 pyrosequencing [16S (V3–V4)]	China	[121]
D. variabilis	Adults	*Francisella*, *Brevibacillus*, *Arsenophonus*, *Stenotrophomonas*, *Mycobacterium*, *Rickettsia*	454 pyrosequencing [16S (V1–V3)]	America	[108]
	Adults	*Francisella*, *Arsenophonus*	454 pyrosequencing [16S (V1–V3)]	America	[109]
Haemaphysalis bancrofti	Nymphs	*Flavobacterium*, *Pedobacter*, *Propionibacterium*, *Rickettsia*, *Francisella*, *Pseudomonas*, *Stenotrophomonas*	MiSeq [16S (V1–V2)]	Australia	[105]
	Adults	*Francisella*, *Pseudomonas*, *Stenotrophomonas*, *Delfia*, *Ralstonia*, *Rickettsia*, *Sphingomonas*, *Agrobacterium*, *Flavobacterium*, *Pedobacter*, *Propionibacterium*, *Kineococcusi Mycobacterium*			
H. bispinosa	Nymphs / Adults	*Coxiella*, *Rickettsia*, *Bacillus*, *Mycobacterium*, *Sphingomonas*, *Pseudomonas*	Ion Torrent [16S [V6])	Malaysia	[122]
H. flava	Adults	*Coxiella*	454 pyrosequencing [16S (V1–V3)]	Japan	[106]
H. formosensis	Nymphs / Adults	*Chlamydophila*, *Streptococcus*, *Chlamydia*, *Helicobacter*, *Prochlorococcus*, *Campylobacter*, *Bacillus*, *Clostridium*, *Borrelia*	454 pyrosequencing (Bacteria and Archaea)	Japan	[32]
H. hystricis	Nymphs / Adults	*Coxiella*, *Rickettsia*, *Bacillus*, *Mycobacterium*, *Sphingomonas*, *Pseudomonas*	Ion Torrent [16S [V6])	Malaysia	[122]
H. longicornis	Nymphs	*Mycobacterium*, *Propionibacterium*, *Flavobacterium*, *Pedobacter*, *Staphylococcus*, *Steptococcus*, *Agrobacterium*, *Rasltonia*, *Delfia*, *Coxiella*, *Pseudomonas*, *Francisella*, *Stenotrophomonas*	MiSeq [16S (V1–V2)]	Australia	[105]
	Adults	*Mycobacterium*, *Flavobacterium*, *Coxiella*, *Francisella*			
	Nymphs / Adults	*Lactobacillus*, *Salmonella*, *Grimontia*, *Providencia*, *Coxiella*, *Cyanothece*, *Streptococcus*, *Staphylococcus*, *Bacillus*, *Acinetobacter*, *Mycoplasma*	454 pyrosequencing (Bacteria and Archaea)	Japan	[32]

Tick species	Tick stage	Bacteria detected	Technique of detection	Country	References
H. wellingtoni	Nymphs	*Coxiella*, *Rickettsia*, *Bacillus*, *Mycobacterium*, *Sphingomonas*, *Pseudomonas*	Ion Torrent [16S (V6)]	Malaysia	[122]
	Adults				
R. annulatus	Adults	*Flavobacterium*, *Curvibacter*, *Acidovorax*, *Stenotrophomonas*, *Shigella*, *Variovorax*	454 pyrosequencing [16S (V1–V3)]	Turkey	[119]
R. microplus	Adults	*Achromobacter*, *Staphylococcus*, *Corynebacterium*, *Pseudomonas*, *Bacillus*, *Coxiella*	454 pyrosequencing [16S (V1–V3)]	America	[91]
R. sanguineus	Nymphs	*Rickettsia*	MiSeq [16S (V5–V6)]	France	[123]
	Adults	*Rickettsia*, *Coxiella*, *Bacillus*, *Acinetobacter*			
	Adults	*Coxiella*, *Bacillus*			
	Adults	*Coxiella*, *Bacillus*		Russia	
R. turanicus	Adults	*Propionibacter*, *Bacteroides*, *Ralstonia*, *Serratia*, *Pseudomonas*	454 pyrosequencing [16S (V4–V6)]	Israel	[89]

Table A2.
NGS studies and tick microbiota composition reported in the literature.

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Section 2

Emerging Tick Infestations

Chapter 4

Rickettsial Endosymbionts of Ticks

Markéta Nováková and David Šmajs

Abstract

Rickettsiae are widely known to be human bacterial pathogens transmitted by blood-sucking ectoparasites, such as ticks, fleas, and lice. However, most rickettsial species are nonpathogenic endosymbionts with various groups of organisms, such as arthropods, protists, and other eukaryotes. While attention has been given to rickettsial endosymbionts of insects, rickettsial endosymbionts of ticks have been less well studied. Tick hosts are found across the phylogeny of *Rickettsiae*; hence, the tick was the most probable ancestral host of *Rickettsiae* associated with arthropods. Here, we focus on rickettsial endosymbionts of ticks, describing their role in association with ticks and comparing them to tick-borne vertebrate pathogens.

Keywords: *Rickettsia*, endosymbiont, tick, host-parasite interactions, tick microbiome, tick-borne pathogens, tick-borne diseases

1. Introduction

Rickettsiae are human bacterial pathogens transmitted by blood-sucking ectoparasites, such as ticks, fleas, and lice. Many studies have shown that rickettsioses belong to the oldest known zoonoses. Since they have no pathognomonic signs [1], the association between the disease, the vector, and the causative agent has been described decades apart [2]. With the advent of molecular methods, rickettsial agents are being constantly discovered; however, it is not clear whether these novel tick-borne diseases escaped the attention of physicians or they did not exist [2].

Molecular approaches have also revealed the remarkable diversity of *Rickettsiae* and their host associations ranging from arthropods to plants [3]. Nowadays, the majority of the members of the genus *Rickettsia* are considered nonpathogenic endosymbionts [4]. Multiple serological studies suggest that vertebrates may be possible reservoirs of *Rickettsiae* in nature; however, confirmation of these hypothesized reservoirs requires further study [5]. Nevertheless, in all cases, humans are accidental hosts of tick-borne *Rickettsiae* [6].

While pathogenic *Rickettsiae* have been extensively studied, less attention has been given to nonpathogenic endosymbionts [4, 7]. This chapter presents the current state of knowledge relative to tick rickettsial endosymbionts and focuses on tick-*Rickettsia* interactions and their relationship to tick-borne human pathogens.

2. *Rickettsiae*

2.1 Taxonomy

Rickettsiae are Gram-negative obligately intracellular coccobacilli belonging to the family *Rickettsiaceae* and order *Rickettsiales* in the alpha subdivision of the class *Proteobacteria*. They can be found in the cytoplasm or nucleus of eukaryotic host cells [8].

The term "rickettsia" historically denoted small intracellular bacteria, which could not be identified by cultivation in axenic media due to their obligate intracellular nature [9]. The order *Rickettsiales* contains the families *Rickettsiaceae*, *Bartonellaceae*, and *Anaplasmataceae*. The family *Rickettsiaceae* contains the tribes *Rickettsiae*, *Ehrlichia*, and *Wolbachia*; the tribe *Rickettsiae* includes the genera *Coxiella*, *Rickettsia*, and *Rochalimaea*. The advent of molecular taxonomic methods including *16S rRNA* gene analysis resulted in reclassification of rickettsial taxa, and several genera (e.g., *Coxiella*, *Bartonella*, and *Rochalimaea*) have been removed from the order *Rickettsiales* [10, 11]. Currently, the family *Rickettsiaceae* contains genera *Rickettsia* and *Orientia*. The genus *Rickettsia* traditionally contained two groups of pathogenic *Rickettsiae*: the typhus group and the spotted fever group (SFG). The latter included approximately 20 species, mostly transmitted by ticks. Over several years, a remarkable diversity of *Rickettsiae* in arthropods has been found, which led to a new description of the ancestral group and includes *Rickettsia bellii* and *Rickettsia canadensis*, which are clearly distinct from other *Rickettsiae* [3]. Subsequently, a transitional group containing *Rickettsia felis* and *Rickettsia akari* was established, since these species share molecular features with both the typhus group and the SFG [12]. A recent phylogenetic study, based on whole-genome data, provided a single tree topology that well describes the evolutionary history of the core genome and is, in general, consistent with previous studies [13].

2.2 Endosymbiotic lifestyle of *Rickettsiae*

Rickettsiae are endosymbionts (i.e., organisms living within a host cell), and the level of their dependence on the host is variable. An obligate symbiont is, according to the definition, present in most individuals of a given host species, and the mutualistic relationship is crucial for the survival of both organisms. In such associations, co-cladogenesis between symbiont and host is typical. Facultative symbionts are not essential for the host and vice versa and have variable frequencies of prevalence.

2.3 Host diversity of *Rickettsiae*

A remarkable host diversity has been revealed for *Rickettsiae* [3]. Their common ancestor was presumptively free-living. The estimated transition to an intracellular niche took place 775–525 million years ago. The genus *Rickettsia* appears to have originated more recently, approximately 150 million years ago [3]. Presumably, the primary host for *Rickettsiae* was an arthropod, with some species later shifting to other eukaryotes, such as protists and leeches. It has been estimated that 24% of arthropod species harbor *Rickettsiae* [14].

2.4 *Rickettsiae* associated with ticks

Interestingly, hard tick (Ixodidae) hosts are found across the phylogeny of *Rickettsiae*, and related rickettsial species tend to share related tick host species. This suggests a tick was the most plausible ancestral host for rickettsial species associated with arthropods [14].

Of the approximately 900 known tick species, 81 nonrandomly selected species were tested for the presence of bacterial endosymbionts and 55.6% harbored *Rickettsiae* [15]. The most prevalent endosymbiont in arthropods is *Wolbachia* and in ticks *Coxiella*-LE (*Coxiella*-like endosymbiont) with 52.0 and 60.5% of the known species being infected, respectively. However, these results may be biased by uneven sample collections, e.g., in the study by Weinert et al. [14], the vectors of rickettsial diseases were highly overrepresented, and in the study by Duron et al. [15], the available tick species varied widely.

2.4.1 Perpetuation of Rickettsiae in nature

There are two types of *Rickettsiae* transmission in ticks—vertical and horizontal.

Vertical transmission takes place from female to offspring via egg cytoplasm or from one arthropod stage to another after molting (i.e., from larva to nymph, from nymph to adult) [7, 16]. *Rickettsiae* capable of invading ovarian tissues during oogenesis develop in the interstitial cells of tick ovaries and within oogonia and oocytes. Other tissues of rickettsial endosymbionts of ticks are rarely infected, as reported for *Rickettsia peacockii* and *Rickettsia buchneri* [17]. It has been documented in several pathogenic *Rickettsiae* that bacteria can negatively interfere with tick reproduction. Species reported to use transovarial transmission are shown in **Table 1**.

Horizontal transmission, i.e., transfer among host individuals, may involve several mechanisms. Co-feeding (i.e., several ticks feeding close to each other on the same host individual) seems to be one mode of accidental horizontal transmission of tick rickettsial endosymbionts [18]. Sexual transmission (via copulation) has been reported but probably does not play a significant role in perpetuation of *Rickettsiae* in tick populations.

For successful horizontal transfer of *Rickettsiae* from a vertebrate host under natural conditions, a host must develop rickettsemia with sufficient levels of bacteria in the blood and for a sufficient duration. Since some *Rickettsiae* negatively impact the health of their tick hosts (which is more evident for pathogenic species), a vertebrate host must maintain such *Rickettsiae* in nature (e.g., capybara for *Rickettsia rickettsii* in South America [19]). However, the role of vertebrates in perpetuation of tick-borne *Rickettsiae* remains largely unknown [5].

Rickettsial endosymbionts of ticks are mainly transmitted vertically, while pathogenic Rickettsiae are typically transmitted horizontally [17]. *Occas*ional horizontal transfer allows symbionts to disperse beyond their primary host species, which leads to limited phylogenetic congruence between tick hosts and rickettsial symbionts [14, 20].

2.4.2 Infection of Rickettsiae-free ticks

The initially infected site of a *Rickettsia*-free tick may be the gut when feeding on a *Rickettsiae*-infected vertebrate host [7]. The first interaction with tick cells after *Rickettsiae* ingestion occurs in the midgut, the storage organ [21]. *Rickettsiae* pass through the midgut barrier and escape the ticks' immune response by entering hemocytes present in the hemolymph, then enter the epithelial cells, and replicate. After that, bacteria invade tissues and organs, where they replicate and persist [7].

2.4.3 Strict blood diet of ticks and rickettsial endosymbionts

For decades, it was not fully understood why ticks harbor rickettsial endosymbionts. It was previously suggested that some endosymbionts may manipulate

Species	Pathogenicity	Host species	TOT reported	Ref.
R. aeschlimannii	Spotted fever	*A. variegatum*, *Rhipicephalus* spp., *Hyalomma* spp., *Hae. punctata*	No	[46]
R. africae	African tick bite fever	*Amblyomma* spp., *Rhipicephalus* spp., *Hyalomma* spp.	Yes	[47]
R. amblyommatis	Unknown	*Amblyomma* spp., *Rhipicephalus* spp., *D. nitens*	Yes	[48]
R. argasii	Unknown	*A. dewae*	No	[49]
R. asembonensis	Unknown	*Rh. sanguineus* (mostly associated fleas)	No	[50]
R. asiatica	Unknown	*Ixodes* spp.	No	[51]
R. australis	Queensland tick typhus	*Ixodes* spp.	No	[52]
R. bellii	Unknown	*Amblyomma* spp., *Dermacentor* spp., *Haemaphysalis* spp., *I. loricatus*, *O. concanensis*, *C. capensis*	Yes	[53]
R. buchneri[*]	Unknown	*I. scapularis*	Yes	[17]
R. canadensis	Unknown	*Hae. leporispalustris*	No	[54]
R. conorii subsp. *caspia*	Astrakhan fever	*Rhipicephalus* spp.	No	[55]
R. conorii subsp. *conorii*	Mediterranean spotted fever	*Rhipicephalus* spp., *Haemaphysalis* spp.	Yes	[56]
R. conorii subsp. *indica*	Indian tick typhus	*Rh. sanguineus*	No	[55]
R. conorii subsp. *israelensis*	Israeli spotted fever	*Rh. sanguineus*	Yes	[57]
R. felis	Flea-borne spotted fever	*Hae. flava*, *Rh. sanguineus*, *I. ovatus*, *C. capensis* (mostly associated with fleas)	Yes	[48]
R. gravesii	Unknown	*A. triguttatum*	No	[58]
R. heilongjiangensis	Far Eastern spotted fever	*Haemaphysalis* spp., *D. silvarum*	No	[59]
R. helvetica	Unnamed rickettsiosis	*Ixodes* spp.	Yes	[60]
R. honei	Flinders Island spotted fever, Australian spotted fever (*str. marmionii*)	*B. hydrosauri*, *Ixodes* spp. (str. RB), *Hae. novaeguineae (str. marmionii)*	Yes	[61]
R. hoogstraalii	Unknown	*Haemaphysalis* spp., *Carios* spp., *Arg. persicus*	No	[62]
R. japonica	Japanese spotted fever	*Haemaphysalis* spp., *I. ovatus*, *D. taiwanensis*	Yes	[63]
R. lusitaniae	Unknown	*Ornithodoros* spp.	No	[64]
R. massiliae	Unnamed rickettsiosis	*Rhipicephalus* spp., *I. ricinus*, *Hae. paraleachi*	Yes	[65]
R. monacensis	Spotted fever	*Ixodes* spp.	Yes	[66]

Species	Pathogenicity	Host species	TOT reported	Ref.
R. montanensis	Unknown	*Dermacentor* spp., *A. americanum*	Yes	[48]
R. monteiroi	Unknown	*A. incisum*	No	[67]
R. parkeri	Mild rickettsiosis	*Amblyomma* spp., *D. variabilis*	Yes	[48]
R. peacockii[*]	Unknown	*D. andersoni*	Yes	[17]
R. raoultii	SENLAT	*Dermacentor* spp., *I. ricinus*, *Haemaphysalis* spp., *A. testudinarium*	Yes	[7]
R. rhipicephali	Unknown	*Rhipicephalus* spp., *Dermacentor* spp., *Hae. juxtakochi*	Yes	[7]
R. rickettsii	Rocky Mountain spotted fever (*str. Iowa avirulent*)	*Dermacentor* spp., *Rh. sanguineus*, *Amblyomma* spp., *Hae. leporispalustris*	Yes	[68]
R. sibirica subsp. *mongolitimoniae*	Lymphangitis-associated rickettsiosis	*Hyalomma* spp., *Rh. pusillus*	No	[6]
R. sibirica subsp. *sibirica*	Siberian tick typhus	*Dermacentor* spp., *Haemaphysalis* spp., *I. persulcatus*	Yes?	[7]
R. slovaca	SENLAT	*Dermacentor* spp.	Yes	[69]
R. tamurae	Spotted fever	*A. testudinarium*	No	[70]
R. vini[*]	Unknown	*Ixodes* spp.	Yes	[37]

**Obligate endosymbiont.*
Abbreviations: A., Amblyomma; Arg., Argas; B., Bothriocroton; D., Dermacentor; H., Hyalomma; Hae., Haemaphysalis; I., Ixodes; O., Ornithodoros; R., Rickettsia; Ref., reference; Rh., Rhipicephalus; SENLAT, scalp eschar and neck lymphadenopathy after a tick bite; spp., species (plural); str., strain; TOT, transovarial transmission.

Table 1.
Valid and published Rickettsial species associated with ticks [71].

reproduction or enable survival in changing environments [22]; however, specific reasons remained unclear until recently.

Some of arthropod endosymbionts became obligate mutualists that adapted to host specialization to a restricted diet, e.g., blood or plant sap [22]. It had been found that the rickettsial endosymbiont of *Ixodes scapularis*, *R. buchneri*, was presented only in females of this tick species. As males do not feed with blood, a possible relationship of the rickettsial endosymbiont and the tick blood diet had been suggested [23].

This hypothesis has been confirmed by metabolic reconstructions derived from rickettsial endosymbiont genomes of *R. buchneri* and *Rickettsia* species phylotype G021, which showed that they contain all the genes required for folate (vitamin B9) biosynthesis [24]. This is in accordance with the expected nutritional compounds required for strict hematophagy [15]. Vitamin B9 is not present in a restricted blood diet in sufficient amounts. Moreover, *Rickettsia* species phylotype G021 was shown to massively proliferate after a tick blood meal in all stages [25].

2.4.4 Insights into rickettsial genomes

In the last decade, whole-genome sequences of several rickettsial species (including obligate endosymbionts) were published, which allows detailed analyses of their evolution and host associations [26].

The recurrent biphasic model described in parasitic and symbiotic organisms is characterized by longer phases of genome reduction and simplification, interrupted by shorter phases of episodic expansion [27]. Rickettsial chromosomes and plasmids are in progressive degradation and size reduction and contain numerous laterally acquired genes that display evidence of horizontal transfer between *Rickettsia*, other *Rickettsiae* and bacterial endosymbionts (such as *Cardinium*), and even eukaryotes [9, 13, 28]. For instance, rickettsial plasmids have gained novel metabolic functions that are missing in rickettsial chromosomes and which may fill host-metabolic gaps [29].

A convergent reductive pattern has led to relatively small rickettsial genomes, ranging from 1.1 Mb for pathogenic *Rickettsia prowazekii* and *Rickettsia typhi* to 2.1 Mb for the obligate endosymbiotic *R. buchneri* [26].

2.4.5 Are pathogenic and endosymbiotic Rickettsiae two separate groups?

The phylogenetic position does not define the pathogenicity since tick rickettsial endosymbiotic and vertebrate pathogen species are dispersed along the phylogeny [3]. In the most recent review on tick-borne rickettsioses, it was stated that every member of the SFG should be considered a potential pathogen [6]. Numerous pathogenic tick-borne *Rickettsiae* are vertically transmitted [7]; hence, transovarial transmission is not a sign of nonpathogenicity. The ability of *Rickettsiae* to invade tick host cells seems to be the crucial feature that was lost by endosymbionts. *R. peacockii*, in *Dermacentor andersoni*, is not able to enter hemocytes and salivary gland tissues, which establishes its obligate endosymbiotic nature and prevents infection of vertebrates [30]. The borderline between pathogens and endosymbionts is not sharp since there are avirulent strains of pathogenic *Rickettsiae* that retain the ability to persist in ticks and can be transmitted transovarially, such as *R. rickettsii* strain Iowa [31].

The pathogenic and endosymbiotic lifestyle could probably evolve via various scenarios: First, loss of pathogenicity, as described for strictly endosymbiotic *R. peacockii*, which is closely related to the most clinically severe *R. rickettsii*. The genome of *R. peacockii* contains various deletions and mutations caused by a recombination of transposon copies that extinguished its ability to cause cytopathic effects [32, 33]; a similar situation exists with nonpathogenic *R. buchneri*, which is closely related to pathogenic *Rickettsia monacensis* [17]. However, since rickettsial phylogeny shows repeated occurrences of horizontal transfer, this may lead to the appearance of novel bacterial phenotypes as described in Q fever cases caused by *Coxiella burnetii*, which probably originated from a *Coxiella*-LE that infected vertebrate cells [15]. *Rickettsia vini*, an obligate endosymbiont of ornithophilic *Ixodes arboricola* and *Ixodes lividus* ticks, has repeatedly been detected in *Ixodes ricinus* ticks, which may illustrate horizontal transmission of endosymbiotic *Rickettsiae* via co-feeding [34–36]. Since this species is a member of the SFG and was successfully isolated in vertebrate Vero cells, it may represent a potential candidate for a vertebrate pathogen [37].

2.4.6 Rickettsial endosymbionts in relationship to other maternally inherited bacteria within ticks

Ten distinct genera of maternally inherited bacteria have been recently described in ticks (e.g., [23, 38, 39]). Based on a recent study by Duron, the most prevalent bacterial genera in ticks are *Coxiella*-LE (60.5%) and *Rickettsia* (55.6%), both of which have been identified in more tick species than any other genera [15]. While 43.2% of tested tick species harbored one bacterium, 56.3% were infected

with two or more bacterial genera. *Rickettsia* has also been found to nonrandomly aggregate with *Midichloria*. Such fixed multiple endosymbiotic associations may imply that, collectively, the bacteria can synthesize all the components needed for certain essential pathways and hence are interdependent [15].

Only 2 out of 81 tick species (2.5%) did not harbor any maternally inherited bacteria [15]. In some filarial nematodes, symbiont genes acquired from bacteria via lateral gene transfer have been found in the host chromosome [40]. This could explain why Duron did not detect any bacterial endosymbiont in two tick species. However, such horizontal gene transfer has yet to be reported in ticks [15].

Infection frequencies vary among different geographical populations of a given tick species [4]. Combining maternal inheritance with horizontal transfer allows unrelated bacteria to coinfect one individual host and to form an endosymbiotic community with complex interactions resulting in phenotypic differentiation within tick populations [41]. Recent studies have revealed that relationships among bacterial communities within ticks are more complex than had been previously assumed [4].

2.4.7 Interaction of nonpathogenic rickettsial endosymbionts and pathogenic bacteria

Ticks are exposed to various *Rickettsiae* while feeding on multiple hosts [38]. However, typically only one rickettsial species is observed per individual tick [42]. Transovarial transmission of more than one rickettsial species from the SFG has not been proven. It is believed that infection of tick ovaries could induce a specific molecular response that results in a second infection being blocking [43]. However, the coexistence of *R. bellii*, which belongs to the ancestral group, with SFG *Rickettsiae* has been described [28]. Additionally, interactions of *Rickettsiae* with other pathogens have been reported. The occurrence of *R. bellii* in *D. andersoni* ticks precludes infection of *Anaplasma marginale* [44]. Males of *I. scapularis* infected by *R. buchneri* were significantly protected against infection by *Borrelia burgdorferi* compared to *R. buchneri*-free males [45].

3. Conclusion

Non-pathogenic rickettsial endosymbionts of ticks appear to interact with ticks in complex ways. While some of them are essential for tick survival and reproduction, others may impact multiple tick features, e.g., rickettsial endosymbionts may significantly influence the abundance of tick-borne pathogens, which may help reduce the health risk to humans. The boundaries between categories, such as vertically transmitted pathogen and maternally inherited endosymbiont, are not terribly sharp since transitional states occasionally arise [4].

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Conflict of interest

The authors declare no conflicts of interest.

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Chapter 5

Spinose Ear Tick *Otobius megnini* Infestations in Race Horses

Rupika S. Rajakaruna and Chulantha Prasanga Diyes

Abstract

Spinose ear tick, *Otobius megnini*, has a worldwide distribution causing otoacariasis or parasitic otitis in animals and humans. It mainly infests horses and cattle. It is a nidicolous, one-host soft tick spread from the New World to the Old World and is now distributed across all the continents. Only the larvae and nymphs are parasitic, feeding inside the ear canal of the host for a long period. Adult males and females are free-living and nonfeeding, and mating occurs off the host. Being inside the ear canal of the host allows the tick to be distributed over a vast geographic region through the distribution of the host animals. The presence of infectious agents *Coxiella burnetii*, the agent of Q fever, spotted fever rickettsia, *Ehrlichia canis*, *Borrelia burgdorferi*, and *Babesia* in *O. megnini* has been reported, but its role as a vector has not been confirmed. Human infestations are mostly associated with horse riding and farming through close contacts with companion animals. Control measures involve use of acaricides, repellants, and biological control methods. However, controlling the tick population and its spread is extremely difficult due to its life cycle pattern, seasonal dynamics, and resistance to certain acaricides.

Keywords: *Otobius megnini*, spinose ear tick, horses, otoacariasis

1. Introduction

The spinose ear tick, *Otobius megnini* (Dugès 1883) (*Acari*: *Ixodida*: *Argasidae*), is an economically important soft tick as it parasitizes livestock mostly cattle, goats, sheep, and horses and also infests humans [1–7]. *Otobius megnini* is a one-host soft tick from the New World with a wide geographical distribution. Its original center of distribution is considered to be the southwestern North America from where it spread to Central and South America [5, 8]. Since the larva and the nymph of this tick feed inside the ear canal of the host for a long period, it allows the tick to be distributed over a vast geographic region transcontinentally through the distribution of the host animals. It has distributed far north as Canada where it is reported southeastern parts of British Columbia infesting mountain goats, mountain sheep, mule, white-tailed deer, elk, cattle, and a house cat [9]. Two scenarios have been put forward to explain how the tick reached the Old World: (1) During the Boer Wars (in the late nineteenth century between the United Kingdom and the Boers of the South African Republic), the movement of horses bringing the tick from South America or Mexico to South Africa and (2) after the Boer Wars, importation of cattle from the United States to South Africa [5]. From there it was introduced to many neighboring countries in the African continent: Madagascar, Lesotho,

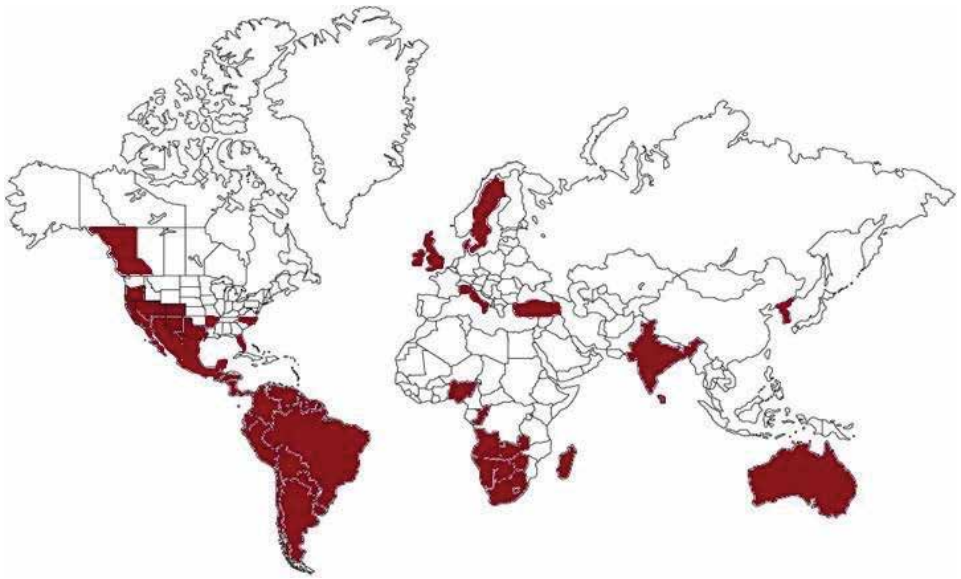

Figure 1.
Geographic distribution of spinose ear tick Otobius megnini (modified from Keirans and Pound [5] with permission).

Botswana, Namibia, Zimbabwe, Zambia, Malawi, Nigeria, and Democratic Republic of Congo [5]. The first report of *O. megnini* from Europe was in 1901 from a US resident who visited the UK with a tick in the ear [10], and there is another unverified report of *O. megnini* from Denmark [11]. Dogs imported from South Africa to Italy [12] and to Sweden carried the tick in the ear canal [13]. In Turkey, it was first recorded in 1988 [14], and it is well established now [15–17]. *Otobius megnini* is thought to have reached India in the mid-1930s together with cattle or horses brought from Southern Africa. This tick species is recorded in race horses brought from farms in northern India for an auction at the Madras Race Club [18]. There is a speculation that *O. megnini* was introduced to Sri Lanka from India via horse trading. The first report of *O. megnini* in Sri Lanka is in 2010 from stable workers and jockeys as an intra-aural infestation [7, 19]. In Sri Lanka, this tick appears to have a limited distribution with no records of it infesting any other domesticated animals other than horses in the racecourses. It has now moved to Far East as Korea [20] and Western Australia [21] and has been recently reported from Iran [22]. **Figure 1** shows the current geographic distribution of *O. megnini* in the world.

The presence of *O. megnini* inside the ear canal is known as otoacariasis or parasitic otitis. It can cause toxic conditions, allergies, paralysis, muscle spasms, irritations, eardrum perforation, and myotonia, and *O. megnini* has been listed as a potential vector for many tick-borne infections [23–25]. Studies have reported deaths of domestic cattle and horses as a result of heavy infestation of *O. megnini* [9, 26–29].

2. Horse otoacariasis

Otobius megnini is the causative agent of horse otoacariasis. This condition can cause serious injury and occasionally death in horses [9, 30], and common clinical signs include abnormal head carriage, head shaking, and head rubbing [31]. Early studies report nervous disease [32] and auricular nerve paralysis [33] due to the presence of *O. megnini* in the ear canal of horses. Intermittent painful muscle

Figure 2.
Spinose ear tick Otobius megnini inside the ear canal of a racehorse.

cramps not associated with exercise were described in horses that were severely infested with *O. megnini* [34]. Infested horses show cramping of pectoral, triceps, and abdominal muscles lasting from minutes to a few hours with severe pain that often resembles colic [34]. Between muscle cramps, horses appear to be normal, and once the ticks are removed, clinical signs are reduced and recovered within 12–36 h [34]. While a neurological pathology that includes muscle tremors and muscle contractions are observed, electromyographic measurements suggest these may be due to increased motor unit activity [34]. No conclusive evidence supports the classification of *O. megnini* as a paralysis tick. The fact that this tick feeds within the ears of its hosts where inflammatory reactions could affect the balance of the host and lead to symptoms that could be interpreted as being neurological in origin should be considered. Recently, from Northern Mexico, a 2-year-old quarter breed was reported having myotonia and colic associated with the infestation of *O. megnini* [35]. **Figure 2** shows larvae and nymphs of *O. megnini* inside the horse ear canal.

3. Life cycle

The life cycle pattern of soft ticks varies considerably among the populations of the same species as well as between species of the family *Argasidae*. *Otobius megnini* has four stages in its life cycle: egg, six-legged larva, nymph, and adult. The number of nymphal instars in the life cycle varies and is controversial. Studies have reported the presence of either one [6, 36, 37] or two nymphal stages [5, 20, 38] and also the presence of a third nymphal instar [39]. Unfed larval stage is highly active showing constant and rapid movements. Nymphs have distinct integument covered with short blunt spines and hence the name spinose ear tick. The adults have spineless-granulated integument. Only the larvae and nymphs are parasitic and stay attached inside the ear canal for extended periods of time, while the adult is a nonfeeding free-living stage [40]. The life cycle pattern of *O. megnini* closely resembles that of a one-host hard tick by having a long parasitic period and a short nonparasitic period.

The larvae and nymphs feed for several days to months [6, 38, 41, 42]. Fully engorged nymphs detach after a long parasitic phase, drop off, and molt on the ground to nonfeeding adults [42]. *Otobius megnini* has a single gonotrophic cycle; hence, females die soon after oviposition. Successful completion of the life cycle depends on the efficacy of the blood meal which is determined by the interactions with their host and environmental conditions [43, 44]. Temperature and humidity have been identified as the main climatic variables that contribute to the nature of the life cycle [45] on which the egg incubation and hatching success, larval and nymphal feeding, survival and pre-molting periods, and female oviposition and survival are dependent [42, 46–48]. Female ticks, compared to males, tend to take a larger blood [42, 49]. Under laboratory conditions, *O. megnini* can feed on rabbits and complete the life cycle successfully (**Figures 3** and **4**) [42].

Ticks developing in temperatures between 21 and 28°C typically have oviposition 6–12 days after dropping as nymphs from their hosts. The number of eggs, which are laid in the nesting grounds of potential hosts, can range from 398 to 1187 depending on the weight of the female [40]. Egg incubation ranges from 14 to 19 days in laboratory studies [50] and 18–23 days in field studies [26]. Once hatching occurs, larvae seek hosts for survival; unfed larvae have been found to survive in the laboratory up to 78 days [51]. Larvae feed on the host for 1–5 weeks and then molt into the nymph. The majority of nymphs feed between 2 and 4 months [50] but some up to 6 months [51].

A tropical population of *O. megnini* successfully completed the life cycle within 123 days [42]. Only the larger larvae and nymphs weighing more than 0.9 mg molt to the next stage. Larvae do not molt if the temperature is below 10°C, and there is a higher survival of larvae at 28°C. Nymphs undergo diapause if the temperatures are below 10°C [42]. Females survive longer (313–629 days) than males (142–321 days). Some females lay eggs without mating. However, parthenogenesis is not confirmed. Apart from the descriptive study on Sri Lankan population of *O. megnini* [42], life history of laboratory populations of Nearctic population of *O. megnini* from the Southwestern USA (duration of the life cycle: 52–248 days; [52]); Texas, USA (92–125 days; [53]); California, USA (62–118 days; [50]); Maryland, USA (60–120 days; [54]); neotropical population of *O. megnini* from Córdoba, Argentina (101.4 days, [9]) and oriental population of *O. megnini* from Madras, India (69–98 days; [36]) and Bangalore, India (118–207 days, [38]) has been reported with considerable variations in parasitic period, molting period, fecundity, and survival. These variations can be attributable to the

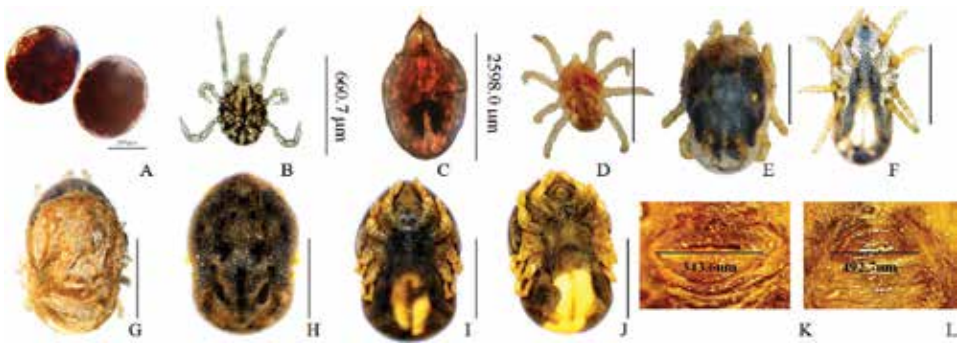

Figure 3.
Life-history stages of Otobius megnini. (A) Eggs. Larvae: (B) unfed free-living larva soon after hatching, (C) Engorged larva. Nymphs: (D) unfed nymph soon after hatching inside the ear canal, (E) dorsal view of the engorged nymph fed on horse, (F) ventral view of engorged nymph, (G) nymph molting. Nonfeeding adults: (H) dorsal view, (I) ventral view of a female, (J) ventral view of a male, (K) female genital pore (L), and male genital pore (scale bar indicating 1 cm).

Figure 4.
Larvae and nymphs of Otobius megnini feed on New Zealand white rabbits under laboratory conditions.

differences in laboratory conditions that the ticks were exposed and the host animals used to feed the immature *O. megnini* (e.g., rabbits, cattle). However, data obtained from these laboratory studies cannot entirely extrapolate to the natural context uncritically because the survival period of *O. megnini* greatly depends on their niche condition (e.g., air temperature, soil temperature, relative humidity and amount of direct light) [45] and the presence of pathogens, predators, and parasitoids [55]. The remarkable survival strategy of *O. megnini* has enabled the global expansion of this tick.

4. Seasonal dynamics

Otobius megnini is adapted to survive in diverse ecological niches in tropics, subtropics, and temperate regions [6, 37, 41, 52]. In nidicolous ticks that have distinct seasonal activity, reproductive diapause with respect to temperature and photoperiod has been suggested as the main mechanism, which controls the seasonal activity. However, ambient climatic conditions needed for better survival of ticks greatly vary from region to region [41, 56, 57]. Therefore, there is a high geographic variation in seasonal population dynamics of ticks. In temperate region where distinct seasonal pattern is pronounced, the activity of different development stages of ticks is directly linked with the changing environmental condition. However, in the tropics, the altering of rainy and dry periods has been identified as main contributing factors of tick seasonal activity [41].

Studies conducted in Argentina [6, 58], South Africa [59, 60], and Texas, USA [61] have reported discordant results for seasonal activity for *O. megnini* indicating absence of a clear seasonal pattern. Further, climatic factors such as annual rainfall, temperature, and altitude appear to have no profound effect on distribution and seasonal dynamics of *O. megnini* populations in Argentina and the Union of South Africa [58, 60, 62]. In the tropics, the larvae show clear seasonal dynamics with a high larval activity during warmer and dryer months [42]. Information about seasonal dynamics of *O. megnini* is important in controlling populations because the sanitary measures and applications of biocides considering the seasonal dynamics have been able to minimize the tick abundance [63].

5. Infectious agents

Since *O. megnini* is a one-host tick and the adult females do not feed, spreading of infectious diseases from one host to another is limited unless the infectious agent shows both transovarial and transstadial transmission like *Rickettsia bellii* maintained in *Ixodes loricatus* [64]. Studies on infectious agents of *O. megnini* are few and mostly carried out retrospectively. First, as early as 1948, infection of *Coxiella burnetii*, the causative agent of Q fever, was recovered from *O. megnini* collected from dairy cattle in Southern California [24]. Q fever, first reported in Australia as an outbreak in 1935 in nine patients [65], has now been listed as a zoonotic disease transmitted to humans primarily through inhalation of contaminated dust or aerosols and through ticks. Infection with *C. burnetii* is therefore recognized as an occupational hazard for people who work with or around waste and birth products of livestock and may include farmers, veterinarians, zoo, and slaughterhouse workers [66]. Although ticks may readily transmit *C. burnetii* in experimental systems, they only occasionally transmit the pathogen in the field. Furthermore, there are many *Coxiella*-like bacterial endosymbionts which are widespread in ticks and may have been misidentified as *C. burnetii*. Desjardins and co-workers in [67] examined the presence of antibodies and DNA of *C. burnetii* in horses, ticks, and equine environment and the potential expression of clinical disease in horses in Southeast France, a region known to be hyperendemic for human Q fever [68–70]. Although few horses (4–12%) reported as seropositive and DNA in ticks and dust being qPCR positive, horse blood was qPCR negative and did not observe any statistical association between seropositive horses and positive ticks. Although the analysis consisted of 149 ticks, none of them were *O. megnini* [67].

One specimen of *O. megnini* collected from a human in Turkey was tested for Rickettsial DNA and reported PCR negative [16]. An early record from Mexico shows two cases of spotted fever *Rickettsia* infested with nymphs of *O. megnini* [23]. Studies have shown that *O. megnini* can be naturally infected with *Ehrlichia canis* but does not transmit the agent [71]. Experimental transmission of *E. canis* by laboratory-reared *O. megnini* was attempted, but neither transstadial nor transovarial transmission occurred [71]. A specimen recovered from a child who had serologic evidence ehrlichiosis was examined microscopically, but no evidence of infection was found [71]. In a study carried out to determine equine Lyme borreliosis in a large horse riding school in Natal Province, South Africa reported *Borrelia burgdorferi* seropositive cases of 3 horse riders and owner of a stable, 71 horses, and 5 dogs, but none of the *O. megnini* specimens collected from these hosts were positive for the infection [72].

A laboratory study carried out on *O. megnini* collected from the ear canal of 11 race horses in Sri Lanka investigated the presence of three infectious agents: *Rickettsia*, *Theileria*, and *Babesia* and reported that the ticks collected from two horses being PCR positive for *Babesia* infections [25]. However, there are no records whether these horses showed any clinical symptoms of babesiosis. The study provides the first record of *Babesia* infections in *O. megnini*. Further investigations to confirm the *Babesia* species and blood samples from horses to verify its vector capacity are important. In 1967 Uilenberg reported that the vector of equine piroplasmosis is unknown and reported the presence *O. megnini* horses together with other tick species [73].

6. Human infestations

Presence of ticks in the human external auditory canal is a common parasitic otopathy reported in many parts of the world including South Africa [4, 74], Chile [3], the USA [75], Nepal [76], Malaysia [77], India [78], and Sri Lanka [79].

However, only few cases are presented with *O. megnini,* most of these are either associated with horse riding or grooming or farmers working closely with livestock [3, 7, 26, 71, 74]. The first record is in as early as 1917 [26]. Seven otoacariasis cases of *O. megnini* infesting human ear canal have been reported in New Mexico, USA [75]. More recent cases came from South Africa where a 15-year-old girl from Pretoria, a keen equestrian visited a riding school east of Pretoria and acquired infestation possibly while she was grooming or riding her horse [4]. Another case of 13-year-old girl reported with *O. megnini* inside her ear canal after a riding holiday in the Eastern Cape, South Africa [74]. Five patients visiting the Ear, Nose, Throat (ENT) clinic in Nuwara Eliya General Hospital in Sri Lanka reported having *O. megnini* in the ear canal. All these patients are horse riders or stable worker from a racecourse nearby [7]. In Turkey, *O. megnini* infections were reported from 29 females and 2 males between the ages from 17 to 72 years involved in agriculture and livestock mostly living in rural area but with no complications [80]. This tick was found in the ear of a woman who had the habit of basking in the sun on the lawn near a sheep shed at the Sheep Breeding Research Station in Sandynallah, India [18].

Tick paralysis is the most widespread and dominant form of tick toxicosis. Usually, the intra-aural tick infestation results facial paralysis, edema [81], otitis externa, bleeding [82], and acute labyrinthitis [83]. Human ear infestations by *O. megnini*, however, do not result in paralysis although irritation and pain was common [75]. However, there is one human case from 1958 reporting tick paralysis following infestation of *O. megnini*. Since having *O. megnini* inside the ear canal can be very painful in humans, development of the larva to next stage nymph is unlikely because the patient in this case becomes aware of the tick after the larva has engorged [84]. For *O. megnini* infestations, differential diagnosis should be followed whenever painful otitis externa with wax and debris is not responsive to conventional treatment [84]. Since the tick feeds intermittently and does not attach firmly, it may be easier to flush it from the ear canal unlike the hard ticks [84]. Tick inside the ear canal is removed using various methods. K.V.G Medical College, India recommends the use of turpentine and xyclocane prior to removing the tick [78]. However, the ENT clinics in general hospitals in Kandy and Anuradhapura in Sri Lanka use two different methods to remove the tick inside the ear canal [85]. In Anuradhapura General Hospital, lignocaine is used as a local anesthetic and the tick is removed immediately after using a suction pump. In Kandy General Hospital, glycerine is used to fill up the ear canal and followed by removal of the tick 2–3 weeks later by using a suction pump [85]. The method using local anesthesia is best for removal of *O. megnini* as the tick does not attach firmly and permanently but feeds intermittently.

In addition to the ear, the *O. megnini* has been found attached to other parts of the body. Larval *O. megnini* in the conjunctiva of a 2-year-old child's eye has been reported from Arizona, USA [2].

7. Control methods

Ticks can be controlled using acaricidal chemicals, natural repellants, and biological control agents. Application of synthetic acaricides: carbamate, organophosphate, synthetic pyrethroid, formamidine, macrocyclic lactone, and pyrazole have played pivotal role in controlling both soft and hard ticks in the world [86]. Combinations of hexachlorocyclohexane, xylol, and pine oil provide protection from *O. megnini* for a minimum of 17 days [87]. Using insecticide impregnated ear tags are shown to be effective [88]. A list of acaricide recommended for tick control including *O. megnini* is given in Spackman and Lloyd [89]. Feeding sulfur to calves

does not have any effect on controlling *O. megnini* in the ear canal of the host [90]. Moreover, ivermectin is effective for controlling arthropod pests of livestock, but it is not an effective control measure for nymphs of *O. megnini* in the ears of cattle and horses [61]. However, control of *O. megnini* is challenging, due to its nidicolous lifestyle, abundant progeny and site of attachment deep in the ear canal [41]. Although acaricides are the best control and eradication effort because they offer quick and cost-effective suppression of tick populations, long-term use has developed acaricide resistance in many tick species worldwide and thereby reducing their effectiveness in controlling ticks [91, 92], impaired environmental and human health with negative effects on non-target organisms, and poor quality in animal products (e.g., milk, meat, and hide; [93]). Regular monitoring of the ticks for development of resistance against the acaricides used is therefore important. Detection of resistance level of an acaricide in a tick population is important before applying it as a control measure. Susceptibility of larvae of *O. megnini* to four acaricides, Permethrin, DDT, Malathion, and Flumethrin, has been tested in an *O. megnini* population in the stabled horses in Nuwara Eliya racecourse in Sri Lanka [94]. Flumethrin is the most susceptible acaricide against *O. megnini*, while the presence of resistance for DDT and possible presence of resistance to other three acaricides tested have been reported. Prevalence of the mutations in the resistant gene/genes has to be investigated to conclude the extent of resistance in *O. megnini* for these chemicals.

Use of alternative and more sustainable control measures as biological control and host immunization are therefore increasing rapidly [95], and the application of acaricide substitutes such as the extracts of plants like *Azadirachta indica*, *Calotropis procera*, and *Nicotiana tabacum* [96] is also being promoted. Although plant extractions have been used in general tick control, there are no studies conducted specifically for *O. megnini*.

In the biological control of ticks, Samish and Rehacek [55] have listed three types of potential natural enemies including pathogens like bacteria, fungi, and nematodes that infect ticks, predators like birds and ants, and parasitoid dipterans and hymenopterans that deposit eggs on ticks. Later, Samish et al. [95] have shown that these natural enemies can be used as potential candidates in controlling some hard and soft tick species under field and laboratory conditions. Bacterial species such as *Rickettsia* sp., *Cedecea lapagei* sp., and *Proteus mirabilis*, which are pathogenic to *Dermacentor andersoni*, *Amblyomma hebraeum*, and *Hyalomma marginatum* [97] may change the tick behavior, interfere with the development, cause changes in salivary and ovarian tissues, and also induce abnormalities in subsequent generations. Among protozoans, *Nosema ixodis* and *Babesia bigemina* cause deaths and minimize egg production of *Rhipicephalus microplus*, respectively [98]. Six out of 57 major genera of entomopathogenic fungi are known to infect ticks [99]. Of these fungi, *Metarhizium anisopliae* and *Beauveria bassiana* are shown to be effective in controlling *R. microplus* and *Rhipicephalus appendiculatus* [55, 100]. Even though nematodes have been listed as potential biological agents against ticks, these pathogens have never been reported in ticks in nature. However, under laboratory conditions some nematodes infest *Rhipicephalus annulatus* [55].

The role of predators in controlling ticks has been well documented. So far, predator-tick relationship of 28 arthropod families has been recognized of which many are ants (*Hymenoptera*: *Formicidae*), followed by carabid beetles (*Coleoptera*: *Carabidae*) and some spiders (*Araneae*: *Lycosidae*; [101]). Other than arthropods, some vertebrates like the amphibians (*Bufo parcnemis*; [55]), birds (oxpeckers, egrets, domestic fowl; [95]), and mammals (shrews, rodents; [55]) occasionally feed on ticks. Among the ants, 27 species belonging to 16 genera including *Solenopsis* sp., *Pogonomyrmex* sp., *Iridomyrmex* sp., *Aphaenogaster* sp., and *Monomorium* sp. have been identified as potential biological control agents of ticks [55, 95]. They target different developing

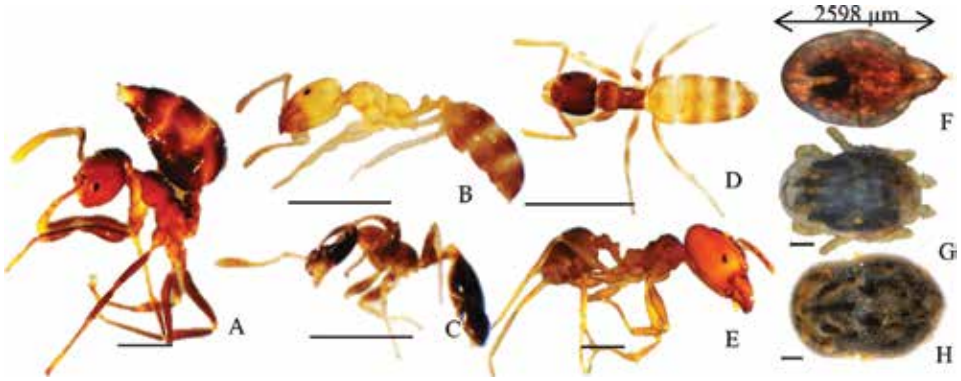

Figure 5.
Ant species that infest different life-history stages of Otobius megnini. (A) Crematogaster sp., (B) Monomorium sp. 1, (C) Monomorium sp. 2, (D) Tapinoma melanocephalum, (E) Pheidole sp., (F) engorged larvae, (G) nymph, and (H) adult of Otobius megnini. Scale bar represents 1 mm.

stages of the ticks. However, many of these ants occasionally target ticks as their main food source but are natural predators of tick species including *Argas miniatus* [102], *R. microplus,* and *R. annulatus* [102], *O. megnini*, and *Ornithodorus moubata* [53]. Five ant species, *Tapinoma melanocephalum*, two species of *Monomorium*, one species of *Pheidole*, and one species of *Crematogaster* feed on eggs fed and unfed larvae and adults of *O. megnini* (**Figure 5**) [103]. Among these, *T. melanocephalum* is the best predator as it feeds all free-living stages (eggs and adults) [103].

Among the opportunistic parasitoid dipterans, *Megaselia scalaris* and *Megaselia rufipes* (Family: Phoridae) have been identified infesting hard and soft ticks successfully [55, 95, 104]. *Megaselia scalaris* actively infests laboratory colonies of *O. megnini* [104] and other tick species [105]. It is a cosmopolitan fly, 2–3 mm long with medical, forensic, and veterinary importance commonly known as scuttle flies or hump-backed flies due to their erratic movement on surfaces and morphological features of the thorax, respectively (**Figure 6**) [104, 106]. These flies are capable

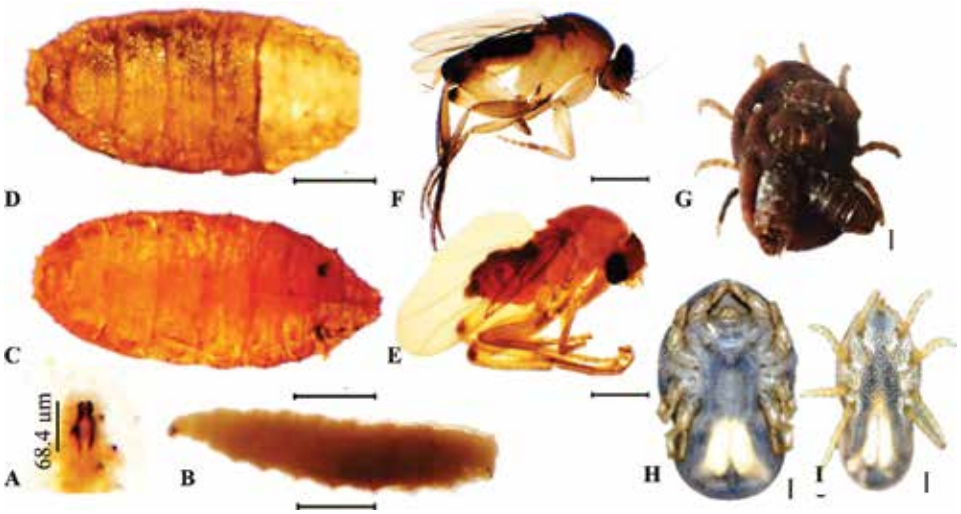

Figure 6.
Life-history stages of Megaselia scalaris and Otobius megnini. (A) Cephalopharyngeal structure of second instar larva, (B) second instar larvae, (C) pupa, (D) open pupal case, (E) female imago, and (F) male imago of M. scalaris. (G) O. megnini adult female with several pupa attached on dorsal side. (H) Ventral side of a healthy O. megnini female. (I) O. megnini nymph. Scale bars represent length of 1 mm.

of exploiting diverse ecological niches in tropics and subtropics [107]. *Megaselia scalaris* has adapted to polyphagous lifestyle, feeding, and breeding in wider spectrum of plant and animal matter [107–109]. They are attracted to putrid odors and lay eggs on decaying organic matter. The larva (maggot) undergoes two molts leading to three larval stages. Saprophagous (feeding on decaying organic matter), sarcophagous (feeding on flesh), and necrophagous (feeding on carrion) modes of feeding, as well as parasitic behaviors of *M. scalaris* larvae are well documented [107, 110]. Larvae of *M. scalaris* feed on larvae and nymphs of *O. megnini*, and when the development of the fly is completed, pupae attach to adult ticks, and all nymphs were found dead [103].

8. Conclusions

Infestation of *O. megnini* has become a problem worldwide. Controlling tick populations is hard because of its life cycle and seasonal dynamics and development of acaricide resistance. Among horses, *O. megnini* infests only well-groomed horses but not those with hairy ears. If the horses are left without trimming the hair in and around ears during racing off season, together with integrated pest control methods, the infestations can be effectively controlled and will alleviate the painful experience and other complications in the horse having the ticks inside the ear. The presence of many infectious agents has been detected in the tick; however, whether *O. megnini* acts as a vector or a reservoir in spreading the infection needing to be substantiated.

Conflict of interest

None.

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Chapter 6

Murine Gammaherpesvirus 68 (MHV-68), a Newly Discovered Tick Borne Virus

Marcela Kúdelová and Iveta Štibrániová

Abstract

MHV-68, closely related to human gammaherpesviruses (Epstein-Barr virus and Kaposi's sarcoma herpesvirus), is a natural pathogen of murid rodents commonly infested with ticks. After the first finding of MHV-68 in immature *Ixodes ricinus* ticks removed from wild green lizards, its occurrence was proved in free-living *Dermacentor reticulatus*, *I. ricinus*, and *Haemaphysalis concinna* ticks. Next, finding of live MHV-68 in salivary glands, intestine, and ovaries of *D. reticulatus* ticks strongly supported the idea that MHV-68 could be transmitted from infected to uninfected host via blood-feeding ticks. Recently, experimental transmission of MHV-68 between *I. ricinus* ticks and mouse and *vice versa* proved that MHV-68 could be vertically and horizontally transmitted from F0 to F1 tick generation, and thus, MHV-68 is a tick-borne virus (arbovirus). Therefore, ticks commonly attack humans transmitting important pathogens (e.g., tick-borne encephalitis virus and the Lyme disease spirochete); there is the speculation that MHV-68 can also infect humans *via* ticks. Earlier studies documented antibodies to MHV-68 in the sera of laboratory workers, hunters, and general population as well. In future, we need to carefully test whether people bitten by ticks are at real risk of infection with MHV-68 that normally infects murid rodents, and what effect it may have.

Keywords: MHV-68, gammaherpesvirus, blood-feeding tick, tick-borne transmission, arbovirus

1. Introduction

This review attempted to summarize the results of the work that contributed to the recognition of murine gammaherpesvirus 68 as a novel tick-borne virus in the context of to date known viruses found in ticks of species *Dermacentor reticulatus*, *Haemaphysalis concinna*, and *Ixodes ricinus* focusing on the territory where MHV-68 was first discovered.

2. MHV-68 and rodent gammaherpesviruses

Murine herpesvirus 68 (MHV-68 or γHV68) belonging to a group of dsDNA viruses of large genome was originally isolated from the bank vole *Myodes glareolus*

(formerly *Clethrionomys glareolus*) during a study on the ecology of arboviruses in Slovakia [1]. Four other murine gammaherpesviruses were isolated at the same time, two from bank voles and two from the yellow-necked field mouse *Apodemus flavicollis* trapped in west Slovakia. Later, three further gammaherpesviruses were isolated from the latter species in Bohemia and Slovakia [2]. Very early studies on murine herpesvirus neutralizing antibodies were identified in the sera of 20.7% individuals of five reservoir animal species (i.e., wood mice, bank voles, field voles, yellow-necked mice, and wild mice) [3]. By molecular methods, the presence of MHV-68 DNA was also confirmed in the blood of 34.4% of *M. glareolus* and *A. flavicollis* mice trapped in Slovakia [4]. Even, antibodies against MHV-68 have been detected in sera of at least 13 different mammalian species such as red deer (*Cervus elaphus*), fallow deer (*Dama dama*), hare (*Lepus europaeus*), wild boar (*Sus scrofa*), sheep, and foxes that share the biotope of infected rodents. Neutralizing antibodies to MHV-68 have also been detected in humans, but they are considered to reflect antigenic cross-reactions with human gammaherpesviruses (reviewed by [5]). Studies on MHV-68 *in vitro* showed that MHV-68 could replicate in as much as 16 cell cultures of different origins (e.g., mouse, chick, rabbit, hamster, mink, swine, monkey, and/or human origin, including T and B cells) [6]. The following molecular studies on MHV-68 genome including its full-length sequencing confirmed suggestions of a close genetic relationship of MHV-68 to primate saimiri herpesvirus-2 (SaHV-2) and human gammaherpesviruses—Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV) [7, 8]. During the 1990s, the infection of mice with MHV-68 and some other murine gammaherpesviruses inducing lymphoproliferative diseases (LPD) have been intensively studied (reviewed in [9]). Compiling all the results, in the year 2000, MHV-68 was classified into a new species, as murid herpesvirus 4 (MuHV-4) (synonyms murine gammaherpesvirus 68 and mouse herpesvirus strain 68), the genus *Rhadinovirus* and the subfamily *Gammaherpesvirinae* [10]. Its genome contains 118,237 bp of unique sequence flanked by multiple copies of a 1213 bp terminal repeat. Of the 80 ORFs identified in the MHV-68 genome, 63 are homologs of SaHV-2 genes, all of which are also present in the KSHV genome and many of which are present in the EBV genome [8]. The discovery of MHV-68 provided a tractable laboratory infection model for investigating human gammaherpesvirus reactivation from latency as well as host immune response mechanisms involved in persistent infection associated with the development of malignancies such as Burkitt's lymphoma, Hodgkin's disease, and Kaposi's sarcoma [11–13]. A huge work was done on MHV-68 that is recently the most amenable animal model virus for studying the pathogenesis of human gammaherpesviruses [14].

It has been speculated rodent gammaherpesviruses are geographically widespread and may occur throughout the mouse and vole subfamilies [15]. The epidemiological surveys in the UK, Germany, France, and Peru found several other gammaherpesviruses in free-living rodents. Two novel gammaherpesviruses were isolated in the UK and France, one from the wood mouse (*Apodemus sylvaticus*), designated wood mouse virus (WMHV) (classified as MuHV-7), and the other from the white-toothed shrew (*Crocidura russula*) designated Brest herpesvirus (BRHV) [16, 17]. WMHV showed similarity with MHV-68 in the growth in cell culture and pathogenesis in its natural host, and its complete genome sequence was determined [17]. In the UK and Germany, the first gammaherpesvirus infecting house mice, *Mus musculus* (*Mus musculus* rhadinovirus 1 [MmusRHV1]), was described as a member of a newly discovered group of rodent herpesviruses. This virus, designed as MHV-68-like rodent gammaherpesvirus, is distinct from MHV-68 the most

probably diverged from the other gammaherpesviruses soon after the evolutionary separation of EBV-like lymphocryptoviruses from KSHV 8-like rhadinoviruses [18]. To date, the latest rodent gammaherpesvirus was isolated from pygmy rice rat (*Oligoryzomys microtis*), designated rodent herpesvirus Peru (RHVP). Analysis of its full-length genome sequence confirmed that it shares conserved genes and genome organization with MHV-68 and the primate gammaherpesviruses but is phylogenetically distinct from MHV-68 [19].

Although the MHV-68 belongs to the best-characterized murine gammaherpesviruses and it has been documented that is mainly transmitted in the rodent population via intranasal routes and through body fluids, such as saliva, urine, tears, breast milk, and also vertically, it is not yet fully understood how this virus spreads in nature (reviewed by [9]). Following intranasal inoculation of laboratory mice, the virus spreads to the lungs, and viremia appears due to virus replication in the alveolar epithelium and endothelial cells of alveolar septa. The productive virus growth within lung epithelium ceases at 7–10 days p.i. During the viremic phase, mature B cells as well as macrophages become infected. At the acute stage, an infectious mononucleosis-like syndrome develops, analogous to that induced by EBV, associated with the establishment of MHV-68 latency and splenomegaly. After primary infection, the MHV-68 spreads to host organs via blood, in which it remains for roughly 15 days. As with other gammaherpesviruses, it causes lifelong infection of its host. It establishes a long-term latency not only in B lymphocytes (spleen, lymph nodes) and macrophages but also in lung endothelial cells that may lead to lymphoproliferative disorders (LPDs) [20–23]. Besides LPDs, also solid tumors (lymphomas) were described in infected Balb/c mice [23, 24].

Furthermore, as typical for all herpesviruses, based on various conditions (stress, gravidity, immune deficiency, and others), the virus can reactivate to a state of repeated lytic infection and reappear in host blood. Taking into account the properties of MHV-68, including its extreme stability at a wide range of pH and temperature values and nature of its spreading (via urine, breast milk, and other body fluids), a relatively high host reinfection rate should be considered [25, 26]. This suggests that MHV-68 can exist for a relatively long time in the blood of murid rodents, which undoubtedly feed hard ticks, including spp. *Dermacentor* and *Ixodes*. Both tick species mentioned, the most common in Slovakia, were identified as vectors of many tick-borne pathogens. Based on these data, a hypothesis was formed suggesting that blood-feeding ticks might transmit the virus from infected to uninfected animal host.

3. Ticks and tick-borne pathogens

Rodents, including *Apodemus* spp. mice and *M. glareolus* from which some murine herpesviruses were isolated, were found displaying the infection along with numerous pathogens, viruses, and also nonviral pathogens (bacteria, protozoa, and helminths) from the ticks which fed on them. Rodents play a role in the enzootic cycles of the so-called tick-borne pathogens that are transmitted from the ticks to vertebrates of which most have a life cycle that requires passage through the vertebrate host, thus being important reservoirs for these pathogens [27–30]. The most extensively characterized viruses that have rodent hosts in the family Muridae are the members of the family Herpesviridae. These include mouse cytomegalovirus and rat cytomegalovirus, which are classified in the genus *Muromegalovirus* of the subfamily *Betaherpesvirinae* and MHV-68 of the family *Gammaherpesvirinae*.

Hard ticks are highly specialized obligate hematophagous ectoparasites of wild and domestic animals and humans. There are over 900 species of ticks in the world, and many of them are capable to transmit disease-causing pathogens, including viruses, thus having significant medical and veterinary impact by causing serious diseases in humans and animals [31–33]. Less than 10% of known tick species were identified to act as virus vectors. Many unique features of ticks make them inevitably suitable to host and to carry different viruses as well as act as long-term virus reservoirs. Among hard ticks, virus vectors have been found mostly in the genera *Ixodes*, *Haemaphysalis*, *Hyalomma*, *Amblyomma*, *Dermacentor*, and *Rhipicephalus*. Moreover, some tick species are known to be vectors of a few TBV species (e.g., *I. ricinus*, *A. variegatum*), while others can transmit many different TBV species (e.g., *Ixodes uriae* is the vector of at least seven TBVs) [33, 34].

In Europe, there are two important hard tick spp., *Ixodes* and *Dermacentor* (Acari: Ixodidae) [35], which act both as important arthropod vectors and reservoirs for a series of wildlife zoonotic pathogens such as bacteria (e.g., *Rickettsia* spp., *Coxiella burnetii*, *Anaplasma phagocytophilum*, *Ehrlichia* spp., *Borrelia burgdorferi sensu lato*, *Francisella tularensis*, and *Bartonella* spp.): protozoa (e.g., *Babesia* spp.) [36–38]: and viruses (e.g., tick-borne meningoencephalitis virus, Colorado tick fever virus, Kemerovo virus, Crimean-Congo hemorrhagic fever virus) [39, 40].

Tick-borne viruses (TBVs) belong to the largest biological group known as arboviruses with unique mode of transmission by blood-feeding arthropods (ticks, mosquitoes, sand flies, biting midges, etc.) to a susceptible vertebrate host. They are different from other viruses in their ability to replicate in both vertebrate and invertebrate cells. Tick-borne viruses are causative agents of several important human diseases. Since the discovery of the first tick-borne pathogenic virus which was identified as being responsible for severe encephalitis in sheep in 1918 [41], diversified TBVs with global distribution have been discovered and isolated belonging to at least 2 orders, 9 families, and 12 genera [42]. Most of them belong to orders Bunyavirales and Mononegavirales and families of Flaviviridae, Asfarviridae, Reoviridae, and Orthomyxoviridae [43]. In recent years, the rapid development of next generation sequencing (NGS) has boosted the discovery of novel TBVs, many of them still unassigned to families (reviewed by [44]). At present, more than 16 specific tick-borne diseases (TBDs) of humans and 19 TBDs of veterinary importance have been described [42, 45]. The tick-borne encephalitis virus (TBEV) is the most medically prominent and important arbovirus (arthropod-borne virus) in Europe and Northern Asia, causing more than 10,000 clinical cases of tick-borne encephalitis annually. Other relevant tick-borne viruses which cause encephalitis in humans are the Powassan virus, Tribeč virus, Kemerovo virus, and Colorado tick fever virus. Of all the routes of human infection by tick-borne viruses, those of TBEV have been described in the most detail. The latest emerging TBD, caused by Bourbon virus, was reported in 2014 [46].

With two exceptions, all arboviruses are RNA viruses. The only established DNA tick-borne virus, African swine fever virus (ASFV), belongs to the Asfarviridae family with a single genus *Asfivirus* [47]. The ASFV genome consists of a single molecule of linear, covalently close-ended, dsDNA varying in length from 170 to 190 kbp. ASFV is the causative agent of African swine fever. ASFV is maintained in the sylvatic transmission cycle of ticks in Africa [48, 49]. Widely distributed ixodid ticks in Europe such as *I. ricinus* and *D. reticulatus* are unable to support ASFV replication and presumably do not contribute to disease spread [50]. The spread of ASFV has been primarily caused by human activities including long-distance transport of livestock. The presence of a susceptible wildlife host, wild boar, has further complicated efforts to control the disease, and it is likely that it will continue to

spread across the continent. Recent transmission studies have demonstrated the evidence for a role of the hard ticks *Amblyomma hebraeum* and *Rhipicephalus appendiculatus* in mechanical and transstadial transmission of the second DNA virus, lumpy skin disease virus (LSDV), a member of Poxviridae family of large enveloped viruses with linear double-stranded DNA [51, 52]. This virus belongs to the genus *Capripoxvirus*, causing lumpy skin disease of cattle in Africa and the Middle East [53, 54].

4. Findings of the MHV-68 in ticks

As mentioned above, MHV-68 is currently recognized as a natural pathogen of murid rodents that host blood-feeding ticks. The first evidence of MHV-68 in ticks was found in nymphs and larvae of *I. ricinus* feeding on 116 individuals of a temperate lizard species—the green lizard *Lacerta viridis* captured in the Slovak Karst National Park (48°57' N, 20°44′ E, ∼200 to 400 m above sea level). In this study [55], MHV-68 was detected in 10 of 649 nymphs and in 5 of 150 larvae, respectively. We found 15 of 799 (1.8%) nymphs and larvae as virus positive when 9.6% of green lizards fed at least one MHV-68-infected immature tick. These results provided two possible explanations. The first was that lizards could be infected via direct contact with jointly occupied holes and paths with rodents contaminated with infected animals. Although experimental data describing contact infection with MHV-68 *in vivo* are still missing, the routes of natural infection of murid rodents with this virus (intranasal or via body fluids) and relatively extreme stability of murine herpesvirus at wide range of pH and temperature [26] made contact infection with MHV-68 probable. The second possible source of MHV-68 infection of lizards represented feeding of infected hard ticks in the past (though in this study, no hard ticks were found on lizards). However, the following studies were evoked to obtain the evidence that the MHV-68 is able to escape from the gut after feeding and move through the tick to the salivary glands where it could be transmitted during a second feeding. Anyhow, finding of MHV-68 in immature *I. ricinus* ticks supported the hypothesis that ticks may play a mediating role in circulation of MHV-68 in nature.

In Slovakia, *Ixodes ricinus*, *Dermacentor reticulatus*, *Dermacentor marginatus*, *Haemaphysalis concinna*, *Haemaphysalis inermis*, and *Haemaphysalis punctata* tick species are common and widespread [56], where they had been found to be infected with numerous nonviral and viral pathogens [57–59]. In the following studies, three of the most common tick species in Slovakia were examined for the presence of MHV-68, *D. reticulatus*, *H. concinna*, and *I. ricinus*, to take a position on the hypothesis that the ticks could be a vector in the transmission of MHV-68 from infected wild mice to other mammals.

Dermacentor reticulatus (Fabricius, 1794) (Acari: Ixodidae) is the three-host meadow tick that parasitizes primarily wild and domestic mammals and, infrequently, humans. It is widespread throughout Europe and is expanding its range in several European countries [60, 61]. Recent comparative analyses have revealed changes in the distribution and abundance (almost doubled) of *D. reticulatus* ticks in some European countries, implying a higher risk of the transmission of tick-borne diseases. In Slovakia, the *D. reticulatus* tick had a focal distribution in Slovakia in the past [62], occurring mainly in the southwest and southeast along the Morava, Dunaj, and Latorica rivers. Of late, *D. reticulatus* has extended its former geographical distribution by at least 200 km further to the north and by approximately 300 m into higher altitudes up to 520 m above sea

level [63]. Rubel et al. [40] recently described geographical distribution of this tick in Europe.

D. reticulatus tick is associated with a number of different pathogens and currently considered the second most significant reservoir and vector of numerous pathogens causing bacterial, protozoal, rickettsial, and viral diseases in its hosts [38, 43, 64–68]. Its role in the transmission of disease to humans is currently small; however, it might play an important role in the maintenance of pathogens in enzootic cycles [69–72].

Until 2014, *D. reticulatus* tick was proven as a vector of only one viral pathogen, Omsk hemorrhagic fever virus, identified in Western Siberia [73]. However, two other viruses, Kemerovo virus and tick-borne encephalitis virus, have been identified in this tick collected in Western Siberia and Eurasia [74, 75]. In *Dermacentor* sp. several other tick-borne viruses were identified such as Colorado tick fever virus, Burana orthonairovirus, Lanjan virus, Razdan virus, Dhori virus, and Sawgrass virus (reviewed in [64]).

Kúdelová et al. [76] by nested PCR examined the presence of MHV-68 within a group of 432 adult *D. reticulatus* ticks collected near the river Dunaj in two sites in southwestern Slovakia from 2011 to 2014. Analyses showed MHV-68 positive as much as about 23.3% (28/120) and 40% (125/312) ticks from Gabčíkovo (47°54′00″ N, 17°35′00″E) and Vojka nad Dunajom (47°58′35″N, 17°22′50″E). The infecting virus was confirmed analyzing amplified products via sequencing. Thereto, the salivary glands, intestines, and ovaries of five females were examined for live MHV-68 using an explantation and cocultivation procedure used to achieve spontaneous reactivation of latent herpesviruses [77, 78]. These methods allowed to use tick organs to determine the presence of viruses capable of replicating in VERO cells and producing CPE. The VERO cells seemed to be a good choice because a relatively long cultivation was needed to properly detect the replication of the virus. A finite amount of virus from tick organs should be considered because it was impossible to predict virus dose in each tick. However, live MHV-68 capable of replication in mammalian cells was identified in all organs of two ticks (**Figure 1**) suggesting that MHV-68 found at least in salivary glands might be transmissible from infected to uninfected host. In the following study, MHV-68 was identified in *D. reticulatus* ticks collected from other two sites in Slovakia in 2014, while viral incidence in adult tick was 53.3% in a group of 30 ticks from Komárno (47°45′48″N 18°07′42″E) and 62.5% in a group of 40 ticks from Vysoká pri Morave (near the Morava river) (48°19′50.51″N, 16°54′15.38″E), respectively [79].

The next study on adult *D. reticulatus* ticks collected in Vojka nad Dunajom in spring 2013 provided the first evidence of MHV-68 transcripts in field-collected ticks suggesting that MHV-68 might replicate in their bodies. The transcripts of M3 gene (known to be expressed during both the lytic phase and latent infection of the animal host) were identified in as many as ten out of eleven questing ticks by nested RT-PCR method. As one might expect, the transcription of MHV-68 previously limited to evidence from tick organs after virus propagation in vitro was evidenced to have different amounts of M3 gene transcripts (**Figure 2**).

In this study, the amount of MHV-68 genome copies per *D. reticulatus* tick was identified in samples of 38 virus-positive ticks. An infectious dose of MHV-68 in ticks quantified by qPCR varied from 2.2×10^4 to 8.6×10^6 [80].

Haemaphysalis concinna (Koch, 1844) (Acari: Ixodidae) is the second most abundant tick species after *I. ricinus* collected from birds and third most abundant tick species flagged from vegetation in Central Europe. It is widely distributed in France, Germany, Poland, and alongside the rivers Danube and Morava in Hungary, Bohemia, Slovakia, and Austria, in Russia and temperate Eurasia, and in China [81]. In some areas of Slovakia, *H. concinna* has been found to co-occur with

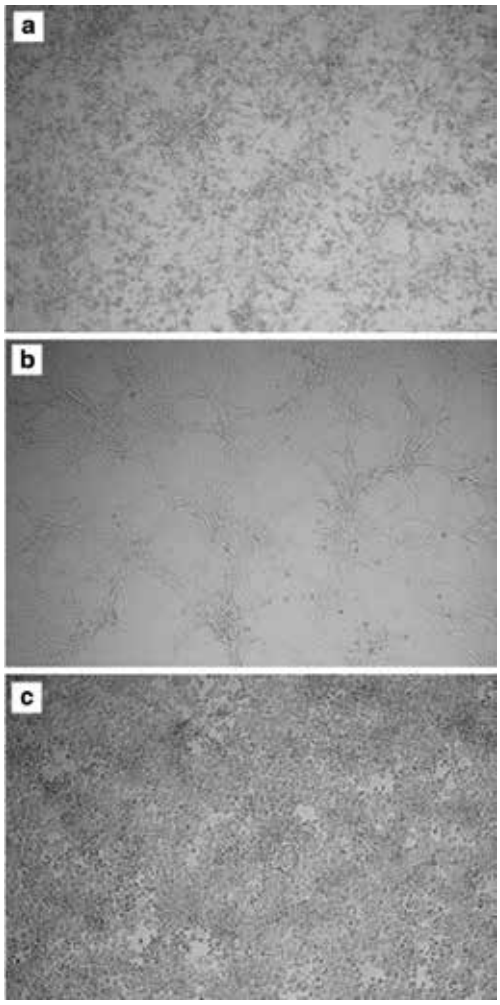

Figure 1.
Detection of infectious MHV-68 in the explanted salivary glands of the D. reticulatus *tick. Infectivity of MHV-68 as determined by plaque formation (CPE) in VERO cells 10 days after inoculation with explantation medium coming from salivary glands of tick No. 1 observed by light microscopy. Magnification, ×10, (b) uninfected VERO cells (negative control), (c) VERO cells infected with MHV-68 (MOI = 0.001 PFU/ml) (positive control); for details, see Kúdelová et al. [76].*

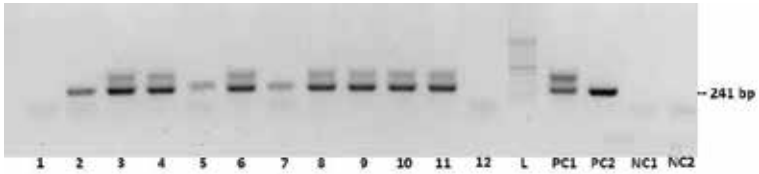

Figure 2.
Detection of M3 gene transcripts from MHV-68 in D. reticulatus *ticks collected in Slovakia in 2013 by nested RT-PCR. Lanes: 1–11—ticks nos. 49–59; 12—uninfected tick from the breeding station (negative control); L—100 bp plus DNA ladder (Thermo Fisher Sci); PC1—MHV-68 BAC DNA (nested PCR; positive control); PC2—MHV-68 BAC DNA (the first PCR with nested primers; positive control); NC1—no template (nested PCR; negative control); NC2—no template (the first PCR with nested primers; negative control); for details, see Kúdelová et al. [80].*

I. ricinus and *D. reticulatus* ticks which feed on small- and medium-sized mammals [82]. *H. concinna ticks* have been found to transmit nonviral pathogens such as *Coxiella burnetii*, *Borrelia* genus spirochetes, *Rickettsia* and *Babesia* spp., *Anaplasma*

phagocytophilum, and *Neoehrlichia mikurensis.* Two tick-borne viruses were established in *H. concinna* ticks, tick-borne encephalitis virus (TBEV), and Burana virus [83, 84]. In *Haemaphysalis* spp. also other viruses were identified such as Kyasanur Forest disease virus, three strains of Burana orthonairovirus, Nairobi sheep disease orthonairovirus, SFTS phlebovirus, Barur ledantevirus, Yongjia ledantevirus, New Minto virus, and to-date ungrouped viruses (Bhanja virus, Kaisodi virus, Silverwater virus, and Kwatta) [64]. In 2016, MHV-68 was firstly identified in adult *H. concinna* ticks collected in Gabčíkovo from May 2013 to May 2014. Virus incidence in ticks was 38.3% (18/47), and its genome copy number per tick varied from 2×10^2 to 9.6×10^3 [85].

The castor bean tick *Ixodes ricinus* (Linnaeus, 1758) (Acari: Ixodidae) is commonly found in Europe. Its distribution covers most of the continent, extending from Southern Italy up to northern Scandinavia. Rodents, natural hosts of MHV-68, are important hosts for several *Ixodes* ticks especially for larvae, to some extent for nymphs, and in the case of host-specific species also for adults. Therefore, following studies on MHV-68, a novel potential arbovirus concerned on *I. ricinus* ticks. As mentioned above, the first evidence of MHV-68 in ticks was found in immature *I. ricinus* ticks infesting *Lacerta viridis* green lizards, from which 1.8% (15/799) nymphs and larvae were virus positive [55]. It should be noted that ixodid tick species have multiple life stages with each feeding off a different host and often a different host species. *Ixodes* ticks are the most important arthropod disease vector, long been acknowledged as an important vector for a wide variety of pathogens of medical and veterinary importance, particularly the tick-borne encephalitis virus and the Lyme disease spirochetes of the genus *Borrelia* [86–88]. Their vectorial capacity is due to long-term coevolution with the pathogens that they transmit, an extended life span (up to years), and long-lasting blood feeding by all parasitic life stages [89]. *I. ricinus* ticks, considered as vectors and reservoir hosts, were collected from different localities in Slovakia [90]. As mentioned above, *Ixodes* ticks serve as reservoirs for a series of nonviral pathogens. Describing the results of their occurrence so far is beyond the scope of this review. One latest for all is recent study on diversity of *Coxiella*-like and *Francisella*-like endosymbionts, *Rickettsia* spp., and *Coxiella burnetii* in the tick populations, including *I. ricinus* ticks, collected in Slovakia [91]. Besides TBEV, several viruses were identified in *I. ricinus* ticks (e.g., Louping ill virus, Langat virus, Eyach virus, and at least 20 other viruses in *Ixodes* sp. ticks) [64, 92].

Recent studies assessing the occurrence of MHV-68 in *I. ricinus* ticks proved that nymphs collected from the vegetation (in Vysoká pri Morave; May 2014) [79] and also adult ticks could be infected with MHV-68. The viral incidence in adult ticks collected in the spring of 2014 near waterworks in Gabčíkovo was 38.1% (21/55), and the viral load varied from 1.5×10^3 to 2.85×10^4 genome copies per tick. These results suggest that the *I. ricinus* ticks became infected with MHV-68 from biting infected rodents; thus, *I. ricinus* ticks may also play a role in the spread of this virus in nature [93].

As described, there are a large number of pathogens found in ticks, but the low number of experimental transmission studies, that proved or disproved tick vector competence. To determine tick vector competence, the following conditions must be fulfilled: acquisition of the virus during blood-feeding on an infected host and transmission of the virus to a host by the tick after its molting to the next development stage. Vertical transmission of pathogens between generations of ticks has been observed (transovarial transmission) for viruses such as TBEV [94] and ASFV

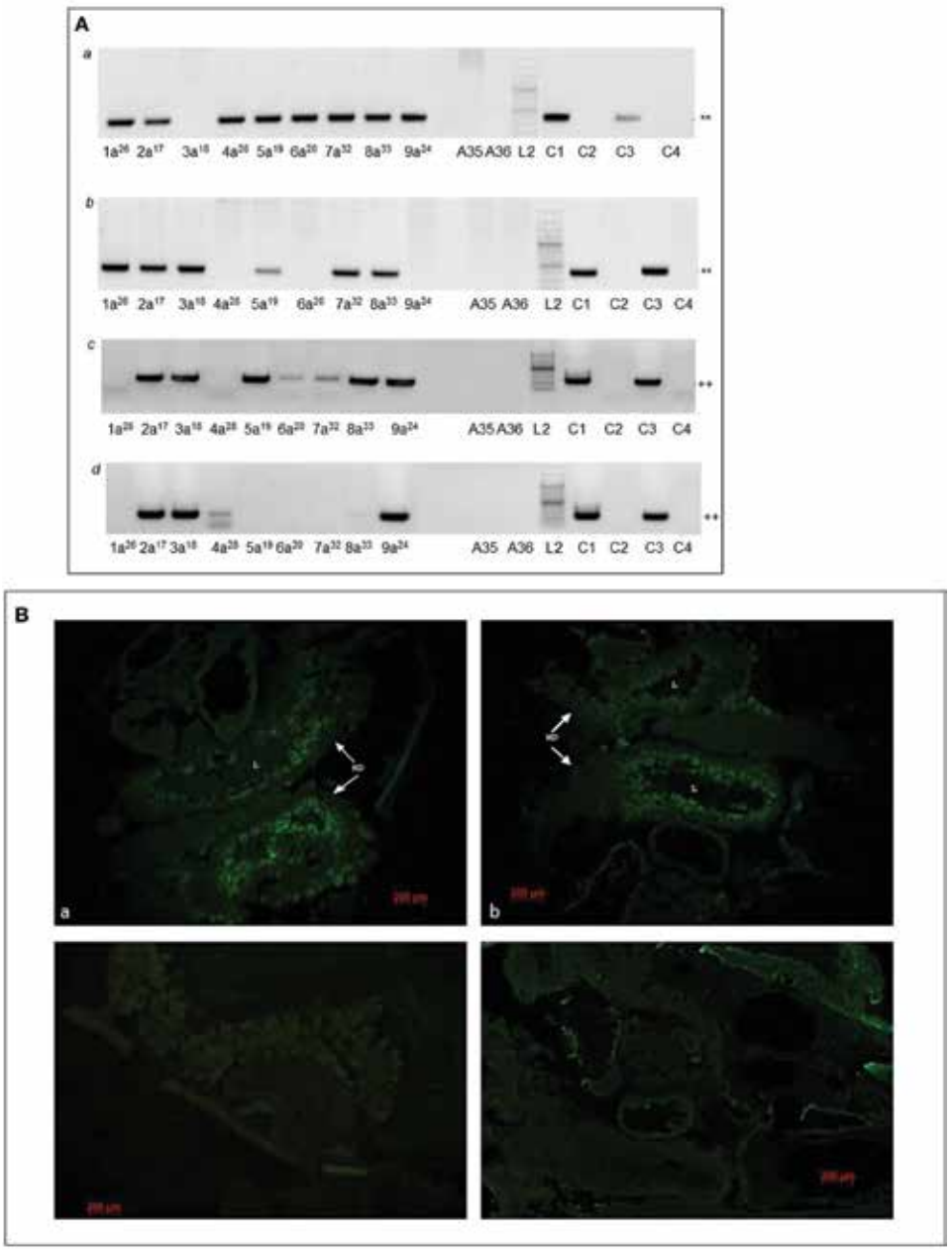

Figure 3.
MHV-68 detection in lung and spleen samples from mice infested with F1 infected adults and in F1-infected female ticks. (A) Lung (a,c) and spleen (b,d) samples of mice infested with F1-infected adults examined by nested PCR (a,b) and RT-PCR (c,d). Lanes 1a26, 2a17, 3a18, 4a28, 5a19, 6a20, 7a32, 8a33, and 9a24 samples of mice infested with F1-infected adult ticks; A35, A36, samples of control mice infested with F1-control adults. Lanes L2, C1–C4 as for ***Figure 1A****. ** Indicates MHV-68 ORF 50 gene nested PCR product of 382 bp; ++ indicates MHV-68 M3 gene nested RT-PCR product of 241 bp. (B) Semi-thin sections of frozen whole body of F1-infected females fed for 4 days. (a,b) F1-infected tick from mice 3a18 and 5a19 stained with anti-MHV-68 rabbit polyclonal serum; (c) uninfected tick (F6 generation of breeding) stained with anti-MHV-68 rabbit polyclonal serum; (d) F1-infected tick from mouse 3a18 stained with rabbit polyclonal serum against PB1-F2 protein of influenza virus A (H1N1) (negative control). MD, cells of midgut diverticula; L, lumen of midgut diverticulum. Scale bar, 200 μm; for details, see Hajnická et al. [96].*

[95]. Last but not the latest study of the MHV-68 in ticks submitted evidence of virus transmission via *I. ricinus* ticks. Hajnická et al. [96] studied experimental vertical and horizontal transmission of MHV-68 between *I. ricinus* ticks and their host—mouse—and vice versa investigating whether MHV-68 is a tick-borne virus. Uninfected *I. ricinus* ticks were shown to acquire the virus by feeding on experimentally infected laboratory mice. The virus survived tick molting, and the molted ticks transmitted the virus to uninfected laboratory mice on which they subsequently fed. MHV-68 was isolated from the tick salivary glands, consistent with transmission via tick saliva. The virus survived in ticks without loss of infectivity for at least 120 days and subsequently was transmitted vertically from one tick generation to the next, surviving more than 500 days. Furthermore, the F1 generation (derived from F0-infected females) transmitted MHV-68 to uninfected mice on which they fed, with MHV-68 M3 gene transcripts detected in blood, lung, and spleen tissue of mice on which F1 nymphs and F1 adults engorged. The presence of MHV-68 in the body of female tick of F1 generation was verified using anti-M3 monoclonal antibody (**Figure 3**). All results confirmed vertical transmission of MHV-68 in *I. ricinus* ticks. These experimental data fulfilled the transmission criteria that define an arthropod-borne virus (arbovirus).

5. Conclusions

Little is known of the natural history of MHV-68 that was discovered in 1980 to infect murid rodents trapped in Slovakia. About 20 years ago, the finding of neutralizing antibodies to MHV-68 in sera of at least 13 different mammalian species including humans sharing the same biotope with infected rodents gave rise to the hypothesis that MHV-68 might spread in nature also via tick biting. However, up to 10 years later, the first evidence of MHV-68 in ticks appeared, namely, in immature *I. ricinus* ticks, which feed on lizards. The following field studies have reported MHV-68 in free-living ticks of three species: *D. reticulatus*, *H. concinna*, and *I. ricinus*. They confirmed that MHV-68 belongs to few tick-borne viruses that have been detected in three tick species. Taking into account the nature and pathogenesis of MHV-68, it is not surprising that its incidence in ticks depends on, among other factors, in particular its incidence in natural host—murid rodents. Recent experimental transmission study submitted inevitable evidence that MHV-68 is capable of transmitting from infected to uninfected hosts via *I. ricinus* ticks; thus, MHV-68 is a novel arbovirus. This finding is of importance because herpesviruses were till now believed not to infect arthropods, and vector-mediated transmission of herpesviruses was unreported hitherto [97]. More interestingly, MHV-68 is the first herpesvirus and also a gammaherpesvirus among tick-borne viruses known to date. Further studies are needed to determine if neutralizing antibodies to MHV-68 detected 20 years ago in mammals sharing the same biotope with infected rodents are the result of tick-borne transmission of MHV-68 in nature and whether humans are at risk of infection.

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Section 3

Tick Economic and Control

Chapter 7

Economic and Health Impact of the Ticks in Production Animals

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Abstract

Nowadays there is no doubt about the importance of production animals in the economy and food security of the population throughout the world. For an animal to be productive (cattle, small ruminants, swine or poultry) is needed to be in adequate health conditions. The health of these animals can be altered by the direct and indirect effects of ticks, causing significant losses in the production of meat, milk, eggs, leathers, and in many cases the death of the affected animals. The direct losses are related to the damage produced by the ticks when feeding on the blood of their hosts, while the indirect losses are related to the infectious agents transmitted by the ticks, and the costs associated to the treatment and control. It is important then, to know what are the economic and health impacts of ticks on the main production animals.

Keywords: cattle, diseases, impact, poultry, small ruminants, swine, ticks

1. Introduction

Ticks are external, temporary and obligate parasites of vertebrate animals (birds, mammals and reptiles), which need to feed on blood in order to live. The hot and humid climates favor their survival, while the low temperatures inhibit their development [1]. Ticks belong to two main families, Ixodidae and Argasidae. The most important is the Ixodidae, also called hard ticks, due to the presence of a rigid chitinous shield, which covers the entire dorsal surface of the adult male. In the adult female and in the larva and the nymph it extends only by a small area, which allows the abdomen to swell after feeding. The other family is the Argasidae or soft ticks, so called because they lack of a shield [2]. There is a third family (Nuttalliellidae) to which only one species belongs [3].

Within the hard ticks *Ixodes* is the largest genus, which contains 217 species. Other genera of veterinary importance include *Dermacentor*, *Haemaphysalis*, *Rhipicephalus* (which now includes the genus synonym *Boophilus*), *Hyalomma* and *Amblyomma* (genus synonym *Aponomma*) [2]. On the other hand, the most important soft ticks belong to the genera *Ornithodoros*, *Argas* and *Otobius* [3].

Ticks are one of the biggest public health and veterinary problems in the world [4]. These ectoparasites can impact the production and health of the animals, either directly by the effect of their bites or by the infectious agents they transmit [1], which include viruses, bacteria, rickettsiae and protozoa [2].

Ticks and the pathogens they transmit have co-evolved in equilibrium with wild animals that serve as hosts, and reservoirs at the same time. Normally situations of instability only occur when these reservoirs come into contact with domestic animals, either by the introduction of uninfested animals to infested regions, or by the movement of infested animals to non-infested regions [3].

Ticks are periodically fed with blood, with long intervals between meals. When they bite their hosts, they injure the tissues of animals at their feeding site, causing irritation, inflammation or hypersensitivity [2]. Massive infestations of ticks can cause anemia, as a result of blood loss [5]. Each time a tick bites its host to feed it causes stress and weakens its immune response affecting its productivity, which results in losses in the production of meat and milk, increased morbidity and in many cases mortality, in addition to the indirect economic losses for producers related to prevention and control costs. Affected skin loses its commercial value [1].

Sites bitten by ticks cause lesions that may predispose to localized dermatitis, secondary bacterial infections, or invasion by flies (miasis) that are attracted to bloody areas [6]. Certain ticks contain paralyzing toxins in their saliva (for example *Dermacentor andersoni*, *Ixodes rubicundus*, *I. holocyclus*) that can even cause the death of affected animals. The saliva of *Hyalomma truncatum* can also cause toxicosis that manifests as widespread eczema in African livestock species [3].

The negative impact of ticks is especially important in production animals, and to a lesser extent in equines and companion animals, where pathogens causing tick-borne diseases can limit the international trade and the presence of ticks in sporting events. On the other hand, and not less important, is the role of ticks in the transmission of zoonotic diseases, which cause high morbidity and mortality in people [3]. In this regard, Betancur et al. [7] conducted a literature review highlighting the role of ticks in the transmission of zoonotic agents, and some prevention and control measures to protect the health and well-being of people at risk to get in contact with these ectoparasites.

2. Economic and health impact of the ticks in cattle

2.1 Direct and indirect losses in cattle

Babesiosis, theileriosis and anaplasmosis are the main parasitic diseases transmitted by ticks and that generate important economic losses in cattle production around the world [8], being especially relevant in different countries of Asia, Africa and Latin America [3].

Common signs associated with hemoparasitic disease are: fever, anemia, decreased appetite [9], reduction in milk production [9–11], lower weight gain [12, 13], loss of body condition, reproductive effects in males and females, abortions in the last third of gestation [9], lower pregnancy and birth rate [13], death in some animals [9–12].

Ticks affect 80% of the cattle population of the world. Specifically, *Rhipicephalus microplus* (formerly *Boophilus microplus*) is the tick that has the greatest economic impact [12], due to its wide distribution, vector capacity, blood-sucking habits and the number of cattle that affects [14]. Ticks usually prefer places on the body of animals where the skin is thin and short, and have abundant blood supply, such as the inguinal region and external genitals. Ticks grow and develop best in hot and humid climates [15]. Due to its great capacity for adaptation and propagation, ticks of the genus *Rhipicephalus* have been able to spread in various geographical areas around the world. Approximately 1 billion bovines are in areas at risk of being affected by these parasites [4].

The economic impact is strongly linked to the epidemiology of the disease and can be distributed in direct and indirect losses [9]. Its direct effect on production, results in damage to the skins by biting, especially in highly infested cattle [4, 11, 13, 16]; blood loss associated with high parasitic loads, anemia [4, 13, 16]; severe immunological reactions by the inoculation of toxins (antigens and coagulants in saliva) [4, 13]; permanent stress that affects the behavior and welfare of the animal [9, 13, 16] which also leads to depression of the immune function [17]; loss of energy associated with the constant movement that occurs in response to infestation [13].

Indirect losses are related to the effects of hemoparasites and other diseases that they can transmit [4, 9, 11, 17]. Other indirect losses correspond to the cost of treatment for clinical cases; expenses incurred in the control of ticks; unearned income or inefficiencies in the production system: use of genetically resistant breeds to ticks but less productive; confiscation by acaricide residues in meat or milk; trade restrictions of animals between areas and countries [9]. The economic losses by ticks include not only the price of animals of high genetic value, but the impossibility of these animals to contribute to the genetic improvement (productive potential) of an entire herd or even a region [18].

Betancourt [19], mentions that the losses caused by the infestation with *R. microplus*, the associated diseases and the control of it, have been calculated at USD $13.9–18.7 billion per year worldwide. In Colombia the losses could amount to COP $480,000 million per year (approximately USD $168 million). In Brazil, potential annual losses due to the infestation of *R. microplus* have been estimated in USD $3.24 billion [20]. The same exercise performed in Mexico, indicate losses of USD $573.61 million derived from the potential losses in meat and milk as a result of the infestation by *R. microplus* [21]. Another report estimates that the losses for Mexico are up to USD $942.23, not including the losses produced by the death of animals infected by hemoparasites, nor the expenses in medicines, which could double the annual losses [14]. According to FAO [22], the average total financial losses (production losses plus control cost) per animal per year are USD $7.3.

The effects of ticks on weight gains are quite negative. On average, each engorged female tick is responsible for the loss of 1.37 g of body weight in *Bos taurus* cattle. The comparable value for cattle *B. taurus* × *B. indicus* is 1.18 g per fattened tick [23]. It has been observed that animals infested with ticks reduce their feed intake (4.37 kg) compared to animals not exposed to ticks (5.66 kg). These effects cause losses of several billions of dollars in the global livestock economy [24].

The direct effect of ticks on dairy cattle can reduce total milk production by approximately 90 l/lactation/cow. Each fattened female tick can be responsible for up to 8.9 mL of milk reduction [25]. Other estimates indicate that losses in milk production reach 23% [10].

2.2 Tick-borne pathogens in cattle

2.2.1 Tick fever (babesiosis and anaplasmosis)

Rhipicephalus microplus, is considered the most important tick of cattle in the world, acting in the transmission of pathogens such as *Babesia bigemina*, *B. bovis* and *Anaplasma marginale* [17], developing the clinical disease known as "tick fever" [9]. This disease is endemic in tropical and subtropical areas [16]. These tick-borne hemoparasitic diseases affect the export and import trade of live animals and products of animal origin (meat, milk, leather and skin) [8]. Ticks negatively impact milk production in cattle, both in quantity and quality [1].

Bovine babesiosis is a disease that affects erythrocytes and is characterized by fever, hemolytic anemia, anorexia, lethargy, hemoglobinuria, tachycardia and icterus. In severe cases it can cause seizures, hyperesthesia and paralysis, which can lead to death due to shock and respiratory distress [26]. The two most important species in cattle are *Babesia bovis* and *B. bigemina* [27]. The disease caused by *B. bovis* is usually severe and a large number of sick animals die. The disease caused by *B. bigemina* is usually less severe but can develop very fast [16]. *Rhipicephalus microplus* is the most important and widespread vector, but in southern Africa, a closely related tick, *Rhipicephalus decoloratus*, interferes with its dissemination in drier and colder areas [27]. In Europe there is babesiosis caused by *Babesia divergens* that is transmitted by the *Ixodes ricinus* tick, which is restricted to that continent [9].

Bovine anaplasmosis is caused by *Anaplasma marginale*; affects erythrocytes and causes an acute infection characterized by fever, high levels of bacteremia, anemia, weakness, reduced growth and milk production, abortion and in some cases death [26]. The severity of clinical signs varies considerably, depending on the species and age of the infected animal, with adult cattle being the most severely affected [28]. It is an infectious disease but not contagious. *Anaplasma marginale* can be transmitted by three methods: biological: infected erythrocytes are ingested by ticks; *A. marginale* replicates within the intestine of the tick and the salivary glands and is subsequently transmitted through the saliva of ticks to uninfected ruminants; (ii) mechanical: infected erythrocytes are transferred from infected cattle to susceptible by biting flies or contaminated fomites with blood, including needles or surgical instruments, without this implying the amplification of *A. marginale*; and (iii) transplacental: the infected erythrocytes move through the placenta from the infected cows to their offspring, without the amplification of *A. marginale* [29]. Anaplasmosis is currently classified in List B of the Terrestrial Animal Health Code of the International Office of Epizootics [30] because of its socio-economic importance and its importance in terms of restrictions on the international trade of animals and products of animal origin [31].

It has been reported that at least 20 different species of ticks transmit *A. marginale* throughout the world. In general, tick vectors of *A. marginale* include *Boophilus* spp., *Dermacentor* spp., *Ixodes ricinus* and *Rhipicephalus* spp., while *Amblyomma* spp. they do not seem to transmit *A. marginale* [32]. The soft ticks *Argas persicus*, *Ornithodoros lahorensis* have also been mentioned as capable of transmitting them. The transestadial transmission is the usual mechanism of *Rhipicephalus* species of a single host. Tick males are particularly important as vectors, being able to be permanently infected and serve as reservoirs for infection [30]. Under favorable conditions of adequate vegetation and preserved moisture that protect ticks from drying out, male ticks can persist in the environment for several months to more than 1 year, thus serving as a reservoir of *A. marginale* in the wild [33]. *Rhipicephalus* species are clearly important vectors of anaplasmosis in countries such as Australia and countries in Africa and Latin America [30]. In North America, *A. marginale* can be transmitted by the *Dermacentor* tick species, including the ticks of three hosts as *D. andersoni*, *D. variabilis* and *D. occidentalis*, as well as the single-host tick *D. albipictus* [29].

Animals from childhood that have permanent contact with ticks, usually never develop a clinical episode of tick fever, but become carriers of *Babesia bigemina*, *Babesia bovis* and *Anaplasma marginale* subclinically [9], and therefore, livestock is immune to later challenges as adults. Cattle breeds that are indigenous to endemic regions often have a certain degree of natural resistance to the disease and the consequences of infection are not as severe as when they are exotic breeds of *Bos taurus* [27]. In situations of enzootic stability, when the animals through a natural selection process have become tolerant (but non-refractory) to the infection, as a

consequence of the prolonged exposure to the ticks and infectious diseases they transmit, and the number of ticks keeps in balance with the amount of animals, it is possible to find a 100% infection prevalence without clinical evidence of the disease [3]. The problem and the negative effect occur when tick populations increase and when it corresponds to the first contact with the hemoparasite. Thus, in situations of first introduction or enzootic instability and in susceptible animals, direct and indirect economic losses are greater [9].

2.2.2 Theileriosis

Another important disease that derives from the tick bite is theileriosis [10]. *Theileria* species of economic importance that infect cattle and small ruminants are transmitted by ixodid ticks of the genera *Rhipicephalus*, *Amblyomma*, *Hyalomma* and *Haemaphysalis*. The stages of development of the parasite occur in the tick and pass transestadially through the larval, nymph and adult stages, but there is no transovarial transmission. As a result, larvae or nymphs become infected and transmit infections such as nymphs or adults. Adults are more efficient vectors than nymphs [15]. Globally, *Theileria annulata* (cause of tropical theileriosis) and *Theileria parva* (cause of east coast fever) are the most economically important tick-borne pathogens that cause bovine theileriosis [34].

Tropical theileriosis is a risk to approximately 250 million cattle and acts as a major limitation in the production and improvement of livestock in many developing countries [8]. This disease causes high morbidity and mortality in exotic cattle, which inhibits the introduction of improved cattle in endemic areas. The consequence is that the quality of livestock in endemic areas remains low, which prevents the development of the livestock industry. *Theileria annulata* causes serious financial losses due to the decrease in live weight, a decrease in milk production, abortions and in some cases deaths, in addition to the high costs for treatment. The mortality rate in the introduced breeds fluctuates from 40 to 90%, while the mortality rate in native cattle can be only 3% [34]. It has been estimated a decrease in weekly milk production of 2.76 L/day/cow, which corresponds to 31.92% of total milk yield [35].

On the other hand, infection by *T. parva* represents a major threat to the livestock sector in two ways: through the economic impact of the disease due to livestock morbidity and mortality and production losses in all production systems, as well as the cost of measures to control ticks and disease [8]. Morbidity and mortality vary with host susceptibility and parasitic load. The lethality rate in untreated animals can reach 100% in cattle from non-endemic areas. In contrast, the morbidity rate is close to 100% in native cattle, but the mortality rate is usually low [34].

2.3 Control of ticks in cattle

In bovine cattle, the main tool for the control of ticks is still the use of acaricides (chemical control). Chemical control methods have the function of breaking the life cycles of ticks through the application of ixodicides [24]. However, for years it has been suggested that the exclusive strategy of chemical control is inadequate due to the possible development of resistance [36]. The incorrect use of pesticides such as the use of sub-doses, inadequate preparations and erroneous applications cause the failure of the treatment. With this, whenever the ticks survive the applications of the different products used, they transmit to the later generations genetic information about the active principle of the drugs, causing resistance to subsequent generations [37]. The excessive use, the incorrect dosage and the decrease in the

interval between the applications, has generated in addition to problems of resistance, the presence of chemical residues, both in the meat and in the milk, as well as the increase in production costs [38].

In order to reduce the possibility of resistance, Rodriguez-Vivas et al. [11] recommend an integral control of ticks whose strategies include: rotation of acaricides, with active ingredients that have different mechanisms of action, and without cross-resistance potential; correct application of acaricides, in recommended doses and intervals of time. According to Betancur [39], it is always recommended, to use products with proven effectiveness; in addition, the author recommend to reduce the selection pressure of the toxic compound on the pest species, in other words, the complete elimination of ticks in cattle by the pesticide should be avoided. Knowledge of the biology of the tick, its epidemiology, climatic conditions (such as soil temperature), as well as knowledge of the pesticide to be used, are necessary to understand the effectiveness of the products applied over time, and establish the best application strategies [40].

Different classes of acaricides (organochlorines, organophosphates, carbamates, pyrethroids and amidines) have been used successfully to control ticks in cattle, but some factors such as environmental damage, adverse effects on health (carcinogenic effects), as well as problems of resistance have caused that in some cases its use is limited [41]. The rational use of the traditional and new generation chemical molecules, through the correct dosage and rotation of the active ingredients on the market, allow extending the use of this control alternative, avoiding the resistance on the part of the ticks [38]. In order to improve the effectiveness of tick control, it is now possible to find on the market, products that mix different active ingredients. It is reported that some pesticides such as macrocyclic lactones (ivermectin, doramectin, moxidectin), fipronil, spinosad and fluazuron are very effective in ticks control [41]. Some studies have shown that fluazuron is a molecule with efficiencies greater than 99% in the control of the tick *Rhipicephalus* (*Boophilus*) *microplus*, without resistance problems [37, 42].

Other strategies of an integral control program of ticks are: manual elimination (only practical on farms with small number of infested animals); use of breeds resistant to ticks and the pathogens they transmit; release of sterile male ticks; sowing of plants that are unfavorable for ticks; rotation of pastures with forced breaks in order to interrupt the life cycle of the tick; burning of pastures, exposing the different stages of the ticks at high temperatures, and eliminating the vegetation that protects them; quality animal nutrition to improve resistance to ticks; use of plant extracts and essential oils with acaricidal activity; vaccination; biological control with nematodes, entomopathogenic fungi, ants, birds, among others [11]. The use of chickens as biological controllers of cattle ticks has been suggested, leaving them in the meadows where they consume the ticks that are found in the vegetation [43].

3. Economic and health impact of the ticks in small ruminants

3.1 Direct and indirect losses in small ruminants

Small ruminants are an important source of meat and milk in different countries and play a vital role in food security, in addition to the income earned from the sale of skins and wool. However, as with other species, ticks can limit the production systems of small ruminants, causing direct and indirect losses [44]. Although no tick is a specific host for sheep or goats, both hard and soft ticks parasitize these ruminants [45].

Some species of ticks cause paralysis while others cause toxicosis. Intensive lameness has been noted in the goats, where ticks adhere around the coronary band [46]. Ticks cause substantial financial losses in the livestock industry of some countries such as Ethiopia, for the damage to leathers and skins of sheep, goats and cattle. Lamb skins are particularly susceptible to damage. Secondary bacterial infection after tick bite increases the severity of the damage [47]. Some infestations by ticks such as *Otobius megnini* and *Ornithodoros coriaceus* can generate irritations and injuries at the ear level, which can lead to permanent nerve damage and death from meningitis [45].

Ticks generate indirect damage due to their key role in the transmission of a large number of infectious agents [44]. As mentioned in Bilgic et al. [48], in recent decades, the socioeconomic impact of small ruminants has grown worldwide, and therefore more attention is now being given to the pathogens that affect sheep and goats. As in the case of bovines, the main tick-borne diseases are babesiosis, anaplasmosis, theileriosis and heartwater [3]. Losses attributed to these diseases include mortality, production losses, diagnostic, veterinary treatment and control costs of ticks [48]. In China, it is estimated that *Anaplasma*, *Babesia* and *Theileria* species infect about 35 million of small ruminants. As the per capita economic loss of sheep or goats infected by these tick-borne pathogens is at least 2 USD, the total annual loss of small ruminants due to tick-borne diseases is estimated at around 70 million USD [49].

3.2 Tick-borne pathogens in small ruminants

The etiologic agent of ovine anaplasmosis in most cases is *Anaplasma ovis*. The disease is related to hemolytic anemia in goats and sheep. *A. ovis* is transmitted biologically by ticks of the species *Rhipicephalus bursa*, *Dermacentor silvarum*, *D. marginatus*, *D. andersoni* and *Haemaphysalis sulcata* [50]. In China it has been confirmed that *D. nuttalli*, *Hyalomma asiaticum* and *R. pumilio* are vectors of *A. ovis* [49].

The so-called tick fever in sheep is produced by *Anaplasma phagocytophilum*, whose symptoms include fever, neutropenia (predisposing to secondary bacterial and viral infections), cough, loss of appetite, fatigue, weight reduction and milk production loss. In goats, *A. phagocytophilum* can cause fever and a severe reduction in milk production. Complications often include abortions and alteration of spermatogenesis in rams for at least 2 months. In rare cases it is fatal unless there is a complication with other infections. *A. phagocytophilum* is transmitted by Ixodidae ticks. In Europe, is transmitted mainly by *Ixodes ricinus*, while in the United States the main vectors are *Ixodes scapularis* and *Ixodes pacificus* [50]. It is suggested that *Amblyomma maculatum* has the potential to transmit *Anaplasma* sp. in sheep [51].

Small ruminants are also affected by babesiosis caused by *Babesia ovis*, *B. motasi, B. crassa, B. foliata, B. taylori,* and *Babesia* sp. (China) [52]. *Babesia ovis* is considered highly pathogenic with mortality rates of 30–50% in susceptible sheep. Regarding to babesiosis caused by *B. motasi*, the parasite appears to be of moderate virulence, but it can be fatal. Ticks of the genus *Rhipicephalus* (especially *R. bursa*), *Haemaphysalis*, *Dermacentor* and *Ixoides* are responsible for the transmission of the disease [50].

Theileriosis in sheep and goats is a hemoprotozoan disease transmitted by ticks caused by *Theileria ovis, T. lestoquardi*, *T. luwenshuni*, *T. uilenbergi*, *T. recondita* and *T. separata* [52]. In susceptible sheep, the disease can be highly pathogenic, especially when it is caused by *T. lestoquardi*, causing a lymphoproliferative disease with mortality and high morbidity. *T. lestoquardi* can be transmitted by *Hyalomma* spp., and *Rhipicephalus bursa* [50]. According to Yin et al. [53], *Haemaphysalis qinghaiensis* efficiently transmit *Theileria* sp. to sheep and goats.

Heartwater is a rickettsial disease of domestic and wild ruminants caused by *Ehrlichia* (formerly *Cowdria*) *ruminantium*, which represents a significant obstacle to the improvement of livestock production in the tropics and subtropics with mortality rates ranging from 20–90% in susceptible animals. The organism is transmitted by ticks of *Amblyomma* spp., and small ruminants are particularly at risk of acquiring the disease [54].

3.3 Control of ticks in small ruminants

Control strategies against ticks should be aimed at cutting the biological cycle of these [44]. Although there are several useful options for the control of ticks by means of chemical products, it is difficult to achieve a long lasting control, for that reason it is suggested to consider an integrated approach that incorporates cultural, physical and chemical methods [45]. In small grazing units, ticks can be manually removed from the animals. Rotary grazing has been recommended as a means to control tick infestation. Although burning of heavily infested pastures is practiced in some countries, it is not widely recommended due to its damaging effects on the environment. Tillage of the grazing land exposes different stages of the ticks in the soil to sunlight and also buries them in deep layers of the soil thus preventing their development [46]. In most cases, the protection of sheep and goats from ticks still depends mainly on the direct application of acaricides to the animals. The treatment should be scheduled to protect the animals during the peak of tick activity [45].

4. Economic and health impact of the ticks in swine

Domestic pigs are also susceptible to tick infestation, however, under modern production conditions, they hardly come into contact with these ectoparasites. The most important species of ticks in the United States are *Dermacentor*, *Ixodes*, *Amblyomma*, *Ornithodoros* and *Otobius*. Its main economic impact is due to the ability to transmit pathogens, such as the African swine fever virus [55].

4.1 African swine fever

African swine fever is a viral disease that generates large economic losses in swine production, being transmitted by several species of soft ticks of the genus *Ornithodoros* [56]. This is the only known DNA virus that is transmitted by arthropods. The virus is endemic in many parts of the world [57], mainly spread in sub-Saharan Africa, Eastern Europe and the Caucasus and the Italian island of Sardinia [58]. African swine fever is a highly deadly and contagious hemorrhagic disease that restricts the international trade of pigs and their derivatives [59].

The virus is very well adapted to survive and persist in the tick, with minimal harmful effects on this host. The virus enters the tick when it feeds on an infected animal, then reaches the middle intestine where it replicates, then enters the hemocele and infects the major secretory gland, the salivary and coxal glands; finally when the tick feeds the virus is transmitted by means of the fluids of these glands [60]. Tick populations can remain infected and infectious for long periods due to transestadial, venereal and transovarial transmission of the virus in the tick population, which allows the virus to persist even in the absence of viraemic hosts. Infected ticks play an important role in the long-term maintenance of the disease, surviving for months in burrows and up to several years after feeding from an infected host [58].

All members of the pig family (Suidae) are susceptible to infection, but clinical disease is only observed in domestic and wild pigs, as well as in the closely related European wild boar. It affects pigs of all ages and induces a hemorrhagic fever. It can appear in a variety of forms ranging from peracute, acute, subacute, chronic and non-apparent. It is recognized more frequently in the acute form with an associated lethality of up to 100 percent [58]. The high lethality in domestic pigs, the introduction of mass slaughter campaigns and restrictions on swine movement contribute to the high socio-economic impact of the disease on swine production, global trade and livelihoods of the people. The impact is usually greatest for low-income farmers in developing countries, who depend on pigs as an additional source of income and a relatively cheap source of protein [61].

It is difficult to find global data on the economic costs of African swine fever and, therefore, estimates can vary substantially. As a result of outbreaks of African swine fever in 2014 and 2015 in Poland, Lithuania, Latvia and Estonia, the value of exports of pork and pork products was reduced by USD $961 million, which represents up to 50% of exports [61]. The introduction of African swine fever in Denmark could generate losses of USD $12 million in direct costs and USD $349 million in exports [62]. In Russia, it was estimated that African swine fever had cost USD $267 million in 2011. The further spread of African swine fever to China could have disastrous consequences, recognizing that China contains more than half of the world population of pigs [61].

When the populations of ticks are very low, they can be eliminated manually, removing the animals from the infected zone, and in other cases acaricides can be used [55]. In the case of African swine fever, as there is currently no vaccine or effective treatment, the best strategy for countries or areas that are still free of the disease is to prevent the entry of the virus through improved border control, adequate awareness and better biosecurity. For infected countries, improved awareness and biosecurity are also applied, along with rapid control of outbreaks through movement restrictions and sanitary slaughter policies [58]. According to Fasina et al. [63], a full implementation of biosecurity will result in a reduction of 9.70% in the total annual benefit, but is justified in view of the substantial costs incurred in the event of an outbreak of African swine fever.

4.2 Tularemia

Another disease that can be transmitted to pigs through tick bites is tularemia. This disease is caused by a bacterium (*Francisella tularensis*) and is zoonotic in nature. Ticks are true reservoirs, as well as vectors, and can transmit the bacterium to their offspring (transovarial and transestadial) or horizontally to other healthy hosts. Multiple species are included, particularly *Amblyomma americanum*, *Dermacentor andersoni*, *D. variabilis*, *Ixodes* spp. Repeated isolations of *F. tularensis* from ticks have been reported in the United States, Europe, Asia and Japan. In adult pigs the disease is usually subclinical, while in young, fever, dyspnea and depression are observed [64]. However, this disease may be unimportant in domestic pigs, while wild pigs behave as reservoirs of the bacteria, putting hunters and consumers of infected pork at risk [65]. Prevalence has been found in wild pigs of 1.3% [66].

5. Economic and health impact of the ticks in poultry

Ticks are associated with bird production systems. In modern poultry production, there are not many cases of tick infestation. The two most common species considered as pests of poultry are the ticks *Argas persicus* and *Argas radiatus*. Wild

birds are usually the source of infestation [67]. Most infestations occur in backyard birds, where the environment is more compatible with ticks. Adult soft ticks spend most of their lives in cracks and other hiding places outside the bird; their feeding habits on the bird are nocturnal, so that an infestation can be easily overlooked, and only a nocturnal inspection can make them noticeable [68]. At the opposite, larvae of *Argas persicus* adhere to poultry and feed for a few days [69].

The most important tick in poultry is *Argas persicus*, known as bird tick (sometimes called "blue bug"), although many species of hard ticks feed intermittently on poultry [68]. It is widely distributed in tropical and subtropical areas [70]. It affects poultry, turkeys, ducks, pigeons and canaries [71]. In commercial birds, infestations by *Argas persicus* occur with irritation, drowsiness, ruffled feathers, weight loss, decreased egg production, and anemia that can be fatal in heavy infestations [6, 70, 72]. The larval forms of these ticks also cause paralysis [73].

Insertion of the tick hypostoma into the skin of the host causes damage to the epidermis and rupture of the blood vessels. Tick bite causes skin damage consisting of edema, cell infiltration, and extensive hemorrhage. These injuries predispose the animals to decrease the absorption of food and lose body weight. In addition, the poor appearance of the carcass reduces marketability and this is a point that must be carefully considered in the poultry industry [74]. Soft ticks have many nymphal instars, each of which must be fed with blood, so repeated feeding by large populations of soft ticks can cause blood loss, wasting and deadly anemia [68]. Khan et al. [75], quantified up to 3.5 *Argas persicus* per bird, each sucking an amount of 18.57 mg of blood per day and 0.06 g per bird, which translates into huge economic losses due to production losses.

5.1 Tick-borne pathogens in poultry

It has been shown that the avian tick has the potential to transmit a significant number of pathogens in many parts of the world [68]. *Borrelia anserina*, *Staphylococcus aureus*, *Salmonella* Pullorum and *Escherichia coli* have been isolated from *Argas persicus*, and it is considered that they may play an important role in the epidemiology of these diseases [76]. Other microorganisms isolated include *Pseudomonas pyocyanea*, *Bacillus subtilis*, *Salmonella* Gallinarum, *Streptococcus gallinarum*, *Sporosarcina lutea*, *Serratia marcescens*, *Flavobacterium indothefcum*, *Bacillus anthracis*, *Aerobacter cloacae*, *Proteus vulgaris*, *Proteus rettgeri*, *Aerobacter aerogenes*, *Staphylococcus albus*, *Streptococcus zooepidemicus*, *Streptococcus pyogenes*, *Vibrio cholerae*, *Clostridium botulinum*, *Klebsiella aerogenes* and *Flavobacterium* spp. However, even when different pathogens can be isolated from ticks, not all are capable of promoting clinical disease in birds [77]. Different reports of literature mention that the ticks that transmit certain pathogens that cause diseases such as salmonellosis, mycoplasmosis, leukocytozoonosis, aegyptianellosis, pasteurellosis, avian encephalomyelitis, borreliosis and avian cholera [72, 75, 78].

Although salmonellosis is a disease of great impact in the poultry industry, and there are reports that indicate that *Argas persicus* has the possibility of transmitting at least experimentally *Salmonella* Pullorum [79], and *Salmonella* Gallinarum [80], vector role for ticks with respect to *Salmonella* remains speculative [81]. In any case, it is suggested to take sanitation measures that aim to eliminate ticks in poultry farms, since ticks are able to harbor these viable bacteria for 8 months, excreting it through the feces [79].

Argas persicus transmits *Borrelia anserina*, an important avian pathogen that causes spirochetosis [3, 69]. Spirochetosis has an important economic impact, since it causes a high mortality among birds that can reach up to 100%, in addition to its effect on the reduction of egg production in layers and the reduction of production

in broilers. The clinical signs of spirochetosis vary according to the virulence of the strain, but it is characterized by weight loss, drop in egg production, drowsiness, ruffled feathers, pyrexia, greenish diarrhea, pallor of the crest and chins, paralysis of the wings and lateral desquamation that is observed in the last stage of the disease [82]. The bird can also become infected as a result of ingestion of ticks, their eggs, contaminated droppings and cannibalism [83].

The adult argasides are highly resistant to starvation, which allows them to survive without feeding for more than 1 year in the absence of a host, which confuses the eradication of the infested facilities. All cracks and crevices that can harbor ticks should be thoroughly treated with an appropriate acaricide to successfully eliminate a tick infestation, and it may be necessary to repeat the treatment to suppress ticks that are born from the remaining eggs [68].

6. Economic and health impact of the ticks in equines and companion animals

Pets, particularly dogs, suffer the consequences of tick-borne diseases. Babesiosis and ehrlichiosis are the most important, being the infection by *Ehrlichia canis* frequently fatal [3].

As in cattle, ticks are an ectoparasite of sanitary importance in equines, due to their potential role in the transmission of pathogens. The *Dermacentor*, *Ixodes* and *Amblyomma* species are the most common hard ticks in horses. The severity of the symptoms will depend on the level of infestation, being able to develop a localized or generalized hypersensitivity reaction, in addition, at the bite site appear nodules, erosions, papules, scabs, ulcers and hair loss [84]. Two of the main diseases derived from tick infestation in horses are equine granulocytic anaplasmosis and equine piroplasmosis. The first has its origin in the bacterium *Anaplasma phagocytophilum*, while the second is caused by the hemoparasites *Theileria equi* and *Babesia caballi*. These pathogens have been detected in various parts of the world by molecular techniques [85]. The presentation of piroplasmosis generates a restriction in the international mobilization of horses, preventing their participation in sporting events [3].

6.1 Control of ticks in equines and companion animals

Tick control on dogs in particular is advocated by the use of acaricide-impregnated collars, whereas individual treatment for horses usually consists of synthetic pyrethroid pour-on compounds [3]. Numerous studies have been conducted to evaluate the efficacy of various acaricides such as amitraz, fipronil and permethrin against ticks infesting dogs. While product efficacy is often excellent in most studies, significant variation in efficacy can occur and 100% control is rarely achieved [86]. In recent years, some last-generation acaricides have come on the market, such as the lotilaner, which has been shown to be highly effective (up to 100%) and with excellent residuality for the control of ticks in dogs [87] and cats [88]. However, for best results, it is suggested restricting pet access towards tick infested environments [86].

7. Conclusions

Ticks are important ectoparasites that cause great economic and health losses in production animals, such as cattle, small ruminants, swine and poultry. The feeding

habits of ticks cause stress in animals affected by bites, blood losses that can lead to anemia and even death. Animals that are severely affected by ticks, or that do not have immunity against them or the infectious agents they transmit, decline in their capacity to produce meat, milk, eggs or leathers. The economic importance of ticks in equines and companion animals is relatively "minor", but its health impact is very relevant. Different tick control methods have been proposed, and the best approach is always an integral management that considers physical, chemical and biological controls. The present literature review can help professionals and producers to know, in a general way, what are the direct and indirect effects of ticks in animals, as well as the main infectious agents they transmit. It is recommended to deepen in each of the animals and ticks species, according to the needs of the interested people.

Conflict of interest

The authors declare that they have no conflicts of interest that may have inappropriately influenced them in writing this chapter.

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Chapter 8

The Enigma of Identifying New Cattle Tick Vaccine Antigens

Ala E. Tabor

Abstract

Several reviews have summarised cattle tick *Rhipicephalus (Boophilus) microplus* vaccine candidate discoveries by comparing efficacies and localisation characteristics. However, few have re-analysed all the reported proteins using modern bioinformatics tools. Bm86 was developed as a successful vaccine in the 1980s; however, global efficacies vary from 45 to 100%. Subsequent vaccines, including four published patents, were discovered by targeting enzymes important for blood digestion and/or metabolism or by targeting genes shown to disrupt tick survival following RNA interference experiments. This chapter analyses published vaccine candidates using InterPro, BLASTP, SignalP, TMHMM and PredGPI tools to confirm whether each reported protein is likely to be secreted, membrane associated or intracellular. Conversely, these proteins are considered as 'exposed', 'exposed' and 'concealed' or 'concealed', respectively. Bm86 was always described as a 'concealed' antigen; however, the protein has a confirmed signal peptide and GPI anchor which suggests it is anchored to the cell membrane and exposed on the surface of gut cells. It is the only tick vaccine with a GPI anchor. Secreted vaccine candidates appear to have promise and exhibit higher efficacies if delivered with an 'intracellular'/'concealed' antigen. Improvements in tick genomics and bovine immunomic resources will assist to identify robust new cattle tick vaccines.

Keywords: cattle tick, vaccines, bioinformatics, Bm86, review, *Rhipicephalus microplus*

1. Introduction

Cattle ticks (*Rhipicephalus* (*Boophilus*) *microplus*) and the diseases they carry affect almost 80% of the world's population of domestic cattle at an economic burden approximately $US 25–30 billion per annum [1]. The *R.* (*B.*) *microplus* taxonomic status is based upon *Cytochrome c oxidase I* (COX1) mitochondrial gene sequencing. There are three clades of *R.* (*B.*) *microplus*, plus *R.* (*B.*) *australis* and *R.* (*B.*) *annulatus* which are monophyletic with a different *R.* (*B.*) *microplus* clade [2, 3]. A recent study expanded this analysis and showed that *R. (B.) australis* is most similar to a large *R. (B.) microplus* clade (A) which has worldwide distribution, whereas *R. (B.) annulatus* is similar to *R. (B.) microplus* clade B predominantly from China [4]. An additional *R. (B.) microplus* clade C consists of Malaysian and Indian isolates [3]. Separation of species from several continents using morphological characters was not consistent with the above COI sequence clades and suggested that in some regions there exists a mixture of both *R. (B.) microplus* and *R. (B.) australis* [4]. It has been noted that more crossing studies need to be undertaken using geographically diverse wild strains

and preferably not 'inbred' colony isolates of *R. (B.) microplus* before conclusions on clades and species relationships can be confirmed. Publications and sequences reviewed here are most likely to be from different *R. (B). microplus* clades and *R. (B.) australis* but will be referred to collectively as *Rhipicephalus microplus*.

Regardless of the above seemingly complicated taxonomic status, the treatment of cattle tick infestations is either addressed by vaccination using Bm86-based vaccines: TickGARD^{PLUS} (now discontinued) or GAVAC™ and most commonly through the application of chemical acaricides [5]. Bm86 vaccines have diverse efficacies reported worldwide (45–100%), but in a few isolated countries, the vaccines have worked well apart from the need for multiple annual boosts to achieve adequate efficacies [1, 5, 6]. Ticks are also quite capable of developing resistance to acaricides; thus vaccine research continues globally [7] to identify conserved and immunogenic alternatives to Bm86.

The first notion that tick guts could be the source of viable tick vaccines was reported in 1979 [8] where native tick gut and organ extracts protected guinea pigs and cattle from *Dermacentor andersoni* ticks. The authors also suggested that this vaccine would affect tick feeding and reproduction and would be ideal for '*Boophilus microplus*' as all tick stages feed on the same host [8]. A gut protein named Bm86 was discovered in the 1980s as a protective antigen isolated from *R. microplus* in Australia [9]. The most notable characteristics at this time was the presence of epidermal growth factor (EGF) domains which are highly conserved extracellular domains associated with membrane-bound or secreted proteins (https://www.ebi.ac.uk/interpro/entry/IPR000742).

Bm86 is also a glycosylphosphatidylinositol (GPI)-anchored protein and as such is modified post-translationally [10]. It has been proposed that Bm86 is secreted and anchored to gut digestive cells through its C terminus [11]. Using immunogold labelling Bm86 was found to be located on the microvilli of gut digest cells [12]. The immune response induced by Bm86 was hypothesised to be mediated through host complement and anti-Bm86 antibodies which damage the tick gut surface affecting egg viability [13, 14]. However, the actual function of this tick protein has never been determined. Nonetheless, the early successes of Bm86 vaccines such as TickGARD^{PLUS} in Australia and GAVAC™ in Cuba provided researchers with the necessary fervour to identify alternative vaccine candidates to potentially be either 'broad spectrum' (i.e. cross protective for different tick species) or with a longer duration of immunity compared to Bm86-based vaccines.

2. Methods

Previously reviewed antigen types were summarised as 'secreted', 'intracellular' or 'membrane associated' [1]. In this review, each antigen was analysed in silico to confirm previously described localisations. Each ORF was submitted to InterPro to determine if the candidate antigen had domains or motifs representative of conserved protein families including the predicted GO Terms associated with 'biological process', 'molecular function' and 'cellular component' (https://www.ebi.ac.uk/interpro/) [15]. InterPro also predicts the presence of signal peptides and transmembrane helices; however these were examined separately using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/) [16, 17] and the TMHMM server v. 2 (http://www.cbs.dtu.dk/services/TMHMM/). GPI anchor predictions were undertaken using PredGPI (http://gpcr.biocomp.unibo.it/predgpi/pred.htm) [18]. The BLASTP server was employed to confirm published sequence identities (https://blast.ncbi.nlm.nih.gov/Blast.cgi). This analysis was limited to vaccine candidates reported as screened against *R. microplus* ticks in cattle challenge trials.

3. Results and discussion

Table 1 summarises BLASTP and InterPro analyses of published *R. microplus* recombinant vaccines which have cattle (or other ruminant) trial data. **Table 2** summarises localisations of these vaccine candidates analysed through SignalP, TMHMM and PredGPI and provides known trial data, references and patents (if applicable).

3.1 Secreted antigens

Most tested antigens are predicted to be secreted with no membrane-associated moieties (transmembrane helices or GPI anchors) (**Table 2**). The idea of selecting secreted proteins may have been cultivated to identify putative antigens that are more immunogenic in comparison to Bm86 and therefore boosted by natural tick challenge. The latter is usually associated with the injection of proteins by tick salivary glands. Studies have also shown that tick gut proteins also elicit host antibody responses; however perhaps gut protein-based vaccines are less immunogenic, that is, Bm86, which requires multiple annual boosts.

Two secreted proteins were also isolated from salivary gland and gut fractions similarly to how Bm86 was originally derived: **5′ nucleotidase** [19] and **Bm91 angiotensin converting enzyme-like** protein [20, 21]. However, neither demonstrated notable vaccine efficacies to warrant further development (**Table 2**).

In other studies, successful vaccine candidates were identified in other tick species, that is, *Ixodes ricinus* (sheep tick) **Ferritin-2** at 96% efficacy [22]. The researchers subsequently mined the *R. microplus* (BmGI) database for a *R. microplus* IrFerritin-2 homologue [22, 23], and RmFerritin-2 was patented at 64% vaccination efficacy [24]. Ferritin-2 was discovered in the sheep tick when studying iron homeostasis and it was found to be required for optimal tick feeding. In addition, unlike other tick ferritins, it was found to be unique without functional orthologs in vertebrate hosts [25].

Metalloproteases were targeted as vaccine candidates as these proteins were considered crucial for the maintenance of blood meal-related functions in other tick species [26, 27]. After an examination of five *R. microplus* metalloprotease GenBank sequences (AAZ39657.1-AAZ39661.1; Untulan et al., 2005, *unpublished*), it was found that Bmi-MP4 (AAZ39660.1) was expressed in female organs and male ticks and exhibited potential antigenic properties in comparison to other *R. microplus* metalloproteases [28]. A Bmi-MP4 metalloprotease vaccination study in Brazil yielded 60% efficacy as reported in 2015 [29], with no patent published (**Table 2**). A different Brazil-based study identified an unrelated **metalloprotease** Rm239/Sequence 82 (31% identity with Bmi-MP4, data not shown) as a component of a cocktail vaccine of four proteins achieving 73% protection in a tick challenge trial [30]. These proteins were identified through a salivary gland transcriptome study; thus in this instance the researchers were targeting secreted salivary proteins. Interestingly, the proteins selected were highly up-regulated in male ticks found on tick susceptible cattle which were not known to induce antibodies in naturally infected bovines [30]. Note that these two metalloproteases (Bmi-MP4 and Rm239/Sequence 82) and the Bm91 angiotensin converting enzyme-like protein described above all possess the GO:0008237 pertaining to 'metallopeptidase activity' (**Table 1**). As metalloproteases are members of a large protein family [31], this may lead to differences between strains or clades of *R. microplus* causing variable vaccination responses. Metalloproteases have been considered as vaccine candidates for other parasite species such as hookworm and human amebiasis, but no commercial products have emerged [32, 33].

Antigen description	GenBank accession/ BLASTP hit	InterPro analysis	GO term predictions (InterPro)[3]		
			Biological process	Molecular function	Cellular component
Secreted					
Angiotensin converting enzyme-like (Bm91)	AAB04998.1	Family peptidase M2, peptidyl-dipeptidase A	GO:0006508 proteolysis	GO:0008237 GO:0008241 metallopeptidase and peptidyl-dipeptidase activity	GO:0016020 membrane
[1]Extra-cellular matrix protein (Rm39)	No significant hit	No significant hit	—	—	—
Ferritin-2	CK190528[2]	Ferritin homologous superfamily	GO:0006826 iron ion transport GO:0006879 cellular iron ion homeostasis	GO:0008199 ferric iron binding	—
[1]Immunoglobulin G-binding protein C (Rm76)	AAB68803.1	GM2-AP, lipid-recognition domain superfamily	GO:0006689 ganglioside catabolic process	GO:0008047 enzyme activator activity	—
Metalloprotease Bmi-MP4	AAZ39660.1	Metallopeptidase homologous superfamily	GO:0006508 proteolysis	GO:0008237 metallopeptidase	—
[1]Metalloprotease (Rm239)	BAF43574.1		—	GO:0008237 metallopeptidase	—
5' Nucleotidase	AAB38963.1	5'-Nucleotidase/apyrase	GO:0009166 nucleotide catabolic process	GO:0000166 nucleotide binding GO:0016787 hydrolase activity GO:0016788 hydrolase activity-ester bonds GO:0046872 metal ion binding	—
[1]Proteinase inhibitor domain (Rm180)	XM_011553087.1	Pancreatic trypsin inhibitor Kunitz domain superfamily	—	GO:0004867 serine-type endopeptidase inhibitor activity	—
'SILK'	No significant hit	No significant hit	—	—	—
Membrane associated					

Antigen description	GenBank accession/ BLASTP hit	InterPro analysis	GO term predictions (InterPro)[3]		
			Biological process	Molecular function	Cellular component
Aquaporin	AIT69684.1	Aquaporin-like	GO:0055085 transmembrane transport	GO:0015267 channel activity	GO:0016020 membrane
Bm86/Bm95	M29321	EGF-like domains	—	—	—
Intracellular					
60S acidic ribosomal protein P0	AGQ49465.1	Ribosomal protein L10P	GO:0042254 ribosome biogenesis	—	GO:0005622 intracellular
Glutathione S-transferase *Haemaphysalis longicornis*	AAQ74441.1	GST, Mu class homologous superfamily	GO:0008152 metabolic process	GO:0004364 GST activity GO:0005515 protein binding	—
Subolesin and akirin chimeras	ABZ89745.1 AGI44632.1	Akirin protein family	—	—	—
Trypsin inhibitor 1-BmTI-6	P83606.2 CK186726[2]	Pancreatic trypsin inhibitor Kunitz domain superfamily	—	GO:0004867 serine-type endopeptidase inhibitor activity	—
Vitellin	AAA92143.1	Lipovitellin-phosvitin complex, lipid transport protein	GO:0006869 lipid transport	GO:0005319 lipid transporter activity	—

[1]*Four proteins conform a cocktail vaccination [30]; see* **Table 2** *for vaccine efficacies.*

[2]*Sourced from the BmGI database [23].*
[3]*Nil predictions denoted by a dash.*

Table 1.
Reported Rhipicephalus (Boophilus) microplus antigens with published vaccine challenge efficacies[1] analysed using InterPro (GO terms, domains, protein family identification) including relevant GenBank accessions[2].

Antigen description	Published efficacy[2]	Signal P[3]	TMHMM[3]	PredGPI[3]	References and patents[4]
Secreted					
Angiotensin converting enzyme-like (Bm91)	7% reduction egg viability	Secreted	—	—	[20, 21]
'Extracellular matrix protein' Rm39/ Sequence81[1]	~73% in mix of four proteins	Unknown	—	—	[30, 37][4]
Ferritin-2	64%	Secreted	—	—	[22–24][4]
Immunoglobulin G-binding protein C Rm76/Sequence76[1]	~73% in mix of four proteins	[1]Incomplete ORF (likely secreted)	—	—	[30, 37][4]
Metalloprotease Bmi-MP4	60%	Secreted	—	—	[29, 74]
Metalloprotease Rm239/Sequence82[1]	~73% in mix of four proteins	[1]Incomplete ORF (likely secreted)	—	—	[30, 37][4]
5' Nucleotidase	No protection	Secreted	—	Weakly probable	[19]
Proteinase inhibitor domain Rm180/ Sequence79[1]	~73% in mix of four proteins	[1]Incomplete ORF (likely secreted)	—	—	[30, 37][4]
'SILK'	62%	Secreted	—	—	[38, 39]
Membrane associated					
Aquaporin	73%	—	Four transmembrane helices	—	[40][4], [41]
Bm86/Bm95	45–100%	Secreted	—	Highly probable	[53][4], [75, 76]
Intracellular					
60S acidic ribosomal protein P0—peptide	96%	—	—	—	[55, 56][4], [77]
Glutathione S-transferase *Haemaphysalis longicornis*	57%	—	—	—	[60]
Subolesin and akirin chimeras	83% (deer) 60–75%	—	—	—	[39, 53][4], [76]
Trypsin inhibitor 1-BmTI-6	32%	—	—	—	[23, 67]
Vitellin	Native protein 68%, recombinant 0% (sheep)	—	—	—	[70]

[1]Four proteins conform a cocktail vaccination with 'Rm' names [30] and 'sequence' names from corresponding patent [37].

[2]Efficacy from cattle tick challenge trial unless otherwise stated in parentheses.

[3]Nil predictions denoted by a dash.
[4]Denotes published patent record.

Table 2.
Reported Rhipicephalus (Boophilus) microplus antigens with published vaccine challenge efficacies analysed using SignalP (secretion), TMHMM (transmembrane helices) and PredGPI (GPI anchor) including relevant references and patents.

The second protein in the above-described cocktail with Rm239/Sequence 82 metalloproteinase was Rm180/Sequence 79 which has a **proteinase inhibitor** domain (IPR002223: pancreatic trypsin inhibitor Kunitz domain) similar to a **trypsin inhibitor** on the 'intracellular' list (**Tables 1** and **2**), also tested in Brazil. Rm180/Sequence 79 in contrast is likely to have a signal peptide based on its top BLAST hit, and this new proteinase inhibitor does not appear to have any homology with known tick proteins (data not shown). Trypsin inhibitors are serine protease inhibitors potentially involved with tick blood meal digestion through the inhibition of trypsin (a serine protease which hydrolyses proteins).

The third protein within the cocktail was Rm76/Sequence 76 (also secreted) which is an **immunoglobulin G (IgG)-binding protein C** possessing domain IPR036846 ganglioside GM2 activator associated with lipid recognition function (**Table 1**). The top BLASTP hit for this protein is AAB68803.1 *Rhipicephalus appendiculatus* IgG-binding protein C at 88% identity. Tick immunoglobulin-binding proteins have been examined previously in several other tick species including *R. appendiculatus*, *Rhipicephalus haemaphysaloides* and *Ixodes scapularis* [34–36] and are thought to function as tick defences against host antibodies. Rm 239/Sequence 82 (metalloprotease) and Rm76/Sequence 76 (IgG-binding C) were shown to be the most immunogenic proteins in the cocktail vaccine based on antibody titres, predicted T cell epitopes and antibody boosting during tick challenge [30]. The fourth protein in this cocktail Rm39/Sequence 81 did not return any significant hits using BLAST or InterPro thus could not be examined using the parameters in the tables. The vaccine cocktail consisting of the trypsin inhibitor (Sequence 79), IgG-binding protein C (Sequence 76), metalloprotease (Sequence 82) and the unknown protein (Sequence 81) has been patented [37]. All sequences were published in the associated patent [37] without signal peptide regions.

'**SILK**' protein was predicted from an expressed sequence tag (EST) library prepared from male *R. microplus* ticks in response to *Anaplasma marginale* infection, and it was thought to be similar to arachnid flagelliform silk proteins [38]. However, no significant hits of the *R. microplus* EST to a 'SILK protein' sequence could be confirmed in this study. The protein has not been exploited further as an anti-tick or anti-*Anaplasma* transmission vaccine; however, at 62% efficacy [39] perhaps further study is warranted. No patent has been published.

3.2 Membrane-bound antigens

Apart from Bm86, the only other published antigen with a membrane association was **aquaporin**. Aquaporin does not have a GPI anchor as Bm86 but has four transmembrane helices predicted by TMHMM (**Table 2**). A reported 73% trial efficacy has been published and the data patented [40, 41]. The protein was identified in tick gut transcriptome studies and predictably functions as a water-conducting channel. An aquaporin was previously suggested as vaccine candidate for the human blood fluke *Schistosoma japonicum* with six predicted immunogenic epitopes and an integral membrane structure [42]. No further testing has been reported which is common for many human vaccine candidates. Perhaps the tick aquaporin vaccine will inspire further investigations of similar orthologs in human parasite infections.

Bm86 is thus the only protein with a confirmed GPI anchor that has been examined as a tick vaccine candidate. GPI-anchored proteins are conserved in eukaryotes and are luminal secretory cargo proteins with several functions across mammals and parasites [10, 43]. Notably, the *R. microplus* **5′ nucleotidase** (listed as a 'secreted protein') was predicted to have a 'weakly probable GPI anchor', and it is known that mammalian 5′ nucleotidases possess GPI anchors [10]. In terms

of vaccine candidates, GPI-anchored proteins have been investigated in several parasite species such as *Leishmania amazonensis* [44], *Plasmodium falciparum* [45], *Schistosoma mansoni* [46], *Theileria annulata* [47] and *Babesia bovis* [48] and have appeared to be associated with host invasion. In mammals, certain GPI-anchored proteins are cytokines with complement regulation functions [10]. Further studies pertaining to the discovery of tick salivary or gut proteins with GPI anchors have not been reported.

3.3 Intracellular antigens

Although Bm86 is cited as a 'concealed antigen' [49, 50], it appears to be a combination of 'exposed' and 'concealed' based on localisation predictions including a signal peptide (**Table 2**). Antigens in the 'intracellular' category do not have predicted signal peptides, GPI anchors or transmembrane helices and thus perhaps should be considered as truly 'concealed'. Several intracellular antigens have been investigated as tick vaccine antigens; however, results have been variable and seemingly dependent on delivery mechanisms as host antibodies need to target the protein that resides intracellularly.

Subolesin from the akirin protein family (**Table 1**) has been investigated in several tick species as a putative vaccine candidate [51] with the first *R. microplus* ORF described in GenBank as accession ABZ89745.1 (Shao et al. 2008, *unpublished*). Studies have confirmed that subolesin is involved in blood ingestion and reproduction in 2006 [52]; however, no predicted GO terms or other localisation predictions were identified in this study to confirm any of these putative functions (**Tables 1** and **2**). Subolesin was recently patented with Bm86 as a dual vaccine emulsion at a reported patented efficacy of 100% [53]. This dual vaccine is currently being testing by the CATVAC consortium in Morocco [7]. It is unknown if the varied efficacies of Bm86 will affect the activity of this dual vaccine or whether the short duration of immunity will continue to be an issue as for Bm86-based vaccines. Previously, a strong phenotypic knockdown of *Rhipicephalus sanguineus* ticks was observed using RNA interference through the silencing of subolesin and Rs86 (*R. sanguineus* Bm86 homologue) [54].

The **60S acidic ribosomal protein P0** has demonstrated 96% efficacy using a peptide fragment in cattle tick challenge trials in Cuba [55]. This is otherwise a conserved ribosomal protein, and the peptide region selected had significant sequence differences from the host ortholog. This vaccine has been patented and is under further trial testing also through CATVAC [7, 56]. Previously, gene silencing of this intracellular protein was found to be lethal to *Haemaphysalis longicornis* ticks [57]. Ubiquitin (also an intracellular protein) when silenced is also found to be lethal to *R. microplus* ticks [58] but was not found to be an effective vaccine candidate [59].

Haemaphysalis longicornis **glutathione S-transferase** (GST) showed some cross protection against *R. microplus* in a cattle trial [60]; however, further investigation as a tick vaccine candidate has not been reported. GSTs have been examined by several researchers as candidate parasite vaccines, for example, for hookworm, schistosomiasis and trichinellosis [61–63], at varying degrees of efficacy. GST proteins are considered as common 'housekeeping' genes forming a large protein superfamily present in eukaryotes and prokaryotes [64]. They function as detoxifying enzymes and thus in ticks may function in response to acaricides or in response to tick-borne pathogens and or stress [65, 66].

Trypsin inhibitors are serine protease inhibitors potentially involved with blood meal digestion as described above. A BmTI-6 sequence was identified in the BmGI database [23] and while native protein vaccine efficacies were high (73%), the corresponding recombinant protein efficacy was poor at 32% [67, 68] (**Table 2**).

This particular trypsin inhibitor is not predicted to be secreted (**Table 2**) thus may have a function different from gut digestion. The protein sequence reported by Andreotti et al. [67] is identical to BmTI-6 P83606.2 [69]. Alternatively, a 'secreted' trypsin Inhibitor showed promise within the cocktail vaccine described above [37]. As stated for metalloproteases, trypsin inhibitors are also members of large dynamic protein families which may circumvent host immune responses if administered as vaccines.

Vitellin was investigated as a native vaccine candidate showing some promise in sheep trials through a reduction in female ticks and their weights and a reduction in tick oviposition [70]. However, the recombinant form had no vaccine effect (**Table 2**), and no further studies were conducted. Vitellin is a high molecular weight yolk lipoglycoprotein, and in ticks and insects, it is synthesised in female fat bodies as a large precursor polypeptide—vitellogenin [70]. In insects, vitellogenin is processed into vitellin polypeptides by specific proteolytic cleavages during passage into haemolymph and/or upon receptor-mediated endocytosis by the developing oocyte [71, 72]. Tick vitellogenins are crucial for egg development and oviposition as demonstrated when silencing of three *H. longicornis* vitellogenin genes [73]. There are no reports of vitellin or vitellogenin as vaccine candidates in other species to date; however, this could be because they exist in arthropods (ticks and insects) rather than other 'pathogenic' species of parasites.

The investigation of intracellular vaccine candidates appears to less likely lead to a successful outcome. Perhaps some of these proteins could be delivered in dual emulsions as shown above for Bm86 and subolesin for a strong vaccination effect. It seems prudent to suggest that an intracellularly localised vaccine candidate requires a mechanism whereby host antibodies are able to access cells internally in order to be active against feeding ticks.

3.4 Other potential protein features

G protein-coupled receptors (GPCRs), also known as 'seven-(pass)-transmembrane domain receptors' are associated with many diseases and as such are the targets of several treatments. They are receptors for pheromones, hormones and neurotransmitters and could potentially be targeted as tick vaccine candidates [78]. Most literature associated with GPCR studies in ticks to date are acaricide-related and not associated with vaccines.

3.5 Protective immune response

The identification of tick vaccine candidates since the discovery of Bm86 appears to be haphazard in that selection has involved either targeting an enzyme involved with feeding or metabolism or to target a gene that showed diminished tick survival following RNA interference silencing. Neither of these approaches is directly linked to the development of a protective immune response which is fundamental for a protective vaccine. Many different experiments have been undertaken describing effective tick immune responses in different breeds of cattle including different mixtures of *Bos indicus* (naturally tick resistant) and *Bos taurus* (innately tick susceptible) cattle. These studies have also been undertaken in many different geographic regions with the use of highly divergent tick infestation protocols. The latter is particularly problematic where in some instances tick-naïve cattle cannot be sourced, and researchers treat the cattle for ticks prior to artificial tick infestations and subsequent immune studies. This topic has been reviewed in detail elsewhere and will not be repeated here [79]. The latter review summarised that there are different immune responses in tick-susceptible and tick-resistant breeds of cattle.

Perhaps different *R. microplus* tick vaccine candidates will need to be developed for different cattle breeds and crosses? Is the tick host response in tick-resistant breeds of cattle a result of superior immune function or genetic differences or a combination of both? One theory is that naïve tick-resistant breeds are readily primed with epithelial γδ T cells able to respond to larval ticks, whereas susceptible breeds need to recruit these T cells to the larval bite sites [80, 81]. This immune cell recruitment phase seems to manifest in an inefficient immune response in susceptible breeds. It has been a challenge to demonstrate this phenomenon in all immune studies due to the common practise of studying previously exposed cattle in several published experiments, reviewed previously [79].

3.6 Further research

Reverse vaccinology or genome-based approaches have been reviewed elsewhere, and promise in this approach has been reported [1]. Studies have used EST and transcriptome sequence databases to mine for potential tick antigens using a variety of approaches [1, 30]. Tick genomics has only recently become possible due to the availability of new 'long read' sequencing technologies and a dramatic decrease in the cost of sequencing large repetitive genomes [82, 83]. Bovine-specific immunology resources are also increasing [84, 85] with earlier research relying on human models for the major histocompatibility complex predictions. In combination with new genome sequences and bovine immunomic resources, a modern approach to identify robust tick candidates could perhaps finally be developed.

4. Conclusions

Although several approaches have been examined, one way to determine the true significance of a particular antigen or protein is to examine the current-published patents associated with cattle tick (*R. microplus*) vaccines. Upon examination of all patents and publications with cattle trial data to date, there are mixed features for *R. microplus* vaccine candidates with either secreted, membrane-bound or intracellular localisations which can also be described as 'exposed', 'a combination of exposed and concealed' and 'concealed, respectively. Intracellularly localised antigens are truly 'concealed' and in comparison to 'secreted' antigen types have highly variable outcomes. The key to identifying efficacious vaccine candidate(s) is to determine how best to stimulate a long-term protective immune response. This may also be feasible through new vaccine delivery options such as nanotechnologies or liposomes which may enhance the immunity to previously identified vaccine candidates.

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It is vital to understand ticks and tick-borne pathogens as well as their impact on humans. This book is intended for students in parasitology, biologists, parasitologists involved in molecular diagnostics of tick-borne diseases, practicing veterinarians, and for others who may require information on ticks and tick-borne diseases. Here we have put together a collection of chapters focused on different aspects of ticks and tick-borne diseases mainly to provide the reader with novel information in the field, but not the basic generalised information provided by many textbooks. This book includes topics such as high-throughput technologies in diagnosis, discovery of novel tick vaccines, identification of new pathogens transmitted by ticks, and new epidemiological information of certain well-known ticks and tick-borne diseases. These chapters were authored by parasitologists from all over the world, giving an insight to the reader about significant ticks and tick-borne diseases prevalent in those particular geographical regions with the local expert's point of view. Each of the chapters has separate reference lists, making it easier for the reader to find additional reading material related to their topic of interest.

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