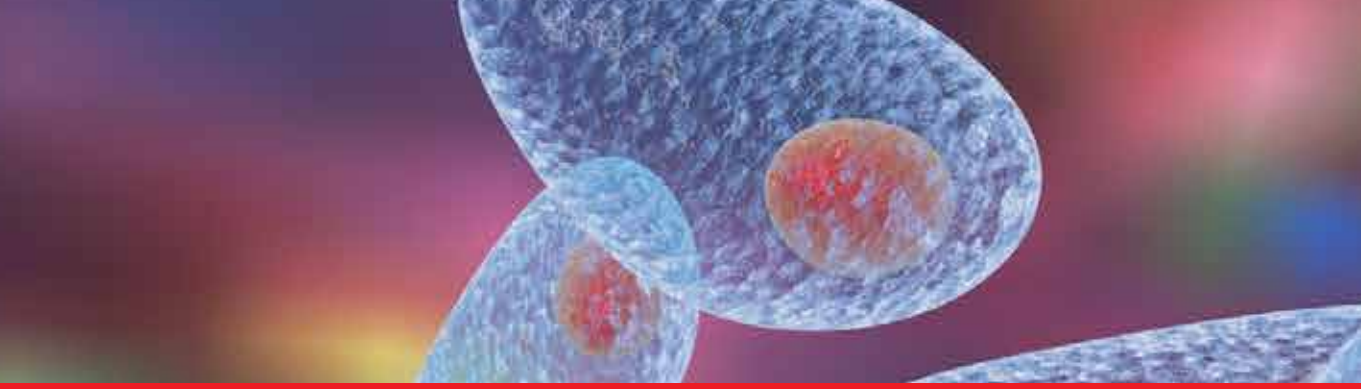

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Toxoplasmosis

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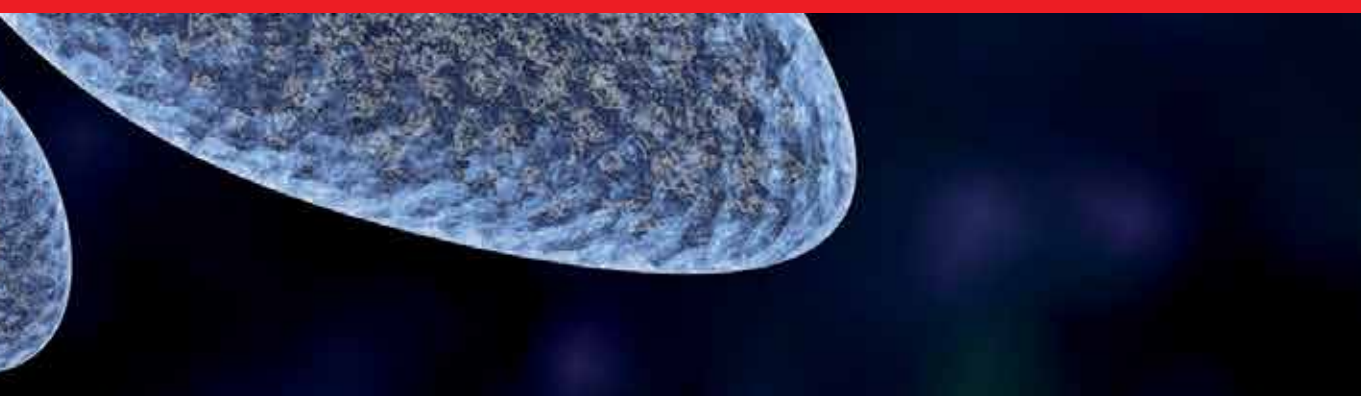

TOXOPLASMOSIS

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Meet the editor

Dr. Isin Akyar graduated from Cukurova University School of Medicine, Adana, Turkey, in 1989. She completed her Medical Microbiology specialty training in Gazi University School of Medicine Department of Microbiology and Clinical Microbiology in Ankara, Turkey, in 1999. She joined Acibadem Labmed Clinical Laboratories in 2004. She became coordinator of Microbiology Services in 2007. In 2008, she joined Acibadem University School of Medicine Department of Medical Microbiology in Istanbul, Turkey. Since 2014, she is working as an associate professor. Her special interests are laboratory quality control, molecular microbiology, parasitology, mycobacteriology, and proteomics studies. And also she is interested in developing rapid diagnostic tests. Currently, she is serving both as a microbiology coordinator in Acibadem Labmed Medical Laboratories and an associate professor in Acibadem University School of Medicine Department of Medical Microbiology.

Contents

Preface

Toxoplasma gondii, a ubiquitous protozoan parasite, was discovered 100 years ago. Although toxoplasmosis is a worldwide matter, approaches to diagnostic strategies considerably differ among countries. Its wide distribution may be attributed to complex transmission patterns and parasite coevolution with multiple hosts. Although *T. gondii* infections of immunocompetent people are generally considered asymptomatic, infections in immunocompromised individuals, such as those with AIDS or organ transplant recipients, can result in severe consequences. Diagnosis of toxoplasmosis had been established by several methods including the isolation of *T. gondii* from blood or body fluids, demonstration of the parasite in tissues, detection of specific nucleic acids with DNA probes, and serological tests for detection of *T. gondii*–specific immunoglobulins synthesized by the host in response to infection.

This book, composed of a series of articles, from diagnosis of laboratory in toxoplasma infection to the microparticle vaccines, presents a wide open point of view for toxoplasmosis.

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Introduction

Chapter 1

Toxoplasma gondii Tissue Cyst: Cyst Wall Incorporation Activity and Matrix Cytoskeleton Proteins Paving the Way to Nutrient Acquisition

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Marialice da F. Ferreira-da-Silva,
Erick V. Guimarães and Helene S. Barbosa

Additional information is available at the end of the chapter

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Abstract

Toxoplasma gondii is an intracellular parasite that causes chronic infection by the development of bradyzoites housed in tissue cysts, preferably in the muscles and central nervous system. The composition and the function of the cyst wall are still not fully understood. Are *T. gondii* cysts able to incorporate nutrients through its wall? If so, how would these nutrients be traversed to cross the cyst matrix to reach the bradyzoite forms? Herein, we tested the uptake capacity of the Toxoplasma tissue cyst wall by employing some fluid-phase endocytosis tracers as peroxidase (HRP) and bovine serum albumin (BSA). Fluorescence images revealed these molecules on the cyst wall as well as in the cyst matrix. The subcellular localization of the tracer was confirmed by ultrastructural analysis showing numerous labeled vesicles and tubules distributed within the cyst matrix in close association with intracystic bradyzoite membrane, suggesting the cyst wall as a route of nutrient uptake. Furthermore, we confirmed the presence of cytoskeleton proteins, such as tubulin, actin, and myosin, in the tissue cyst matrix that may explain the nutrient input mechanism through the cyst wall. A better understanding of the nutrient acquisition process by the cyst might potentially contribute to the development of new therapeutic targets against chronic toxoplasmosis.

Keywords: *Toxoplasma gondii*, tissue cysts, endocytosis, macromolecules, cytoskeleton

1. Introduction

Toxoplasmosis is a worldwide human protozoan infection caused by an intracellular protozoan, *Toxoplasma gondii.* Characterized by the presence of slow replicative bradyzoites surrounded by a thick wall, the tissue cysts have a striking predilection for infecting the central nervous system and skeletal muscle cells of the host [1]. It is widely accepted that the chronic phase of the disease in an immunologically competent person is normally asymptomatic and self-resolving. However, *T. gondii* has been associated with an increase in the incidence of many relevant psychiatric disorders. This relates to the fact that the brain is an immune-privileged site for lifelong existence of *T. gondii* tissue cysts [2]. Although the current pharmacological treatments have been ineffective against the cyst form, it is possible that the tissue cyst wall acts as a biological barrier preventing the interaction of drugs with intracystic bradyzoites [3].

Despite the clinical relevance of chronic toxoplasmosis, the biology of the cyst wall as yet has not been completely elucidated [4–9]. A 116-kDa glycoprotein termed CST1 [7] and two lectins, *Dolichos biflorus* and succinylated wheat-germ agglutinin, that bind specifically to the cyst wall, were identified, and it was suggested that chitin probably represents an important component of the cyst wall [10]. There are few evidences of the composition of the cyst matrix. Parmley et al. [11] described a cyst matrix antigen named MAG1, which was also found inside the parasitophorous vacuole matrix, forming a filamentous-like protein material.

Our previous ultrastructural analysis demonstrated that the cyst wall displays endocytic activity through the engulfing of negatively charged molecules in the cystic wall [8]. These molecules are incorporated by tubules and vesicles formed from the membrane that delimits the cyst wall and localized in the granular region and posteriorly in the cyst matrix. Within the cyst, the presence of vesicles containing the tracer in close contact to the bradyzoite membrane or in its neighborhood suggests that it could be one of the incorporated molecule pathways from the host cell cytoplasm to intracystic parasites [8]. The current knowledge of mechanisms involved in the process of nutrient uptake by this parasite still presents many gaps, restricted to few reports [12, 13]. As an obligate intracellular parasite, *T. gondii* actively invades and replicates in a wide variety of nucleated vertebrate cells where it resides in a parasitophorous vacuole. The ultrastructural analysis also demonstrated that *T. gondii* is able to invade host cell nuclei, and this invasion could reflect an alternative parasite route for immune evasion or may be a nutrient source when scarce in the host cell cytoplasm [14]. The vacuole membrane is the interface between the host and parasite, playing a role in the nutrient acquisition from the host cell by the parasite. The physiological significance of the endocytosis in the *T. gondii* lifestyle with tachyzoite forms is in the incipient stages of elucidation [15, 16], as the basic mechanisms delineating endocytosis in tachyzoites, bradyzoites, and tissue cysts are still poorly understood [17].

In all eukaryotes, endocytosis and intracellular vesicle traffic are events mediated by several proteins including a complex cytoskeleton network. Cytoskeleton proteins are indispensable components of a number of vital parasite structures and functions, like cell division, membrane and cytoplasmic architectures, parasite gliding motility (glideosome), and host-cell invasion [18–20]. Besides conventional cytoskeleton elements, many components of *T. gondii* are novel proteins. Two examples are Actin-Like Protein 1 (ALP1), which plays a role during

cell division [21], and a protein called TgSIP, a structural cytoskeleton component of the parasite inner membrane complex [22].

Herein, the putative mechanisms employed by *T. gondii* cysts involved in nutrient incorporation and the cytoskeletal protein network in the tissue cysts were investigated, which could explain the endocytic activity of the tissue cyst wall and vesicle traffic in the cyst matrix. The endocytosis process and the cytoskeleton network are crucial to *T. gondii* replication, motility, invasion, and maintenance, indicative as excellent drug targets for antiparasitic therapies.

2. Experimental design

2.1. Parasites

Mice infected with ME-49 strain *T. gondii* and maintained for 4–8 weeks in C57BL/6 were euthanized and the brain cysts isolated as described [23]. License CEUA LW 50/14 from Fundação Oswaldo Cruz Committee of Ethics for Use of Animals was authorized.

2.2. Endocytosis assays

Cysts were incubated with bovine serum albumin (BSA) labeled with fluorescein isothiocyanate (BSA-FITC), a fluid phase endocytic tracer, for 2 or 3 h at 37°C and processed for analysis in a confocal laser scanning microscopy FV300/BX51 Olympus and differential interference contrast (DIC) microscopy. Serial optical sections of each cyst incubated with BSA-FITC were converted into a volume performing 3D reconstruction. Additionally, the ultrastructural analysis was performed with two fluid phase endocytic tracers, BSA and peroxidase (HRP), both conjugated with colloidal gold (Au) particles.

2.3. Identification of cytoskeleton proteins

Brain cryosections of *T. gondii*-infected mice as well as *ex vivo*-isolated tissue cysts (pre-fixed with 4% PFA) were processed for immunolabeling. Briefly, the samples were incubated with polyclonal primary antibodies (anti-actin 1:500, anti-tubulin 1:200, anti-myosin 1:200, and microtubule-associated proteins (MAPs) 1:200), followed by incubation in CY3-conjugated secondary antibody (goat anti-rabbit IgG, 1:1000) and processed for fluorescence microscopy analysis. Control samples were processed omitting primary antibodies. Brains of uninfected animals were the controls for the experiment.

3. Results

3.1. *T. gondii* is able to incorporate macromolecules through the tissue cyst wall

T. gondii tissue cysts incubated for 2 or 3 h with BSA-FITC at 37°C were analyzed by DIC and confocal microscopy, **Figure 1A–C** and **D–F**, respectively. The BSA-FITC presented a strong

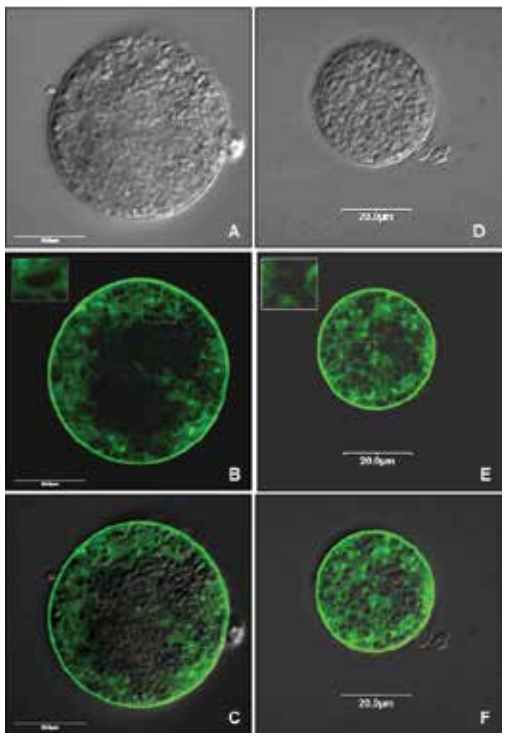

Figure 1. Confocal microscopy of *Toxoplasma* cyst incubated with BSA-FITC. (A–C) *Toxoplasma gondii* tissue cyst incubated at 37°C for 2 h and (D–F) 3 h with BSA-FITC. (A and D) Interferential microscopy (DIC) showing the cyst wall and numerous parasites inside the cyst. (B and E) Cyst fluorescence microscopy after 2 and 3 h of BSA-FITC incubation showing the tracer in the matrix, fulfilling the free spaces between the bradyzoites and throughout the cyst wall. (C and F) Overlay of fluorescence and DIC images showing the matrix and cyst wall labeled with BSA-FITC. Note that the cysts incubated for 3 h presented higher tracer incorporation. Scale bars: 20 μm.

labeling throughout the cyst wall and also located in the cyst matrix between the intracyst bradyzoites (**Figure 1B**, **C**, **E**, and **F**). These features were reinforced by merge obtained from DIC and fluorescence images (**Figure 1C** and **F**). The incorporation of BSA-FITC by tissue cysts was time dependent (**Figure 1C** and **F**). During the 3D reconstruction performance of a whole cyst from a set of 351 serial sections of 0.3 μm thickness, cyst wall transparency was strategically maintained in order to permit the visualization of the intracystic tracer (Video 1, supplementary material).

3.2. Ultrastructural analysis confirmed distinct molecule incorporation through the cyst wall

In order to examine whether the predicted protein labeling was in fact throughout the cyst wall, an ultrastructural analysis was conducted, incubating cysts with electron dense tracers, such as BSA-Au and HRP-Au. The incubation for 30 min at 4°C with BSA-Au revealed the marker at the surface as well as within invaginations of the cyst wall (**Figure 2A**). The internalization of BSA-Au occurred after 2 or 4 h of incubation at 37°C. Colloidal gold particles could

be found within small vesicles and tubules localized at the granular region independent of the time of BSA-Au incubation (**Figure 2B** and **C**).

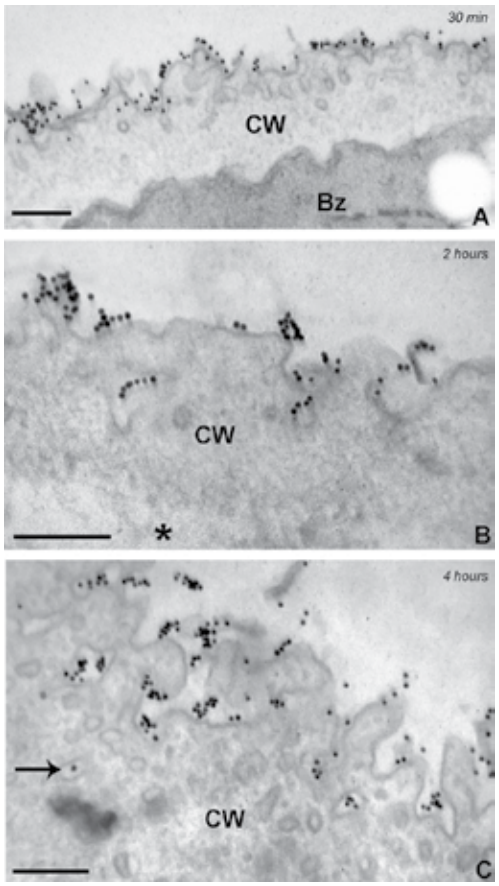

Figure 2. Transmission electron microscopy of *Toxoplasma gondii* cysts incubated with BSA-Au. (A) Tissue cyst presents many BSA-Au particles on cyst wall membrane after incubation for 30 min at 4°C. (B) Tissue cysts incubated for 2 h at 37°C displaying gold particles lining the cyst wall membrane and within the cyst membrane invaginations. (C) Tissue cysts incubated for 4 h at 37°C present BSA-Au particles in the uncoated vesicles and within tubules full of particles (arrow). Scale bars: 0.2 μm.

Similar results were obtained with HRP-Au by incubation of tissue cysts for 30 min at 4°C (**Figure 3A**). When the temperature was elevated to 37°C, the HRP-Au particles were observed within uncoated vesicles and tubules localized in the granular region of the cyst (**Figure 3B** and **C**).

3.3. Tissue cysts display a cytoskeleton network both in vivo and ex vivo

Aiming to reveal the cytoskeletal proteins in *T. gondii* tissue cysts, immunofluorescence assays were carried out with different polyclonal antibodies. Anti-myosin demonstrated the protein in

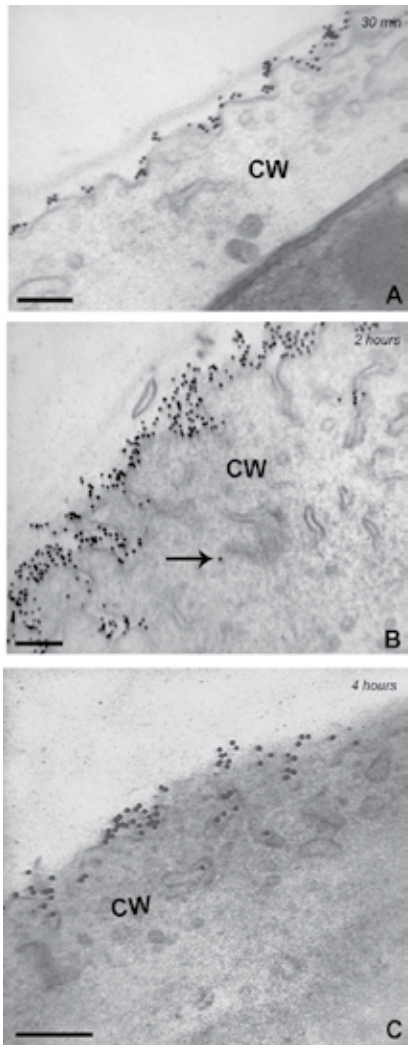

Figure 3. Transmission electron microscopy of *Toxoplasma* cyst incubated with Au-HRP. (A) Tissue cyst incubated for 18 h at 37°C with HRP-Au displaying gold particles lining the cyst membrane (arrow) and within the cyst membrane invaginations (large arrow). Scale bar: 0.2 μm. (B) Cysts present different regions of their surface with the distribution of HRP-Au (arrows) lining the cyst wall followed by spaces without any labeling. Absence of the marker in the cyst interior. Scale bar: 0.5 μm.

the cyst matrix as a diffuse staining and on the tissue cyst wall as intense heterogeneous fluorescent dots (**Figure 4A–C**). Brain cryosections from C57BL/6 mice chronically infected with *T. gondii* were incubated with the anti-myosin antibody, revealing that the distribution in tissue cysts *in vivo* followed the same pattern as *in vitro* (**Figure 4D** and **E**). For positive control, bradyzoites were incubated with the same antibody, and the myosin was localized mainly at the anterior pole (conoid region) or diffused through the parasite plasma membrane and cytosol (**Figure 4F**).

DIC microscopy (**Figure 5A**) and the immunofluorescence with the anti-tubulin antibody revealed a homogeneous labeling along the cyst wall in an area correspondent to the

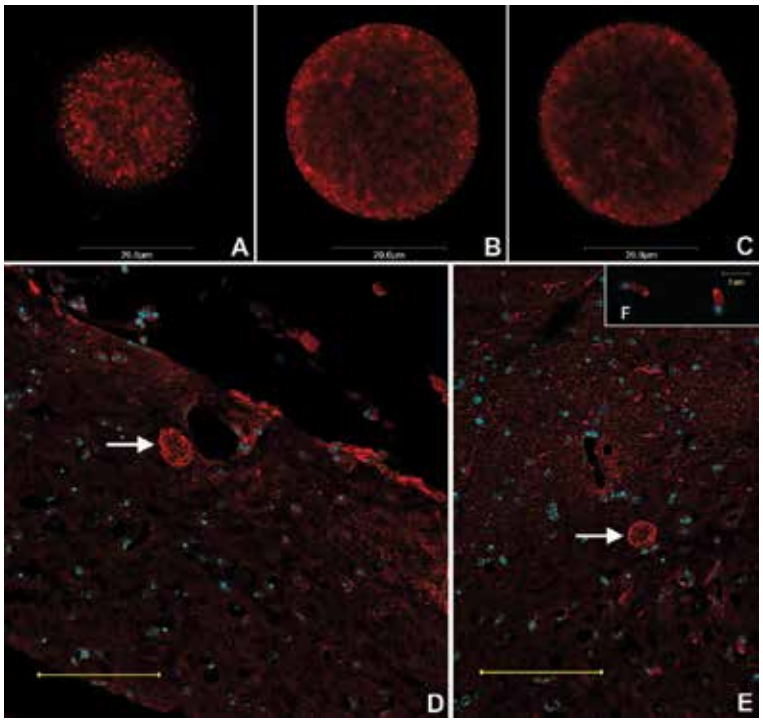

Figure 4. Immunofluorescence for detection of myosin in *T. gondii* tissue cysts. (A–C) Different confocal sections of the same isolated tissue cyst show that myosin was located along the tissue cyst wall with a dotted distribution, and the labeling was seen inside parasites as well. Scale bars: 20 μm. (D) Cryosection of a chronically infected brain showed that *in vivo* the tissue cyst wall had the same myosin distribution pattern as *in vitro*. (E) Different brain area labeled for myosin. Scale bars: 100 μm. (F) Positive control of myosin staining in *T. gondii* bradyzoites confirms the localization of the protein at the anterior pole and associated with the plasma membrane, similarly as described for tachyzoites. Scale bar: 5 μm.

granular region (**Figure 5B**). This intense wall labeling was better visualized in the merged figure (**Figure 5C**). Different virtual confocal sections of the same cyst disclosed the presence of tubulin inside bradyzoites (**Figure 5D–I**). In *T. gondii* chronically infected brains, the pattern of tubulin distribution was the same as in isolated tissue cysts (**Figure 6A**). In bradyzoites, tubulin was strongly labeled in the region corresponding to the subpellicular microtubules (**Figure 6B**), conferring the typical elongated shape of the parasite and the apical polarity of apicomplexan phylum. Microtubule-associated proteins (MAPs) form bridges linking with themselves and other structures. Tissue cysts in infected brain cryosections incubated with the anti-MAPs antibody colocalize with tubulin distribution (**Figure 6C** and **D**).

The most intense fluorescence labeling was for actin detection in *T. gondii* tissue cysts with homogeneous distribution along the cyst wall of isolated cysts (**Figure 7A**), presenting the same pattern of labeling as in the infected brain cryosections (**Figure 7B**). The revelation of actin inside the cyst (**Figure 7A**) suggested the detection of bradyzoites. To confirm this distribution, isolated bradyzoites were incubated with the same antibody, the labeling apparent on the conoid and extending below the apical region of the parasite body (**Figure 7C**).

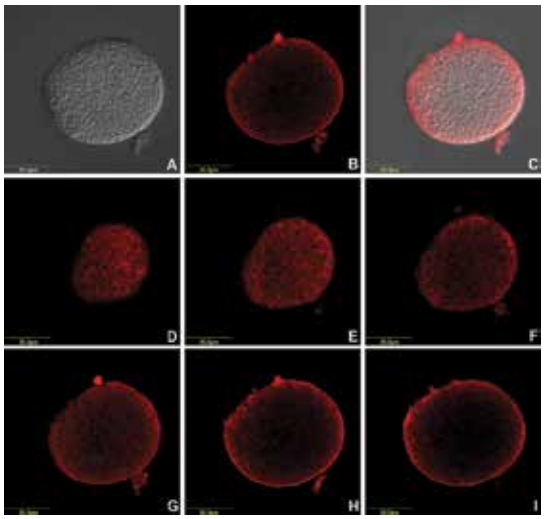

Figure 5. Immunofluorescence for detection of tubulin in *T. gondii* tissue cysts. (A) DIC microscopy, (B) fluorescence, and (C) a merge of figures A and B of an isolated tissue cyst labeling for tubulin detection showed an intense and homogeneous staining around the tissue cyst wall as well as parasite staining. (D–I) Different confocal sections of the same isolated tissue cyst confirm this distribution. Scale bars: 20 μm.

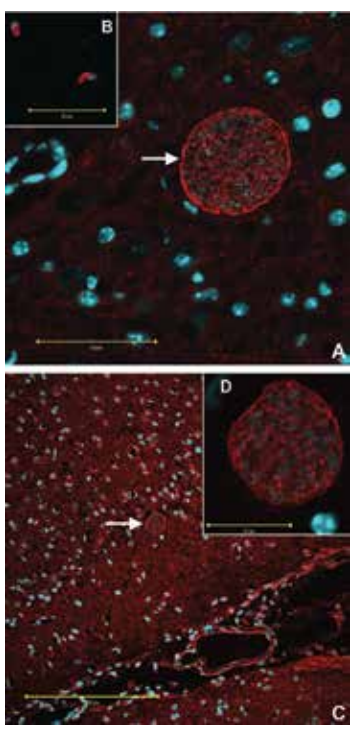

Figure 6. Immunofluorescence for detection of tubulin and microtubule-associated proteins (MAPs) in *T. gondii* tissue cysts. (A and B) Cryosections of chronically infected brains showed that, as *in vitro, in vivo* staining for tubulin and MAPs was intense and homogeneous around the tissue cyst wall. Scales bars 50 and 200 μm, respectively. (A′) Positive control for tubulin staining in bradyzoites of *T. gondii* revealed the population of subpellicular microtubules of the parasite. Scale bars: 20 μm. (B′) Detail of tissue cyst in cryosection. Scale bars: 20 μm.

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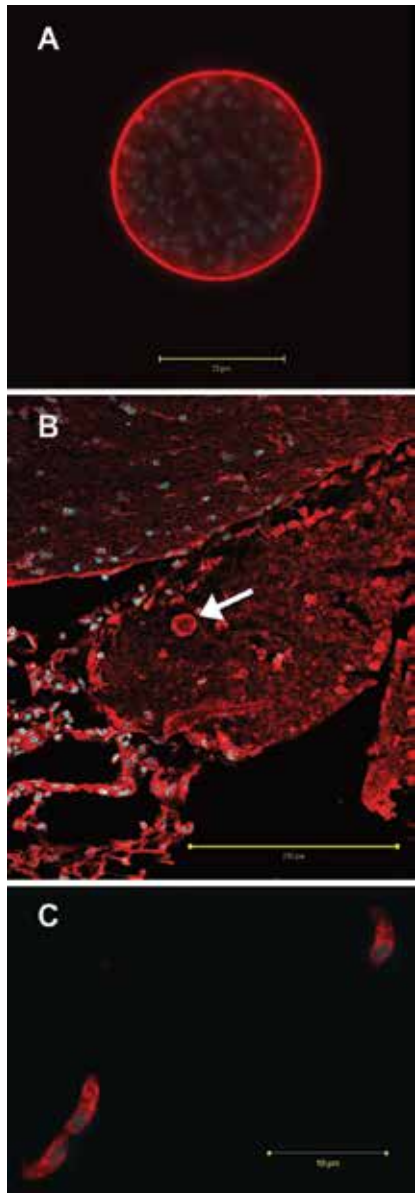


Figure 7. Immunofluorescence for detection of actin in *T. gondii* tissue cysts. (A) In isolated tissue cysts, actin detection was more intense than tubulin but still homogeneous around the tissue cyst wall. Scale bars: 20 μm. (B) In cryosections of chronically infected brains, the staining was the same as *in vitro*. Scale bars: 200 μm. (C) In positive control, anti-actin antibody labeled bradyzoites in the apical region and appeared as a diffuse staining in the parasite cytosol. DAPI stained both the cell and parasite nuclei (in blue). Scale bar: 10 μm.

3.4. Ultrastructural localization of cytoskeleton proteins through the cyst matrix

The detection by ultrastructural immunocytochemical of actin and tubulin proteins in tissue cysts revealed many gold particles distributed along the granular region and dispersed in the cyst matrix of tissue cysts (**Figure 8A**). In bradyzoites, some particles were located in the

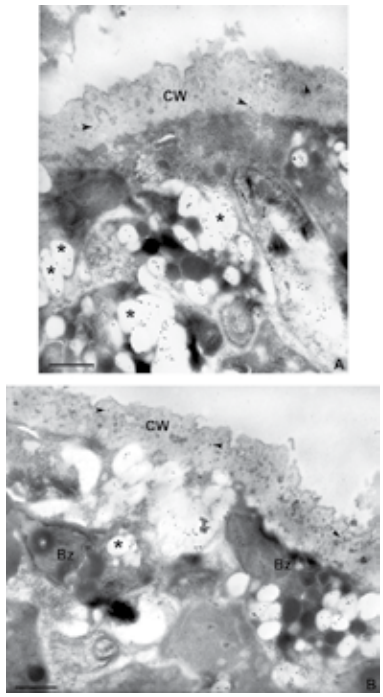


Figure 8. Transmission electron micrographs (TEM) of *T. gondii* tissue cyst labeled for actin by immunogold assays. (A) In tissue cyst, many gold particles were distributed along the granular region (arrowhead) and in the matrix (circle). In bradyzoites, some particles were located in the cytoplasm (arrows), most of them inside amylopectin granules (asterisk). (B) In bradyzoites, the gold particles were seen along the cyst granular region (arrowhead) and in clusters in the parasite cytoplasm (circle). Cyst wall (CW) and bradyzoites (Bz). Scale bars: 0.5 μm.

cytoplasm, most of them inside amylopectin granules, along the cyst granular region (arrowhead) and in clusters in the parasite cytoplasm (**Figure 8B**). The immunogold reaction for tubulin identified some gold particles on the cyst wall membrane along the granular region and in the matrix (**Figure 9A**). In bradyzoites, tubulin gold particles were detected mainly inside and around the amylopectin granules as well as in the cytoplasm and membrane (**Figure 9B**).

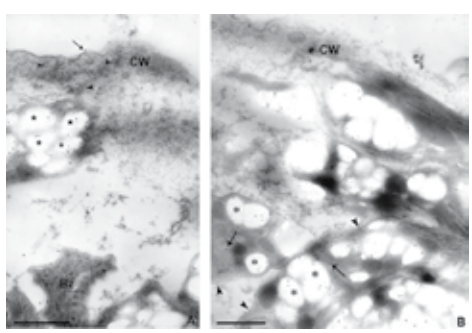


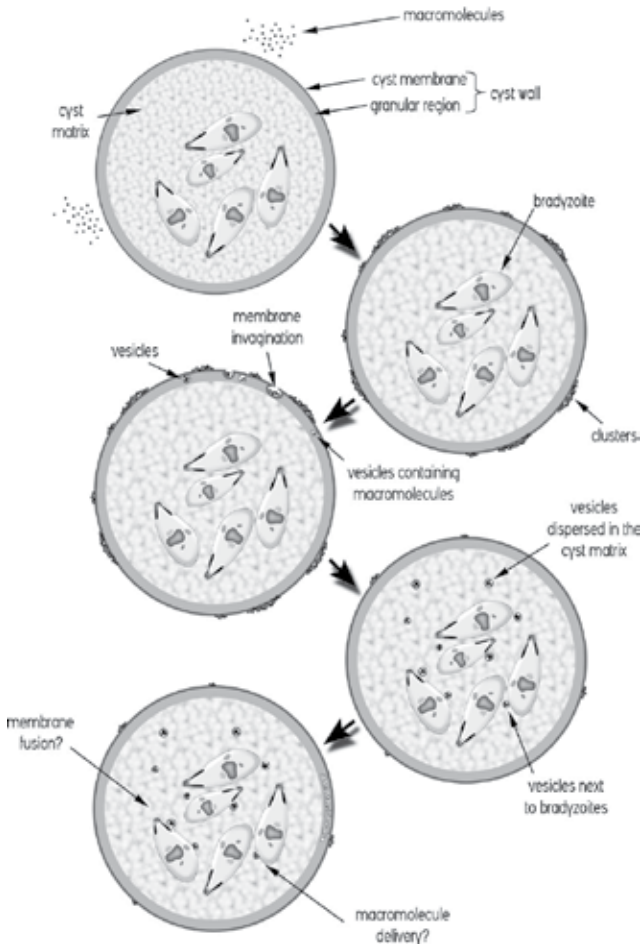
Figure 9. Transmission electron micrographs (TEM) of *T. gondii* tissue cyst labeled for tubulin by immunogold staining. (A) In the tissue cyst, gold particles are seen on the cyst wall membrane (arrows) along the granular region (arrowheads). In the bradyzoites, gold particles were present mainly inside and around the amylopectin granules (asterisk). (B) Bradyzoites cytoplasm (arrow) and membrane (arrowhead) showed some gold particles. Cyst wall (CW) and bradyzoites (Bz). Scale bars: 0.5 μm.

4. Discussion

This study reveals the ability of the *T. gondii* cyst wall to incorporate macromolecules *in vitro* as well as the presence of cytoskeleton elements in tissue cysts. A critical question in the lifelong persistence of host *Toxoplasma* cysts is how these intracellular parasites are able to survive for long periods within the cyst. The nutrients required by bradyzoites surrounded by the cyst wall may be necessary for its maintenance, multiplication, and amylopectin granule synthesis [23, 24]. The cyst wall, due to its limiting, highly invaginated membrane, provides an enhancement of the cyst surface area, affording the traffic of material between the parasite intracyst and the host cell cytosol [25–27]. Our results show that the *T. gondii* cyst wall is able to incorporate fluids and macromolecules from the external environment via the formation of vesicles and tubules, corroborating findings [8], when there is endocytic activity of the cystic wall, a dynamic process that occurs in *ex-vivo* cysts. These properties might be an alternative method for parasite survival, remaining enclosed within cysts for a long period of time or, as proposed, for the entire life of the infected host [28, 29]. These data point to the endocytosis, described for mammalian cells [30, 31] as well as the endocytic pathway of other pathogenic protozoa [32], as a mechanism of tissue cysts during intracellular development for nutrient acquisition from the host cell.

The exposure of Toxoplasma cysts to BSA-FITC for 2 or 3 h exhibited for the first time, the incorporation dynamics of this endocytic marker located at and attached initially to the cyst wall and afterward, the transit within vesicles toward the cyst matrix. Ultrastructural images from cysts incubated with the two different fluid-phase endocytic tracers (BSA-Au and HRP-Au) demonstrated that they were capable to associate with the cystic wall and be internalized via vesicle and tubules. The incubation of tracers and cysts at 37°C for periods 1–4 h displayed a heterogeneous labeling with formation of clusters on the cyst wall. Guimarães et al. [8] obtained similar images after cyst incubation with cationized ferritin. This labeling type may be due to the motility of cyst wall components or even the presence of different surface micro domains. Some images suggested fusion between the vesicles originating from invagination of the cyst wall with the parasite membrane. However, we have been unable to visualize vesicles discharging the marker or the marker inside the parasite. The present results corroborate previous data of our group with cationized ferritin showing vesicles and tubules containing particles in close contact with the membrane bradyzoite intracystics [8]. The previous data added together with those presented in this work suggest an active process of membrane fusion involved in the bradyzoite macromolecule uptake. It could be one of the pathways for parasite nutrient acquisition through the molecules available in the host cell cytoplasm (see **Scheme 1**, supplementary material). Moreover, we believe that the tubules and vesicles between the filaments of the cyst wall play a key role in delivering internalized molecules from the cyst wall to the intracellular bradyzoites and vice versa.

This cyst wall property opens new perspectives to the investigation of cytoskeletal element involvement in the process of nutrient incorporation by *T. gondii* tissue cysts. Thus, research was initiated concerning the molecular motors that move vesicles and tubules along actin filaments or microtubules, which might mediate the movement from the wall to the matrix cyst. Immunofluorescence of infected brain cryosections and isolated tissue cysts revealed that the tissue cyst wall and matrix are surrounded by a network of cytoskeleton proteins containing at least actin, tubulin, and myosin. Previously, our group demonstrated that the cyst wall is



Scheme 1. *Toxoplasma* cyst endocytosis. Schematic representation illustrating macromolecules endocytosis by *Toxoplasma* cyst showing each step of this process: (**A**) *Toxoplasma* cyst exposed to macromolecules; (**B**) binding of macromolecules on the cyst wall and cluster formation; (**C–E**) macromolecule incorporation through the cyst membrane invagination followed by the formation of vesicles which migrate from the granular region toward the cyst matrix where they were visualized next to bradyzoites.

formed by negatively charged molecules, and that these molecules are incorporated in the matrix through an endocytic process, involving tubules and vesicles formed from the membrane delimiting the cyst wall [8]. Additionally, we verified that these vesicles and tubules were at different locals of the cyst wall, intimating that they are certainly moving from the outer membrane of the cyst toward the bottom of the cyst wall. Moreover, some vesicles were seen in the matrix very close to the bradyzoite plasma membrane.

Until now, there has been no evidence of the fusion of these vesicles with the parasite membrane. However, we suggest that it might be an important pathway for host cell nutrient acquisition. Nutrient required for bradyzoites is extremely important, as the parasite is confined inside the tissue for long periods and can persist throughout the life of the

host [1]. Di Cristina et al. [33] observed that fluorescence molecules, such as D-luciferin, have access to bradyzoites within intact cysts both *in vitro* and *in vivo*. Lemgruber et al. [9] described that the tissue cyst wall incorporation is dependent upon the molecular weight of the tracers. Our data are therefore consistent with both of these studies.

In this way, our interest was to explain how these molecules are incorporated by the cyst wall and also how they can move within the cyst matrix considering that the cyst is not one cell but a structure that maintains various parasites in its interior. The endocytic activity of the cyst wall requires a stimulus induced by molecular signaling which involves cytoskeleton proteins as described for eukaryotic cells. Both actin and tubulin contribute to intracellular vesicle trafficking and endocytic activity in a series of dynamic events in the endomembrane system. The strong positive staining of tubulin and actin, revealed with polyclonal mammalian antibodies throughout the cyst matrix, indicated the presence of a cytoskeleton network within the cyst. Further investigation would be needed to evaluate the origin of these proteins. Eukaryotic cells exhibit tubulin dimers that can be easily altered by posttranslational modifications that differentially mark distinct microtubule subpopulations [34, 35]. Each microtubule subpopulation is organized for specific functions, such as the mitotic spindle, tracks for vesicular transport plus the basal body, and associated flagellar axoneme. In *T. gondii*, there are at least five discrete tubulin-based structures including the conoid, cortical microtubules, intraconoid microtubules, and centrioles, as well as a spindle in replicating parasites [19]. Studies may be required to confirm whether or not tissue cyst matrix cytoskeleton proteins originate from the parasite or from mammalian host cells during the parasitophorous vacuole formation.

Numerous protozoan parasites have the ability to form the cyst stage that normally allows them to survive under adverse environmental conditions. Chávez-Munguía et al. [36] showed that during the encystation process of *Entamoeba invadens*, the cyst wall components are concentrated and transported in specific vesicle, which contain a fibrillar material. This material is then deposited on the parasite plasma membrane through the aperture of the vesicles. Vesicles with a similar appearance may also be present in the cytoplasm of *E. invadens* mature cysts and in the parasite *Acanthamoeba castellanii*. Benchimol [37] also described this structural mechanism involved in the deposition and transport of material to the *Giardia lamblia* pseudocyst wall. After releasing their contents, the membranes may reseal to form empty vesicles, which may be endocytosed by the parasite and/or remain as empty vesicles. Hausen et al. [38] suggested that *G. lamblia* cysts treated with the benzimidazole derivative drug Albendazole, which binds to α-β-tubulin heterodimers of assembling microtubules, link to cyst tubulin, strongly interfering in the excystation process of the parasite. The demonstration of actin, tubulin, and myosin in the composition of the *T. gondii* cyst wall and their distribution in the cyst matrix, as shown here, by immunofluorescence in the *ex-vivo* and *in vivo* systems, is a potential key to the understanding of the incorporation and traffic of molecules by the *T. gondii* cyst wall.

Actin is a highly conserved microfilament protein that plays an important role for gliding motility and in *T. gondii* tachyzoites host cell invasion [39]. By indirect immunofluorescence, actin has been reported to be primarily concentrated in the anterior third of the tachyzoite and in a diffuse staining pattern throughout the cytoplasm [40, 41], our immunofluorescence for

actin in the bradyzoite stage revealing the same staining pattern. We also considered that the strong fluorescence staining along the cyst wall could be a false positive and may be caused by adsorptions of the antibody. This hypothesis was discarded when an infected brain was sectioned, and the cyst stain pattern was the same as for intact isolated tissue cysts.

In conclusion, *T. gondii* cyst wall endocytic activity has been demonstrated to provide insight in the parasite cell biology. The relationship of the cyst wall dynamics and cytoskeleton elements maintained in *ex-vivo* cysts corroborate the hypothesis that the cyst wall incorporates and circulates molecules, which could guarantee the parasite survival and persistence during *T. gondii* host infection. In addition, this data potentially provides prospects for new strategies and drug design for toxoplasmosis treatment.

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Chapter 2

Prevalence, Genetic Diversity, Tissue Distribution, and Risk Factors Contributing to *T. gondii* Burden in Domestic Pigs

Malgorzata Jennes and Eric Cox

Additional information is available at the end of the chapter

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Abstract

Toxoplasma gondii is an obligatory intracellular parasite of mammals, including humans and domestic animals. The infection with this parasite has severe clinical consequences, as it causes abortion or fetal abnormalities, encephalitis in immunocompromised humans, ocular toxoplasmosis with chorioretinitis, and it may contribute to Alzheimer disease. Therefore, an efficient control of *T. gondii* by prevention of the transmission to humans is strongly recommended. Pork is considered as an important source of toxoplasmosis, due to the frequent consumption of the raw or undercooked porcine meat products, a high susceptibility of pigs to the infection, and because of the numerous risk factors, contributing to the prevalence of toxoplasmosis in the pig population. The cellular and humoral immune responses, such as IgM, IgG, IFN-gamma, and interleukin-10 or -12 production, associated with the acute and chronic infection in pigs, do not prevent development of the tissue cysts, which persist lifelong within the intermediate host. Therefore, the prevalence of *T. gondii* in the pig population might be an useful indication of the risk associated with the consumption of the porcine meat.

Keywords: *Toxoplasma gondii*, pigs, meat, risk factors, parasitic burden

1. Introduction

Toxoplasma gondii is an obligate and worldwide-distributed intracellular parasite, causing disease in all warm-blooded animals, which includes aquatic and terrestrial mammals and birds. The prevalence of toxoplasmosis in humans reaches, depending on the study, 33–50% of the worldwide population, regardless of the geographical location or the economic status of the inhabitants [1, 2]. The pig is, among other domestic and wild mammals, a common

intermediate host for *T. gondii*. At the same time, it can trigger human infection via the consumption of raw, undercooked, or cured porcine meat products [3]. Therefore, pork is indicated as one of the major meat sources associated with human food borne *T. gondii* infection, together with *Salmonella* and *Campylobacter* [4].

Although the exact prevalence of this food pathogen, in consumption of meat, is difficult to establish, the subsequent infection rate of humans has been estimated as an average of 300 consumers per 1 infected animal [5]. In addition, nearly all tissues from a pig are used directly for the consumption or processed in other meat products without freezing or cooking, increasing in that way the chance of the transmission of the disease [1].

The naturally infected domestic pigs are considered one of the sources for toxoplasmosis in humans. The lack of an obligatory screening method for the detection of antibodies or the viable parasite in the edible tissues, respectively on farm level or in slaughterhouses, increases the risk that infected animals enter the food chain. The worldwide prevalence of 0.4–93% varies significantly between continents and countries, being determined by diverse serological techniques using different cutoff values. Overall, the results strongly depend on the detection method, the age, and the size of the sampled population. In the developed countries, a spectacular drop in the prevalence of toxoplasmosis was observed in pigs, raised indoor in a strict confinement. Interestingly, next to the management system, many other factors can contribute to the increased risk of infection in pigs in the modern farms. The rodent control is of pivotal importance, together with the proper carcass disposal. The increasing age of the animals, small size of the herd, free range or backyard pigs rather than the strict confinement housing, source of water, and feeding of goat whey to the pigs are frequently listed risk factors. Reversely, organic or biofarms increase the chance of the transmission to pigs, and indirectly, toward humans.

2. Prevalence of *T. gondii* in domestic pig

The global incidence of the porcine toxoplasmosis per continent and country is shown in **Table 1**, taking into account the origin and the age of the animals, the farm management, and the serologic assay applied [48, 49]. According to the report from European Food Safety Agency published in 2012 [50], describing the number of the foodborne zoonotic outbreaks between 2008 and 2010, the highest proportion of positive samples in PCR or serology for *T. gondii* across all countries was reported for sheep and goats. However, the clinical manifestation of toxoplasmosis is particularly obvious in these two species due to abortion, so it is also more likely to be confirmed, compared with other animal species, in which the more subtle signs of infection (particularly in the acute phase of the disease) may be missed. Referring to the same report, the prevalence of *T. gondii*-specific antibodies in the porcine serum samples collected in all the EU-states was 2.2% [50].

In general, the seroprevalence of *T. gondii* dropped significantly during the last decades in Europe and in the USA. Corresponding to that, a low (0.0–0.4%) to moderate (36%) prevalence was estimated on conventional pig farms in European countries [43, 48]. In the USA, a higher

Country	Origin	Prevalence (%)	Assay	Reference [No.]
Argentina	Sows	37.80	MAT	Venturini et al. (2004) [6]
Brazil	Farm	25.50	In-house ELISA	de Sousa et al. (2014) [7]
	Slaughter	19.50	IFAT	Cademartori et al. (2014) [8]
	Indoor raised	11.50	In-house ELISA	Luciano et al. (2011) [9]
	Free range	20.60		
	Farm	13.40	MAT	Piassa et al. (2010) [10]
Canada	Finisher	0.74	ELISA kit[4]	Poljak et al. (2008) [11]
Chile	Slaughter	8.80	ELISA kit[4]	Munoz-Zanzi et al. (2012) [12]
China	Finishers	4.60	IHAT	Chang et al. (2013) [13]
	Farm	29.60	In-house ELISA	Du et al. (2012) [14]
	Slaughter	12.00	IHAT	Wu et al. (2012) [15]
	Finishers	24.50	In-house ELISA	Tao et al. (2011) [16]
	Mixed farm	53.40	ELISA kit[5]	Yu et al. (2011) [17]
	Sows	14.40	IHAT	Huang et al. (2010) [18]
Czech Republic	Slaughter	36.00	ELISA kit[2]	Bartova and Sedlak (2011) [19]
France	Slaughter	2.00	ELISA kit[3]	Roqueplo et al. (2011) [20]
Germany	Finisher	4.10	In-house ELISA	de Buhr et al. (2008) [21]
	Sows	16.50	IFAT	Damriyasa et al. (2004) [22]
Ireland	Finishers	4.70	LAT	Halova et al. (2013) [23]
Italy	Indoor raised	16.10	IFAT, 1/16	Veronesi et al. (2011) [24]
	Slaughter	16.30	ELISA kit[1]	Villari et al. (2009) [25]
Latvia	Finishers	0.40	In-house ELISA	Deksne and Kirjusina (2013) [26]
	Free ranged	6.20		
Malaysia	Sows	0.00	IFAT	Chandrawathani et al. (2008) [27]
Mexico	Backyard	17.20	MAT	Alvarado-Esquivel et al. (2012) [28]
	Farm	0.50		
Nepal	Slaughter	11.70	In-house ELISA	Devleesshouwer et al. (2013) [29]
Panama	Indoor raised	32.10	IFAT	Correa et al. (2008) [30]
Peru	Slaughter	27.70	Western blot	Saavedra et al. (2004) [31]
Poland	Slaughter	26.40	MAT	Sroka et al. (2008) [32]
Portugal	Farm	9.80	MAT	Lopes et al. (2013) [33]
Romania	Backyard	30.50	IFAT	Pastiu et al. (2013) [34]
	Sows	12.40		
	Finishers	0.00		

Country	Origin	Prevalence (%)	Assay	Reference [No.]
Serbia	Slaughter	9.20	MAT	Klun et al. (2011) [35]
Slovakia	Slaughter	2.16	ELISA kit[1]	Turcekowa et al. (2013) [36]
	Sows	4.26		
Spain	Finisher	9.70	MAT	Garcia-Bocanegra et al. (2010) [37]
	Sows	24.20		
Switzerland	Finishers	14.00	ELISA kit[3]	Berger-Schoch et al. (2011) [38]
	Adult	3.60		
	Free range	13.00		
Taiwan	Slaughter	10.10	LAT	Tsai et al. (2007) [39]
The Netherlands	Organic	10.9	In-house ELISA	Kijlstra et al. (2008) [40]
	Indoor raised	0.40		van der Giessen et al. (2007) [41]
	Organic	2.70		
	Free range	5.60		
	Organic	3.00	ELISA-kit[3]	Meerburg et al. (2006) [42]
	Indoor raised	0.00	LAT	Kijlstra et al. (2004) [43]
	Free range	4.70		
	Organic	1.20		
USA	Indoor raised	2.60	ELISA kit[4]	Hill et al. (2010) [44]
	Outdoor raised	6.80	In-house ELISA	Gebreyes et al. (2008) [45]
	Indoor raised	1.10		
	Free range	25.00	ELISA kit[4]	Dubey et al. (2008) [46]
	Slaughter	16.40	Western blot	Saavedra et al. (2004) [31]
Vietnam	Finisher	23.00	MAT	Huong et al. (2007) [47]
	Sows	32.30		
	Free range	35.70		

Adapted from Guo et al. [48].

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Table 1. Global seroprevalence of *T. gondii* in domestic pigs.

(25%) prevalence was detected in the free range animals than in the outdoor- (6.8%) or indoor-ranged finishers (2.8%) [46, 51]. In the Netherlands, a spectacular drop was seen for the finishers (from 54 to 1.9%; Ref. [72]) and for the seropositive sows (30.9–5.6%; Ref. [41]). In the most industrialized countries, the prevalence is not higher than 5% [2]. Nevertheless, the low prevalence cannot however, prevent the high transmission rate to humans, as even as low as 1% is equal to 1 million *T. gondii* seropositive animals, infecting potentially 300 million

humans [52]. To date, there is no obligatory screening or notification system for the detection of seropositive animals on farms or in slaughterhouses within the EU-states. These observations and the possible reasons of the decreased prevalence are discussed in detail under Section 5 (Risk factors associated with porcine toxoplasmosis).

3. Genetic diversity

Because of the high clinical relevance, the parasite has been isolated from many naturally infected animals and humans, and molecular analysis of the parasitic genome was performed using restriction fragment length polymorphism (RFLP), PCR, or random amplified polymorphism DNA [53]. The obtained data led to genetic identification of the isolated parasites; and therefore, allowed to distinguish three major multilocus genotypes of *T. gondii*. The genetic lines named I, II, and III correspond to the genetic analysis of the polymorphic surface antigen 2 locus (*SAG2*) [54] and are characterized by a different degree of virulence and pathogenicity in LD50 in mice. Type I is considered as the most virulent genotype, followed by type III and type II. The latter is the most prevalent type present in animal reservoir and human population throughout Europe. Additionally, next to the typical three lineages, some of the strains obtained from South America, Africa, and Asia show an atypical genome or a mixed genotype of the existing ones. These strains are often characterized by an increased virulence in mice [2, 55]. Concerning the genotypes of pigs' isolates, the majority of the strains circulating within the porcine population in North America belong to the genotypes II and III or has a mixed allele composition, while in Europe, genotype II seems to be the most predominant clonal type [56, 57]. In South America, on the contrary, genotype I or mixed types are prevalent in domestic pigs [58].

4. Immune responses and tissue distribution upon acute and chronic toxoplasmosis

Upon infection with the parasite, the humoral and cellular immune responses are initiated, while clinically toxoplasmosis in pigs proceeds mainly asymptomatic. Only occasionally, pigs develop clinical signs. Following experimental inoculation of pigs with any infectious stage of *T. gondii* (oocysts, tissue cysts, or tachyzoites), a range of antibodies develops against the parasitic antigens. The time interval between the inoculation and the first detection of the immunoglobulins or cytokines varies depending on the virulence of the used strain, the infectious stage, and the applied dose [37, 59–62].

Secondly, a significant Th1-immune response can be observed as an increase of IFN-γ production after inoculation of pigs with a well-defined number of the infectious *T. gondii* strains [61, 63–65]. This increase is positively correlated with the duration of the experiment and can be detected in the serum collected from the infected animals in the supernatant from cultured PBMCs, and also as IFN-γ mRNA expression in PBMCs and intestinal lymphoid tissues. The IFN-γ production associated with the induced toxoplasmosis in pigs was in this

case the result of the activation of the innate and acquired immune system. These immune responses were investigated *in vitro* by determining the cytokine profile until 14 [61], 40 [63], or 56 dpi [66].

Next to IFN-γ, other cytokines are involved in the immune response. The infection of pigs with the VEG-strain oocysts induced a Th-1 immune response shortly after the inoculation, with the production of IL-15 and TNF-α mRNA [61]. Subsequently, a Th-2 response profile with IL-10 as anti-inflammatory cytokine was detected after the acute phase of the infection, dominated by IFN-γ production [63].

During the acute phase of the infection, the immune responses are directed against the disseminating tachyzoites, while throughout the chronic toxoplasmosis they also target the latent cysts with bradyzoites in the variety of tissues. It is well described that the parasite can persist within the intermediate host for a life span and can be found in all internal organs and muscles [67–69]. For instance, in pigs, viable *T. gondii* was recovered from porcine brain, heart, tongue, diaphragm, liver, and kidneys. Additionally, all edible commercial cuts of meat tested are also positive, representing thereby, a potential risk for consumers [4, 70]. It is a subject of discussion and ongoing research whether the host can clear the tissues during the chronic infection phase. In pigs, there is a scientific evidence that tissue cysts can gradually decrease in number and show a decline in viability, as tested by bioassay [66].

5. Risk factors associated with the porcine toxoplasmosis

Several factors can potentially modify the risk of *T. gondii* infection in pigs, such as the presence of cats on farms, rodent control, age of the animals, size and type of the herd, outdoor access, the carcass disposal, and feeding of unprocessed animal products such as goat whey to the pigs [41, 44, 49, 71].

The cat is responsible for the direct transmission of toxoplasmosis to farm animals such as pigs by entering the stables and shedding the oocysts in the animal facilities, or by contaminating the environment, and indirect spread of oocyts by animals entering the stables like dogs or birds. The free access to stables for people, without the application of strict hygienic measures (e.g., disinfecting foot bath or protective footwear and clothes for the exclusive use in the animal facilities) can also contribute to the dissemination of the oocysts within the herd or its transmission to the stables from the contaminated environment. The lack of these measures is especially important if domesticated or feral cats live in the close neighborhood of the animal facilities.

The second factor, namely the insufficient rodent control in the stables drives the persistence of the parasite on farm level in two ways: the rodents serve as a constant reservoir of the parasite for the cats, maintaining directly the infection risk within the herd; additionally, the rodents can directly be involved in the infection transmission by the predation or accidental ingestion of the mice by pigs [40, 56].

Age as a risk factor is strictly associated with a longer exposure and evidenced by an increased seroprevalence in older animals in comparison with piglets. As colostral antibodies disappear by 120 days of age, piglets and young raising pigs do not have any maternal immune protection, and are, therefore, exposed to the infection [71]. However, the

humoral immunity alone is not sufficient in combating the parasite. Indeed, the antibodies facilitate the cytotoxicity by the innate immune cells toward the extracellular parasite, but the intracellular *T. gondii* can escape from that. The age-dependent increase in seroprevalence in pig farms have been described in numerous studies, showing a higher incidence in adult pigs (19.5%) than in young animals (10.9%) [28], or in breeding sows than in finishers (30.9% and 1.9%, respectively, in the Netherlands [72] and 24.2% and 9.7%, respectively, in Spain [28].

The age of the animals at slaughter has also an important implication for the transmission of the disease toward human consumers and the epidemiology of human toxoplasmosis. Indeed, the younger the animals, the more chance that the meat will be consumed fresh and unprocessed, while the meat derived from older animals such as sows, will undergo processing to different pork products, which is harmful for the parasite and thus, safer for the consumer in terms of parasitic load [49].

Two major factors contributing to porcine toxoplasmosis are the size of the herd and the management type of the herd with a reverse correlation between the size and the on-farm prevalence: small herds showed a higher rate of seropositive animals (4.1%) than medium (1.9%) or large (0.6%) herds [51]. The reason for that would be the higher exposure per animal in smaller farms due to the lower density of the pigs. Even more critical for the risk for toxoplasmosis is the management type of the farm. The recently observed decrease in seroprevalence of toxoplasmosis in the pig population in the developed countries might be due to the implementation of the modern management system in porcine herds, with a visible shift from housing of a smaller number of animals in less strictly confined establishments or outdoor, to large scale facilities with a high output and a fast turn-over, characterized by all-in-all-out or farrow-to-finish models [44, 48, 73]. The extensive pig production expressed by the increasing number and size of porcine herds is driven by the high consumption of porcine meat in the developed countries in Europe and in the USA. In Belgium for instance, the total yearly number of pigs comprise 6.5 million animals, housed in 5000 conventional farms, ranging between on average 700–1300 pigs, as estimated by the National Institute of the Calculations, Federal Public Service Economy, (Actualization of the Industrial Study on Pork, 2015). Consequently, nearly 12 million of animals are slaughtered each year, due to an excessive consumption rate per inhabitant (35 kg/year), in comparison with other countries.

Summarizing, in the modern large-scale herds, the risk of *T. gondii* infection and thus, the prevalence of seropositive animals can be substantially reduced by the employment of a strict confinement housing with restrictive biosecurity regulations [1, 44, 45, 48].

However, in the last years, a new tendency in animal husbandry deserves the attention, namely the animal-friendly herds, housing organic or free range animals, providing daily a permanent or a temporary outdoor access to the animals. The term 'organic' refers to the quality and safety of the porcine products, with constraints about the chemical compounds originating from the feed or drug treatment, while 'free range' stands for the life quality of the animals during the production round. Hence, organic pigs are mainly reared outdoor, receive an organic feed, are provided with an animal friendly living space, the piglets are weaned at the later age than 3–4 weeks as on the intensive farms, and undergo a restrictive use of the antibiotics. The free range pigs differ from the regular pigs by the outdoor access and straw bedding, but are fed with a standard porcine feed and may receive drug treatment, if necessary, without losing a label

as in case of the organic pigs [43]. In both types of farming, pigs are continuously exposed to the parasite by contact with contaminated soil or ground water, and can easily transmit the infection further in the food chain [43, 44, 74].

As indicated above, an appropriate rodent control is of significant importance for the reduction of the risk for porcine toxoplasmosis; in addition to the latter, a proper carcass disposal seems to be equally essential, since both measures are intended to avoid the ingestion of formerly infected tissues [44, 56]. The cases of cannibalism by the accidental access to preliminary dead animals, especially when animal tissues are buried or composted are considerably common [41, 44, 49, 71]. Similarly, providing drink water of unknown quality to the pigs, possibly contaminated with the oocysts and feeding of raw animal products such as goat whey is also a potential risk, if made from unprocessed milk containing tachyzoites from a recently infected animal undergoing the dissemination phase [42, 75].

Finally, the prevalence of porcine toxoplasmosis may be influenced by the climate and geographical factors, for example, in altitude, temperature, and humidity, since they have a direct impact on the survival of *T. gondii* oocysts in the environment. As established previously, the oocysts remain infectious for a long period of time in a humid and cold environment, as long as they are not dried out or frozen. Hence, the prevalence of toxoplasmosis in pigs is higher in the mountains (32.1%) than in semi desert areas in Mexico (14%) [28], or in the coastal and northern regions of the USA (2.9–3.2%) than in the south continental part of the country [76].

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Chapter 3

Toxoplasmosis and Public Health Genomics

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Additional information is available at the end of the chapter

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Abstract

Toxoplasma gondii infection generally causes flu-like symptoms in healthy individuals; however, immunosuppression of the infected individual causes reactivation of the pathogen to its active form and relapse of the toxoplasmosis. Today it is known that toxoplasmosis triggers psychiatric disorders such as schizophrenia as well as behavioral changes such as suicide attempts. Although dermatological manifestations are very rare, the dermatological lesions are not unique. In addition, previous toxoplasma infection also causes congenital infections because of placental infection and causes birth defects and spontaneous abortion. *T. gondii* strains are mainly divided into three main clonal lineages, yet higher recombination rate causes unusual population structure and heterogeneous distribution of the pathogen. Both genetic variations, of the pathogen and the patients, are important for virulence property and success of the therapies. The scientist focuses on the genetic variations of the pathogens and individuals to achieve effective treatment and developed tailor-made medicines. Thus, understanding the molecular basis of the disease and the link of molecular mechanism with host immunity is important to fully know the disease and related disorders. In this chapter, we would like to evaluate the current knowledge on genetic, molecular characteristics of toxoplasmosis in view of public health genomics.

Keywords: *Toxoplasma gondii*, schizophrenia, public health genomics

1. Introduction

Toxoplasma gondii is a life-long infectious protozoan parasite and thus understanding the immunological and molecular pathways during toxoplasmosis is important for effective treatment and vaccine development. *T. gondii* infection and related neurological, behavior,

dermatological, and ocular manifestations are an important area of current research. In addition, the molecular life cycle of the pathogen, immunologic, and genetic changes during the infection is also important to diagnose and improve new treatment strategies. The other important research area related to toxoplasmosis is development of safe and efficient diagnostic assays for early detection of pathogens during pregnancy period.

It is thought that *T. gondii* has a role in the etiology of schizophrenia and that parasites have special genes that can be inherited. However, there are some limiting factors. In schizophrenia, it is difficult to show the cause of toxoplasmosis in brain tissue. *T. gondii* can also be found in brain tissue in immunocompetent individuals. Moreover, not all of the *T. gondii*-infected individuals develop schizophrenia. Increased risk of exposure to *T. gondii* has been shown in schizophrenic patients, but the exposure relationship is often studied by case-control studies. Despite the cohort-type studies that show the best relationship between the etiologic agent and the disease, researchers prefer to work in case-control type because of the latent course of the disease, not being a common disease in society, and difficulties in follow-up. It should be kept in mind that genetic factors as well as environmental exposures may increase the risk of this disease and that the presence of the agent before disease development may better explain the causal relationship.

We need a perspective of public health genomics to better understand the relationship between schizophrenia and toxoplasma, which is one of the most frequently used topics in recent years, and also in order to better understand the public health effects of parasites. The clear understanding of the histological, biochemical, and genetic characteristics of parasites can also explain the mechanisms of disease development. For this purpose, in this chapter, genotypic features of *Toxoplasma gondii*, other molecular changes, and mechanisms of schizophrenia development are discussed and suggestions are given in terms of public health.

2. Molecular basis of toxoplasmosis

The first line of defense against *T. gondii* compose of dendritic cells (DCs), monocytes, and macrophages, and *Toxoplasma* induces toxoplasmosis-specific $CD4^+$ and $CD8^+$ T cells, which are the secondary defense systems. The ligands expressed by *T. gondii are* recognized by toll-like receptors (TLR) [1–3] and stimulate IL-12 production via MyD88 signaling [4, 5]. GPI-anchored proteins of *T. gondi* is important for both adhesion to host and regulate host immune system via TLR2 and TLR4 on macrophage surface [2, 3] aside profilin that secreted from *T. gondii* binds to TLR11 on dendritic cells [5]. Pro-inflammatory IL-12 secretion triggers IFN-γ production by NK cells; thereafter, $CD4^+$ and $CD8^+$ T cells join to release IFN-γ [6], and then IFN-γ binds to a receptor tyrosine kinase (IFN-γ-R) and activates the JAK/STAT1 cascade by JAKs phosphorylation (**Figure 1**).

Activation of JAKs phosphorylates the tyrosine residue of STAT1 and cause dimerization of the molecule hence translocate the nucleus [7] then binds to IFN-γ-responsive gamma-activated site (GAS) consensus sequence and initiate the transcription of IFNγ-inducible genes Irf-1 and Lrg47 [7, 8]. In addition to T-cell mediated immunity, STAT1 induces nitric oxide (NO) and reactive oxygen species (ROS) production in monocytes and macrophages [9]. Lüder et al. [10]

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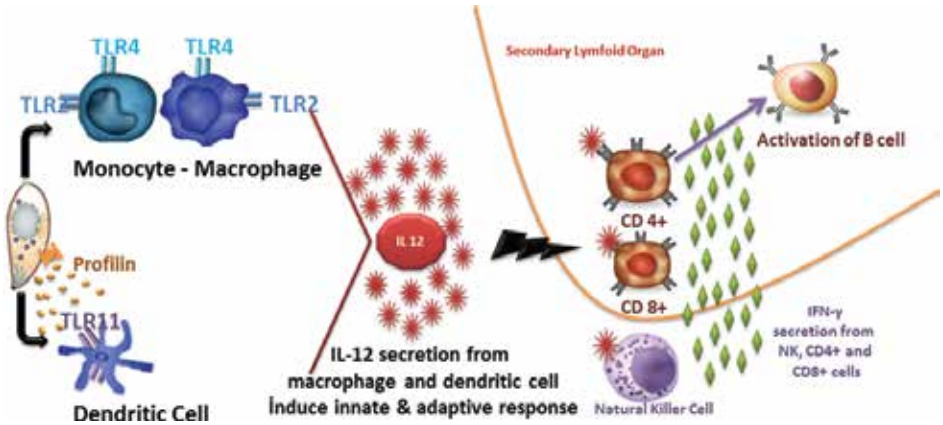

Figure 1. Immune response to *Toxoplasma gondii* infection in humans.

have reported the toxoplasma IFN-gamma-induced gene expression via STAT1 pathway. In addition, STAT4-dependent IFN-γ has been reported in dendritic cells and macrophages [11]. Many reports have been shown the *T. gondii* success of toxoplasma's immune evasion strategies [10, 12, 13]. The molecular basis of parasite-host interaction is important in understanding the disease mechanism and developed successful treatment strategy (**Figure 2**).

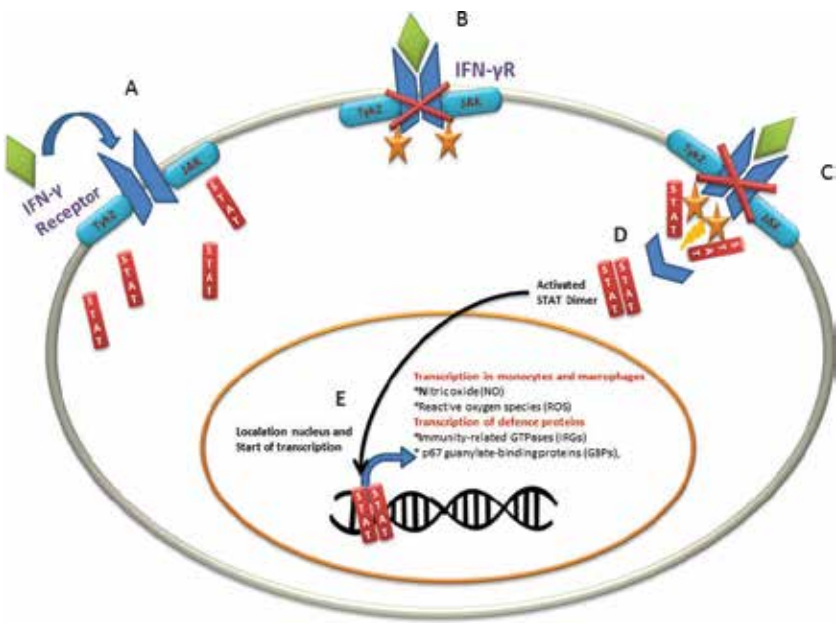

Figure 2. JAK-STAT pathway is important to induce toxoplasma-related response genes. INF-γ binds to its receptor (A) and activates the tyrosine kinase activity of receptor (B) and hence the phosphorylate SH2 domain of the STAT (C). Phosphorylation of the STAT triggers dimerization of the molecule (D) and conformational changes cause nuclear localization of the STAT which then binds to the nuclear response sequence on promoter region (E) to induce expression of target genes.

3. Genetic variation of *Toxoplasma gondii*

The complexity of an organism is directly related to its genome, and the variation on parasite genome is crucial for their success during evolution. In addition to natural selection, the pharmacy industry also forces parasites for evaluation; thus, genomic diversity also means successful pathogenicity.

Toxoplasma gondii consists of three main clonal lineages (types I, II, and III) among North America and Europe [14], and type II is the major strain that causes human infections in North America [14] and Europe [15, 16]. Early studies that performed multilocus enzyme electrophoresis (MLE) had limitations to show biological and epidemiological diversity [17].

Nowadays, high throughput technologies allow the scientist to understand the genetic diversity of *T. gondii*. In contrast, genetic analyses that were performed by multilocus markers have been shown that isolated samples from South America were highly diverse from each other and also from European and North American strains [15–18] due to a higher recombination rate [19]. The recent researchers have been shown that *T. gondii* has unusual population structure with different growth rates [20], frequency of differentiation, motility [21] virulence, persistence, and migration capacity [15, 21–23]. Recent developments in molecular tools allow enhancing their success to identify the genetic diversity of the parasite [24, 25]. Su et al. [25, 26] have analyzed the nuclear and plastid genome of 950 isolates from all over word and reported six major clades dividing 15 haplotypes with 138 unique genotypes. Ning et al. [22] have reported that toxoplasma dense granule protein 20 (GRA20) may be useful for intraspecific phylogenetic analyses. Wang et al. [27] have reported that rhoptry protein 47 may be used as a genetic marker for a phylogenetic relationship. In contrast, genes with low sequence variation such as superoxide dismutase (SOD) are not useful for variation analyses [28, 29].

4. Importance of human genetic variation for toxoplasmosis infection

Nowadays, researches are focused on the personalized medicine by using genetic variations of the patients to develop tailor-made therapeutics for appropriate and optimal therapies. The most important part to develop tailor-made therapeutics understands the relation between molecular basis of the disease and phenotypic screening. In addition, genomic variation is also important to understand personal immune response to a specific disease or therapeutic efficiency.

Toll-like receptor (TLR) is a single-pass transmembrane, non-catalytic receptor family, which is crucial for an early innate immune response during evolution. TLR expression differs in the stage of pregnancy, and a different subtype of TLR is activated or deactivated in placental tissues during pregnancy period [30, 31]. Nishimura and Naito have shown that TLR3 expression was the highest in placenta [32]. TLR is linked to infertility [33], pregnancy disorders, and placental dysfunction [34]. In toxoplasmosis TLR2, TLR4, TLR9, and TLR11 have been found important for recognition of ligands expressed by *T. gondii* [1, 35–37]. Andrade et al. [38] have demonstrated *TLR7 and TLR9 sense T. gondii*-associated nucleic acids (DNA or RNA) while TLR11 and TLR12 sense Toxoplasma profiling. Thus, understanding genomic variations in TLR is important to highlight immune defense against *T. gondii* [30, 39, 40]. NOD-like receptor

(NLR) families are important for immediate responses against structures that are present in *T. gondii* like TLR receptors. Dutra et al. [41] have reported that NOD2 gene polymorphism (rs3135499) increases the ocular toxoplasmosis by its effects on IL-17A production. Witola et al. [42] have reported that NALP1, a member of the NLR family of proteins, is crucial for immune responses to *T. gondii* infection and pathogenesis. Variation in immunity-related genes is also associated with toxoplasmosis. Shimokawa et al. [43] have shown that HLA-DQA1 and DQB1 alleles are increasing the risk of congenital toxoplasmosis. In conclusion, human genomic variation influences the defense against *T. gondii* as well as its manifestations of congenital infection and ocular toxoplasmosis.

5. Immunohistochemistry of toxoplasmosis

Toxoplasmosis can cause severe fetal conditions during pregnancy, and this fetal influence is related to the trimester of pregnancy. *Toxoplasma gondii* infection in the first trimester of pregnancy (4th week) can cause serious neurological and ophthalmological damage as well as spontaneous termination of pregnancy [44]. Infection of the nervous system leads to central nervous system injuries including hydrocephalus, microcephaly, motor mental retardation, and intracranial calcifications. Microphthalmia can also be observed in the first trimester. *Toxoplasma gondii* infection in the second and third trimester may result in chorioretinitis, visual defects, and mild neurological sequelae [45]. Dermal symptoms of *Toxoplasma gondii* in fetus are nonspecific and differs in each trimester.

Histopathological report attracted attention to especially parasitic infiltration and also inflammatory cell infiltration in affected tissue during toxoplasmosis. Immunosuppressive patients because of reduced inflamatory response due to immunne suppressive agents. Thus, immunohistochemically staining with appropriate antibodies will be helpful in these cases [46, 47]. Pathologic changes especially visible in skin lesions are pseudoepitheliomatous hyperplasia and perivascular lymphohistiocytic infiltration. Parasites can be seen in macrophages by hematoxylin eosin staining or as its single parasitic form by basophilic staining. Bradyzoites form that contains tissue cysts can be visualized by hematoxylin-eosin, Giemsa, Mallory, Biondi, and PAS reagent [48]. Parasites can also be visualized immunohistochemically by marking them with antigen-specific antigens inside the host tissue [49]. Tachyzoites are able to be visualized by light microscopy from samples taken from infected tissues [48, 50]. In addition to light microscopy, immunofluorescence methods can be used to detect parasites and to show them microscopically in samples taken from infected tissues with parasites [50].

6. Toxoplasmosis and psychiatric patients

6.1. Behavioral changes and toxoplasmosis

Kozar has shown the relationship between toxoplasma infection and psychiatric disorders for the first time [51] *T. gondii*, present in one-third of the world's population, may be associated with an increase in suicide rate [52]. *T. gondii*, a neurotrophic parasite, plays a role in the

development of schizophrenia and causes behavioral changes, suicide attempts, and neuropathological degenerations in the brain tissue [53–56, 74]. *T. gondii* has a role in the development of behavioral disorders via changes in neuroimmunomodulation and neurotransmission, yet pathophysiological mechanisms are not fully understood by scientists [57, 58].

6.2. Schizophrenia and toxoplasmosis.

Several studies and later metaanalyses have been reported the association between *Toxoplasma gondii* infection with schizophrenia [55, 59, 60]. It has been shown that *T. gondii*-infected individuals increase the risk of schizophrenia when compared to healthy individuals, yet these relationships were only examined by case-control studies. Epidemiologically, the best way to show the relationship between the agent and the disease is to work in cohort type. In contrast, the researchers prefer to work in case-control type studies because of the latent course of the disease, the difficulties encountered in the follow-up, and the life cycle pathogen and rare nature of disease. It should be kept in mind that genetic factors as well as environmental exposures may increase the risk of this disease. Previous toxoplasma infection before neurological disease development may better explain the causal relationship.

Schizophrenia susceptibility genes are associated with life cycle of *T. gondii* [61]. The factors that play a role in the etiology of the disease time of infection, genetic variation of *T. gondii*, and strain of the parasite [60]. In addition to the genetic characteristics of infected individuals, immigration or being the offspring of immigrants is also related with schizophrenia among *T. gondii*-infected humans [62].

T. gondii and schizophrenia relations have been linked in many studies [63], and generally it's accepted that *T. gondii* triggers psychiatric disorders via affecting neurotransmitter secretion [54]. It is known that the *T. gondii* genome contains two aromatic amino acid hydroxylases that can directly affect dopamine and/or serotonin biosynthesis [64]. It is unclear how *T. gondii* increased dopamine levels; however, the dopamine is released during inflammation due to the increase of cytokines such as interleukin-2 (IL-2) [65, 66]. Hence, dopamine imbalance in the mesolimbic and mesocortical regions of the brain may play a role in the development of schizophrenia [67].

T. gondii modulates host gene expression by secretion of effector molecules [68] or cause post-translational modification such as acetylation of protein residues in neurons and glial cells [69]. The success of the pathogen is its virulence ability to cross biological barriers such as the blood brain barrier [70]. In a study, Du et al. [68] demonstrated that *T. gondii* terminates the NF-κB pathway by its rhoptry protein that causes p65 ubiquitination for proteasomal degradation. In a study, it has been shown that *T. gondii* changes lysine acetylation in astrocytes [69].

TOXO-specific IgG and IgM antibody levels are elevated in schizophrenia [71–75]. In a study in which other demographic variables might affect age, race, sex, and mortality, it was found that the risk of death in serologically positive individuals was five times higher than *Toxoplasma gondii* seronegative ones [75]. Toxo-specific IgG antibody level was found four times higher in patients with schizophrenia than in individuals without [76].

Torrey has shown that *Toxoplasma* seroprevalence in individuals with schizophrenia is 2.73 times higher than the control population [60]. In a study, Emelia et al. [77] have shown significant serointensity rates of anti-*T. gondii* IgG antibody in schizophrenic patients when compared to psychiatrically healthy volunteers. Tamer et al. [78] have shown that IgG *T. gondii* antibodies were higher in patients with schizophrenia when compared with controls, and in contrast, Karabulut et al. [79] have reported that there is no association between *T. gondii* IgG positivity and schizophrenia.

7. Toxoplasmosis and dermatological disease

Dermatological manifestations are rare and diagnosis of lesion is difficult. Skin lesions in congenital infections are usually exfoliative lesions and hemorrhagic and necrotic papules (small papules in the shape of 'blueberry muffins'). These lesions tend to hold the body. But it can be seen in the entire body, except in the palmoplantar region and face. The skin findings of toxoplasmosis are very varied: the dermatological lesions are reported as roseola, erythema multiforme [80], papular urticaria [80, 84], urticarial, hemorrhagic eruptions, formation of nodules and bullae [81, 82] on palms, soles, hands, legs, trunk, face and chest [83, 84]. Jeffrey and Pollock have shown a 12-year-old boy patient with dermatomyositis and polymyositis and offered to use sulfadiazine, pyrimethamine, and folinic acid for treatment [85]. Fong et al. [81] have reported a 49-year-old HIV-positive Chinese male with hard and painful nodular lesions. Ivanova et al. [86] described a case of 46-year-old male patient with acute toxoplasma lymphadenitis, similar to malignant cervical lymphadenopathy. In another study performed by Marina et al. [87], scientists reported a case of a 43-year-old immunocompetent man who has several erythematous papules and nodules on the body and extremities.

Histopathologic features are common for both acquired and congenital forms. Lymphocytes, macrophages, plasma cells, and superficial and deep perivascular infiltration, which is composed of eosinophils, are seen in the dermis. Tissue forms of *T. gondii* may not always be seen in specimens of biopsy taken from the lesion.

It is important that congenital toxoplasmosis is distinguished from the TORCH group of infectious diseases. Acquired toxoplasmosis may be confused with inflammatory diseases such as viral exanthema, meningococcemia, syphilis, urticarial vasculitis, and erythema multiforme associated with herpes simplex and autoimmune collagen tissue diseases. If active acute infection occurs in organs such as the skin or the eye, or congenital infection occurs, or immunosuppressed patients need treatment, the most effective treatment method is pyrimethamine (from 25 to 50 mg per day followed by 100 mg loading) and sulfadiazine (2–4 mg per day oral, divided into four doses). Patients allergic to sulfonamides may be given clindamycin (300 mg, four times a day).

8. Toxoplasmosis and pregnancy

T. gondii is an important, warm-blooded pathogen that causes congenital infection during pregnancy which results in cardiovascular, cerebral, and ocular damage in newborns by

transplacental infection [87, 88]; *T. gondii* infection also causes birth defects and spontaneous abortion [89–92].

Determination of *T. gondii* infection is crucial during pregnancy to decrease congenital toxoplasmosis [91] and thus companies try to develop new commercial tests. The serologic screening of IgM, IgA, and IgG is important to determine current or past infections of the pregnant women [88, 93]. In addition to serological methods, ELISA IgG avidity is also found safe, efficient, and easy to determine *T. gondii* infection in the first trimester of pregnancy in routine diagnostic treatment [94, 95]. PCR is an effective diagnostic tool to determine the pathogen in a tiny amount of sample with high specificity [96–98]. The scientist's efforts to develop efficient and safe methods to alternates of ELISA, IFA, or modified agglutination test (MAT [88] or to develop these commercial ones to eliminates limitations of previous ones are ongoing [99, 104]. Armengol et al. [100] have compared different commercial assays which use anti-Toxoplasma IgG seroconversion in pregnant women and efficiency of the assays differs.

Pomares et al. [101] have developed new multiplexed *T. gondii* IgG and IgM tests which allow determining the pathogen in ~1 microliter of the serum sample. Mahmoudi et al. [102] have developed ELISA-based interferon-gamma release assay for the early detection of the pathogen. Mohammadpour et al. [103] have been reported that tachyzoites form of *T. gondii* able to detect via its soluble crude antigens by ELISA. Robert-Gangneux et al. [104] have been developed efficient toxoplasmosis assay which able to diagnose from non-cell-rich or non-hemoglobin-rich samples by real time PCR.

The development of diagnostic tests is particularly important in addressing the difficulties encountered during routine follow-ups by the population. Common guidelines are needed to guide both patients and clinicians in the subsequent processes, especially when toxoplasmosis is diagnosed in pregnancies. Sensitive tests to be developed for definite diagnosis are very important because there are problems related to diagnosis in pregnancy. *T. gondii* scans should be done serologically in cats and dogs at the same time. There is a need for regional studies on toxoplasmosis, especially in developing countries because socio-cultural features play a role in the development of the disease. As in many countries, studies in Turkey are continuing. There are studies on seroprevalence of toxoplasmosis and schizophrenia reported from the University and State Hospital in our research area Canakkale [105–108].

9. Conclusion

Attention should be paid to veterinary basic health services in combating and protecting zoonoses, which are thought to be an important risk factor in schizophrenia etiology such as *T. gondii*. While the risk of being infected with *T. gondii* by individuals living in urban centers increases risky behaviors such as cat feeding, cleaning of cat external feces, and contact with street cats; these risks are the factors such as contamination of animal products, consumption of raw meat and meat products, general hygiene conditions and inadequate

water hygiene in the people living in rural areas and living with agriculture and animal husbandry. Therefore, it is more effective and useful to plan the health trainings and surveys that are to be carried out, considering the socio-demographic and environmental characteristics of these target groups.

Data obtained in screening studies should be recorded with geographic information systems, and risky areas should be identified and individuals at risk (primarily pregnant women, children, people with psychiatric health problems) living in these areas should be regularly monitored. Trainings should be given on healthy lifestyle behaviors, such as personal and environmental hygiene. In clinical practice, in order to prevent *T. gondii* from being missed, in-service trainings should be made to raise awareness and responsibility for health personnel.

In order to overcome the question marks in the schizophrenia mechanism, a gene pool can be created on serum samples obtained from regional and national studies, and the present and changing immunogenetic structure of parasite can be monitored. This important point of public health and genetics is that infectious agents are monitored in a large pool of genes to monitor the changes they show and to help them analyze the distribution of health problems caused by infectious agents in their populations and risk factors. We also believe that the molecular monitoring of parasites such as toxoplasma will help to improve the early diagnosis and treatment of parasitic infections, which are the most important of public health problems, and the prevention and control of these infections.

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Section 2

Toxoplasmosis Clinical Studies

Chapter 4

Congenital Toxoplasmosis: *In Vivo* Impact of *Toxoplasma gondii* Infection on Myogenesis and Neurogenesis

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Additional information is available at the end of the chapter

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Abstract

Congenital toxoplasmosis (TC) from *Toxoplasma gondii* positive mother to child transmission results in fetal death, abortion, or infantile neurologic and neurocognitive deficits as well as chorioretinitis. This study aims to analyze the morphological changes in brain and skeletal muscle cells of Swiss mouse embryos during experimental congenital toxoplasmosis. Swiss mice, before mating, were gavage inoculation infected with approximately 25 or 50 cysts of ME-49 strain *T. gondii*. Eighteen day postcoitus maternal and embryonic muscle and brain samples were collected and processed for histopathological analysis. The muscle tissue from embryos of infected mothers, in comparison with healthy muscle myofibers, exhibited discontinuous and shorter myofibrils, more interfibrillar space and immature cells with fewer stained and poorly defined striated profiles. These *in vivo* findings might be related to an adhesion protein decrease, observed *in vitro*, where myogenesis was completely affected during *Toxoplasma* infection. The neurogenesis was severely affected with irregularly arranged cells, reduced cell density, and a significant intercellular space increase. The brain tissue presented ischemia, cell death, necrosis, and thrombi, increasing according to the degree of the acute infection, which compromised the neurogenesis, thereby justifying brain size decrease in these embryos.

Keywords: *Toxoplasma gondii*, experimental toxoplasmosis, myogenesis, neurogenesis

1. Introduction

Toxoplasma gondii is a parasite that persists during the chronic phase of the disease in the form of tissue cysts mainly in brain and skeletal muscle. *T. gondii* has been implicated in the pathogenesis of inflammatory myopathies and brain diseases, which are of great medical

importance due to congenital toxoplasmosis (CT) [1, 2]. Prevention is an essential measure against *T. gondii* infection from mother to child [3, 4].

The ability to transmigrate through the placenta to replicate in various fetal tissues, evading the immune system of the fetus, makes Toxoplasma a major cause of prenatal complications. This infection can seriously interfere with the development of the fetus, possibly leading to abortion or serious pathologies after birth with severe consequences in childhood, adolescence, or adulthood [3–7]. Among the most common brain afflictions are encephalitis, altered mental status, seizures, weakness, cranial nerve disorders, sensory abnormalities, movement disorders, psychomotor or mental retardation, and behavioral alterations. Acute retinocorioidite is a severe condition, which may develop, involving vitreous hypervascularization and retinal necrosis with a total loss of vision [6, 8–12]. With regard to muscle tissue, the *T. gondii* infection can decrease and even inhibit the formation of new fibers by negatively modulating adhesion proteins such as cadherin [13–15]. Toxoplasmosis also causes myocarditis and polymyositis either by recent infection or by reactivation of tissue cysts in immunosuppressed, immunodeficient, and even immunocompetent individuals [1]. The study of conditions resulting from Toxoplasma infection in brain and muscle tissue has been developed from infected adult mice [7, 16–20]. The influence of Toxoplasma in these tissues during embryonic development in the murine model has yet not been explored despite the predilection of this parasite for these tissues in the chronic phase of the disease. The focus on skeletal muscle as a model for experimental toxoplasmosis opens new prospects for parasite biology understanding during interaction with one of the *T. gondii* target cells for parasite establishment in the chronic phase of the disease [13, 21–29]. Therefore, we decided to investigate the role of *T. gondii* in myogenesis and neurogenesis processes in murine embryos from adult females, that were infected before mating.

2. Experimental design

Three experimental protocols were developed in an attempt to establish trans-chromosome (TC) in mice, premating infection, imminently postmating, and during pregnancy. Swiss mice during the mating period (five females and one male per experiment, $n = 5$) were divided into two groups and inoculated via gavage with approximately 25 (Group 1) or 50 cysts (Group 2) of ME-49 strain *T. gondii* (type II). After infection, the mice were placed in cages for 24 h with a suitable male of the same species and age. Thereafter, the animals were visually monitored daily (morning, afternoon, and evening) on the general aspects of lethargy, vaginal bleeding, and mortality. The dead animals were collected immediately and their embryos examined for macroscopic aspects of the peritoneum, spleen, liver, and lung. Pregnant females that sustained pregnancy underwent cesarean section approximately 18 days postcoitus, their embryos, alive or not, collected.

For the diagnosis of possible morphological changes in muscular and cerebral tissue caused by parasite presence in embryos from *T. gondii* cyst-infected mothers, embryonic thighs and brains were collected, sectioned longitudinally, and fixed in Millonig-Rosman solution

(10% formaldehyde in phosphate buffer). The tissues were then processed for impregnation in paraffin, and 3 μm thick sections were stained as routine with hematoxylin and eosin (H&E) for the analysis of morpho-structural and parasitological parameters. Alternatively, part of the material was processed by freezing in liquid nitrogen (−196°C). Five-micrometer thick sections were obtained at −25°C in Leica CM1800 cryostat (Germany), attached to slides treated in poly-L-lysine and fixed in 4% PFA in PBS.

3. Results

From three experimental protocols for *T. gondii* cyst mouse infection, postcoitus and during pregnancy promoted 100% in the number of abortions, embryo reabsorption, or adult female death. The most successful protocol was premating adult female mouse infection. However, some animals did not carry the pregnancy to term and this complication arose soon after conception.

The severity of the *T. gondii* infection in adult females and embryos was directly proportional to the concentration of parasites (cysts) inoculated. Group 1 displayed fetal reabsorption, miscarriages, and stillbirths with a loss rate of over 50% (**Figure 1A**). This group was separated from animals with clinical signs of TC according to the increasing severity scale (**Figure 1B**). The stillborns presented different degrees of morphological impairment. The live embryos of this group were born underdeveloped and with little reaction to external stimuli (**Figure 1B**). They presented extensive liver disease characterized by necrosis, abdominal bloating with high fluid retention, and apparent swelling of the spleen as well as cutaneous and pulmonary hemorrhaging (data not shown).

In addition, by macroscopic analysis, besides the swelling of some organs, there was malformation of the anterior and posterior limbs as well as significant decrease in skull size characterizing microcefalia in these animals (**Figure 1B**). These embryos from infected mothers were extremely swollen, both brain and muscle tissues retaining so much fluid that it was impossible to obtain samples for histological analysis (**Figure 1B**). Some of these embryos exhibited a very committed development such as malformation of the trunk (**Figure 1C**) and even total absence of hindlimb formation (**Figure 1C** and **D**).

Group 2 females inoculated with 50 cysts, although fertilized, did not terminate pregnancy, dying in few days. Many embryos were reabsorbed. In this experimental condition, there was nearly a total loss of females and their embryos.

Longitudinal sections of pregnant mouse muscle tissue infected with 25 cysts of *T. gondii* (Group 1), showed similar cyst structures between muscle fibers (**Figure 2A**). In various sections, the images suggested that parasites were evading the cysts and invading other cells (**Figure 2B** and **C**). The healthy mouse embryo leg muscle tissue demonstrated high density of mature myofibers arranged lengthwise, reduced interfibrillar space, and a well-organized striated profile (**Figure 3A**, **C**, and **E**). Even where these fibers were spaced, the amount of mononuclear cells from the connective tissue or inflammatory infiltrate was markedly small

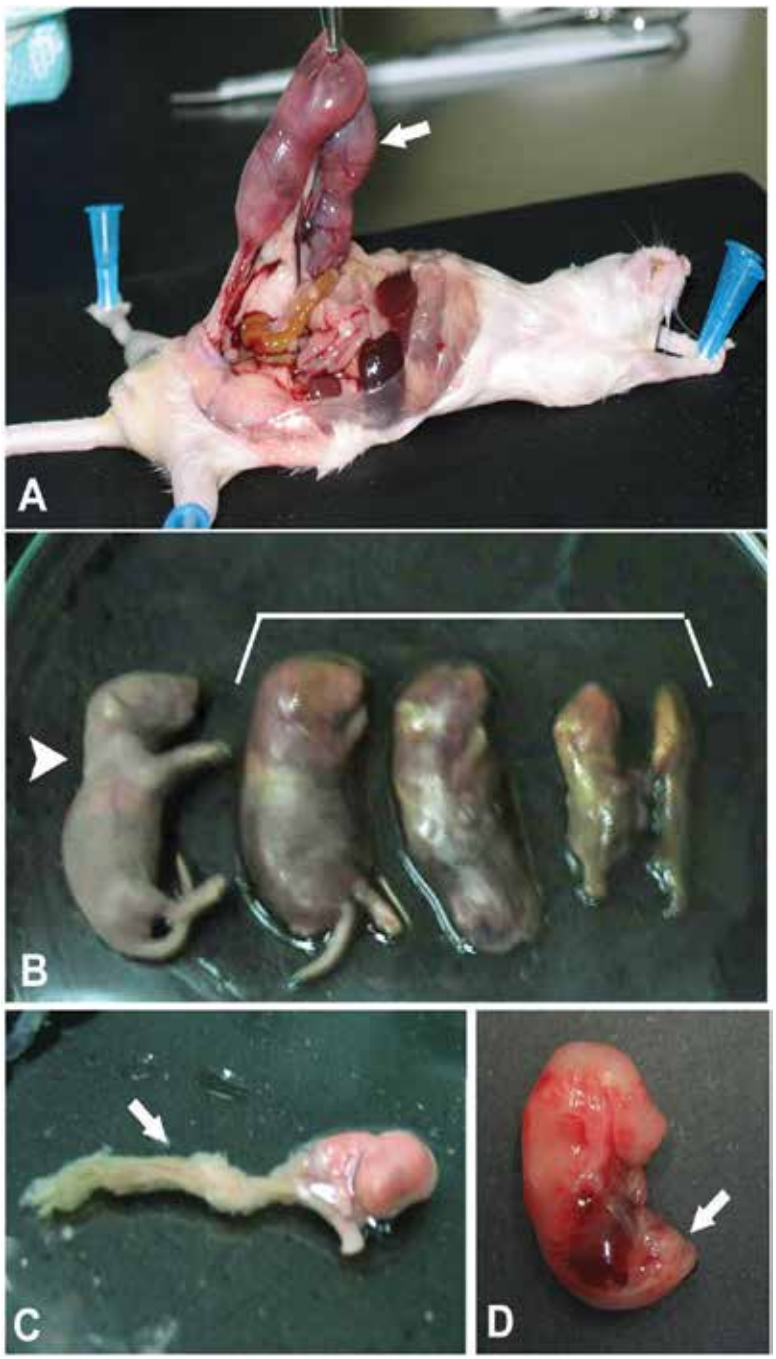

Figure 1. Swiss mouse embryos from experimentally infected mothers with *T. gondii*. (A) Harvesting of embryos by cesarean section (arrow). (B) The infection differentially affected pups in the same litter. In the extreme left of this picture is shown one pup apparently unaffected by the infection, whereas its littermate present different degrees of morphological alterations and decrease of skull size characterizing microcephaly in these animals. (C) Stillborn embryos show congenital malformation, including compromise of the trunk and hindlimbs (arrow). (D) Embryo present atrophy of the hindlimbs (arrow).

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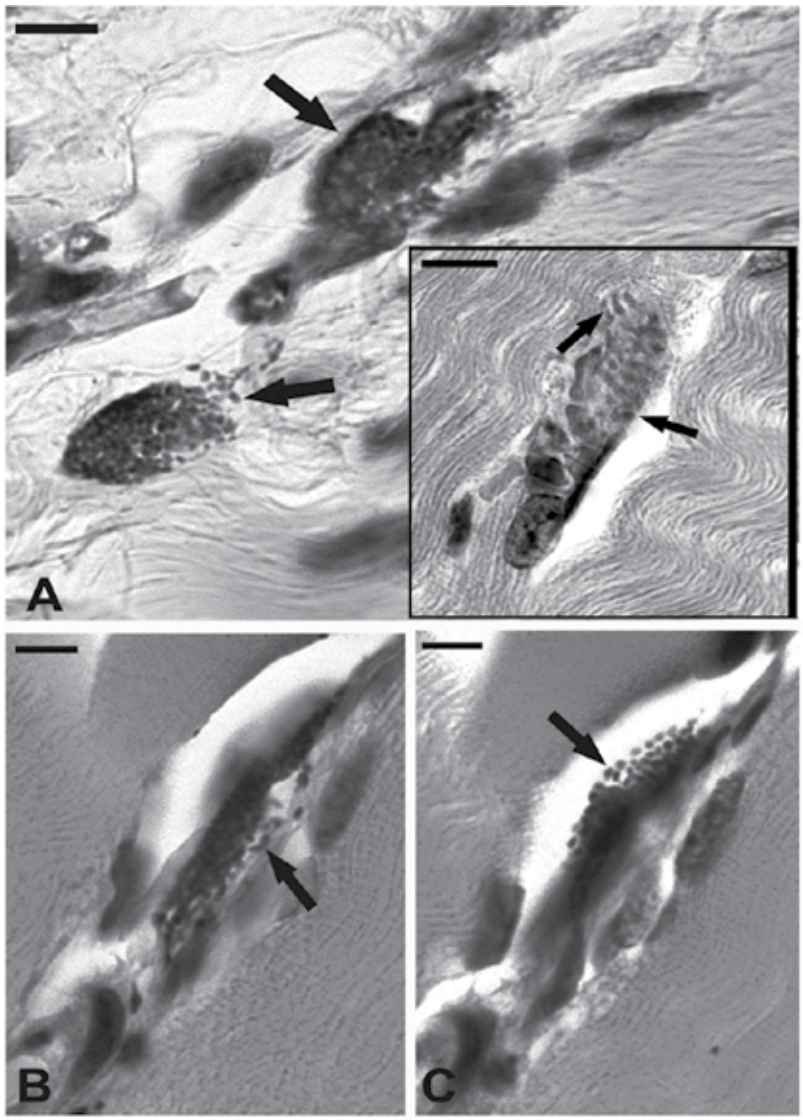

Figure 2. By light microscopy, longitudinal sections of adult muscle tissue of pregnant female infected with 25 cysts of *T. gondii* (Group 1). (A) Cyst-simile structures can be seen between myofibers (arrows), Bar: 15 μm. Note that the parasites seem to evade these structures (inset), Bar: 20 μm. (B and C) The same cell can be seen in two distinct focal planes showing small structures similar to Toxoplasma (arrows), Bars: 10 μm.

(**Figure 3A**, **C**, and **E**). However, the thigh muscles of embryos from *T. gondii* cyst-infected mothers, as compared to tissue from uninfected mice, contained a significant reduction in myofiber density and a great enlargement of the interfibrillar space (**Figure 3B**). Connective tissue cells filled this space, probably mononuclear cell inflammatory infiltrates such as macrophages, lymphocytes, mast cells, and eosinophils (**Figure 3B**, **D**, and **F**). Besides the shortening of mainly mature myofibers, muscle tissue analyzed of embryos born from infected mice presented several bipolar cells possessing small and elongated nuclei characterizing myoblasts

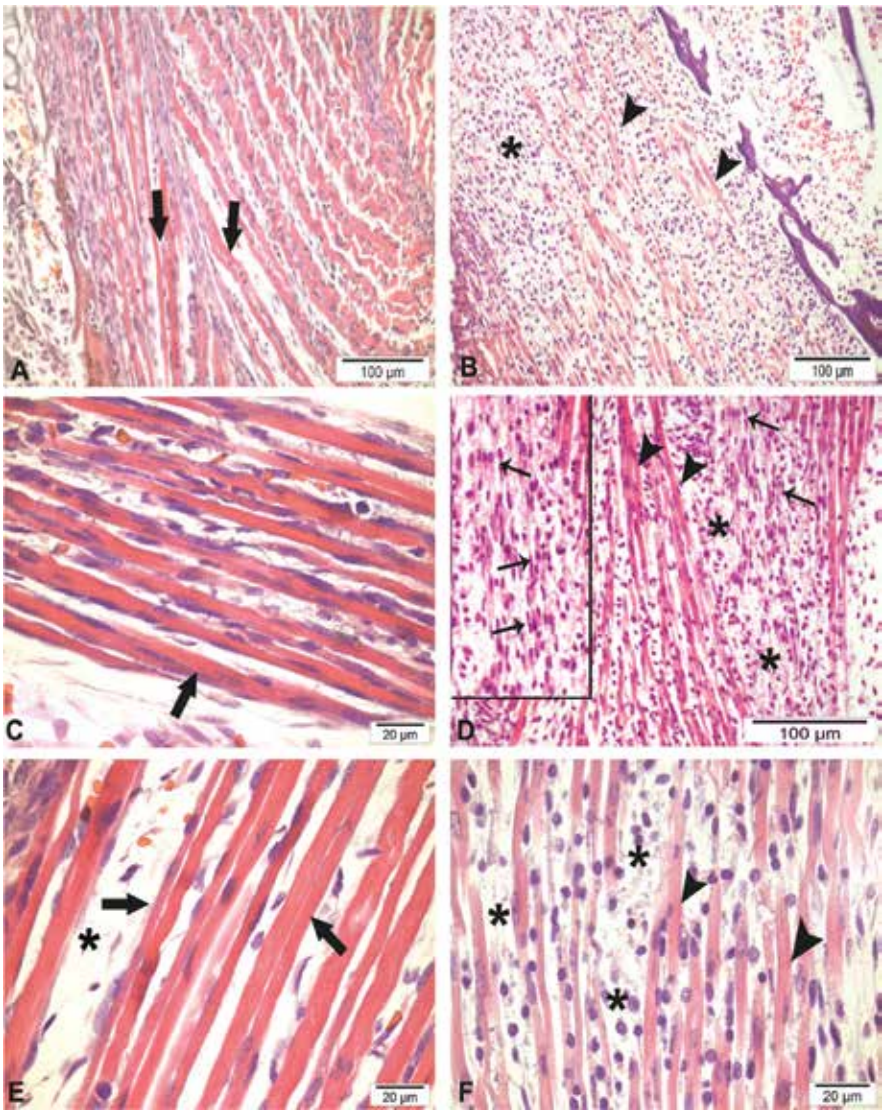

Figure 3. Longitudinal sections of the muscle tissue of thighs of murine embryos normal and from infected mother. (A, C, and E) Healthy embryo: note the high density of mature myofibers and small interfibrillar space (arrows). (B) Embryo muscle from infected mother: smaller density of myofibres (arrowhead) and greater spacing between them, probably filled by cells of connective tissue and cell inflammatory infiltrates such as: macrophages, lymphocytes, mast cells, and eosinophils (asterisks). (C) Healthy embryo: at higher magnification, dense and mature myofibres are observed with parallel arrangement and many nuclei. Note limited interfibrillar space and few mononuclear cells between myofibers. (D) Embryo from infected mother: muscle with scarcity of myofibers (arrowhead). Besides greater interfibrillar space, presence of a large number of mononuclear cells (asterisks). Note several bipolar cells with nuclei small and elongated (fine arrows). Inset: more detail these of aligned cells (myoblasts in the process of alignment and fusion). (E) Detail of healthy embryo myofibers. Higher cytoplasmic density of the myofibres with a striated profile and more organized. Interfiber space with number of cells, mononuclear cells markedly smaller (asterisk). (F) Embryo from infected mother: myofibers are smaller, less dense, less stained, and more spaced (asterisk). Apparently they are immature cells (less nuclei and cytoplasmic) and discontinuous in their extension (arrowhead).

in the process of alignment and fusion (**Figure 3D**). These embryos presented discontinuous, shorter, less dense, and slightly colored myofibrils with more interfibrillar space and immature cells with fewer stained and poorly defined striated profiles (**Figure 3B**, **D**, and **F**).

Brain samples of control embryos and infected mothers were examined. All control embryo brain tissues did not show any changes, the brain tissue normal and cell density characteristics of healthy tissue. Tissue section showed arranged cells in a pink color with the cytoplasm unaltered in purple, the nuclei with no visible changes in the chromatin arrangement and almost no intercellular space (**Figure 4A**). In contrast, the brains of embryos from infected mothers exhibited irregularly arranged cells with higher concentrations at apparently less injured points, reduced cell density, and a significant increase of intercellular space, possibly due to the decrease of the adherent junctions (**Figure 4B–D**). In addition, there was weak

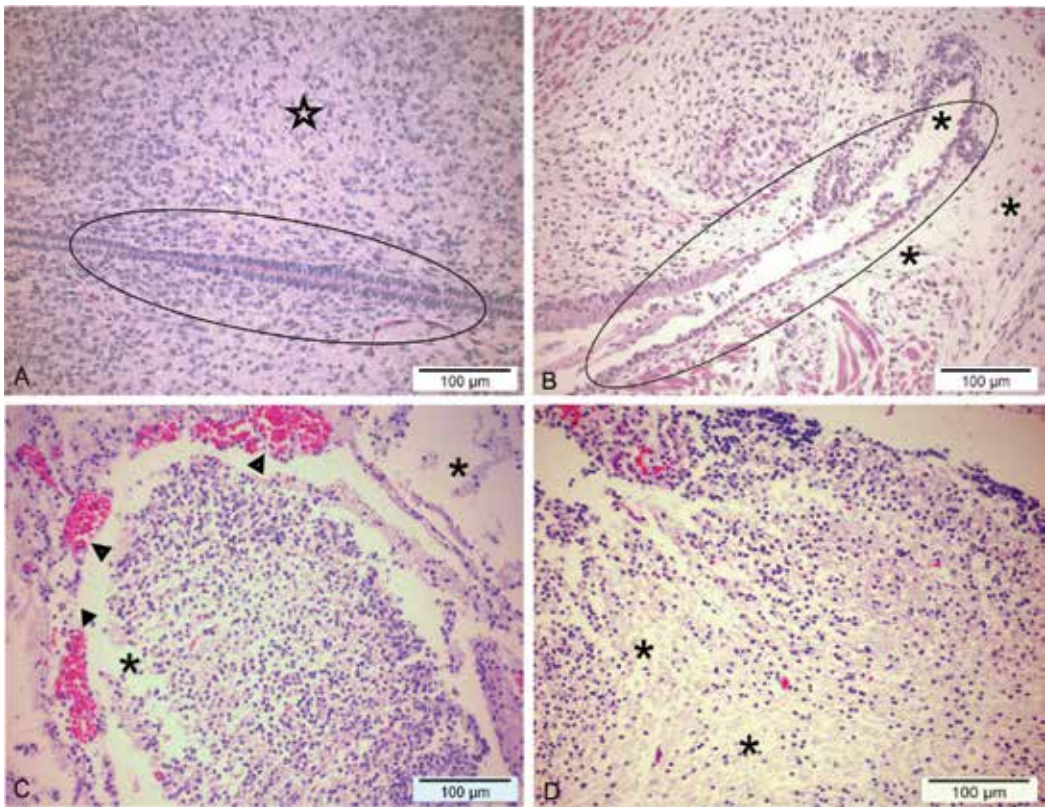

Figure 4. Using H&E staining, brain samples were examined for histopathological damages caused by Toxoplasma in embryos from infected mother. (A) Brain of an uninfected control, histology without any pathological changes. Regularly arranged cells of similar size (circle), cell density characteristic of healthy tissue, well stained, cells arranged in a pink color, with the cytoplasm unaltered in purple, the nuclei with no visible changes in the chromatin arrangement and almost no intercellular space (star). (B–D) The neurogenesis was severely affected and cerebral cortex of these embryos was compromised. Irregularly arranged cells, more concentrated at a point apparently less injured, reduced cell density, with a significant increase of intercellular space (asterisks). Note several points with recent thrombi of different sizes due to the massive presence of red blood cells (arrowhead).

cytoplasmic labeling and nuclear marking disparity, some larger nuclei almost transparent, others much smaller, marked. The presence of mononuclear cell inflammatory infiltrates was noted, such as macrophages, lymphocytes, mast cells, and eosinophils. There were several points with massive presence of red blood cells, implying recent thrombi in all analyzed sections (**Figure 4B** and **C**). In addition, different levels of brain tissue impairment were apparent (**Figure 4D**), such as presence of cells similar to astrocytes and neurons, which possibly suffered karyolysis (weak nuclear staining) and eosinophilia (**Figure 5B** and **C**). Some of these cells presented nearly transparent cytoplasm resulting from the loss of basophilia and others an apoptotic process characteristic (**Figure 5C**). No analyzed sections revealed parasites.

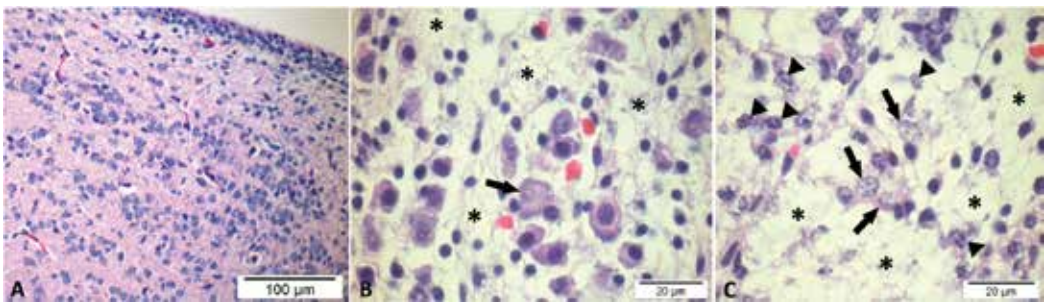

Figure 5. Using H&E staining and light microscopy embryonic brain cell lesions with CT was analyzed. (A) General aspects of normal embryonic brain tissue. (B and C) Observe the lower density and cellular organization in the brains of embryos from *T. gondii*-infected mothers (asterisk). Some cells, similar to astrocytes, possibly suffered karyolysis (weak nuclear staining) (arrow) and eosinophilia (asterisk), with the almost transparent cytoplasm resulting from the loss of basophilia (asterisk). (C) Presence of cells with a nucleus characteristic of the apoptotic process (arrowhead).

4. Discussion

This study investigated the role of *T. gondii* infection in neurogenesis and myogenesis of skeletal muscle tissue of murine embryos from infected mothers. Our previous results *in vitro* disclosed significant parasite interference in the myogenesis process, hence the motivation for the development of this work.

Our first experimental strategy was gavage infection of the female with cysts aiming to mimic *T. gondii* primary transmission in nature through ingestion of raw or poorly cooked meat containing these cysts [30]. The developmental commitment of embryos from Toxoplasma-infected mothers was incontestable, and the *T. gondii* pathogenicity degree is determined by many factors such as susceptibility of host species, infection stage (acute or chronic), and parasite strain virulence [17, 30, 31]. Macroscopic images clearly expose the malformation or not of anterior and posterior limbs as well as other malformations during the development of ME-49 strain *T. gondii*-infected mice.

Our experimental model with Swiss mice involving infection of the female prior to mating demonstrated that during the first week of pregnancy, there was significant fertility reduction,

embryos with low birth weight, fetal reabsorption, miscarriages, and stillbirths, presenting a loss rate of over 50% for Group 1 (inoculated with 25 cysts) and almost 100% in Group 2 (inoculated with 50 cysts). However, since each cyst can vary in bradyzoite numbers (which may die or survive), it was not possible to define the parasite concentration that generated a certain vertical transmission rate. This would explain the loss of more than 70% of the embryos, considering that bradyzoites differentiate to tachyzoites that can damage the placenta, leading to loss of the embryo by spontaneous abortion or absorption, as proposed by Vargas-Villavicencio et al. [20]. These data confirm that the severity of Toxoplasma infection in females and embryos depends upon the concentration of parasites [18–20, 31]. TC is one of the most serious consequences of acute Toxoplasma infection, the greatest severity observed in early pregnancy as demonstrated experimentally in this study.

Embryos from infected mothers presented brain tissue with possible levels of ischemia and morphological aspects compatible with cell death mechanisms. Inflammatory reactions were apparent suggesting the focal presence of mononuclear cell infiltrates, necrosis, and thrombi, which increased according to the degree of infection development, compromising the neurogenesis. It is known that during apoptosis, the cells lose their adherent junctions [32], and lesions, due to ischemia or hypoxia together with other stimuli, also triggering cell death (necrosis and apoptosis) [33, 34]. Some cells, similar to astrocytes and neurons, underwent karyolysis (weak nuclear staining) and eosinophilia with the nearly transparent cytoplasm resulting from the loss of basophilia [35, 36]. The results described here during the cerebral histological analysis point to neuropathogenesis induced by the *T. gondii* infection. Sun et al. [37] demonstrated that this infection in early gestation might inhibit the proliferation, differentiation, and migration of neural stem cells in rats. It has been reported that *T. gondii* induces apoptosis of neural stem cells via the endoplasmic reticulum stress pathway [38]. Although we did not detect the presence of the parasite at the lesion site, some authors suggest that parasite proteins may directly interfere with neuronal function, either in infected or neighboring cells [18, 39–41]. These data corroborate our observations implying a *T. gondii* proapoptotic effect in cerebral cells and also justify the embryonic brain size decrease as described here.

The systemic inflammatory response induced by the parasite (toxoplasmosis sepsis) witnessed in our experiments may have caused multiple organ failure of these embryos, as proposed earlier [17]. Despite the already known involvement of muscle tissue in the development of the toxoplasmosis chronic phase [42], no studies involving embryos from *T. gondii*-infected mothers have ever investigated the influence of this infection in muscle tissue development. Our histological analysis demonstrated that muscle tissue from such embryos, in comparison with the healthy muscle myofibers, possessed lower density and higher interfibrillar space. Connective tissue cells and probably mononuclear cell inflammatory infiltrates such as macrophages, lymphocytes, mast cells, and eosinophils fill this space [1, 16]. Besides the shortening of myofibers and absence of mature myofibers, muscle tissue from *T. gondii*-infected embryos presented several bipolar cells with small, elongated nuclei characterized as myoblasts, still in the process of alignment and fusion (myogenesis). Myofibers from these embryos displayed, besides small size, lower density of cytoplasmic material (characterized by a slight staining, H&E), discrete striation, and a discontinuous profile. These apparently immature cells with few nuclei directly influenced the length of myofibrils thus interfering with the myogenesis

process. Healthy embryos have the muscle tissue with many satellite cells attached to the myofibers, myoblasts in the process of fusion, and mature myofibers parallelly aligned dense and continuous [43]. The histological staining technique of H&E showed well-defined myofibers with striated characteristics of this cell type, dense cytoplasmic material, and greater proximity between them. This high cytoplasmic density characterized by intense staining of myofibers must be associated with an increased myocyte fusion process in the early development of the muscle fiber. However, it has been well established that *T. gondii* can cause myositis either by recent infection or reactivation of tissue cysts, affecting the homeostasis of muscle tissue [1]. Regarding the perspective of this study, our results confirm that *T. gondii* primary infection of premated mouse females affects the embryonic development, inhibiting the process of in the skeletal muscle *in vitro* model myogenesis [13]. In this study was demonstrated that *T. gondii* infection in skeletal muscle cells downregulate the M-cadherin mRNA expression, leading to molecular modifications on the host cell surface that disarray at the contact sites between myoblasts and myoblasts-myotubes, promoting the instability of the junctions. This progressive process interferes with membrane fusion and consequently inhibits the myogenesis process. This set of data justifies the results by histology that point out significant changes in the formation of embryonic myofibers from infected females before mating, corroborated by macroscopic images, showing some embryos with no formation of anterior and posterior members. These changes could lead to the modulation of other molecules contributing to toxoplasmosis pathogenesis in the murine muscle tissue as described here in the *in vivo* system. Additionally, recent research has reported that a virulence factor secreted by *T. gondii* rhoptries (ROP18) may contribute to neuronal apoptosis through the ER stress-mediated apoptosis pathway, which result in neurological diseases by reduction of these cells [41]. In rat brain cells with the same *T. gondii* ME49 strain the apoptosis induction was described, but the cell type involved in this process was not specified [39]. Therefore, we believe that the disorders in muscle tissue during our assays *in vivo* may be also related to a program of cell death, for example, apoptosis with the decrease of the adherent junctions, as also observed in the brain cells of embryos from infected mothers. More detailed histopathological studies are in progress considering that our preliminary analyses clearly indicated that embryos from the same litter had a differentiated influence in the degree of embryo development commitment induced by Toxoplasma.

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Chapter 5

Experimental Models of Ocular Toxoplasmosis

Aléx Martins Nasaré and Roberto Carlos Tedesco

Additional information is available at the end of the chapter

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Abstract

First described in *Ctenodactylus gundi* and simultaneously in rabbit, *Toxoplasma gondii*, an etiological agent of toxoplasmosis, affects different species of vertebrates and invertebrates, presenting different manifestations depending on the host. Ocular toxoplasmosis is one of the mean manifestations of toxoplasmosis in humans, affecting 2% of infected individuals in Europe and North America. Otherwise many aspects of ocular toxoplasmosis still await answer. One of the major factors limiting this process is the difficulty to obtain human samples, doing necessary the use of experimental models. By the way, animal models do not express reality of human disease. The present study defines a compilation of report cases and results that supports the choice of an ideal experimental animal model of ocular toxoplasmosis. Actual literature bears new researches contributing in the choice of a specific experimental animal model. Moreover, the choice must consider behavior, period of life, and maintenance in captivity and ocular toxoplasmosis manifestation. Previous studies contribute for a best-chosen experimental animal model, by the way fragmented information makes difficult to compare mostly animal models picked that do not present efficiency enough. In conclusion, experimental animal models are able to bring relevant information about the course of ocular toxoplasmosis.

Keywords: experimental model, ocular toxoplasmosis, animal model

1. Introduction

Toxoplasma gondii was first described in 1908 by Nicolle and Manceaux, presented into tissues of a north-African rodent, known as *Ctenodactylus gundi*, which after was mentioned to describe the specific epithet that compose specie. Simultaneously in Brazil, Splendore identified same parasite in rabbit tissues. *T. gondii*, etiological agent of toxoplasmosis and an obligatory intracellular parasite, affects different species of vertebrates and invertebrates. In addition, *T. gondii*

presents three different infective forms that can infect hosts by ingestion of raw meat containing cysts or, food and water containing oocysts [1]. Infection can also affect the embryo, during pregnancy; with different levels of severity, depending on the specie and the period that infection occurs. Toxoplasmosis can present different manifestations, as fever, increase of lymph nodes, intraocular inflammation [2], encephalitis, and other systemic infections [3].

Ocular toxoplasmosis, a vision-threatening ocular disease, is the most common cause of infectious uveitis [4]. It is one of the mean manifestations of toxoplasmosis in humans, affecting up to 30% of the humans worldwide. In Europe and North America, development of ocular toxoplasmosis is around 2% of infected individual. In Brazil, uveitis is estimated to be caused by toxoplasmosis in 60–85% of lesions cases [5]. Highlighting Erechim, Southern Brazil with a high frequency of humans infected usually transmitted by ingestion of uncooked meat containing cysts [6]. Ocular toxoplasmosis was first described in 1923 by Jankû, and subsequently by Levaditi in 1928. Whereas it was just in 1952 that *T. gondii* had its relation to the retinochorioiditis Foerster in Weiss and Kim [7]. Accordingly, some authors, retinochorioiditis is a major cause of visual impairment in the USA, where it accounts around 30–50% of all cases of uveitis [8]. Otherwise many aspects of ocular toxoplasmosis still await answer.

One of the major factors limits is the difficult to obtain human infected material. Once *T. gondii* had a predilection by nervous tissues, locating itself in immune competent individuals in brain and eyes. So, it does necessary experimental animal models. But, animal models do not express reality of human disease. Therefore, the establishment of an ideal experimental model, that mimic the course of ocular toxoplasmosis, is fundamental as a springboard for finding answers of the disease. Considering particularities of each experimental animal model for each specific conduced study.

2. Ocular toxoplasmosis

Accordingly literature, the *T. gondii* reaches the bulb of the eye using the blood, normally causing a focal lesion and asymptomatic, but not well comprehended [9]. The eye is an immunologic privileged environment, as the brain. But, brain barrier is more studied than ocular barrier [10]. In previous studies, ocular toxoplasmosis infection has been formed principally by lymphocytes B. This increase in migration of lymphocytes is associated to high levels of IFN-γ and IL-6 and low levels of IL-10, suggesting that cytokines alterations provide inflammation and retinochorioiditis. Other studies indicate that the severity of ocular toxoplasmosis is close related to intensity of inflammation and presence of IL-17. Thus toxoplasmosis infection relates pathogenesis to Th1 and Th17 cell. While IL-10, TGF-β, and IL-27 are cytokines with an important role into immune regulation of maintenance and inflammation of important sites, as eye environment [5, 7].

The most affected structure in ocular toxoplasmosis is the retina. Retina is the nervous tissue of the bulb of the eye, responsible for transduce photon stimulus into electric impulse. And

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for that transduction occurs, it is necessary that the integrity and well function of the 10 layers of retina tissue, identified in **Figure 1**, from the inner tissues to outer in the following order: inner limiting membrane, nerve fibers layer, ganglion cells, inner plexiform, inner nuclear layer, outer plexiform and nuclear layers, outer limiting membrane, segments of photoreceptors, and retinal pigmented epithelium. In evidence, toxoplasmic retinochorioiditis affects straight the retina as a structure, but most damaged layers are nuclear plexiform and ganglion layers, as well as the segments of photoreceptor, once RPE presents a migration, causing a discontinuity forming great interstitial lacunae. Also is observed inflammatory cells in the inner plexiform layers, also responsible for none well function of retina tissue [11].

The retina is the first area involved, furthermore choroids, vitreous humor, and anterior chamber might be also involved, but never will it happen before retina. The most common sign of acute ocular toxoplasmosis is a focal and in major times, unilateral retinal lesion [12].

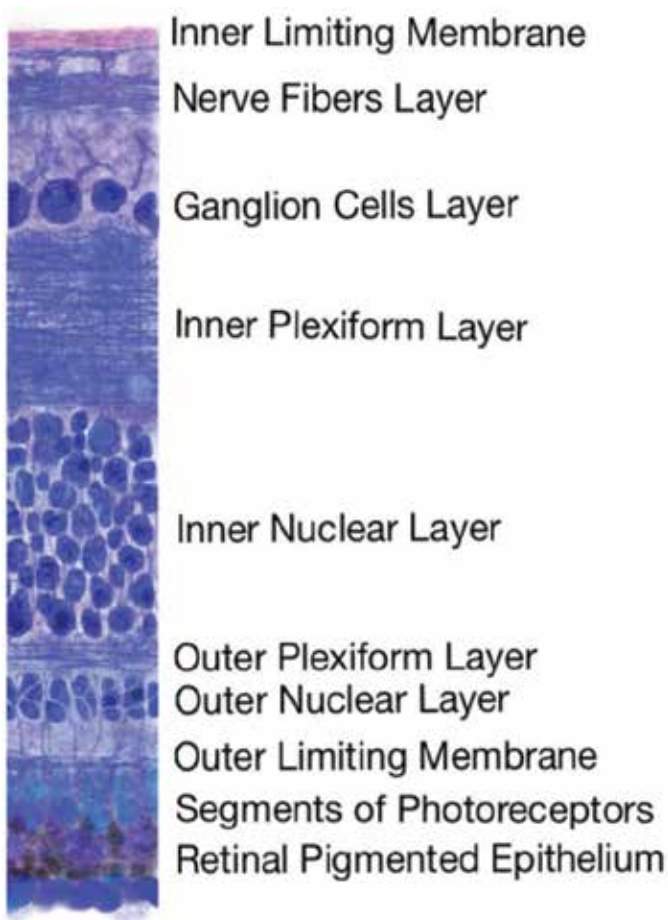

Figure 1. Retinal scheme containing 10 layers that compose this tissue.

But, in some individuals it was observed too, bilateral lesion, occlusive vasculitis, diffuse necrotizing retinitis and scleritis. Retinochorioiditis is commonly observed in congenital and acquired toxoplasmosis [13] resulting in acute disease and infection reactivation. It is possible to affect one or both eyes, presenting it in three different forms. Forms can be didactically differentiated (**Figure 2**) as punctual, multiple, and satellite lesions. In other worlds, punctual lesion might indicate primary infection, while multiple and satellite lesions, might indicate reactivation of disease. In addition, satellite lesions it is characterized by a combination of an active lesion with a healed retinal scar [10]. By the way, it is not dependent if it is acquired or congenital toxoplasmosis [4].

Clinical finds may be influenced by clonal type of *T. gondii*. There are three predominant clonal types, based on virulence studies [14]. But virulent strains appear to have their origin in one genetically homogeneous lineage. Studies based one murine model infers that type II would be responsible for the majority of ocular lesions. Furthermore, recent studies shows that type I, is atypical and with an important role in ocular lesions [14–16]. In Europe, type II strains are responsible for majority of human infections. In South America, a recombinant strain is associated to ocular toxoplasmosis [4]. Studies of Howe and Sibley, in 1995 and Belfort-Neto and collaborators in 2007 correlate type II with an important role in acquired infection, while type I clonal lineage may be responsible for the congenital toxoplasmosis, even so type I and atypical strains may play a role in acquired infection [14]. For instance, type III has been reported only 9% of toxoplasmosis patients in France and United States [17]. However, even with few information relating clonal type with virulence and frequency, just an insufficient part is known, needing more studies in this area.

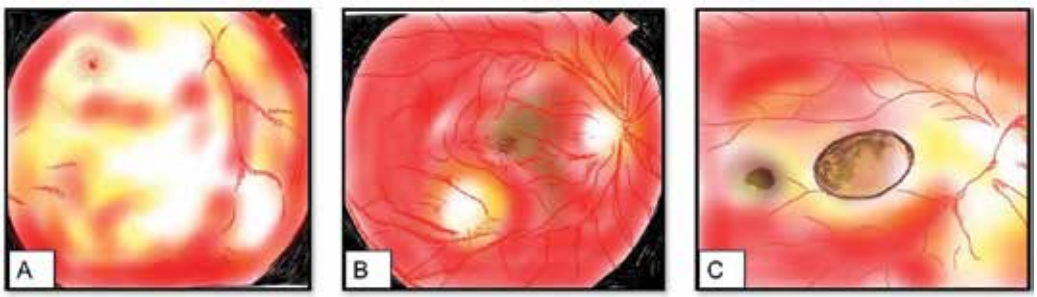

Figure 2. Ophthalmoscopy scheme differentiation of retinal lesions; (A) punctual lesion; (B) multiple lesions; and (C) satellite lesion.

3. Acquired toxoplasmosis

Acquired toxoplasmosis occurs principally for ingesting of raw meat, vegetables, and water contemned with oocysts of *T. gondii*. Acquired toxoplasmosis normally can affect the eye in acute form, beyond 10% of the cases, but the majority is naïve, transitory, and frequently asymptomatic [18]. Loss vision in acquired toxoplasmosis is normally due from destruction of retina tissue, specially the macula. Furthermore, might be worse by reactivation, months or years after [19].

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4. Congenital toxoplasmosis

Congenital toxoplasmosis is a result of an infection in prenatal presenting clinic morphologies before birth, or just along the first quarter of gestational period, been present in the forms of anencephaly, hematological abnormalities, loss vision, and blindness or even death [4, 20]. But the majority of manifestations are rare, been subclinical at birth. It can be possible to present lesions along the life. Congenital ocular toxoplasmosis can present itself neonatal, or lately, but it is more common in the last quarter of gestational period. Retinal destruction is a very frequent sequel of congenital disease [8] presenting a predilection for macular involvement [4]. A congenital ocular lesion appears to be the highest risk of systemic infection when compared to acquire ocular toxoplasmosis. Congenital ocular toxoplasmosis has been estimated to affect 3000 kids born in the USA each year. It affects 70–90% of patients, been the most common manifestation of disease. By the way, great majority of kids appears asymptomatic. Report cases indicate that some of them develop chorioretinal lesions, from scars until vision loss [7].

5. Experimental models of ocular toxoplasmosis

First animal model of toxoplasmosis was guinea pig, established by Markham in 1937. But just in 1951, the first animal model for ocular toxoplasmosis was established by Hogan, using intracarotid injection in rabbits. Following same lineage, in 1953, Frenkel managed an intraperitoneal injection in hamster. Lately nonhuman primates, cats, pigs, and dogs started been used as model [4]. Accordingly history of experimental animal models for toxoplasmosis, author and method used exposed in following time line (**Figure 3**).

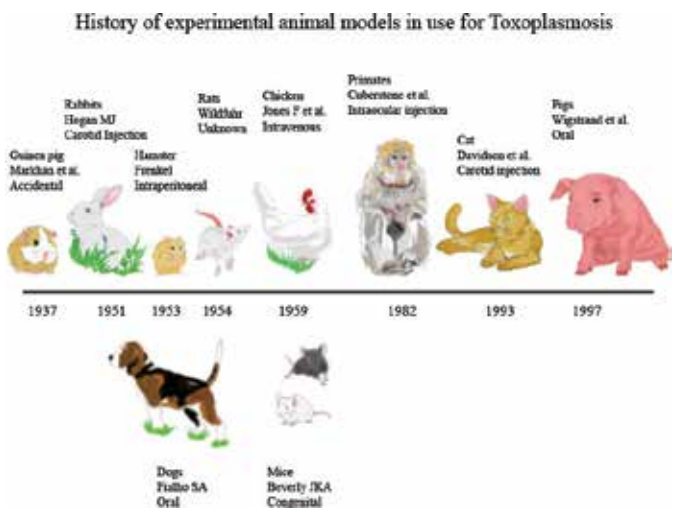

Figure 3. Schematic time line with historic of experimental animal models for toxoplasmosis, independent on development of ocular toxoplasmosis.

In impossibility to obtain sample from human with acute ocular infection, animal models has been developed to supply and find answers [4]. In spite of, any animal model of ocular toxoplasmosis must consider some criteria: parasite entry, onset of disease and manifestation, self-limitation of ocular inflammation, and model of recurrence, parasite strain used, and experimental manipulation of disease processing and treatment [10]. Ocular toxoplasmosis animal models should answer how disease happens in humans, as close as possible, from entry of parasite to development of disease. Furthermore, strains of parasite is so important as route of infection, once some strains show certain defect achieving system of host. The most used strains are: RH, ME-49, Beverly, PLK, and Fukaya. Strains from type I was isolated from patients and animals. Type II lesser extent type I strains are used in animal models of ocular infection.

T. gondii parasitizes the host, without producing clinical disease. After penetrating intestinal epithelial cells. *T. gondii* may spread to other organs by lymphatics and blood system, multiplying in any cell of the organism. As long *T. gondii* may cause an infection and severe disease in animals, causing great losses in some species, like sheep and goats, generating embryonic death, abnormalities, and others.

It is important to highlight that toxoplasmosis affects individuals in different forms, and in some cases do not express the disease how it is described in literature, otherwise it is of major importance to express how parasite is able to hand on the intermediate and definitive host. In immunosuppressed host, toxoplasmosis may lead them to death, unless accomplished of correct treatment. Actually known as corticosteroid, sulfadiazine, and pyrimethamine. Those medics relapse disease, doing necessary in some experimental models. Besides, pathogenicity of *T. gondii* is determined by virulence of the strain and resistance of the host, in some cases even hosts habits.

Nowadays, it is known that some variation of mice is more susceptible than other, seeing the severity of infection course. Adult rats do not become ill, differently from the youngers from the same species, which can dies of toxoplasmosis. Ungulates are the most resistant hosts to toxoplasmosis, whereas some marsupials, and nonhuman primates are highly susceptible to *T. gondii* infection [21]. In literature animals like pig, ordinarily there are not many descriptions as experimental models, moreover as case report. Like young pigs, which have high mortality, while adult pigs are resistant. Notwithstanding, infected pigs are described with pneumonia, myocarditis, encephalitis, and placental necrosis. Other species as mink, rabbits, birds, and other domesticated and wild animals, show sporadic and widespread outbreaks of toxoplasmosis [22]. Many authors have described toxoplasmosis in different species along the years, and this allows conclude how spread is toxoplasmosis.

Retinochorioiditis in laboratory animals may vary, because interferences with parasite proliferation and viability, and the inoculation route (perioral, intraocular, intraperitoneal, conjunctival, or parenteral administration [23].

6. Methods of infection

Any experimental animal model of ocular toxoplasmosis has a set of specific features: specie, parasite strain, stage and concentration of parasites per milliliter of the inoculum, and infection

route. Also important, parasite entry must be rigorously chosen. Parasite entry groups: intracarotid injection; intraperitoneal and oral infection, and presents different recurrences of the disease. Intracarotid injection is performed in bigger experimental models like nonhuman primates, cats, and rabbits. Animals must be anesthetized, accordingly Institutional Animal Care and Use Committee, and the right common carotid artery has to be isolated surgically. Using a 27-gauge hypodermic needle, the inoculum must be performed [24].

Intraocular injection allows purport an ocular toxoplasmosis, and has been used in nonhuman primates, guinea pigs, rabbits, and mice. It is possible to perform intracamerally, periretinally, or intraretinally. The disadvantage of this method is that it can cause mechanical damage to retina, which may interfere in results of the course of the disease. First experiments, using this method consisted on retinal injections through the pars plana, crossing the vitreous cavity. Before introducing needle it was necessary a sclerotomy in the pars plana. Place where the needle was introduced [25]. Notwithstanding, administration by instillation, consist of an application over the surface of the eye, and it has shown close results to intraocular injection and in addition maintains integrity of ocular tissue [2]. Instillation is conduced using one drop of solution containing a determined concentration of parasites directly on eye surface.

The most used method, intraperitoneal injection consists of an inoculum inside the peritoneal cavity. This method is easy to perform and successfully leads to ocular toxoplasmosis and, it has many reports in hamsters and mice. Perioral via is described in literature, by introducing a solution containing oocytes in determined concentration inside the mouth of the animal. Equally important gavage is executed with an instrument that certified that experimental animal model received the right dosage, introducing a solution containing oocytes direct into the stomach of experimental model. But it does not reproduce the ocular toxoplasmosis in majority of cases, further results, or depending on the strain no result. Nevertheless performing perioral demonstrated promising results. For congenital ocular toxoplasmosis experimental models, still been an inoculum in pregnant mice, or embryonated eggs. Normally, pregnant mice are infected via intraperitoneal or perioral. Alternatively, an injection of Tachyzoite free forms in embryonated eggs, allow simulating directly embryo infection.

7. Diagnose in experimental model

In experimental model, identification of toxoplasmosis can be made finding *T. gondii* cysts into the biopsies, with a specific color reaction and immunohistochemical, or polymerase chain reaction (PCR) technique [13]. Nowadays, PCR is used even when is not found the cysts on biopsies. Diagnose of toxoplasmic retinochorioiditis in experimental animal model, is already described in literature, as a combination of three techniques: ELISA, PCR, and immune-staining. By the way, PCR has been replaced by real-time PCR (qPCR), that increases the sensibility of diagnose in 97% and considers that results are further than PCR [14]. Eventually, enzyme-linked immunosorbent assay (ELISA) is one of the most used tests, identifying immunodominant antigens during *T. gondii* infection. However, it can be used only as a complementary diagnose, once identify antigens and not the parasite. Actually, ELISA has been replaced by

flow cytometry. Flow cytometry allows to measure the concentration of substances further and in a less sample dilution. Besides, nowadays there are some specific commercial kits that provide good results, multianalyses in a short period.

8. Guinea pig model

Guinea pigs were one of the first experimental animal models used for this purpose, in an accidental experiment [26]. While working with the submaxillary gland virus of guinea pigs, Markham had noted that some of animals were dying after experiments. Asymptomatic and temperature abruptly fell. However, they have rarely been used for further investigation of the disease after first experiments, with little information about. In a study of acquired transmission, a conjunctiva penetration of tachyzoites in this model was preceded, and authors found invasion of conjunctiva epithelia and goblet cells within 15 minutes post infection [27]. Replicating study within 4 hours was possible to identify a high concentration of antibodies. In conclusion, *T. gondii* evokes a mild to moderate inflammatory response. In spite of, the instillation and infection perioral have associated lymphoid tissue, been considered part of the mucosal immune system, and invasions mimic the three forms of parasite forms (tachyzoites, bradyzoites, and sporozoites). In accordance with previous studies, guinea pig might be a good experimental animal model of ocular toxoplasmosis, once it is susceptible to *T. gondii* infection and also it is resistant to great number of parasites [28].

9. Rabbit model

Rabbits are known to have a genetically determined low resistance against infection. With exacerbated inflammation accordingly to virulence and antigenicity of the parasite strain. Toxoplasmosis in this animal model was described first by Hogan in 1951 [29]. Infection with RH strains is able to produce chorioretinal lesions similar those described in cats and humans, however, concomitantly meningoencephalitis and rapid death. This describe how susceptible is this model. The most common route of infection in this experimental animal model is intravitreal inoculum, developing retinochorioiditis, equally in nonhuman primates. In rabbits as the same in nonhuman primates model is possible to observe pigmented scars after disease remission.

Intraocular infection combined with ophthalmoscopy in rabbits is easy to perform. The infection is described as a dissemination of retinochorioiditis, vitreal infiltration, and retinal detachment [30]. First affecting retina before choroid, differing from *Felidae* model. By the way, this route of infection might cause some trauma in the ocular tissue interfering on results. Instillation on the ocular surface must supplant best this method. In studies, which were described perioral infection, animal model presented low seroprevalence, and did not presented parasites, when histologically analyzed. Tissues like brain, diaphragm, and heart are considered the most damaged. But with no ocular signal described.

10. Hamster model

As experimental model, hamsters had experimental ocular toxoplasmosis, described by Frenkel in 1953 [31], using RH strains showed necessity of therapy while CJ did not generate ocular lesions. In fact the use of ME-49 strains do, intraperitoneal or perioral may influence on ocular toxoplasmosis [7].

Golden Hamster, when infected with RH or CJ strains of *T. gondii*, normally presents sporadic ocular disease, and without treatment with sulfadiazine and pyrimethamine, animals succumb to encephalitis. In contrast, when using ME-49 strain, Golden hamster became an excellent model for ocular toxoplasmosis [32]. Disease may vary among hamsters, but all develop ocular disease, usually limited to retina with loss of layers. All the animals can develop in both eyes the disease among 2 or 3 weeks post inoculum, resolving spontaneously over time, as in humans. As an advantage, eye of this model are large enough to allow fundus photography, in spite of lesions are different from humans, becoming bilateral and multifocal. Hamsters infected orally show development of ocular toxoplasmosis within 4–8 weeks after infection even when intraperitoneal infection is performed, causing retinochorioiditis. Therefore, hamster's disease does not result in pigmentation, showing itself atrophic and bilateral [7].

11. Dog model

There are few studies in experimental dog model, but Fialho's study is one of the first studies using dogs as experimental models, with mean alterations: ocular lesions [33]. In dogs are present retinitis, choroiditis and pseudocyst in ciliary epithelium [34]. During days post inoculation, there was only as a clinical sign a submandibular lymph node enlargement. Considering that gavage, intraperitoneal and intravenous demonstrated similar finds. With nonrelevant difference. Indicating that the immune response was observed in all animals. Similar to humans, dogs present ocular toxoplasmosis evolution from simple papillary edema to blindness and ocular necrosis. Morphological alterations in this experimental model are highly considered, once in humans are described in literature as late manifestation of toxoplasmosis, acquired or congenital. It is also demonstrated in experimental dog model [34]. Accordingly author's results can be reproduced in only 30 days. Turning dogs excellent experimental models. By the way, its maintenance and costs turn this experimental animal model expensive and labored. Once it is seem as a pet animal. Not been easily accepted as an experimental model, for the society and committee for the use of laboratory animals.

12. Murine model

In majority toxoplasmosis in murine model, shows itself as asymptomatic, and widely prevalent, but clinically is relatively rare [21]. They are easily accessible, with complete genome sequenced, exist many knock-outs and a large number of immunological reagents available.

Beverly JKA introduced mice as animal model in 1959, for congenital experiments [35]. Furthermore, it presents certain resistance to toxoplasmic encephalitis that is controlled by gene action. On the other hand, some vary lineage, like C57BL/6 do not have this haplotype generating spontaneously eye lesions [8]. C57BL/6 shows a high parasite load after 7 days of reinfection associated with deterioration of retinal architecture. When mice are infected by instillation or intraocular injection, both methods unleash ocular toxoplasmosis. Highlighting that instillation must replace with excellence infection of bulb of the eye, obtaining in a short period of time an infection, approaching in 7 days [2]. Considering period of obtaining results, NMRI mice infected, with same strain of *T. gondii* demonstrate bilateral ocular toxoplasmosis in 14–21 days, while Swiss-Webster mice, parasite loads did not peak upon reinfection. BALB/c is considered resistant to the infection, showing itself as asymptomatic. In the same way, it must be noticed that diagnosis of migrant of retinal pigmented epithelium is more difficult in albino mice. Otherwise murine congenital toxoplasmosis there is significant opaque cataract formation [7].

Rats as an experimental model described by Wildfuhr, in 1954 [36], but with little information about this author's studies, present high resistance to toxoplasmic infection, because of this, that experimental animal model is used only when compared to human adults disease.

Murine model mimics likely time of exposure, establishment of cysts in humans and is easy of manipulation. Instead, it has the disadvantages of producing different degrees of severity and ocular lesions and hard monitoring, because of size of the eye [24]. Hence with many advantages, murine model may offer a great spread of methods and variable results, but it is necessary such a great number of animals to perform statistically relevant analyses.

13. Bird model

As a natural host of *T.* gondii, chicken was first described as an experimental model for toxoplasmosis in 1959 by Jones et al. [37]. Level of infection in chicken may be under detectable levels, and low parasitemia makes diagnosis in this model by microscopy difficult, needing ELISA or more sensible methods [38]. In some studies, already has been demonstrated that dose of parasites influence on studies, finding parasites in liver and heart, but none was found in brain cells. Doses higher than 10^3 parasites per milliliter, showed high mortality, indicating in these case lower dosages. Many species of birds can act as a reservoir for *T. gondii*, apparently with or without symptoms [39]. But some studies relating to gender prove that female chicken present more seroprevalence than male, probably associated to behavior manner [38].

On the other hand, embryonated eggs have actually shown itself as good model to test pathogenicity of strains of *T. gondii*, but not all animals in study have demonstrated alteration in ocular structure. Embryos of chicken are able to reproduce projections of retinal layers, detachment of retinal pigmented epithelium, alterations in disposition of photoreceptor, inflammatory infiltrate and alteration in gene expression, important for the development of retina [40].

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It is also possible to see migration of RPE, suggesting a potential role of RPE phagocytosis. Evolution of retinochorioiditis in embryo models is related to the period of infection and strain of parasite, and it has a major tachyzoites proliferation in last periods [23].

Chicken embryo has shown itself, as an excellent performance when compared to other congenital ocular toxoplasmosis animal model, although does not exclude them. Its maintenance, usability, stereo environment, and easy to obtain, qualify this model as a great experimental model [40] and can present great studies when correlated to the control of environmental contamination, by controlling prevalence of infected chickens [38].

14. Nonhuman primate model

This experimental animal model of ocular toxoplasmosis, was first described in a bid of replace rabbit model proposed by Nozik and O'Connor in 1968 [25]. First experiment in this model was described using intraocular inoculum containing RH strains of *T. gondii*, even so it was possible to mimic a retinochoroidal inflammation, there was not retinal necrosis, with no evidence of systemic illness, not even detachments of retinal layer. In nonhuman primates, it is possible to see a remission of the disease [10]. Furthermore, intraretinal infection is easy to perform in monkeys, and ophthalmoscopy can easily be done due to the size of eye of this experimental model. In spite of, it is already known that New world monkeys, *Carabide* are highly susceptible to clinical toxoplasmosis when compared to Old world monkeys, as *Cercopithecide*, that present itself resistant. Monkeys from the Old world seem resistant to infection comprehending that it might not been a good experimental model for ocular toxoplasmosis, whereas monkeys from New world look like useful. In contrast, as a disadvantage, these experimental animal models are costly and require proper housing conditions, which sometimes cannot be granted in most laboratories.

15. *Felidae* model

Clinical toxoplasmosis has been diagnosed in wild *felidaes* but, in great majority of cases, the parasite was identified only in feces. First report of cats as experimental model was made in 1993 by Davidson et al. Cats were infected via carotid injection containing ME-49 strains of *T. gondii*. This experiment induced an increase in rectal temperature, and focal areas of choroidal and retinal inflammation, highlighting bilateral lesions. Equally important within 4 weeks, blood-retinal barrier has been established in most posterior segments lesion. Unlike humans, choroiditis is more pronounced in this model than retinitis during the course of the disease. Another relevant information about this model is that oocysts are detectable in feces from 24 days post inoculation but with a low reproducibility [24].

In addition, an advantage of this method of infection for this experimental model is the low concentration of tachyzoites inoculated via carotid, showing retinal inflammation, different

from human. In humans, this characteristic is about a reactivation of congenital infection, multifocal, and self-limiting.

Routes of inoculation as subcutaneous and intraperitoneal, performed with a large number of tachyzoites of RH strains, leads cat too fast to death. Oral administration produces only sporadic ocular lesions, but has shown as not a good via. After exposure, there is dissemination of *T. gondii* to various extraintestinal tissues, like in humans, but most cats present immunity development and express disease only when immune-suppressed or immune-competent. It can present itself as a good model for understanding biology of parasite, but do not understand disease in humans [24].

16. Pig model

Pigs present a potential infection for humans, from eating under-cooked meat [22]. This experimental animal model infected orally [41] or by intramuscular injection presented apathetic and lethargic among 2 and 9 days post infection. Besides, it is possible to see some aggressiveness attitude during chronic phase. By the way, nonlesion was allowed to identify. Using RH strains of *T. gondii*, animals can present eye discharge and temperature embracing form of *T. gondii*. When using ME-49 strains, this experimental animal model demonstrated increase in band neutrophils. Even those neutrophils are not crucial for toxoplasmosis control; but seem to exert some important role during ocular toxoplasmosis infection, when compared to RH strains immune response. RH strains trigger a lower immune response than ME-49. On the other hand, ME-49 strains reach blood system faster by perioral method than RH via intramuscular inoculum, and can mimic the biological activity of parasite as in immunocompromised humans chronically infected, indicating that the pig experimental model may be a good model to understand chronic toxoplasmosis in immune competent humans [42].

17. Sea mammals model

Many sea mammals are described with toxoplasmosis. In Philadelphia, at a zoo, a case of a sea lion that has died was discovered during necropsy, with cysts of *T. gondii* in heart and stomach. In 1985, a fur seal died of toxoplasmic encephalitis. More after serological studies reported a prevalence of 7.6% of toxoplasmosis in harbor seals and dolphins in Canada. Thereafter, congenital toxoplasmosis was reported in sea mammals in Australia and in Beluga whales from an estuary at St. Lawrence. In general, there are many case reports in this model. Furthermore, there are no relevant study using sea mammals as experimental models for ocular toxoplasmosis, but they might be, an important fount of information for controlling marine infection of environment. Previous studies suggest that coastal pollution from human activities is a source of *T. gondii*. Actually, concentration of oocysts by shellfish can occur, and once sporulated, oocysts can survive in marine water for a long period of time, turning shellfish a source of infection to marine environment [22].

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18. Ungulates model

Since 1992, ungulates and nondomestic ruminants have been reported clinically; at the same time, nonexperimental study describes clinically infection by toxoplasmosis. Actually, reports of a *Saiga antelope*, deer, and other animals showed fatal in these cases. Otherwise, there is no report of ungulates as pronghorn, once it is highly susceptible to *T. gondii* infection [22]. Expressing that ungulates are not good experimental models for toxoplasmosis, once it is extremely sensible to this protozoan infection.

19. Marsupials model

Toxoplasmosis is a serious disease of Australian marsupials, local zoo report countless death, as suddenly die, without clinical or neurological signs, loss of vision, diarrhea, or respiratory distress. Virtually any organ can be affected [43]. Even so, some species of wallabies were identified with high levels of antibodies, like adult black-faced kangaroos, this allows to comprehend that not all exposed wallabies die of toxoplasmosis. But, some tests of vaccine (S-48) with live, or modified and nonpersistent strain of *T. gondii* showed itself lethal in Tammar wallabies, describing how susceptible is this specie. Marsupials as New World Monkeys look to be unusually susceptible to toxoplasmosis [7]. Actually the approach is preventing contamination of food and water, needing more studies that are able to immunize those models, with no founds until now [22].

20. Conclusion

Experimental animal models do not express disease how it happens in humans. However, they are able to bring relevant information about the course of ocular toxoplasmosis. Mice in general are the most usual models nowadays. Adult birds do not express the disease, being a reservoir with no clinical manifestation, but avian embryos allow studies about congenital ocular toxoplasmosis, while hamsters provide information about infection. Nonhuman primates, instead the route of infection, may present important facts of the disease, while cat as the definitive host also presents ocular alteration. Other models do not have experimental description, by the way, case report help to fill the blanks for new contributions of methods, experimental model, and strain of parasite. The choice of an ideal experimental animal model is extremely important to understand and contribute for improvements on prospective treatments.

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Section 3

Diagnosis of Toxoplasmosis

Chapter 6

The Laboratory Diagnosis in *Toxoplasma* Infection

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Additional information is available at the end of the chapter

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Abstract

The diagnosis of toxoplasmosis is of great importance due to the damage caused by this parasite in immunosuppressed people or in pregnant women, the diagnosis of an active toxoplasmosis represents a sign to initiate a pharmacological treatment immediately. The diagnosis of *Toxoplasma* can be performed with direct methods through intraperitoneal inoculation of serum or cerebrospinal fluid, in susceptible mice evaluating the survival and detection of tachyzoites of biological samples. Indirect methods detecting the IgM and IgG isotypes against *Toxoplasma* have been the tools mostly used and had leaded to discriminate between an active and acute, from a chronic toxoplasmosis. Molecular methods actually *Toxoplasma*-DNA identification by molecular biology tests like the polymerase chain reaction (PCR) allow the direct detection of the parasite. Polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLPs) have been used to identify three strain linages (type I, II and III). Recently, a high-resolution melting method was described to determine the genotype of the infection by *Toxoplasma gondii* directly from biological samples.

Keywords: *Toxoplasma* infection, diagnosis, toxoplasmosis, methods

1. Introduction

The diagnosis of toxoplasmosis represents a very important decision to whether to initiate or not an anti-parasitic treatment. The immunological methods that detect the IgM and IgG isotypes against *Toxoplasma* have been the tools mostly used and had leaded to discriminate between acute and chronic toxoplasmosis. In immunosuppressed people or in pregnant women, the diagnosis of an active toxoplasmosis represents a sign to initiate a pharmacological treatment immediately.

Several methodologies have been applied historically, including the isolation of the parasite in biological specimens and the xenodiagnostic evaluation through intraperitoneal inoculation

of serum or cerebrospinal fluid, in susceptible mice evaluating the survival and detection of tachyzoites in cerebrospinal fluid. Serologic diagnosis is frequently performed for the detection in serum of IgM and IgG anti-*Toxoplasma* antibodies, and recently *Toxoplasma*-DNA identification by molecular biology tests is the polymerase chain reaction (PCR) that allows the detection of just 10 or less parasites in the reaction volume.

2. Direct methods

2.1. Isolation of the parasite or bioassay

The diagnosis of toxoplasmosis could be performed by the inoculation of suspicious blood samples, cerebrospinal fluid, lymph node or other corporal fluids or tissues, in the peritoneal cavity of the immunosuppressed mice or in a susceptible strain like BALB/c. The presence of tachyzoites in the peritoneal fluid of the mice is analysed by phase contrast microscopy from 6 to 10 days after inoculation. The parasite is observed in its characteristic half-moon fixing the specimen with methanol and stain with Wright, Giemsa or May-Grunwald and observed by a microscope. The parasites present sometimes a granular membrane, dyed blue cytoplasm and a red nucleus at the center of the cytoplasm.

In cases of new-borns with toxoplasmosis, inoculation of mice with blood of the child obtained during the first week of birth reaches 75% of positive results; however, if the sampling is delayed by 1 month, the positive results are reduced to 52%. A drawback of this method is the need of specialized personal because of the risk of contamination by the handling of *Toxoplasma*, as well as the lapse of time to get the results [1–5].

2.2. Cellular culture

Cellular culture is more utilized nowadays for the diagnosis and isolation of parasite *in vitro* [5, 6], the parasite has been isolated from lung [5, 6] and cerebrospinal fluid from patients with acquired immunodeficiency syndrome (AIDS) [7]. Diagnosis by cellular cultures has also been utilized in ocular toxoplasmosis [8, 9]. Cellular cultures are also used for research purposes, like the study of host-parasite interaction, identification of factors involved on innate resistance, molecular and genetic characterization of *Toxoplasma* strains [10] and for the evaluation of candidate molecules on vaccine and treatment development [11].

The cell culture to identify tachyzoites from biopsy or corporal fluid of infected patients, leads to the destruction of the cellular monolayer and is related with the initial amount of tachyzoites in the biological sample. The sensitivity of parasite detection by cell culture from intraocular liquid (aqueous and vitreous humor) is 91%, compared with antibodies detection by ELISA with 67%.

2.3. Histology

The detection of tachyzoites in histological sections from suspicious biopsies indicates an acute infection; on the contrary, the detection of tissue cysts that contain bradyzoites in histological

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samples only confirms a chronic toxoplasmosis infection. The presence of cysts in the placenta, foetus or new-born indicates a congenital infection, according to the number of cysts in histological sections, can be suggested as an active infection and needs immediate treatment. In biopsy or autopsy material, the encysted tachyzoites and bradyzoites are usually demonstrable with wright, hematoxylin and eosin dyes, where the bradyzoites contain glycogen vacuoles. The size of the cyst is important in histological sections of the brain (**Figure 1**). There are other stain techniques like Schiff-periodic acid, specific immunohistochemistry stained with inmunoperoxidase that are equally efficient. These procedures have great diagnosis value but are limited due to the difficulty to grow parasites *in vivo* as well as the identification of tachyzoites by histochemistry methods [3, 5, 8].

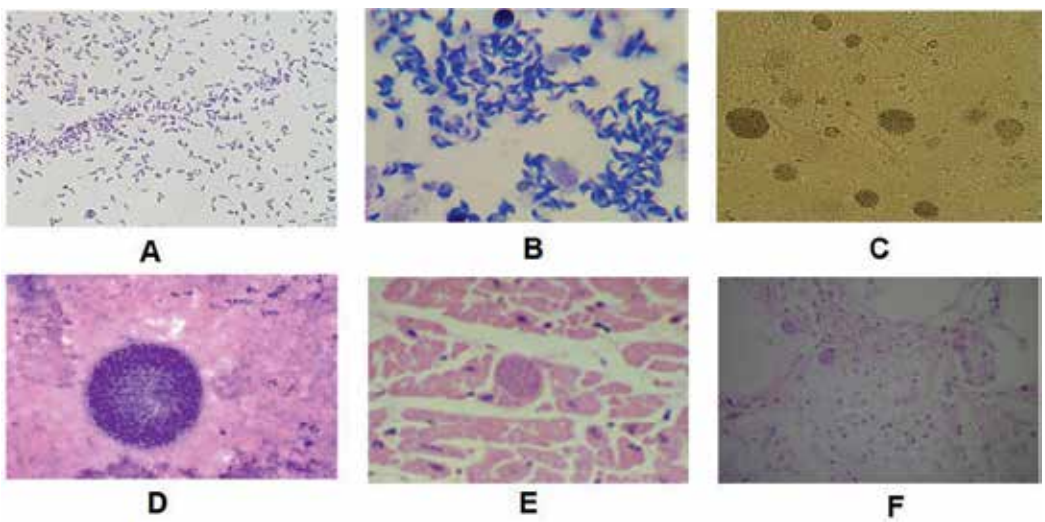

Figure 1. (A) Tachyzoites from mice peritoneal exudate, Diff-Quick stain, 100×. (B) Tachyzoites from mice peritoneal exudate, alkaline metilen blue, 10×. (C) Multiply tisular cysts en mouse brain, without stain, 20×. (D) Tisular cyst in mouse brain, Diff-Quick stain, 40×. (E) Histological section of the necropsy from patient (400×) cyst of *Toxoplasma gondii* in the sarcoplasm of a cardiac muscle fiber. (F) Histological section of the necropsy from patient (400×) H&E stain, in which some cysts of *Toxoplasma* are observed in the alveolar epithelium, necrosis in the adjacent cells, detritus, some inflammatory cells and proteinaceous material H&E stain. (A–D) Courtesy of Gustavo Salas Lais. Student of Doctoral Science in Biomedicine and Molecular Biotechnology, ENCB-IPN.Mexico. (E and F) Courtesy of Dr. Ramón Franco Topete: Medical Pathologist, Department of Microbiology and Pathology CUCS and the New Civil Hospital of Guadalajara, Mexico.

3. Immunodiagnostics'

3.1. Sabin and Feldman test

The Sabin and Feldman reaction, also known as the dye test [12], is considered as golden standard by detection of *Toxoplasma*-specific antibodies. The patient's serum is mixed with a suspension of live tachyzoites, and then methylene blue dye is added. If the patient's serum has anti-*Toxoplasma* antibodies, by the effect of complement factors, the parasites are lysed losing the property of capturing the dye, and as a result they are observed without colour under the

microscope (positive result for toxoplasmosis). When there are no anti-*Toxoplasma* antibodies in the assayed serum, the parasites remain with their cellular layer intact and thereby capture the dye showing a blue colour under the microscope (negative result to toxoplasmosis). The problem with this test is the use of live tachyzoites, a potential risk to laboratory staff conducting the test. Also, it requires specialized infrastructure for the maintenance of the parasites, so only research and reference centres perform this diagnostic tool [12]. Interpretation of results shows that the parasites dyed blue (negative) and more than 50% non-dyed (positive) parasites are reported with the highest serum dilution with the positive result, and the values that reflect infection with the parasite are >1:16 IU.

3.2. Hemagglutination

The hemagglutination assay was first described by Jacobs L. in 1957. This test is based on the capacity of anti-*Toxoplasma gondii* antibodies to agglutinate sensitive erythrocytes masked with cytoplasmic *Toxoplasma* antigens [13, 14]. This test is useful, rapid and highly sensitive ideal to be used in association with indirect immunofluorescence [15]. Also, it could interfere in the diagnosis of congenital toxoplasmosis, as the identified antibodies are IgG and so on. Interpretation of the test: positive if agglutination is present and negative if the agglutination is inhibited. The result is considered the lecture at the higher dilution. Afterwards Yamamoto in 1991, standardized a test for the determination of IgM antibodies, with a sensibility of 98% and specificity of 95% [16].

3.3. Modified agglutination test (MAT)

For this assay, the tachyzoites are fixed with formaldehyde in microtiter plates in the form of U plates with diluted serum added. In positive serum samples, a thin layer of agglutination is produced, while in negative samples compact sediment is formed due to precipitated tachyzoites at the bottom of the well. This assay was first described by Fulton and Turk [17], with low specificity and sensitivity, due to the union of normal IgM at the surface of the parasite and improved by the preparation of the antigen using a buffer with 2-mercaptoethanol, that eliminates the non-specific IgM. This assay detects IgG antibodies, without limitation of host species, but false negative results could occur during early stages of acute infection [17]. The specificity and sensitivity of MAT are similar to the Sabin and Feldman assay in most species. Also, MAT can be used in corporal fluids, being of much used in field work.

3.4. Indirect immunofluorescence (IFI)

This assay was described by Garin in 1967 [18], consists of fixation of tachyzoites on microscope slides. Upon contact with the antibodies present in the patient's serum, the antibody antigen reaction is carried out, which is visible with a second antibody labelled with fluorescein. The antigen-antibody reaction is evidenced by fluorescence microscope. In positive cases, a bright yellow greenish fluorescence is evident and a red colour without fluorescence is observed in negative cases. The colour of the fluorescence depends on the fluorochrome conjugated to the antibodies. The immunofluorescence is a test that shows high precision when it is adapted for specific antibodies. It is known that when the human is infected, it responds first with the production of IgM that disappears later. The detection of specific

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IgM in the new-born shows difference in the antibodies transmitted from the mother to the product (IgG goes through placental barrier and IgM is not capable to do it). The detection of specific IgG contributes to the diagnosis most of the times; however, considerable titles of antibodies may persist for months or years [18].

3.5. Enzyme linked immunosorbent assay (ELISA)

The enzyme linked immunosorbent assay (ELISA) was described initially in 1971 by Engvall and Perlman, uses antibodies conjugated to an enzyme [19]. The antibody conserves its capacity of specific union to the antigen, while the enzyme is capable of a redox reaction, in which the substrate precipitates in a coloured product. In this system, the antigen or the antibody is absorbed to an insoluble solid phase (micro polystyrene plates). There are several variants of ELISA: direct, indirect, capture and competitive. All of them allow the determination of antigens in biological fluids except the indirect method that only detects antibodies.

The direct ELISA methods involve attachment of the antigen to the solid phase, followed by an enzyme-labelled antibody. This type of assay generally makes measurement of crude samples difficult, since contaminating antigens compete for plastic binding sites. Whereas, indirect ELISAs also involve attachment of the antigen to a solid phase, but in this case, the primary antibody is not marked. An enzyme-conjugated secondary antibody, directed at the first antibody, is then added. This format is used most often to detect specific antibodies in serum.

The captured ELISA assay involves attachment of a captured antibody to a solid phase support. Samples containing known or unknown antigens are then added in a matrix or buffer that will minimize attachment to the solid phase. An enzyme-labelled antibody is then added for detection.

The most used enzymes are horse radish peroxidase (HRP), alkaline phosphatase (FA) and the β-D-galactosidase (BG), the substrates for the HRP enzyme are hydrogen or urea peroxide, which on reducing make certain chromogens oxidized in the enzymatic reaction, developing a coloured product. The oxidized ortho-phenylene diamine remains soluble after the oxidation reaction and is the most used. The FA substrate p-nitrophenyl phosphate that produces colour when degraded by the enzyme at temperatures over 30°C, the 5 bromo-4cloro-3-indoilphosfate can be employed in ELISA in tube or in dot-ELISA. The ELISA method detects from 70 to 80% of cases in congenital infections, a rise in the titles of IgM that extends more over the first week is indicative of acute infection, since the average of duration of maternal IgM is from 3 to 5 days. Also, antibodies can be determined in serum and aqueous humour.

Interpretation of the test: If ELISA is qualitative, a development of colour is observed in seropositive samples. If the test is quantitative, the intensity of the developed colour is measured at a determined wavelength (nanometres) and the lecture of absorbance is proportional to the magnitude of the reaction antigen-antibody [20–22].

3.6. Recombinant antigens

At the final of the nineteenth century, several recombinant antigens of different sites of the parasites were analysed [23, 24]. The dense granule antigen—GRA1, GRA2, GRA4, GRA6,

GRA7 and GRA8, rhoptries protein—ROP1 and ROP2, the matrix protein—MAG1, protein micronemes—MIC2, MIC3, MIC4 and MIC5 or the surface antigen—SAG1 and SAG226, were evaluated as diagnostic markers in human beings or animals by ELISA to detect IgG and IgM specific antibodies [25]. The combinations of recombinant antigens have showed more sensibility and specificity for the use of only one antigen. For example, the combinations of SAG2A, GRA2, GRA4, ROP2, GRA8 and GRA7 are potentially useful to detect antibodies IgG in human beings with the infection recently acquired [26], ROP1, SAG1, GRA7, GRA8 and GRA6 are promising to detect specific IgM antibodies while GRA7 and GRA8 are used to detect IgA specific antibodies. A specific protein of sporozoites related with embryogenesis (TgERP) was identified in a study, it can react with specific antibodies of oocysts and can be used to detect an early infection by sporozoites implying the oocysts as infection source [27, 28].

3.7. Avidity test

The IgG avidity test was described by Hedman et al. [24]. This assay measures the avidity of the binding of specific antibodies to *T. gondii* antigen. The proteins present in the serum are denatured with a solution of urea. The avidity can be variable during the course of infection [29, 30]. In early stages of infection, the values of avidity are low and are increasing with the course of infection [24]. Therefore, the avidity test can distinguish between acute and chronic infection. The assay is performed by different serological procedures, such as ELISA. However, there are limitations for the IgG test in pregnant women, since these antibodies can persist for several months and the treatment of *T. gondii* can affect the results [31].

3.8. Western blot

Western blot is an immunoenzymatic technique, based on the binding of *Toxoplasma* antigens fixed on nitrocellulose paper with specific antibodies present in the serum of infected patients. First, the protein antigens must be separated by electrophoresis in polyacrylamide gel (PAGE-SDS), then transferred and immobilized in a solid phase (nitrocellulose layer). Nitrocellulose paper must be blocked or saturated with non-antigenic protein (bovine serum albumin) to prevent the unspecific binding of the antibodies. Then a second antibody conjugated with an enzyme (horseradish peroxidase or alkaline phosphatase) is added, this antibody binds to the Fc of the first antibody, when the substrate of the enzyme is added. The substrate is transformed to a product and visible specific bands in situ indicate the antigens of *T. gondii* recognized by specific antibodies [32].

Western blot has the advantage to identify specific antigens recognized by IgG, IgM and IgA antibodies from serum and cerebrospinal fluid of patients with chronic and acute toxoplasmosis, specially in immunocompromised patients with low titers of antibodies. In immunocompetent patients this method has been used to identify parasite proteins as markers for toxoplasmosis diagnosis (**Figure 2**) [33]. Also, diagnosis of congenital toxoplasmosis from colostrum samples by Western blot is very useful, since IgA antibodies recognize a greater

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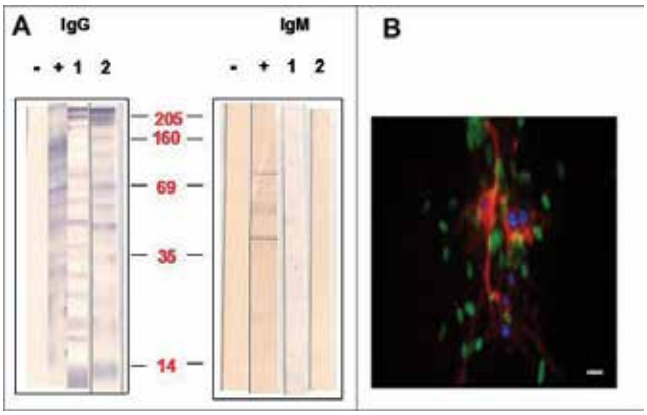

Figure 2. Recognition pattern of antibodies IgG and IgM by Western blot. The antibodies IgG and IgM were detected in samples of serum from a patient with acute infection. Negative control (−), positive control (+), samples 1 and 2 correspond to the bands of antigen *T. gondii* revealed by specific antibodies type IgG and IgM.

number of antigens [34]. This method has also been used with other corporal fluids such as saliva [35].

3.9. Immunocytochemistry and immunohistochemistry

The immunocytochemistry involves detection of epitopes expressed by the antigens of *Toxoplasma* within a tissue sample using a 'primary antibody' capable of binding those epitopes with high specificity. After the epitope-antibody binding event, a 'secondary antibody' capable of binding the primary antibody with high specificity is added. The secondary antibody is coupled to a reporter molecule and after the antibody-antibody binding event, a chemical substrate is added which reacts with the reporter molecule to produce a coloured precipitate at the site of the whole epitope-antibody complex (**Figure 2B**). The immunohistochemistry has the same base as that of the immunocytochemistry, the difference is that this one is realized on tissue slides. Studies have been done for a neuropathological test of cerebral tissue in patients with infection by the virus of human immunodeficiency VHI/AIDS, to evaluate the diffuse and spread brain multifocal lesions [36]. With immunohistochemistry there have been realized studies in different tissues to demonstrate *T. gondii*, such as ocular toxoplasmosis [37–39].

4. Molecular techniques

4.1. Generalities of the genome of *T. gondii*

Molecular biology has permitted the advance in molecular knowledge of *T. gondii*. Its genome contains approximately 65 megabases (MB), distributed in 14 chromosomes which varies from 1.9 to 7.4 MB. It contains 52.3% of GC and codifies for almost 7988 proteins and contains

296 small non-coding genes (Ensemble protists): *T. gondii:* [40, 41]. The complete genome sequence of GT1, ME49 and VEG strains classifies the *Toxoplasma* in three types I, II and III, respectively. Till date, the analysis of the restriction fragment length polymorphisms (RFLPs) with more than 900 strains of the parasite permits its classification in 140 genotypes grouped in 12 main groups. Similar results were obtained by the sequencing of introns of a smaller number of samples and also using microsatellite markers. In spite of these results, it is necessary to sequence more complete genomes for a better precision in the clarification of the linages (information taken in the page of Institute J, Craig Venter) [42].

4.2. Molecular diagnosis by the polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) consists in making a repetitive replication *in vitro* of specific DNA sequences. Amplifying or copying several times a fragment of DNA, the analytical sensibility of the test increases proportionally. The amplified product is analysed by electrophoresis in agarose or polyacrylamide gel. This technique is useful in the detection of the parasite in serum samples, peripheral blood mono-nuclear cells (PBMC), urine, placenta, amniotic and spinal brain liquid. The sensibility and specificity of PCR depends on the technique used in the extraction of DNA, the primers and the parameters of the amplification reaction [43, 44]. The most conserved genes of *Toxoplasma* are B1 with 35 copies in the genome of the parasite, followed by the repetitive region REP of 529 base pairs (bp) with an approach of 300 copies in the genome; these genes are the most selected for the PCR test as diagnostic tool. Other sequences of unique copy, which are used in investigation laboratories mainly for genotyping purposes are SAG1, SAG2, SAG3, SAG4 and GRA4 genes [45]. The polymerase chain reaction (PCR) is a very valuable diagnosis method in acute infections, when the parasite crosses placental barrier and infects the foetus or on infected patients with AIDS and other immunosuppression or reactivation of the parasite [46–48]. Unfortunately, it is difficult to have a confidentially valor in the PCR assays. For a pre-natal diagnosis, the reported sensibility goes from 64 to 97.4% with a predictive negative value of 87.7–99.7%, respectively; in case of patients with AIDS and toxoplasmosis, the reported sensibility goes from 13 to 87.5% [49]. With the detection of the sequence REP 529-bp, a sensibility of 100% has been reported in amniotic fluids [48]. The sequence REP 529-bp is having an increase in the utility for the diagnosis of *T. gondii* by PCR since it demonstrates greater sensibility, possibly the high number of repetitions in the parasites sequence increases the possibilities of detection. The variations in the results are due to different time of infection in the patients, type of sample, storage conditions that affect sensibility. Also, actually there is no agreement in the primers that should be used in the PCR, one of the regions which is more conserved is gen B1 [47], still there exists some polymorphisms and the strains of *Toxoplasma* in clinical samples are not the same genotype, these factors can affect the parasite detection. It requires large number of studies which compare gen B1 and the sequence REP 529-bp to meet and agreement in the selection of primers. Another factor that can affect the results is the variability reported in the techniques used for DNA extraction.

In patients with AIDS, the detection of *T. gondii* by PCR in cerebrospinal liquid has shown different sensibilities, reaching to 87% of sensibility when the sample is taken soon enough

preferably during the first 3 days and a maximum of 7 days after starting the specific treatment [48].

The polymerase chain reaction (PCR) can be in the future, undoubtedly a successful tool in the diagnosis of toxoplasmosis, when a satisfactory correlation exists between the patient's clinical profile and the presence of *T. gondii* DNA aspects which still have to be studied and standardized. Also, in countries with low development and still in many with high development, it has not been implemented for daily use due to its high cost.

4.3. PCR in real time

Unlike the conventional PCR, the real-time PCR using fluorescence probes measures the product of amplification in each cycle and can be quantified with the use of known concentration standards. Real-time PCR has been successfully used to detect the *T. gondii* DNA in human blood, cerebrospinal fluid, aqueous humour, amniotic liquid and other samples [49–51]. Also, it is used to evaluate the progression of the toxoplasmosis and the efficiency of the treatment, since it can estimate the intensity of the infection by *T. gondii*. The PCR assay in real time with REP 529-bp sequence is considered as the technique with greater efficiency for the diagnosis of toxoplasmosis in comparison with the detection of gen B1.

4.4. Loop mediated isothermal amplification (LAMP)

Loop mediated isothermal amplification (LAMP) is a technique of DNA amplification under isothermal conditions, which combines speed, efficiency and high specificity. The amplification of DNA proceeds from the repetition of two reactions of elongation in a structure that has loop form. It uses DNA polymerase with strand displacement activity and two inner primers and two outer primers. The inner primers contain sense and antisense sequences of DNA and initiate the LAMP reaction. The outer primers hybridize a few bases of the inner and initiate the DNA synthesis with strand displacement, forming a loop structure, which initiates a DNA synthesis by auto recognition. In this technique, 10 copies of DNA can be produced in less than one hour [52]. The final products are multiple loop DNA formed by alignment between inverted repeated sequences present in the same chain [53]. It only requires a water bath or thermo block to realize the amplification of DNA and the amplified products can be analysed by real time PCR equipment or by electrophoresis [54]. This technique is more sensitive than conventional PCR but low sensitive than real time PCR [53]. The LAMP assay has been evaluated identifying REP 529-bp, SAG1, SAG2 and B1 *T. gondii* sequences from PBMC [54]. The reported sensitivity was 0.1 tachyzoites and crossed reactivity with other parasites was not found. The detection of SAG2 showed the higher sensitivity (87.5%) followed by SAG-1 (80%) and B1 (80%). The specificity was 100% in all of them [55]. However, all techniques of nucleic acid amplification have a high sensitivity, for that reason a rigorous quality control is necessary to discard false positives.

4.5. Polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLPs)

The PCR-RFLP is based on the capacity of restriction endonucleases that recognize polymorphisms of only one nucleotide (SNPs). Polymerase chain reaction (PCR) products treated with restriction enzymes to generate polymorphic DNA fragments of variable length, that are visualized after electrophoresis in agarose or polyacrylamide gels with a band pattern [55]. In 1995, Howe and Sibley identified three main linages (type I, II and III) from 106 strains of *T. gondii* isolated from humans and animals by PCR-RFLP using six markers [56].

Since then, in different laboratories, several groups of different multi-locus markers have been selected, to characterize by PCR-RFLP *T. gondii* isolates from persons and animals [57, 58]. Currently, there has been developed a method for *T. gondii* classification into genotypes known as multiplex nested PCR-RFLPs (Mn-PCR-RFLPs). This method uses 10 genetic markers including the gens SAG1, SAG2, SAG3, Btub, GRA6, C22-8, c29-2, L358, PK1 and apico [59]. The Mn-PCR-RFLP has been applied broadly in the typing of positive clinically samples and has generated a big amount of data about the genetic diversity of the parasite [60, 61]. As in all techniques, where DNA amplification is performed, several negative controls must be included to have a strict quality control.

The microsatellites (MS) are short tandem repeats (STR) sequences of DNA (2 to 6 pb), that are widely distributed throughout the nuclear genomes of eukaryotes. They are highly polymorphic, the MS length varies depending the numbers of STR. The tandem repetitions in *T. gondii* are often simple, with only repetitions of two nucleotides, which occur from 2 to 20 times [62, 63]. Around 15 markers of MS have been used, including (tub2, W35, TgM-A, B18, B17; M33, IV.1, XI.1, M48, M102, N60, N82, AA, N61, N83) to determine the genotype of *T. gondii* in different laboratories [61, 62]. Ajzenberg et al. developed an easy method to genotype *T. gondii* in one multiplex PCR using 15 MS markers, in which eight MS markers (tub2, W35, TgM-A, B18, B17, M33, IV.1 and XI.1) could differentiate the types I, II and III from all the atypical genotypes, and the other seven markers (M48, M102, N60, N82, AA, N61, N83) could improve the resolution from the genetic differentiation of strictly related isolates in an haplo-group or clonal linage [62, 63]. The multiplex 15-MS assay is the best available tool to characterize *T. gondii* from genetically different or identical isolates during an outbreak, laboratory contamination and from mixed infections. The limitation of this assay is the request of an automatized sequencer with the 'gene scan' tool. The disadvantage is that samples with poor DNA can cause low intensity signals, generating non-specific PCR products [61, 62].

4.6. High-resolution melting (HRM)

In homogeny sample, the HRM method can analyse genetic variations, which allows to characterize polymorphisms based on the melting temperature generated by the DNA length and GC content [62]. On the base of only one SNP from gene B1 which is found in multiple copies, the HRM can classify correctly three different types of strains of *T. gondii* [63]. This assay was developed to determine the genotype of *T. gondii* directly from biological samples, with a better genotyping when use multiple copy genes in comparison to one copy genes, avoiding a cellular culture or bioassay.

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Chapter 7

Effective Diagnostic Marker for Serodiagnosis of *Toxoplasma gondii* Infection: New Developments and Perspectives

Zeehaida Mohamed and Khalid Hajissa

Additional information is available at the end of the chapter

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Abstract

Toxoplasmosis is a prevalent parasitic infection caused by an obligate intracellular parasite *Toxoplasma gondii*. Various methods have been established in the laboratory diagnosis of toxoplasmosis. Among these methods, serological tests are common and provide satisfactory results. However, producing reliable reagents and standard antigen remains difficult and expensive. Replacing native antigens in all current diagnostic kits with standard and reliable reagents are speculated to achieve more sensitive and specific detection that can significantly improve the assay performance. This review provides updated data on toxoplasmosis serodiagnosis. It focuses on the recent trends of producing reliable and standard antigens that have been used in the serological tests of toxoplasmosis, as well as the future direction in this field.

Keywords: ELISA, serodiagnosis, multiepitope peptide, recombinant antigen, sensitivity, specificity, toxoplasmosis

1. Introduction

Toxoplasmosis is one of the most prevalent parasitic infections caused by an obligate intracellular parasite *Toxoplasma gondii.* This parasite can infect almost all warm-blooded animals including humans. The effect of *T. gondii* on public health and animal production is significantly evident worldwide. Therefore, it became one of the well-studied parasites because of its medical and veterinary importance. Although the prevalence rate of toxoplasmosis is

approximately one-third of the world's human population, *T. gondii* infection in immunocompetent individuals is usually asymptomatic, even though some patients may experience fever and other non-specific clinical signs [1]. In immunocompromised patients, this disease may be serious or even fatal; furthermore, primary invasion during pregnancy may endanger the life of the fetus as well as the infected mothers [1, 2]. Accurate diagnosis of the acquired infection by highly sensitive and specific methods is crucial for proper management of animal and human toxoplasmosis and represents the key step in the prevention and treatment of the disease [3].

Various methods have been established in the laboratory diagnosis of toxoplasmosis. Among these methods, serological tests are common and provide satisfactory results. However, producing reliable reagents remains laborious and expensive. Acquiring a specific and effective reagent that can be used in the serodiagnosis is necessary. In the present review, we provide updated data on toxoplasmosis serodiagnosis. The review focuses on the recent trends of producing reliable and standard antigens that have been used in the serological tests of toxoplasmosis, as well as the future direction in this field.

2. Serodiagnosis

Various direct and indirect detection methods have been established to detect *T. gondii* infectious agent or anti-*Toxoplasma* antibodies [4]. In particular, different sets of detection methods have also been developed and evaluated to achieve accurate diagnosis [5]. There are several diagnostic approaches which are applicable to detect the parasitic agents include: histological identification, isolation of the organism in tissue culture [4], and recovery of the parasite DNA by the polymerase chain reaction (PCR) [6, 7] or by a combination of these techniques, whereas the serodiagnostic tests are mainly designated to detect the different classes of antibodies or antigens [8].

Despite the various methods of toxoplasmosis investigation, the routine laboratory diagnosis in both humans and animals depends mainly on conducting various serological investigations to detect specific anti-*Toxoplasma* antibodies in serum samples [5, 9]. It remains the primary approach to achieve satisfactory results [10]. The production and appearance of each antibody isotype are correlated with the immune response after the infection, thus, the determination whether the host has got *Toxoplasma* infection or not can be achieved simply by monitoring the immune response especially humoral immunity. The disease symptoms are non-specific and not enough to characterize accurate diagnosis because of clinical signs mimicry between toxoplasmosis and several other infections [5, 11]. Many serological tests have been designed to measure different types of antibody, which show unique increases and decreases during or after infection [12]. All immunoglobulin isotypes have been used successfully in *T. gondii* serodiagnosis, including IgG, IgM IgA, and IgE [10]. Specific *T. gondii* IgM is considered as early and sensitive diagnostic marker that correlates with the occurrences of acute toxoplasmosis. Particularly, IgM can be detected in serum only 1 week following the infection.

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However, it may remain in the serum for several months or years [8]. Thus, the interpretation of the serology results sometimes becomes more difficult. Furthermore, the presence of IgM antibodies in the maternal circulation even 18 months after invasion influence the accuracy of this diagnostic approach because determining whether an antibody is from active or previous infection is crucial during gestation [13]. If an antibody is from a previous infection, no consequences for the fetus normally occur. If the infection occurs during pregnancy, the clinician should decide on administering anti-parasitic treatment to avoid disease complication in the unborn child [5, 11]. Recent studies have shown that the use of IgM alone for the establishment of acute toxoplasmosis is insufficient [8].

Detection of IgG antibodies in patients may aid diagnosis. IgG antibodies can be detected within 1–2 weeks following infectionacquisition, and normally peak within 1–2 months, and decline at various rates, but usually persist lifelong at residual titers [5, 11]. High levels of this antibody indicate previous infection. Therefore, measuring of IgG antibody is a common diagnostic marker that helps clinicians to decide whether a patient has chronic infection or not. However, this antibody still has difficulty in distinguishing previous infection from a recent infection. Consequently, an auxiliary test based on the IgG avidity has been established to differentiate acute from chronic infection in an asymptomatic patient [5, 11]. In recent years, precise dating and infection have been proven to be obtained by using IgE and IgA. However, they produced during the first weeks of infection, and disappear early [9]. There are various serological procedures have been established for the detection of anti *T. gondii* antibodies; these include Sabin-Feldman dye test (SFDT), indirect fluorescent assay (IFA), agglutination tests, and enzyme-linked immunosorbent assays (ELISAs), or a combination of these methods are also required for determining recent and previous infections [9, 10, 12].

3. Sabin-Feldman dye test (SFDT)

It was a greatest advancement in the field of toxoplasmosis diagnosis when Sabin and Feldman described the dye test as a novel diagnostic test 60 years ago [14]. Though SFDT is the first assay developed for the laboratory investigation of *T. gondii* infection [10], but still considered as "gold standard" with high sensitivity and specificity [15]. The principle of the assay is based on incubating live tachyzoites with patient serum and complement. Subsequently, if the serum contains specific antibodies against *T. gondii*, it will immediately coat the parasite and the tachyzoite will be lysed by the complement system and fail to stain with dye methylene blue. The number of unstained (dead) and stained (live) tachyzoites will be counted and used to establish the end-point titer [10, 15, 16]. In comparison with other diagnostic tests, SFDT has the potential to detect both IgM and IgG. On the other hands, the assay has certain limitations. For example, application of live parasite is considered a biohazard; thus, it can be only applied in a few laboratories. The antibody titers also do not give strong clues to determine whether the infection is acute or chronic [15, 16].

4. Indirect fluorescent assay (IFA)

IFA is one of the most simple, sensitive, safe, and widely used assays in the usual detection of anti-*Toxoplasma* antibodies [17]. It has been established as an alternative diagnostic method in response to overcome the biohazard of using live tachyzoites in SFDT [10]. Diluted serum specimens are incubated with killed *Toxoplasma* tachyzoites fixed on a glass slide to allow specific antigen-antibody interaction [8]. The interaction will then detected by addition of fluorescent-labeled anti-human IgG or IgM antibodies, and the result is viewed under a fluorescence microscope [18]. Although IFA is relatively inexpensive, fluorescence microscope is required, and the individual differences in result reading are considered as one of the test limitation. Furthermore, false-positive results may occur in case the sera contain rheumatoid factors or antinuclear antibodies [10]. Nevertheless, high levels of *T. gondii*-specific IgG in some acute acquired toxoplasmosis patients may interfere with the IgM and cause false negative results [19].

5. Agglutination tests

Different agglutination tests, including direct agglutination test (DAT), indirect hemagglutination test (IHAT), and latex agglutination test (LAT), are applied in toxoplasmosis serology [9, 12, 20]. Development of DAT assists tremendously in identifying of anti-*T. gondii* antibody in humans and animal sera [12]. Fulton achieved this test in 1965; the important features of this test are the absence of secondary antibody and no special equipment needed. The principle of DAT starting with coating of microtiter plates with formalinized *Toxoplasma* tachyzoites. Diluted patient sera will then added. Accordingly, the agglutination will occur if anti-*Toxoplasma* antibodies are present in the tested sera. However, precipitated tachyzoites will be noticed at the bottom of the wells if the sample is negative [21]. The test is very simple and has proved to be very sensitive and inexpensive, although a large quantity of antigen is required. Despite that DAT is only used for detection of IgG antibodies, non-specific agglutination can be induced by occurrences of IgM antibodies [12, 20].

In the IHAT test, red blood cells (RBCs) are sensitized with *T. gondii* soluble antigen. Subsequently, sensitized cells will then incubated with patient sera. Accordingly, the RBCs will agglutinate if the sera contain anti-*T. gondii* antibodies. Furthermore, IHAT is also simple to perform and inexpensive and can be used for both humans and animals [8]. In the LAT, tachyzoite particle is fixed to latex beads. When the beads are incubated with patient sera containing specific IgG antibody, visible agglutination reaction will occur [22]. The disadvantage of this assay is the low sensitivity and specificity, particularly in immunocompromised patients; moreover, non-specific IgM can generate false-positive result [9, 20].

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5.1. Enzyme-linked immunosorbent assay (ELISA)

ELISA is highly sensitive and specific analytical assay for quantitative detection of antibodies or virtually all types of antigenically active molecule. Application of ELISA in the diagnosis of toxoplasmosis has been established since 1976 [23]. Since developed up to now, it has been one of the most common biochemical techniques used in research and clinical laboratories, including detection of anti *T. gondii* antibodies [8, 24]. In this assay, a microtiter plate is coated with antigens, and then diluted patients sera are applied. If the serum contains anti-*Toxoplasma* antibodies, it will bind directly to coated antigen, and the presence of *Toxoplasma*-specific antibody will detect by using anti-human enzyme-conjugate (secondary antibody). Any unbound reagents are removed in the washing steps. Finally, the substrate is added, and subsequently, color reaction develops (which is relatively correlated with the quantity of the antibody). Interpretation of the ELISA results normally depends on the assessment of color change spectrophotometrically [25].

ELISA can easily determine a positive or negative sample by correlating the optical density of the serum with the control after a threshold value is established [25]. In general, the advantages of ELISA test are as follows: it shows high sensitivity which allows quantitative and semi-quantitative antibody measurements, automatically adopted and is inexpensive [26]. Furthermore, the assay is simple and easy to carry out and can be used to investigate a large number of serum samples in a short period of time [4]. Moreover, it can be used to detect both IgG and IgM antibodies [25]. The disadvantage of the ELISA includes standardization of used antigens [26]. A photometer is also necessary to assess the results, otherwise, it is difficult to distinguish between negative reaction and weak positive reaction by visual examination, and this may increase the cost of the test [25]. Generally, detection of anti-*T. gondii* IgM antibody possesses a high degree of accuracy according to the reports; however, it might sometimes give false-positive result [27]. It is most probably due to the presence of rheumatoid factor in serum, and on the other hands, it is common to get false-negative results due to specific IgG competitive inhibition [28].

5.2. Production of reliable and standard antigen for serological diagnosis

Establishing of *T. gondii* serology is an essential diagnostic tool, which gives satisfactory results. However, producing specific and standard antigens is a real challenge. Currently, most commercial serological kits use crude *Toxoplasma* antigens prepared through mouse passages or cell culture systems [29, 30]. The soluble native antigen of *T. gondii* parasite has been applied as a diagnostic marker in various seroepidemiological studies [4], a researcher used crude native antigen of type I and II, for example [1]. Crude *T. gondii* tachyzoite antigen applied in an indirect ELISA yielded sensitivity and specificity of 95.75 and 85.11%, respectively [31]. A significant variation between laboratories exists in the process of producing native antigen. Furthermore, antigen contamination with non-parasitic materials is also possible because of the lack of standard method for antigen purification [32]. Moreover, the use of live pathogens in antigen preparation process needs extra care because of biological hazards

generated by the parasite [33]. Therefore, serological tests that use tachyzoite crude antigens seem difficult to standardize [32]. To overcome this limitation and improve the serodiagnostic tests, recombinant antigen may be used as an alternative diagnostic marker, and it could replace the native antigens [34].

6. Recombinant antigens

Application of the recombinant antigens in serodiagnostic kits reduces the native antigen production time and significantly reduces the antigen production cost. Furthermore, better test standardization is achieved when recombinant antigen is used [35]. Moreover, the particular antigenicity of specific antigen can be easily investigated, and with the ability to combine more than one antigen to evaluate their diagnostic value, definitely this will facilitate the standardization of diagnostic assays [29]. If the synthesis of any selected antigens during the parasite life cycle is related to acute or chronic phase of infection, application of this particular antigen as discriminating tool leads to significant improvement in *Toxoplasma* diagnosis [35]. The use of recombinant DNA technology to obtain identical antigenic protein helps in overcoming the biohazard problem related to native antigens production and is greatly beneficial to reduce time, cost and labor consumption. Interestingly, one of the most advantageous of using recombinant antigen in the development of diagnostic tests is that the potential to determine precise protein composition, with reduced non-parasitic material. In addition, more than one antigen can be combined in a single test [32].

Recently, several *T. gondii* antigenic genes have been successfully cloned and expressed using various expression systems. Many reports indicate the successful use of recombinant antigen in toxoplasmosis serodiagnostic tests [33]. The antigens that have been extensively involved in the progress of *T. godii* diagnosis improvement include the surface antigens (SAGs) [36], dense granule antigens (GRAs) [36], microneme antigens (MICs) [37], and rhoptry antigens (ROPS) [33]. More details about the application of *T. gondii* recombinant antigens in the diagnostic studies are illustrated in **Table 1**. Most previous and current studies approved that recombinant protein technology significantly improves the serodiagnosis of *T. gondii* infection. Moreover, the uses of recombinant antigen allow not only the detection of anti-*T. gondii* antibodies but also differentiation between recently acquired and previous infections, resulting in reduced medical complication risk of the disease in immunocompromised patients, especially pregnant women [32]. The potential ability of specific *T. gondii* recombinant antigen to identify the clinical phases of the disease was extensively studied, in the attempts to discriminate acute from chronic infection. The performance of recombinant antigen seems to be sensitive enough and promising in differentiating acute versus chronic infection [35, 38]. Although there is no clear definition of either chronic or acute infection, identification of recently acquired infection normally relies on the detection of specific IgM in patients serum; however, IgM might persist for a long time after infection. Thus, the results may not be precise to indicate recent infection, unless the serum is subjected to additional method such as the IgG avidity, definitely this will improve the accuracy of identification of the infection occurrence. Performance of IgG avidity assay by using *T. gondii* recombinant antigens may be useful for identification of toxoplasmosis phases.

Antigen	Diagnostic test	Sensitivity %	Specificity %	Reference
SAG1	ELISA (IgG)	100	100	[39]
	Rapid diagnostic test (RDT)	100	99.4	[40]
SAG2	ELISA (IgG)	100	89.4	[41]
SAG3	ELISA (IgG)	95.4	91.2	[41]
GRA1	ELISA (IgG)	100	100	[42]
GRA2	Western blot	100	90	[43]
GRA5	Western blot	100	46.8	[38]
GRA7	ELISA (IgG)	93.2	94.0	
ROP1	ELISA (IgG)	100	100	[44]
ROP8	Western blot	90	94	[33]

Table 1. Recombinant antigens of *T. gondii* as serodiagnostic markers.

7. Epitope-based antigen

The epitope or antigenic determinant is an antigenic part of a protein that possesses the potential ability to be recognized by T and B cells receptors or the antibodies binding sites [45]. The epitope is classified into two categories based on their primary structure: linear (continuous) epitope if the amino acids sequences in the epitope are continuous or conformational (discontinuous) epitope which composed from discontinuous sections of amino acid [46]. Attempts to achieve high sensitive and specific diagnostic assay usually assume that replacing the native antigen in all current diagnostic kits by standard and reliable reagents can significantly increase the assay performance [30]. In recent years, it has been demonstrated that the use of peptide-based antigen can meet the demand to standardize the serodiagostic test of toxoplasmosis and increase the sensitivity and specificity of these assays. Furthermore, the ability to distinguish between previous and recently acquired infection can be also achieved [47].

Theoretically, epitope or multiepitopes show numerous advantages compared with crude native antigen because they apply only the antibody-binding sites of the antigen, which definitely increase the accuracy of the test. The applied antigen composition in the serodiagnostic test will be known precisely, the ability to apply more than one identified epitope, easily standardization of the assays, application of only antigen epitope will reduce the biohazard problems in using live pathogens [30]. Nowadays, the use of a diagnostic marker that contains a high density of antibody binding site increases the chances of antibody detection in serum samples and provides the potential to acquire inexpensive diagnostic methods with a high degree of specificity and sensitivity. Therefore, a great possibility to improve diagnostic tests performance exists if multiepitope peptide is applied [48]. Application of multiepitopes antigen in diagnostic tests has been conducted successfully in several studies, employing multiepitope peptide in hepatitis C [49], influenza virus [50], leishmaniasis [51], leprosy

[52], *Trypanosoma cruzi* [53], leptospirosis, and *Mycobacterium tuberculosis* [54, 55] as well as *T. gondii* infection [30]. In all these studies, multiepitope proteins were proposed to enhance the test sensitivity. Identification of specific peptide epitope targeted by the host immunoglobulin allows better understanding of the immune response toward the parasite, as well as the development of accurate diagnostic tool, for development of diagnostic kit multiepitope antigen, appears as most promising antigens that can be used in routine toxoplasmosis screening [56].

Recently, bioinformatics tools are widely applied for epitope identification in protein analysis. Consequently, various epitope peptides are used to develop diagnostic antigen and epitope-based vaccines [47]. By using software-based prediction techniques, the SAG1 B cell epitope of *T. gondii* has been analyzed. Consequently, 11 selected peptides were successfully synthesized. The potential antigenicity of these peptides was evaluated by ELISA using pig sera collected from different periods of post infection. Four of 11 peptides showed high reactivity and were identified by all sera, whereas the rest of the peptides interacted with selected sera only [57]. When the same procedure was applied to *T. gondii* GRA1, 3 of 11 peptides were successfully recognized by all sera. The study suggested that these epitopes can activate the host immune system and generate constant immune response because its immunoreactivity was not affected when evaluated using sera collected from different time periods following the infection [47].

The involvement of GRA1 immunodominant B cell epitope in inducing antibody production by the human immune system was previously identified [58], whereas the immunodominant epitope on GRA1 was also located and accessed using pig sera. The result indicates high immunoreactivity as well as the capability of the peptide to improve the toxoplasmosis serodiagnostic tests [56]. The newly synthesized immunodominant epitopes of *T. gondii* antigens (SAG1, SAG2, SAG3, GRA5, GRA6, and P35) appear to be the most promising diagnostic reagents can be applied in routine toxoplasmosis screening The demonstrated multiepitope peptides can successfully replace the recombinant antigens for differentiating the recently acquired infection from the previous infection; thus, it can serve as an effective tool for human serodiagnosis [30].

The advantage of using epitope peptide in serodiagnostic tests can easily increase the assay sensitivity by applying different peptides. Alternatively, the use of multiepitope peptide that expresses a high density of conserved antigenic determinant can contribute in achieving a high degree of sensitivity and specificity [48, 53]. Multiepitope peptide has been evaluated to obtain specific and standard antigens for serodiagnosis of *T. gondii* infection. Findings showed promising results for developing more accurate assays with high degree of sensitivity and specificity and can differentiate between chronic and acute phases of infection [30, 46]. Multiepitope antigen expressed in *Escherichia coli* system provides the potential to replace the currently used crude antigen. This antigen can also contribute in developing accurate and inexpensive diagnostic assay with high degree of sensitivity and specificity. Therefore, recombinant multiepitope peptide appears as an attractive starting point for future development of *T. gondii* serodiagnosis and immunization [30, 48].

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8. Concluding remarks

The diagnosis of *T. gondii* infection is still a huge challenge to the medical knowledge and clinical acumen of physicians. Currently, the serological diagnosis plays a vital role in the identification of both human and animal toxoplasmosis. However, the insufficient accuracy of current diagnostic tests due to the lack of standardization in the production of the *T. gondii* whole-cell lysate necessitates the exploration of standard diagnostic reagents. Nowadays, it is speculated that application of recombinant multiepitope antigens in toxoplasmosis serodiagnostic methods would significantly improve the sensitivity and specificity of these methods. Accordingly, the increased usage of the bioinformatics and recombinant DNA technology will dramatically assist in designing and producing of novel epitope-based antigen, which will be crucial to acquire effective serodiagnostic methods for toxoplamosis.

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Section 4

Improvements and Vaccine Studies in Toxoplasmosis

Chapter 8

Effects of Nanoparticles in Cells Infected by *Toxoplasma gondii*

Sprakel-Leyke Silja, Paulke Bernd-Reiner and Presber Wolfgang

Additional information is available at the end of the chapter

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Abstract

Core-shell model drug carriers on two nanoscale size levels have been applied in cell culture studies and focused on Toxoplasmosis therapy. In synthesis, a seed of rhodamin B-labelled polystyrene latex particles was coated by polybutyl cyanoacrylate under physical inclusion of two different new drugs against Toxoplasmosis. Drug-loaded and drug-free core-shell model drug carriers were added to a cell culture of human macrophages, infected by ***Toxoplasma gondii***, following an infection plan. Drug release from the carriers had been studied before by enzymatic degradation by means of pork liver esterase. Particle size decrease by degradation was investigated in a UV/VIS spectrometer via transmission measurements. Drug release profiles were obtained by HPLC studies. The dynamics in the population of infected human macrophages, *T. gondii* as well as model drug carrier numbers were registered by an FACS (fluorescence-activated cell sorter). As main result, the drug-free references in the two series of core-shell model drug carriers achieved ca.85% of the observed maximum in Toxoplasmosis therapy efficiency. These data were correlated with an immune stimulant effect on the side of the human macrophages, caused by the cell uptake of colloidal substrate, foreign to the body.

Keywords: toxoplasmosis, nanoparticles, model drug carriers, human macrophages, cell culture, infection, therapy efficiency, immune stimulation

1. Introduction

Nanoparticles (NPs) are a tool for specifically targeting drugs or diagnostics to selected tissues or cell types [1–3]. Their use in cancer research and therapy is of special interest [4–9] as well as to overcome the blood-brain barrier [10, 11]. At least in theory, they are a tool to optimise pharmacological data such as drug release, tissue specificity and even cell specificity [2].

In some cases, drug crystals have been used successfully as nanocarriers [12, 13]. NPs composed of polymers may be loaded with drugs which they release in a controlled manner, or they are coated with molecules which give them specific surface characteristics to bind and be taken up by certain cells. Despite these attractive goals and 30 years of research, there are no polymeric nanoparticles in practical, pharmaceutical use. There is hope to apply nanoparticles to deliver drugs across the blood-brain barrier [10, 14, 15].

The reasons for this obvious failure include following: first, the unresolved problem of cytotoxicity of several nanocarrier types [16], low drug loading and insufficient delivery of the bound drug and lack of production facilities to supply demand.

The problem of toxicity could be overcome at least partially by high selectivity with regard to the cells by which the NPs are taken up. Such a strategy is especially interesting in the case of cells, infected with microbes which only multiply intracellularly, such as viruses and some parasites, as these often alter the surface of the host cell, thereby offering the specific targets needed. In this case, even toxic NPs could be useful, as they would exert their toxicity mainly or even better only in or against infected cells which would inhibit the multiplication of the pathogens and destroy them.

At the moment, there are some data showing that tissue specificity may be achieved. One of the interesting targets is the endothelial barrier of the brain. Despite the big potential [17], there are no data concerning infected cells. As it is important to develop drugs against intracellular parasites such as *Toxoplasma* or *Leishmania,* therefore the goal is to establish a system which would allow to trace the fate of nanoparticles in infected cell cultures. It should be possible to evaluate the cell and parasite proliferation and the differential uptake of NPs.

A fluorescence-activated cell sorter (FACS) represents a useful tool to measure particles which differ in size and more importantly, in fluorescence [18]. Thus, it is possible to count extracellular parasites and their host cells separately, if they differ in size. To find the intracellular parasites and to differentiate between infected and non-infected cells, green fluorescent protein-transfected parasites were used. In the past, it was shown that it is possible to follow the fate of *Leishmania* in a cell culture which is conventionally treated (by adding drugs at the necessary concentration) [19].

Because the FACS is able to discriminate between fluorescences of different wave lengths, the purpose of this study was to establish a system with stained NPs as an additional reacting partner [20]. In the case of *Leishmania,* only phagocytic cells, mainly macrophages, act as host cells and the parasite becomes internalised through phagocytosis. There are some data that NPs without drugs may even interfere with normal white blood cell functions, including phagocytosis.

As NPs are recognised as foreign structures, they are eliminated by phagocytising cells [21, 22]. Thus, it should be easier to treat microorganisms which multiply within macrophages, because the nanoparticles would be concentrated automatically in the desired target cells. An example for this strategy is *Leishmania* [23]. Another interesting goal of nanoparticle research is targeting NPs to the brain [11, 14, 24]. *Toxoplasma gondii* offers a possibility to combine these aims. *T. gondii* is a parasite which multiplies in different cell types including

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macrophages and phagocytising cells in the brain [25]. The main problems in treatment of toxoplasmosis are infections during pregnancy and *Toxoplasma* encephalitis in the case of immunocompromised patients [25–28]. In normal *Toxoplasma* infections, the multiplication of the parasites is stopped by the immune system, but cysts remain in all formerly infected tissues including the brain, serving as a source for exacerbations. Therefore, efficient treatment of all infected cells to prevent cyst formation would be an interesting goal. Thus, we started our experiments with NPs in *T. gondii*-infected cultures, knowing that *T. gondii* has a completely different mode of penetration which is mostly independent of the support of the host cell [29].

To come closer to in vivo conditions and to make it easy to observe an effect, the culture should work for at least 3 days to allow several cycles of multiplication.

Initially, all experiments with FACS should be controlled by microscopy, to prove that the interpretation of the FACS data is consistent with the images seen under the microscope.

2. Results and discussion

The established and considered system consisted of three components: nanoparticles, parasites and cells which differed in size and shape. However, there were also infected cells, cells loaded with nanoparticles and infected cells with nanoparticles which should be differentiated as well. To trace the fate of parasites and nanoparticles with FACS, they had to be stained with dyes which are distinguishable from each other. Because the green fluorescent protein, *Toxoplasma* was transfected with, shows fluorescence in green and yellow [33, 34] a red dye to stain the nanoparticles gave sufficient results. Of the dyes tested, rhodamine B gave the best results. The intensity of fluorescence corresponds with the amount of dye, fixed inside the core of the NPs and should increase exponentially (to the third) with the increasing radius of the NPs. In larger particles, however, there seems to be a quenching, as light is unable to penetrate from the core of the particle to the surface.

Next, a broader spectrum of NPs of different sizes was tested to see, which gave the best results. To be able to compare the FACS data with microscopical counting, the NPs had to be larger than 100 nm; otherwise, microscopical identification, especially of intracellular particles, would be impossible. In **Table 1**, one of the two best candidates is shown. As the results obtained with the two different NPs in these experiments were identical, only data with MC81cs are shown here.

The nanoparticle suspensions were homogeneous in size, checked by electron microscopy—data not shown—and were shown to be non-toxic, by measuring growth of cell cultures in their presence.

Figure 1A shows an infected cell in the presence of NPs. NPs with diameters between 200 and 300 nm were focused on as they were perfect for these purposes, being visible under a microscope, and therefore allowing the opportunity to compare microscopically the results, obtained with FACS with the situation in the cell culture.

MC81			Core		Core-shell (cs)		Cs + pentamidine	
Z-ave (nm)			133.1		213.4		221	
PI			0.065		0.046		0.071	
ZP (mV)	pH	Distilled water	−93.20	5.52	−84.79	5.5	−79.70	5.55
		10E-3M NaCl	−70.33	5.53	−63.40	5.51	−61.66	5.51
		10E-2M NaCl	−50.85	5.54	−30.60	5.51	−29.00	5.45
Surface charge density (μC/cm²)			−2.54		−0.93		−0.96	
MC81c			**MC81cs**					
			No drug		Pentamidine		Spiramycin	
Size (nm)		133.1	213.4		222.1		198.1	
PI		0.065	0.046		0.071		0.04	

Table 1. Nanoparticle properties (mean particle diameter [z-average] and polydispersity index from dynamic light scattering, zetapotential [ZP] from particle electrophoresis).

The same area photographed using different filters: the arrow points to a macrophage cell infected with one *Toxoplasma* cell.

a. Three channel photography (FITC/PI/neutral): NPs are attached to the macrophage, and some are scattered around.

b. Two channel photography (FITC/PI): the parasite inside the cell is clearly visible.

c. One channel photography (FITC): only the parasite (green) is visible.

d. One channel photography (PI): the parasite is invisible, the cell appears red as a result of uptaken NPs, and some extracellular NPs are visible.

Figure 1B–D demonstrates how different filters are able to distinguish the dyes used. In a first series of experiments, different amounts of NPs added to cell culture were tested with the result that approximately 60,000 particles per cell gave measurable and reproducible results in this system. Most of the NPs were phagocytised by the cells within the first few hours.

As parasites not only infect cells and multiply—increasing in number in an unpredictable way— they will also destroy host cells, thereby diminishing their number.

To calibrate the exact increase or decrease in the different components, 'Perfect Counting Beads' (Caltag Laboratories) were added before FACS scan and all data were adjusted to the beads count.

The particle size (FSC-x-axis) and granularity (SSC-y-axis) were determined. In A, toxoplasmas'and in B, macrophages were counted in relation to beads in order to calculate

http://dx.doi.org/10.5772/67156

absolute numbers. The beads are always seen in the same position. In parallel, the particles were characterised by fluorescence (see **Figure 3**).

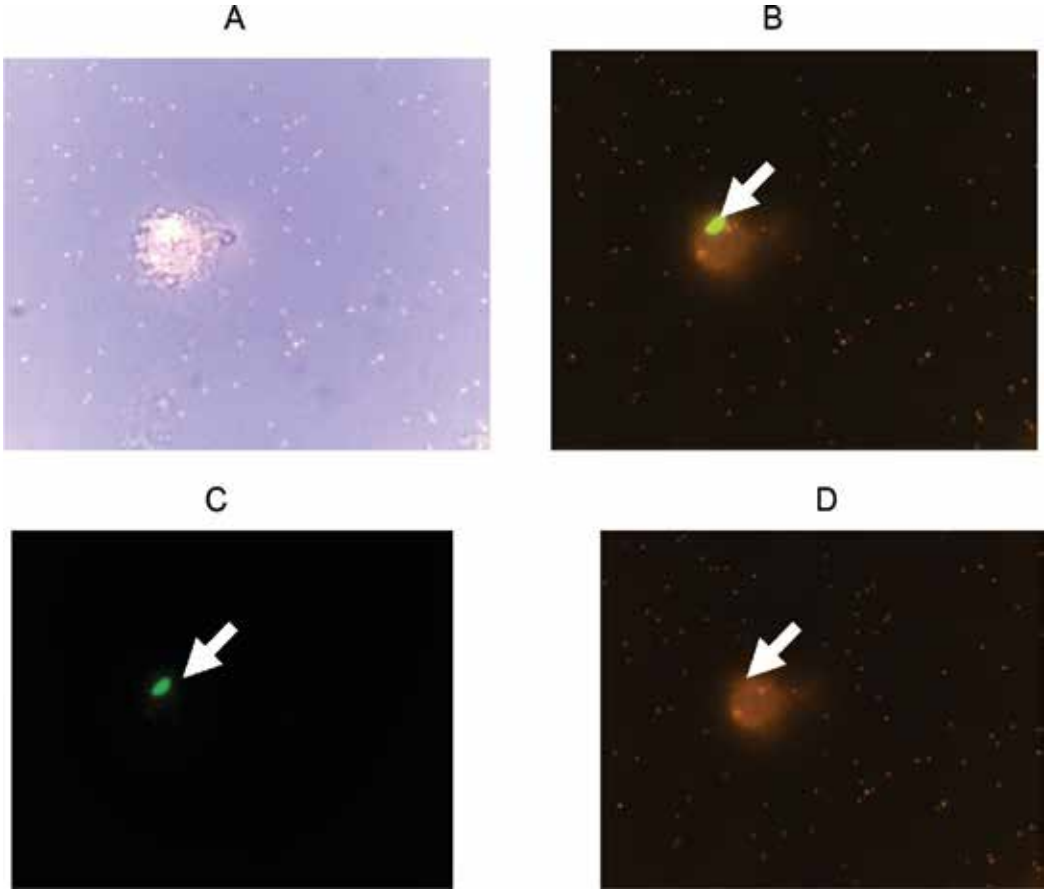
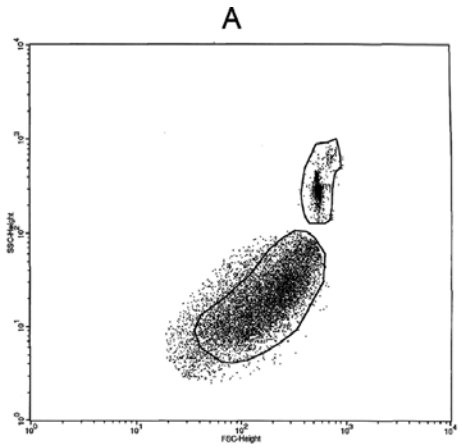


Figure 1. Situation in cell culture: macrophages, parasites and nanoparticles (NPs).

Figure 2 shows an example of data obtained with a culture of J744-A1 cells infected with *T. gondii*. Each sample had to be measured twice: once to count the free parasites (**Figure 2A**) and a second time to count the cells (**Figure 2B**) separated by their size (FSC) and their granularity (SSC).

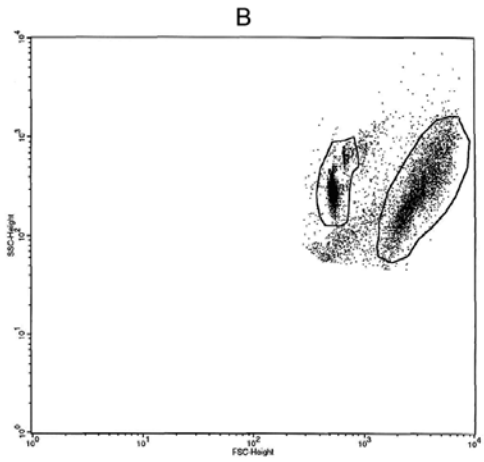
For analysis, the counted cells were sorted by fluorescence then. The series of dot blots in **Figure 3** compares uninfected, untreated control cells (A), infected cells (B), infected cells treated with unloaded nanoparticles (C), uninfected cells treated with unloaded NPs (D) and infected cells treated with pentamidine-loaded NPs (E).

The dot blots show changes in green fluorescence and granularity. In addition, the mean values of the particles in the gates are given in **Table 2**, as the numbers are hard to calculate from the images. The shift in the mean values is often more impressive than the density or position of the dots.



Tube 2 Infection control

Region	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
R1	1846	18.46	18.46	546.01	543.77	329.01	313.71
R2	7183	71.83	71.83	214.94	178.95	24.96	19.97



Tube 1 Cell control

Region	Events	% Gated	% Total	X Mean	X Geo Mean	Y Mean	Y Geo Mean
R1	4933	49.33	49.33	540.11	537.92	344.53	329.27
R2	3968	39.68	39.68	3343.07	3134.86	362.81	286.31

Figure 2. Particle size (FSC-x-axis) and granularity (SSC-y-axis) of (A) Toxoplasma cells and (B) macrophages population.

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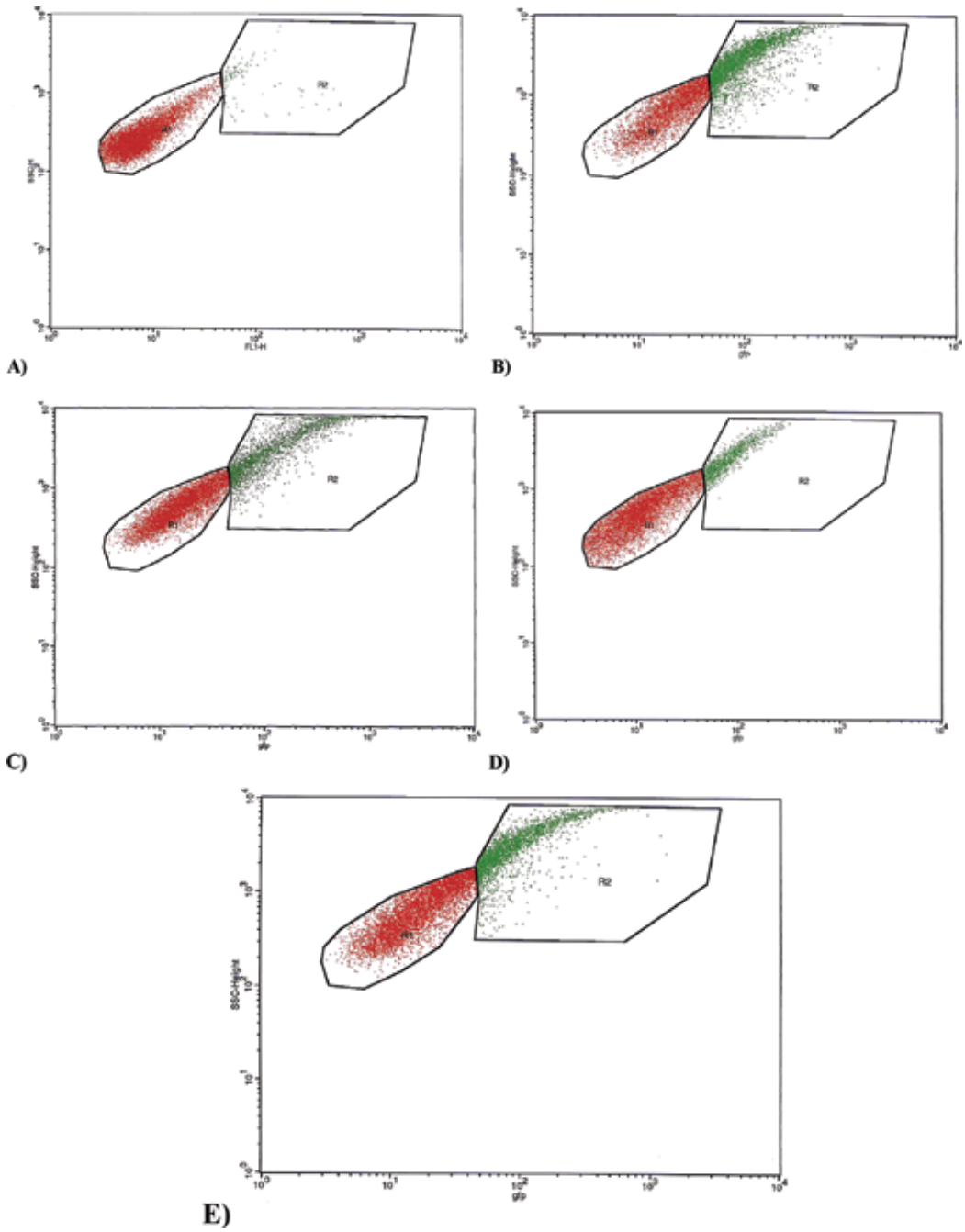

Figure 3. Granularity (SSC) and green fluorescence (FL1) of non-infected, untreated cells (A), infected, untreated cells (B), infected and treated with pure nanoparticles (C), uninfected cells, treated with nanoparticles (D) and infected cells, treated with pentamidine-loaded nanoparticles (E), given after 1 day of incubation.

Figure 3A illustrates the fluorescence of the macrophages of the cell control with its characteristic unspecific autofluorescence. The presence of *Toxoplasma* and/or NPs induces a movement into the area of higher fluorescence (**Figure 3B–E**). Whereas the *Toxoplasma* induces a stronger shift into the green fluorescence (**Figure 3B**), the NPs induced a smaller one (**Figure 3D**) which is an effect of an unspecific stimulation of the cells resulting in an increased autofluorescence [30].

The corresponding **Table 2** shows the percentage of particles in the gate for non-infected cells (R1) and the percentage in the gate of infected cells (R2) as well as the corresponding mean values (mean) and geometrical mean values (G-M).

Green fluorescence is represented on the x-axis and granularity on the y-axis.

Macrophages, activated by nanoparticles, have a basic fluorescence which is at least partially an indicator of their activity [36]. Thus, controls of cells, treated only with nanoparticles, are essential. For data processing, other combinations may be selected, such as granularity or red fluorescence. There is always an overlap of data however, and the interpretation of the blots must be proofread against the mean values of the particles within the gates—here given in the corresponding tables.

In the following series of experiments, a cell system was established in a manner that after infection on day zero, there is a continuous multiplication of Toxoplasma at least until day three. Defined concentrations of cells were put into wells, and parasites in different ratios were added for this purpose. The ratio parasite to cell is given as MOI (multiplicity of infection). All tests were carried out in three wells in parallel, and all experiments were carried out at least twice. The most promising results were obtained with MOI's of 1–10.

Figure 4 shows the results of one of these experiments. MOI's of 10, 5, 2 and 1 were used, and free parasites (A), total cells (B) and infected cells (C) were determined at the first, second and third day after infection. There was a large increase in free parasites over the 3 days with MOI's of 10 and 5, a moderate increase with an MOI of 2 and almost no increase with an MOI of 1 (**Figure 4A**). In parallel, there was no such strong increase in total cell number as with the uninfected controls (**Figure 4B**). With the high MOI's of 10 and 5, there was a decrease at day two and an increase at day three. Only with MOI's of 1 and 2, there was a slight increase in cells over the 3 days. The number of infected cells indicates that with a MOI of 10 and 5, almost all cells were infected, whereas with MOI'of 2 and 1, many cells remained uninfected until day three (compare **Figure 4B** and **C**).

It was shown that MOI 2 is most appropriate for these tests, because there was an increase in the parasites and of the cells over the 3 days.

In a culture of infected cells, running for at least 3 days, there will be individual death of cells and parasites. Some of the free *Toxoplasma* in the culture will die and—as the infected cells disintegrate after liberating multiplied parasites—will shift into the debris. Thus, there will be an unpredictable increase or decrease in cell counts. To differentiate between an increase of non-infected cells and a loss of infected cells, both of which result in an increase in the percentage

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of non-infected cells, it was necessary to determine the total number of cells and the proportion of infected and non-infected cells in relation to the cell input on day zero. Before measuring, cells were fixed with paraformaldehyde, and after 'Perfect Counting Beads' (Caltag Laboratories) were added, the samples were counted.

Experiment	R1 %	R1 X mean	R1 X G–M	R2%	R2 X mean	R2 X G–M
A C	98.84	8.35	7.35	1.16	102.68	77.60
B C + T	50.83	**23.42**	**20.62**	49.17	**108.24**	**91.94**
C C + T + NPs	73.05	**18.68**	**16.16**	26.95	**165.08**	**116.78**
D C + NPs	85.46	13.72	11.16	14.54	88.33	80.10
E C + T + NPs + P	69.55	**19.58**	**16.84**	30.45	**103.65**	**86.43**

Exp.: part of the experiment corresponding to **A** to **E**; R%: percent of counts in this gate; R1 X mean: mean value of counts in gate 1; R1 X G-M: geometrical mean value of counts in gate 1; R2 X mean: mean value of counts in gate 2; R2 X G-M: geometrical mean value of counts in gate 2.

Table 2. Granularity (SSC) and green fluorescence (FL1) with the corresponding values of **Figure 3**.

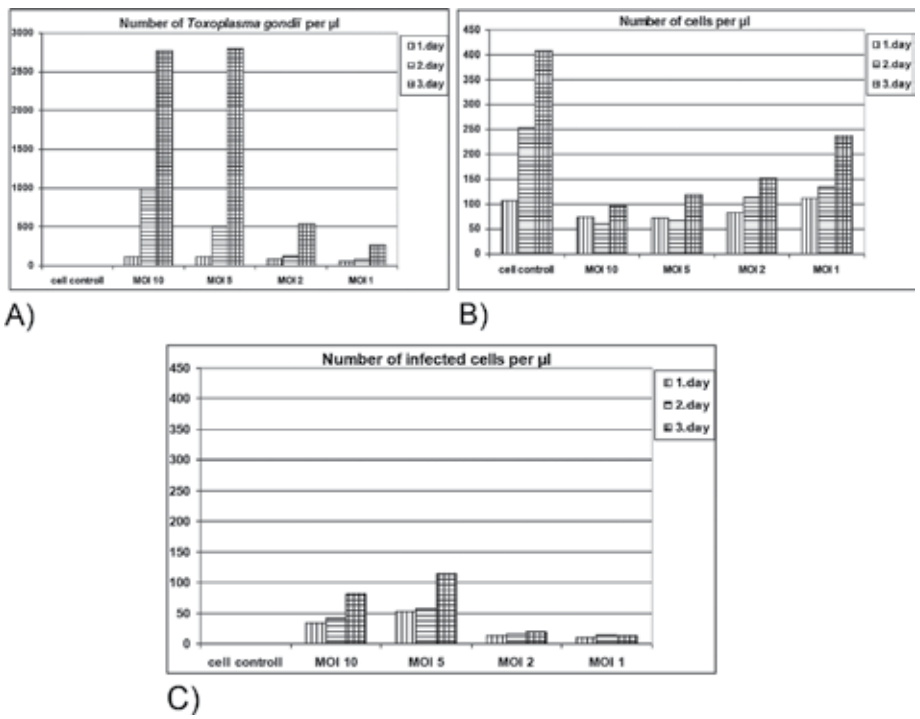

Figure 4. Influence of multiplicity of infection (MOI) 1.36×10^5 cells per ml was infected with different multiplicities of infection (10, 5, 2 and 1). A number of free parasites (A), total cell number (B) and number of infected cells (C) were determined over 3 days.

There was an indication that even unloaded NPs have an inhibitory effect. To prove this, the influence of pentamidine-loaded and unloaded nanoparticles on Toxoplasma-infected cell cultures was determined. **Figure 5** shows the histograms in each case for day 2 of non-infected cells (A), infected untreated cell controls (B), cells only treated with NPs (C), infected cells treated with unloaded NPs (D) and pentamidine-loaded NPs (E). The histograms show the changes in green fluorescence (FL1) and number of counts. One table with all data obtained (**Figure 5A**) is shown as an example of how CellQuest® analyses data. Data of corresponding tables, with the mean values of the gates for all 3 days, are given in **Table 3**. It is shown that the presence of Toxoplasma induced a shift to higher fluorescence. Comparing the data of infected cells, treated with pentamidine-loaded nanoparticles with those treated with unloaded nanoparticles, demonstrates that nanoparticles alone had an effect on *T. gondii* multiplication.

Simply comparing the mean values of M2 (the gate with the infected cells) would lead to a misinterpretation: the geometrical mean value would indicate that NPs even enhance Toxoplasma multiplication (106.69 vs. 86.41-labelled with '1' in **Table 3**), and the median would also suggest this (84.26 vs. 77.04 labelled with '2' in **Table 3**).

By looking at the percentage, one sees that with NPs, there was an increase in cells in the gate of non-infected cells (M1: 71.0 vs. 49.5% labelled with '3' in **Table 3**) and as the mean value of all cells indicates, there was an inhibition by unloaded NPs visible as reduction in green fluorescence (57.48 vs. 63.41 labelled with '4' in **Table 3**). The median indicates that the inhibition was substantially (22.27 vs. 43.32 labelled with '5' in **Table 3**).

The mean value of the green fluorescence marker reflects the parasitic load, as fluorescence is proportional to parasite number. Thus, few cells with high numbers of parasites may increase the mean value despite the fact that there were lots of non-infected cells. The median indicates the shift of the population towards infected or non-infected cells. The closer the median of infected, treated cells come to non-infected control cells, the better the treatment success. The geometrical mean gives some information, concerning the heterogeneity, but avoids the overestimation of exceptionally heavy infected single cells.

By measuring 10,000 cells, FACS offered the opportunity to statistically treat these data in different ways, resulting in a more complex interpretation. In addition to this evaluation of data, one had to take into account that nanoparticles are known to stimulate the macrophages [30–32]. As an increase in unspecific fluorescence was seen, an inhibition of Toxoplasma growth by unloaded nanoparticles was not surprising.

The explanation was that the nanoparticles counteracted the suppression of activities caused by the parasites [33], and this unspecific stimulation of macrophages by nanoparticles was more effective than the inhibition of parasites within downregulated host cells.

As cells, treated with nanoparticles, were always used as controls to estimate the unspecific increase in fluorescence, it was realised that there was a substantial inhibition by unloaded nanoparticles in all experiments.

In an additional series of experiments, the effect of nanoparticles without any drug was specifically proven. Data of an experiment to determine the effect of particle number on the multiplication of *T. gondii* during a period of 3 days are given in **Figure 6**.

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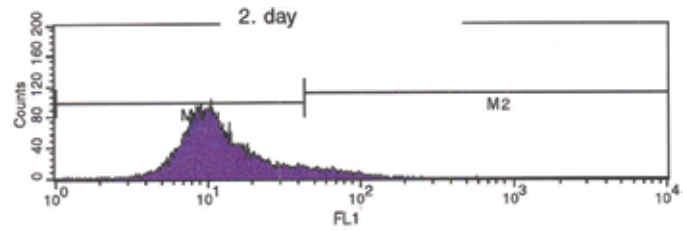
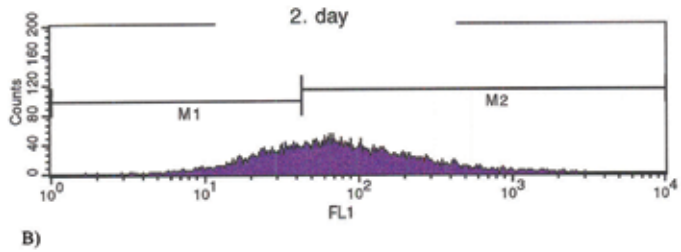
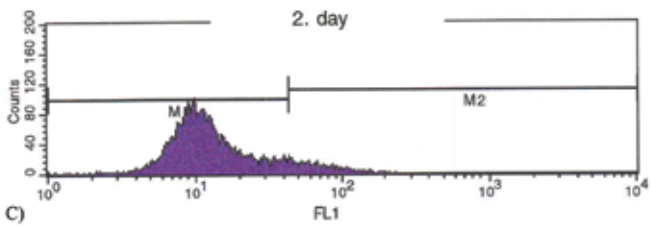
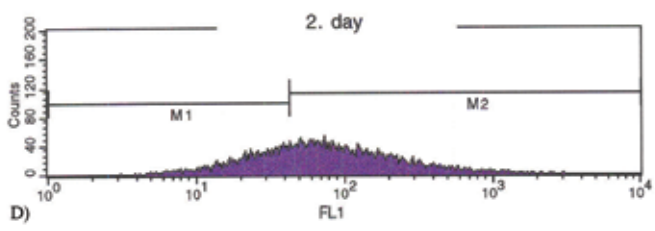


Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 9910	10000	100.00	100.00	16.77	12.57	111.60	10.75	10
M1	1, 43	9311	93.11	93.11	12.73	11.11	58.92	10.37	10
M2	43, 9910	697	6.97	6.97	71.02	66.29	47.17	61.53	63







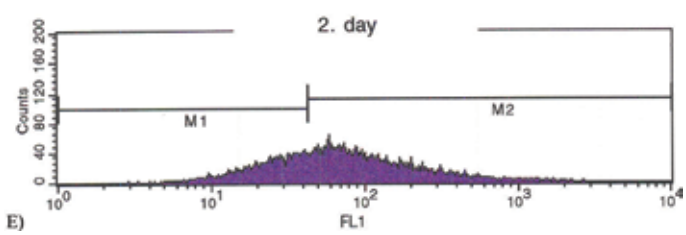


Figure 5. FACS counts of non-infected, infected and NPs-treated cell. Histograms are shown in each case for day 2 of non-infected cells (A), infected untreated cell controls (B), cells only treated with NPs (C), infected cells treated with unloaded NPs (D) and pentamidine-loaded NPs (E). The histograms show the changes in green fluorescence (FL1) and number (counts). M1 indicates the non-infected cells and M2 the infected ones. (T = *Toxoplasma gondii*).

	1. day			2. day			3. day		
	All	**M1**	**M2**	**All**	**M1**	**M2**	**All**	**M1**	**M2**
Cell control [%]		*98.6*	*1.4*		*93.0*	*7.0*		*92.0*	*8.0*
Mean	9.29			16.77			16.14		
Geomean	7.30	7.08	70.91	12.57	11.11	66.29	10.23	8.68	65.62
Median	6.79	6.73	57.25	10.75	10.37	61.53	9.22	8.58	59.89
Infected cells [%]		*35.0*	*65.0*		*33.0*	*67.0*		***49.5***[3]	*50.5*
Mean	160.49			127.46			**63.41**[4]		
Geomean	75.89	18.78	156.81	70.30	23.32	121.25	42.25	20.27	**86.41**[5]
Median	73.63	19.81	145.90	66.12	25.71	103.66	**43.32**[5]	22.07	**77.04**[2]
Cells + NPs [%]		*93.0*	*7.0*		*90.5*	*9.5*		*85.5*	*15.5*
Mean	14.63			18.76			22.96		
Geomean	10.06	8.77	67.19	13.70	11.60	65.49	13.21	9.81	74.96
Median	8.28	7.99	62.08	11.44	10.84	61.53	11.44	9.91	69.16
Cells + Toxopl. + NPs [%]		*34.0*	*66.0*		*33.5*	*66.5*		***71.0***[3]	*29.0*
Mean	159.64			118.23			**57.48**[4]		
Geomean	76.71	19.57	156.14	142.73	23.22	117.34	27.56	15.92	**106.69**[1]
Median	72.34	21.29	143.30	64.94	25.71	101.82	**22.27**[5]	15.96	**84.29**[2]
Cells + Toxopl.+ NPs (pentamidine-loaded) [%]		*37.0*	*63.0*		*35.0*	*65.0*		*69.0*	*31.0*
Mean	157.60			115.67			43.75		
Geomean	73.90	20.18	158.34	65.71	23.49	113.57	27.49	27.49	79.96
Median	67.93	21.48	144.60	61.80	25.71	95.60	25.95	17.31	68.54

[1,2,3,4] and [5] refer to numbers in discussion.

Table 3. Mean values of total cells and gates of **Figure 5**. In addition to the mean values, the percentage of M1 and M2 of total cells for each part is given.

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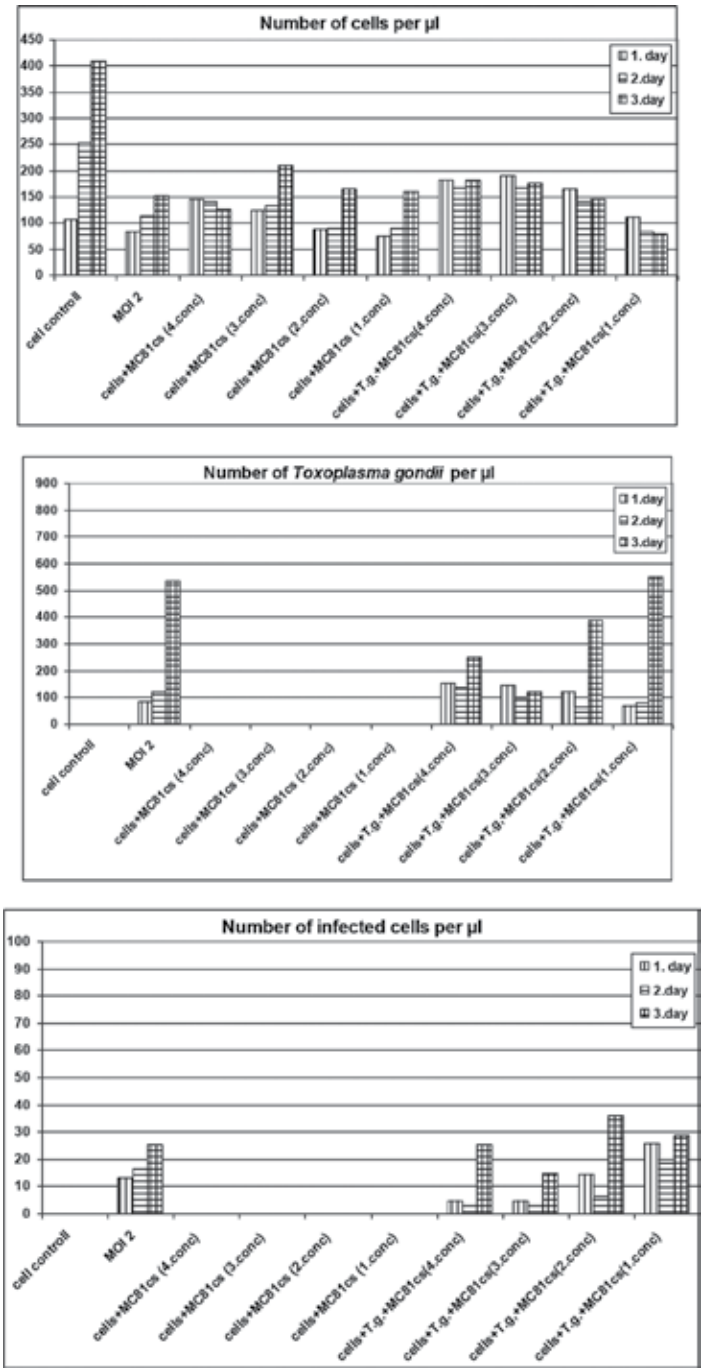

Figure 6. Effect of different concentrations of NPs (MC81cs) on cell proliferation and of *Toxoplasma gondii* (T. g) growth. (A) A number of all remaining cells (infected and non-infected) after 1, 2 and 3 days of infection and treatment. (B) A number of free (extracellular) *Toxoplasma gondii*. There are no *Toxoplasma* in NPs-treated, non-infected cells. (C) A number of infected cells after treatment with different concentrations of NPs.

Part A shows the increase in the infected and non-infected cells with different NPs concentrations. As can be seen, NPs in a high concentration (conc.4) inhibited proliferation over the 3 days. The lower concentrations led to a small increase on day two and a larger increase on day three. With infected cells, there was no difference between conc. 4 and 3, and only a slight decrease in cells with conc. 2. But with the lowest concentration, conc. 1, there was a decrease over the 3 days.

Part B shows the increase in *Toxoplasma* in the presence of NPs. Concentrations 4 and 3 suppressed the propagation of *Toxoplasma*. There was a reduced number of free *Toxoplasma* compared with the infection control. At concentrations 1 and 2 on the third day, there was a large increase in free *Toxoplasma* with concentrations even higher than in the untreated control.

Part C shows the number of infected cells which decreased at all NPs concentrations on day two and increased on the third day. The third concentration led to the smallest number of infected cells over the 3 days.

Cells were infected with an MOI of 2. The concentrations of NPs were as follows:

Conc 1: 118,000 NPs/cell	Conc 3: 24,000 NPs/cell
Conc 2: 60,000 NPs/cell	Conc 4: 12,000 NPs/cell

As discussed above, the effect of NPs was interpreted as activating the functions suppressed by Toxoplasma. On the other hand, there may be negative effects on macrophage functions, too [34]. Thus, in other cases (with other cells or with other parasites), the effect could be different. If there is stimulation, a treatment with nanoparticles alone could have some influence on a Toxoplasma infection, as drug-loaded particles have.

One way to prove this is to repeat these experiments in other, non-phagocytising host cells. The problem is that they will not take up the nanoparticles as easily as the macrophages do. As there are other microorganisms which also multiply within macrophages, there could be other indications for this type of treatment.

In all experiments described so far, Toxoplasma and NPs were added simultaneously to the cells. When nanoparticles influence the activities of phagocytising cells, and *T. gondii* alters the host cell activities, it is interesting to ask, whether the presence of NPs or *T. gondii* interferes with the taking up of the other, is there some type of mutual exclusion? Thus, experiments were carried out with NPs, added four hours after infection with *T. gondii*, or cells were infected 4 h after treatment with NPs. There were no differences in the taking up of NPs or parasites or in the effect of treatment after 3 days. The slope of the parameters was simply shifted (data not shown).

In the future, one will see, whether Leishmania major which in contrast to *T. gondii* are able to grow only in macrophages and are also inhibited by unloaded NPs. A few reports indicate that this should be true [21]. With Leishmania, first data in animal models were published [35, 36]. The fact that Leishmania major multiplies only in phagocytising cells makes them even more interesting for a further use of nanoparticles.

http://dx.doi.org/10.5772/67156

A question to be answered is, whether there is disintegration of nanoparticles by the host cells. There was fluorescence on the third day, what could be the result of dye released after disintegration? Microscopical observation revealed that nanoparticles were still present, at least the non-degradable, fluorescence-labelled polystyrene cores of these core-shell nanoparticles.

With the methods used, a disintegration of the nanoparticles could not be demonstrated in detail as it should start with the cleavage of the ester groups of the polymer shell by esterases which would not alter the structure in a way that could be seen with a microscope. As it only happens inside the cells, this alteration cannot be found with FACS, as there is no change in granularity.

As the core particles remain undigested, granularity will indicate the presence of particles. In the case that there would be no disintegration, this may be an advantage of an in vitro system using phagocytising cells, as the activation will persist. The consequences for in vivo conditions remain to be determined. Allen showed in 1988 that repeated application of liposomes could led to an impairment of Kupffer cells in the liver and fixed macrophages in the spleen which remove most of these NPs [37].

Our aim was to establish a system which allows the observation of the interaction of nanoparticles and microorganisms, infecting a cell culture, because there should be difference between infected and non-infected cells which could be used for targeting. The results are very promising. As various cell lines are able to produce interleukins or other modulators, there is the possibility to characterise the influence of nanoparticles in even more detail.

The data that NPs without any drugs have the same effect as drug-loaded NPs offer the opportunity to use NPs directly for treatment. Perhaps part of the effect of Atovaquone crystal nanoparticles in a Toxoplasma-mouse model should be attributed to the effect of nanoparticles [13], rather than to the direct effect of the drug against the parasite.

3. Experimental part

3.1. Colloid and polymer chemistry

3.1.1. Preparation of nanoparticles

Core-shell latex nanoparticles (MC81cs see **Table 1**) were synthesised by seeded, aqueous polymerisation of butyl cyanoacrylate onto polystyrene cores. Polystyrene cores were prepared by emulsion polymerisation of styrene in a water/ethanol mixture (16:1; v/v) in the presence of rhodamine B and purified by dialysis against deionised water. One gram of these core particles in water with a pH of 2 was used as seed for the polymerisation of 2 g butyl cyanoacrylate [38]. In the case of pentamidine loading, 5 mg pentamidine/g polymer was added to the polymerisation medium. Resulting core-shell nanoparticles were purified by dialysis against de-ionised water after neutralisation with sodium hydroxide (1 M aqueous solution).

3.1.2. Characterisation of nanoparticles

The particle size, determined as Z_{ave} (average particle diameter) and the polydispersity index (PI), was measured by photon correlation spectroscopy (PCS), using a Zetamaster S (Malvern Instruments). Electrophoretic mobilities were measured to reflect the effective surface charge (Zetamaster S, Malvern Instruments). For transformation into zeta potential, the Smoluchowski model for ideal smooth spheres was used. Measurements were carried out in ultra-pure water (Millipore), 0.01 M NaCl solution and in 0.001 M NaCl solution.

Surface charge density was titrated by means of 0.1 mM PDADMAC solution (MW approx. 100,000) at a combination of particle charge detector PCD-03 pH (MÜTEK) and automatic titrator Titrino 716 (Metrohm). Purified, aqueous nanoparticle suspensions were stored in the refrigerator until use.

3.1.3. Beads

'Perfect Counting Beads' (Caltag Laboratories) were used to adjust the cell and *T. gondii* number. Immediately before counting with FACS, a defined number of beads were added and for calculation of inhibition and growth, all cells and *T. gondii* were adjusted to the known bead concentration.

3.2. Biology of Infections

3.2.1. T. gondii GFP growth and experimental infection:

Tachyzoites of the RH (CAT-GFP) strain of *T. gondii* (obtained from D. Soldati; Centre of Molecular Biology, Heidelberg University, Germany) were maintained in vitro in human epitheloid carcinoma cells (HELA) by serial passage into RPMI medium supplemented with 10% FCS (Gibco-BRL) twice a week (Gibco-BRL).

Macrophages and parasites were counted in a Fuchs-Rosenthal chamber and adjusted to the concentrations indicated. In most cases, macrophages of a concentration of 1.36×10^5 per ml were used and the multiplicity of infection (MOI) in most experiments was 2.

3.3. Cell culture work

3.3.1. Cell lines

Macrophages: The intracellular Toxoplasma infection studies were performed on J 774-A1 cells (mouse monocyte macrophages, ACC 170; German Collection of Microorganisms and Cell Cultures). The macrophages were grown in Dulbecco's MEM containing 10% heat-inactivated foetal calf serum (Boehringer, Germany) at 37°C in 5% CO_2 atmosphere.

3.3.2. FACS-analysis

Parasites, infected cells and non-infected cells were counted with FACS-scan analytical flow cytometry and equipped with a 15 mV, 488 nm air-cooled argon ion laser (Becton Dickinson). Multiparametric data were analysed using CellQuest® Software. Ten thousand 'particles', defined by size and granularity, were measured for each analysis.

The optimised instrument parameters for the assay were as follows: forward scatter (E 00, log mode), side scatter (319 voltage, log mode) fluorescence 1 (FL1; 613 voltage, log mode) and fluorescence 2 (FL2; 528, log mode).

Each experiment was carried out in a triple manner and performed in a special laboratory. The equipment was decontaminated with FACS-safe solution (1% active chlorine, Becton Dickinson).

Adding formaldehyde to a final concentration of 0.2% resulted in a preservation of the samples. Samples could be stored for days in a refrigerator and be measured at a more convenient time.

3.3.3. Microscopy and photography

An epifluorescence microscope (Axioskop; Zeiss) was used to view the infection fate of J774 A1 cells with *T. gondii* GFP. The microscope was equipped with a 50 W high-pressure mercury lamp (HB050; Osram) and 10×, 40× and 100× objectives (Zeiss). Narrow band filter sets HQ-F41-007 and HQ-F41-001 (AHF Analysentechnik) were used to analyse the GFP and red signals, respectively, at a magnification of 1000×. Image processing was performed with a standard software package delivered with the instrument (Zeiss Axiovision 3.1).

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Chapter 9

IgY-Technology Applied to Studies of *Toxoplasma gondii* Infection

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Additional information is available at the end of the chapter

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Abstract

In this chapter, we describe relevant aspects of immunoglobulin Y (IgY) technology for *Toxoplasma gondii* applications, including comparison of avian IgY antibody with mammalian IgG antibody, egg yolk IgY production and isolation procedures, important applications for IgY antibody, and state of the art and perspectives for IgY-technology in *T. gondii* studies. *T. gondii* is a worldwide public health problem. IgY-technology provides an alternative antibody (IgY) to mammalian Immunoglobulin G (IgG) antibody. IgY-technology involves the chicken immunization, yolk IgY isolation, antibody characterization, and purified IgY application to several kinds of methods. Immunized chicken transfers a specific IgY from blood to egg yolk. Phylogenetic distance between chickens and mammals influences the generation of antibody repertoires recognizing an antigen profile. IgY is not bound to rheumatoid factor or mammalian complement protein and thus avoids the false-positive results. Yolk IgY isolation is carried out by simple procedures that are accessible for any laboratory and, also, for IgY isolation at large-scale production. IgY-technology provides antibodies for proteomic studies, diagnostic assays, and immunotherapy. Although IgY-technology is promising, there is a reduced number of investigations with IgY and *T. gondii*. Future perspectives involve the use of IgY-technology for the screening of new *T. gondii* antigens for diagnostics, therapy, or vaccine, development of innovative techniques for toxoplasmosis diagnostics and may be an immunotherapy for toxoplasmosis.

Keywords: antibodies, chicken, toxoplasmosis, immunoassay, immunotherapy

1. Introduction

Toxoplasma gondii is the toxoplasmosis agent in human and animals and is a worldwide public health problem. Alternative approach that offers possibility to improve diagnostics, treatment, or control of toxoplasmosis should be accepted as a protective approach to hosts at risk of infection by *T. gondii*. Immunoglobulin Y (IgY)-technology was designed in 1980s with the aim to reduce the suffering procedures to obtain a high-quality antibody, replace animal model for antibody production and refine the kind of applications for purified antibodies. IgY-based antibody production derived from chicken egg yolk antibody is named immunoglobulin Y (IgY). Hens transfer IgY from blood to egg yolk protecting their offspring. Isolation of yolk IgY avoids bleeding the chicken and reduces the number of painful procedures. Hens can lay approximately one egg each day during a period of 2 years. IgY antibody is functional and is equivalent to mammal IgG antibody. Both avian and mammalian antibodies have structure similarity. They have two identical light chains and two identical heavy chains bounded to each other by disulfide bonds. In the amino terminal extremity, they have the fragment antigen-binding (Fab). In the carboxy terminal region, they have the fragment crystallizable (Fc) that is associated to biological antibody functions as interaction with cells or molecules from immune system. Virtually, any substance can be the target of an antibody response and the response to even a single epitope comprises many different antibody molecules, each with a subtly different specificity for the epitope and a unique affinity, or binding strength. The total number of antibody specificities available for an individual is known as the antibody repertoire. The number of antibody specificities present at a particular time is limited by the total number of B cells in an individual, as well as by each individual's previous encounters with antigens. Albeit, they are equivalent in function and structure, the phylogenetic distance and the way to generate antibody specificities produce difference in epitope recognition repertoire between avian and mammalian host. Additionally, modifications in amino acid sequence of IgY antibody result in advantages, comparing to IgG, as follows: IgY does not bound to rheumatoid factor, Fc receptor or mammalian complement proteins that can avoid false-positive results, and many others. Isolation of yolk antibodies begins by removing lipid fraction from yolk and obtaining a water-soluble fraction, and further procedures including salt-based precipitation, gel filtration, or affinity chromatography provide a high-purity IgY-enriched fraction. Purified IgY antibody can be applied to several kinds of techniques including screening new antigens by phage display, enzymatic or florescence immunoassays, diagnostics, and, also, passive immunization for therapy against gastrointestinal pathogens in humans and animal hosts. Future perspective using IgY-technology as a tool for *T. gondii* investigation should include development of new antigen libraries as an innovative source of possible targets for diagnostics, therapy, or vaccination. In addition, create new diagnostic platforms for diagnosing *T. gondii* infection. Finally, the potential protective effect of IgY to be used in prevention or toxoplasmosis therapy by passive immunization should be explored. In this chapter, we describe relevant aspects of IgY-technology to *T. gondii* investigations, including comparing avian IgY antibody with mammalian IgG antibody, egg yolk IgY production and isolation procedures, important applications for IgY antibody, and state of the art and perspectives for IgY technology in *T. gondii* studies.

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2. Overview in IgY technology

2.1. Comparing avian IgY antibody with mammalian IgG antibody

2.1.1. General history

In the early 1893, Klemperer published his observation that there must be neutralizing proteins (i.e., antibodies) in the yolk of eggs laid by immunized chickens. In 1980s, when animal welfare came to be regarded as a serious ethical claim for the scientific community, the extraction of specific antibodies from the egg yolk was considered as an alternative to the blood-taking methods. In this context, phylogenetic distance between chickens and mammals, mechanisms of antibody repertoire diversification, and the way in which chickens deposit IgY immunoglobulin in the egg yolk provide a number of advantages compared to mammals as animal model for antibody production. In an additional manner, the one-step purification of antibodies from egg yolk in large amounts provides a virtually continuous supply; the epitope repertoire recognition of IgY antibodies potentially grants access to new specificities; the absence of cross-reactivity with mammalian epitopes avoids false-positive interference and improves the performance of immunological assays. The generation and application of avian antibodies has caused a surge of interest in a wide variety of applications within the life sciences [1].

2.1.2. Structure characterization of IgY and IgG antibodies

Structurally, avian IgY antibody is similar to mammalian IgG antibody (**Figure 1**). Two identical light chains and two identical heavy chains that are bounded by disulfide bonds compose the antibody's molecular structure [2]. Both chains are formed by the sequences of amino acids (approximately 110 amino acids) named domains which are numerically ordered from the amino terminal region (NH_3^+) to the carboxy terminal region (COO^-). The amino terminal domain, named variable domains, presents a variable amino acid composition sequence by the occurrence of mutational events during the B lymphocyte-produced immune response. For both light chain and heavy chain, variable domains (VL and VH) have three regions with higher variation rate in amino acid sequence named complementarity determining region (CDR). Complementarity determining regions (CDRs) are completely bounded to epitopes (antigenic determinant) during a specific humoral response. In the direction to carboxy terminal extremity, after the variable domain, there are the constant domains. The light chain has one constant domain (CL); however, the number of constant domains for the heavy chain varies with antibody isotypes. IgY heavy chain, named upsilon (Greek letter υ), has four constant domains (CH), and IgG heavy chain, named gamma (Greek letter γ), has three domains (CH) [2, 3].

The fragment antigen-binding (Fab) is positioned in amino terminal extremity and is composed by the entire light chain (VL and CL) and both VH and CH1 domains. The IgY fragment crystallizable (Fc) is formed by CH2, CH3, and CH4 domains and IgG Fc by the hinge region is formed by CH2 and CH3 domains. There was proposed that IgY CH2 domain must be

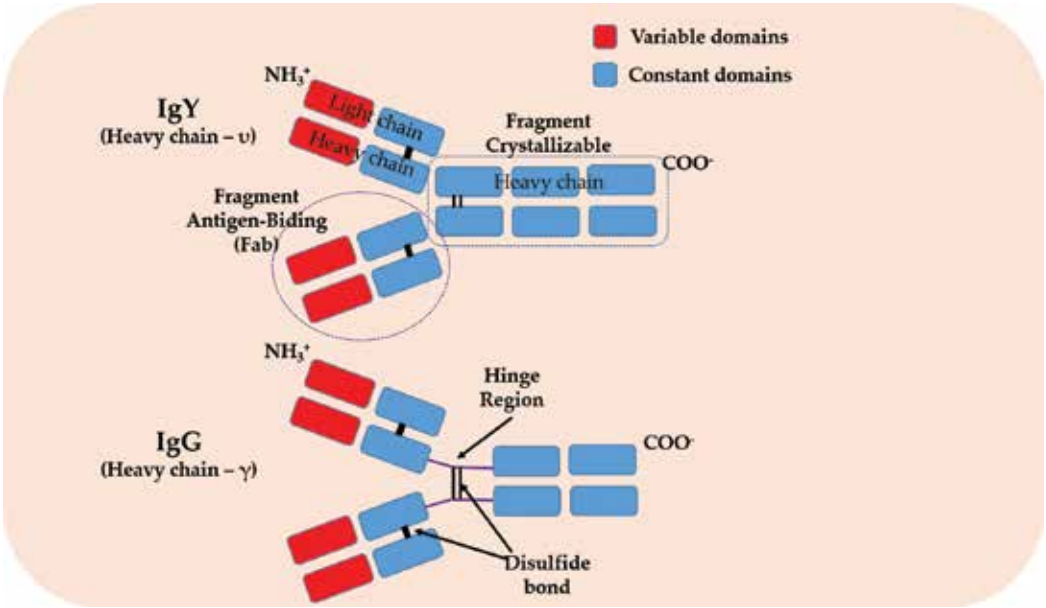

Figure 1. Schematic general structure of the egg yolk antibody (IgY) and the mammalian IgG antibody.

converted to IgG hinge region, which provides a higher mobility to the mammalian Fab than to its avian equivalent. Regarding heavy chain, the chicken has two other classes of antibodies (Immunoglobulin M [IgM], heavy chain μ, and IgA, heavy chain α) and mammals have other four classes of antibodies (IgM, heavy chain μ; Immunoglobulin A [IgA], heavy chain α; Immunoglobulin E [IgE], heavy chain ε; and Immunoglobulin D [IgD], heavy chain σ) [2, 3].

2.1.3. Generation of antigen recognizing repertoire in chicken and mammals

In the genome of a germ-line cell, the genetic information for an immunoglobulin (Ig) polypeptide chain is contained in multiple gene segments scattered along a chromosome. During the development of bone marrow-derived lymphocytes, these gene segments are assembled by recombination that leads to the formation of a complete gene [4]. The vertebrate B-cell repertoire is capable of generating up to 10(9) different antibody molecules. To generate diversity, mammals depend on combinatorial and functional variations that occur during the gene rearrangement events to produce complete heavy and light chain Ig genes. This gene rearrangement process goes continuously in the bone marrow, where each developing B cell assembles a unique heavy and light chain Ig gene from families of functional V (variable), D (diversity), and J (joining) gene segments. In contrast, chickens have only single functional V and J segments for the heavy and light chain loci, and chicken Ig gene rearrangement occurs only during a brief period of embryonic development. A specialized organ involved in avian B-cell development, the bursa of Fabricius, provides the microenvironment necessary for the amplification of B cells that have undergone productive Ig gene rearrangements. Within the bursa, B cells also acquire somatic diversity among the rearranged V gene segments of the

heavy and light chain Ig loci. Somatic diversification of chicken V gene segments occurs by intrachromosomal gene conversion, a DNA recombination process which involves unidirectional transfer of nucleotide sequence blocks from the families of V region pseudogenes into the functional rearranged VH and VL genes [5].

2.1.4. Molecular characterization of IgY and IgG antibodies

Phylogenetic studies have shown that the IgY antibody, homolog of mammalian IgG, has similarities with both mammalian IgG and IgE antibodies. IgY is the predominant isotype in sera, produced after IgM in the primary antibody response, and it is the main isotype produced in the secondary immune response. IgY has different biochemical properties from those of mammalian IgG antibodies and shares homology while functioning with them. Due to the lack of the hinge region, IgY has limited flexibility to its Fab resulting in to precipitate antigens at physiological salt concentrations. Chicken IgY in serum is monomeric, with molecular weight (MW) 165–206 kDa, the mean serum concentration average 5–7 mg/mL and in yolk approximately 25 mg/mL [2]. Mammalian IgG is the Ig found in highest concentration in blood and plays a major role in antibody-mediated defenses. It has a MW of about 180 kDa. Its serum level is approximately 8–16 mg/mL in human and 17–27 mg/mL in cattle [3]. The amount of antibodies obtainable from rabbit is approximately 200 mg, IgG/bleeding (40 mL blood) with 5% of specific antibody, and from chicken is approximately 100 mg/egg (5–7 eggs/week) with 2–10% of specific antibody. IgY sampling is not invasive, which confers advantage on IgG; also, yolk antibody does not interfere with rheumatoid factor or activate mammalian complement [1].

2.2. IgY production

The IgY technology–based layout to produce polyclonal antibodies is displayed in **Figure 2**. First step is the chicken immunization followed by the yolk IgY extraction, the antibody characterization, and finally IgY applications in various kinds of assays. Chickens are a suitable model to produce IgY by immunization with nucleic acid [6], protein [7], lipid, and carbohydrates [8]. In addition, an immune humoral response must be elicited by immunization with recombinant proteins [9] or peptides [10]. To improve the specific IgY response it is demanded the use of adjuvants like Freund's adjuvants for immunization procedures, which is the most frequently used adjuvant. Intramuscularly way is a common way for chicken immunization to produce specific IgY. Alternatively, subcutaneous way provides satisfactory amounts of specific antibody [1].

2.3. IgY purification

Regarding its constitution, yolk can be viewed as an oil-water emulsion with a watery portion containing proteins and a dispersal portion of so-called yolk-granules and lipid-drops [11]. The concentration and distribution of immunoglobulins Y (IgY) in the serum and egg are 6 and 25 mg/ml, respectively. The concentrations of specific antibodies in the yolk are similar to the antibody profile of the serum. To isolate yolk IgY, distinct procedures may be used alone

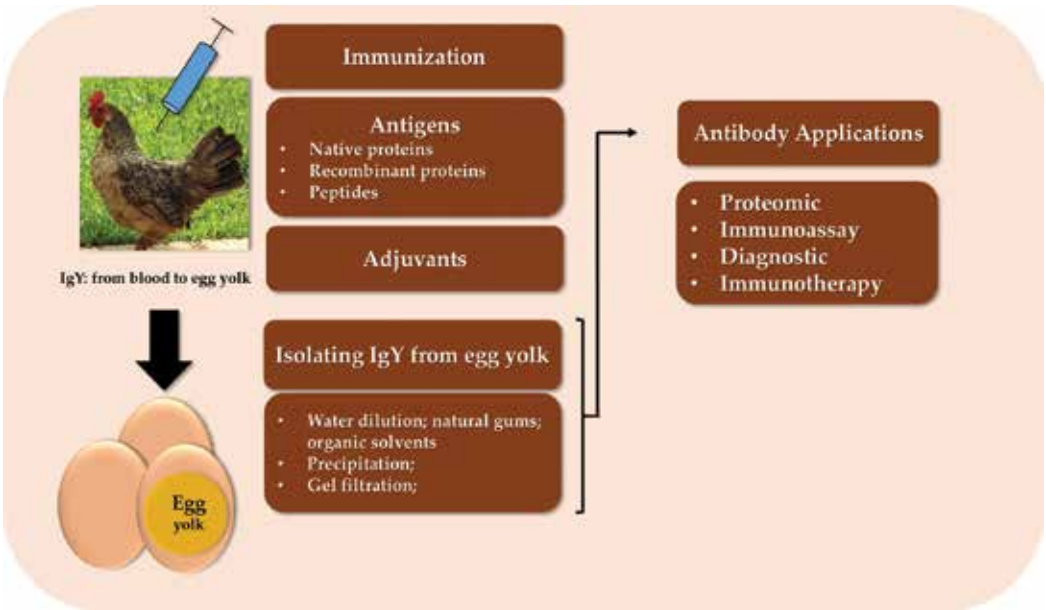

Figure 2. General layout to produce specific IgY antibodies. From immunization with different antigens added to adjuvants, isolating egg yolk antibody to IgY applications.

or in combination according to the following criteria: amount, purity, and biological activity. The yolk antibody extraction begins by separating yolk and egg white. After removal of the vitellin membrane, the yolk is put into a measuring cylinder. The next steps in IgY extraction are devoted to removing the lipid content and obtaining a water soluble phase (WSP) antibodies enriched [12]. The consequent purification procedures vary widely in type and combination and from laboratory to laboratory. The degree of IgY purity depends on desired application; for example, for oral administration, the whole yolk can be used, and on the other hand, for a reagent, isolated IgY from the mixture of yolk molecules is necessary [1].

2.3.1. Removing lipid from yolk

A high quality removing lipid method is the first step to efficiently isolate IgY antibodies from egg yolk. Several natural gums, as carrageenan and xanthan gum, were found to be effective for removal of yolk lipoprotein as a precipitate, resulting in a water-soluble fraction with very low lipid concentration [13, 14]. Optionally, organic solvents (chloroform) have been used as a mean to remove yolk lipid content at the initial step of IgY isolation [1, 14]. The water-dilution method is a widespread procedure for the yolk lipid removal, which provides IgY-enriched water-soluble fraction [14–16].

2.3.2. Isolating IgY by precipitation methods

The extraction of yolk antibodies by the use of polyethylene glycol precipitation (PEG, MW 6000) was, firstly, developed by Polson et al. [17]. The IgY-extraction by means of PEG-precipitation is very cost-effective and results in highly specific antibody with stable titers up

to 1:1,000,000 [18]. Afterwards, PEG 6000 was widely accepted as a standard procedure. Pulverized PEG 6000 is added to WSP until the final concentration of 3.5% is stirred, and the protein precipitate is pelleted by first centrifugation. Finally, PEG is added to supernatant until it brings the final concentration to 12%. The mixture is stirred thoroughly and a second centrifugation PEG method can result in a high IgY antibody concentration per egg; however, it may not be homogeneous when analyzed by SDS-polyacrylamide gel electrophoresis [19]. To improve the quality of extraction, after PEG 12% precipitation, saturated ammonium sulfate solution can be added to IgY enriched fraction [20]. Additionally, by following precipitation procedures, the IgY antibodies may be further purified by DEAE cellulose ion-exchange column chromatography [21] or ionic-liquid based aqueous biphasic systems [22]. IgY-PEG 6000 can be extracted by using three consecutive precipitation steps: 3.5, 8.5, and 12% is a recent adaptation to Polson et al. [17] technique and produces a high amount of specific egg yolk antibodies [23].

Proteins can be precipitated with sodium sulfate (Na_2SO_4, NaS) or ammonium sulfate [$(NH_4)SO_4$, AmS]. Since the precipitation of certain proteins from a mixture by NaS or AmS depends on the concentration of the salt, this procedure can be used not only for isolation of desired proteins but also for elimination of the undesired ones. A precipitate is obtained through centrifuging, taken up in a certain amount of desired buffer or distilled water, and finally dialyzed [1]. NaS and AmS precipitations are economical and environmentally correct procedures for yolk antibody extraction [24]. The major problem in separating IgY is to remove the high concentrations of lipids in egg yolk, which can be obtained by water dilution method [15], followed by IgY salt-based precipitation in the antibody-enriched fraction [25]. NaS or AmS precipitation provides fractions with low protein content compared to original yolk, but with a high specific activity [26]. Salt-based yolk antibody precipitation is promising to be a more efficient and useful purification method for the large-scale IgY from egg yolk preparation [16, 27].

The isolation of IgY from yolk using of combination of different methods results in a high amount of specific antibodies with elevated purity. Sequential precipitation with 31% ammonium sulfate and 12% polyethylene glycol (PEG) produces IgY antibodies with above 95% purity and there is no loss in immunoreactivity [28]. The salt-based egg antibody precipitation combined with thiophilic acid chromatography provides a simple and efficient mean of IgY from egg yolk [29, 30]. Association between AmS and ethanol to IgY precipitation results in a high quantity of antibodies with great purity [31]. Specific antisnake venom IgY with neutralizing effect for therapeutic proposes can be isolated by AmS precipitation followed to ion exchange chromatography [32, 33]. The inhibiting growth activity of IgY anti-*Streptococcus mitis* was preserved by extracting yolk antibodies by precipitation with PEG 6000 and AmS and further isolation by DEAE cellulose ion exchange column chromatography [34]. Similarly, growth inhibition of *Staphylococcus aureus* and *Escherichia coli* was obtained with yolk antibodies isolated by NaS and AmS precipitations [35].

2.3.3. Isolating IgY by gel filtration

The separation of IgY antibodies according to their molecular weight through gel filtration is a further stage of purification of yolk antibodies but rarely reported except in the desalting of

preparation through columns instead through a dialysis [1]. Gel filtration by Sephacryl S300 and Sephadex G50 or G75 provides a high-purity IgY separation but further protein concentration procedures for separated antibody samples are desired [16, 36, 37]. On the other hand, the ion-exchange chromatography is a widespread method to separate proteins from mixtures and it is also used for immunoglobulins isolation. IgY antibodies are bound electrostatically to an ion-exchange matrix with a reverse charge. According to the strength of binding, the antibodies are detached by increasing concentrations of ions of the buffers [1, 32, 33, 34].

2.3.4. Isolating IgY by affinity chromatography

The isolation of yolk IgY antibodies by affinity chromatography consist in attach an antibody ligand on a matrix. The extraction of mammalian IgG-isotypes by affinity chromatography can be obtained by immobilized *S. aureus* A protein or B protein from the *Streptococci* of the C- or G-group. IgY antibodies bind neither with protein A nor with protein G [1]. Recently, protein M (a transmembrane protein from human mycoplasma) has been demonstrated as a promising ligand for purifying polyclonal, monoclonal, or engineered IgY antibodies [38]. Synthetic IgY-ligands were developed to improve yolk antibody isolating mainly by reducing the purification steps and increasing the amount and purity of recovered IgY [39, 40]. Attaching the IgY-induced antigen to matrix provides another way to extract a high-purity IgY by affinity chromatography [41, 42]. The chromatographic thiophilic interaction provides a simple one-step method capable of recovering IgY at concentration close to 100% of initial yolk antibody amount [29, 43].

2.4. IgY applications

2.4.1. Proteomic

IgY antibodies are suitable for a wide application protocols like proteomic, diagnostics, and therapy. The immunodepletion for humans or animal fluids by using specific IgY-attached columns enables the detection of new protein spots, increases resolution, and highly improves the intensity of low-abundance proteins by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) [44, 45]. Other possibility for the use of IgY to proteomic studies is screening random peptide phage display library to detect new epitope candidates to be applied for diagnostic methods to widespread parasitic human diseases [46]. Additionally, using phage-displayed chicken single-chain antibody fragment libraries provides useful diagnostic and research reagents. Libraries constructed, using mRNA from an immune source, are enriched in affinity-matured sequences. Repertoires focused on a number of defined targets can be constructed using lymphocyte mRNA from chickens immunized with a mixture of several different antigens from *Plasmodium falciparum, Trypanosoma* sp., or human viruses [47, 48].

2.4.2. Immunoassay

Development of IgY-based immunoassay is a widespread field to apply yolk antibodies. Enzyme-linked immunosorbent assay (ELISA), western blotting (WB), immunofluorescence

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(IF), and immunohistochemistry (IHC) methods are the most common applications for IgY [1]. Relevant to consider that antigenic targets recognition by the use of antibodies can show some differences according to the method used. Regarding the conformational epitope structure, the performance of antibodies recognizing a given epitope by WB can represent nothing about the same antibody performance in ELISA with the same antigen [49]. IgY antibody shows advantages comparing to the mammal IgG use in immunoassay. In this context, the rheumatoid factor (RF) is a major source of interference in many immunoassays using mammalian polyclonal or monoclonal antibodies by interacting with IgG and causing false-positive results. IgY antibodies do not react with RF avoiding false-positive results in ELISA [50].

2.4.2.1. Enzyme-linked immunosorbent assay

IgY-based ELISA does not demand expressive modifications from the most common procedures and reagents used to carry out this protocol [16, 51]. Egg yolk IgY can be labeled with horseradish peroxidase for use in immunoenzymatic assays [52]. Some possibilities for application of yolk antibodies-based ELISA are: screening molecules related to drug-drug interactions [53]; sandwich ELISA to capture bacterial toxins [54]; screening animal diseases by using recombinant antigens [55]; characterization of maternal IgY transferred to egg yolk [56]; coproantigen to capture ELISA for intestine human parasite [57]; screening antibiotic residues in food samples [58]; detection of blood circulating helminth antigens by immunomagnetic bead ELISA [59]; potential for clinical application diagnosing cancer antigen [60].

2.4.2.2. Immunofluorescence assay

IgY antibodies can be applied to immunofluorescence and flow cytometry assays. IgY-based immunofluorescence experiment allows the detection of antigenic targets on cells and tissues samples [61]. Considering that IgY antibodies do not induce Fc-dependent activation of complement, they must be used to clarify the pathogeny of mammalian autoimmune diseases by immunofluorescence assay [62]. Chicken IgY and rabbit IgG conjugations with fluorescein isothiocyanate (FITC) comparatively used to examine strains of *Campylobacter fetus* revealed that both conjugates have a high percentage rate of detection; IgY has less background due to unspecific fluorescence than IgG. Additionally, IgY is a cheap, bloodless, and very productive method [63]. FITC-labeled anti-*Mycobacterium avium* subspecies *paratuberculosis* can target the pathogen inside the cytoplasm of infected macrophages [64]. Frozen liver fragments were diagnosed with cytomegalovirus antigens by using IgY-based confocal microscopy confirming the yolk antibody as suitable reagent for immunofluorescence assay [65].

2.4.2.3. Flow cytometry assay

IgY antibodies are suitable reagents for flow cytometry and as well as certain monoclonal antibodies, for example, to study human and rabbit platelet physiology [66]. When using phage display-based single chain variable fragments (scFvs), polyclonal IgY production and further the flow cytometry assays it is possible to develop immunoreagents for the isolation and characterization of stem cells, molecular diagnostics and therapeutics of lung cancers [67, 68].

2.4.2.4. Immunochromatographic assay

The immunochromatographic assay (ICA) requires no instruments and has a detection time of less than 10 minutes and it is portable and easy to perform in the field. The development of IgY-based strip could be a promising on-site tool for screening infection or disease outbreaks. IgY-gold complexes depositing onto the conjugate pad as detector reagents showed high specificity [69, 70].

2.4.2.5. Using IgY for passive immunization

The passive immunization is a protective method, which has been tested for many years and shown to be effective [71]. Passive immunization with pathogen-specific egg yolk antibodies (IgY) is emerging as a potential alternative to antibiotics for the treatment and prevention of several human and animal diseases. Laying hens are an excellent source of high-quality polyclonal antibodies, which can be collected noninvasively from egg yolks. The use of IgY offers several advantages such as it is environmentally friendly, nontoxic, reduces the numbers of animals required for antibody production, stability in the orogastrointestinal tract, and its safety profile [71–74]. A major obstacle to its implementation is its relatively high cost, which is dependent, among other things, mainly on two factors: the efficacy of antibody production, and the use of specific pathogen-free (SPF) birds for antibody production to avoid the possible of pathogens transference from commercial layers. Alternatively, treatment of the extracted IgY with formalin can negate the need for SPF birds and shows no interference with the Fab specific antigen-binding or Fc-complement activation of the antibody [75].

Regarding the IgY concentration in egg yolk and blood of laying hens, a recent study provides evidence that there is a significant circaseptan rhythm in yolk IgY and circaquattran rhythm in serum IgY. Additionally, the serum IgY concentration reached to maximum in the morning, decreased to minimum during the daytime, and increased again at night revealing a significant circadian rhythm, which may reflect in yolk antibody concentration [76].

The microencapsulation with a methacrylic acid copolymer may be an effective method of protecting purified yolk IgY from gastrointestinal inactivation, enabling its use for oral passive immunotherapy [77]. The use of chitosan-alginate microcapsules to protect IgY from gastrointestinal environment conditions provides significant resistance to pepsin hydrolysis and may enable intact IgY to reach target microorganisms within the lower digestive tract [78]. Another approach protecting IgY from degradation in gastric pH can be the incorporation of antibodies to hydrogel containing acrylamide and acrylic acid with promising results for IgY oral delivery [79].

Oral administration of IgY antibodies has been tested for many years with promising results [80] to different pathogens as human rotavirus [81]; dental plaque formation by *Streptococcus mutans* [82, 83]; enteropathogenic *E. coli* [84]; *Helicobacter pylori* [85, 86]; *Cryptosporidium parvum* [87, 88]; canine parvovirus [89]; *Porphyromonas gingivalis* [90]; *Pseudomonas aeruginosa* [91]; shrimp's white spot syndrome virus [92]; *Eimeria acervulina* [93]; *E. tenella* and *E. maxima* [94, 95]; H5N1 e H1N1 in mice [96]; *Vibrio cholerae* [97]; rotavirus and norovirus [98]; *Campylobacter jejuni* [99–101]; and botulinum neurotoxins [102]. Immunotherapy as a passive

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immunization method to neutralize venom using purified IgY proved to be efficient for therapy protocol [103–107].

2.5. State of art the use of IgY for Toxoplasma gondii studies

Currently, there are a limited number of studies about IgY antibody production against *T. gondii*, either native or recombinant antigens. *T. gondii* is a worldwide public health and veterinary problem.

Hassl et al. [108] first described the production of anti-*T. gondii* IgY. They used total soluble antigens that are immunogenic to comparatively produce rabbit IgG and chicken IgY. The results indicated differences between the specificities of egg yolk IgY antibodies and rabbit IgG serum antibodies, although both animal species had been immunized with identical antigen preparations. Ferreira Junior et al. [16] have produced comparative mice IgG and chicken IgY against soluble antigens from tachyzoites of *T. gondii*. They purified yolk antibodies by water dilution method, sodium sulfate precipitation, and molecular weight exclusion chromatography. Specific antibody characterization was due to both indirect ELISA and Western blotting methods and applied to immunofluorescence assays by immunohistochemistry and immunocytochemistry assays. Using the Western blotting method, the antigenic bands recognition profile were different between mice and chicken antibodies. IgY antibodies detected parasites in cytoplasm of infected cells and in brain samples of infected mice.

Hoto et al. [109] experimentally infected turkeys with tachyzoites and oocyst, and chickens with oocyst from *T. gondii* and investigated the humoral response against recombinant *T. gondii* antigens (rGRA1, rGRA6, rGRA9, rSAG1, and r SUB1) in a line blot assay. They found that infection with oocyst induced a stronger, permanent long-lasting antibody response compared to tachyzoite-infected animals.

Studying the heat shock protein of *T. gondii* (TgHSP70), a parasite virulence factor that is expressed during parasite stage conversion, Barenco et al [110] produced a polyclonal IgY against the recombinant TgHSP70 and tested it to detect native heat shock protein in brain samples from *T. gondii*-infection resistant (BALB/c) and susceptible (C57BL/6) mice after dexamethasone (DXM)-induced infection reactivation. In parallel, they investigated the TgHSP70-specific humoral response. They found higher specific antibody titers in serum samples of BALB/c compared with C57BL/6 mice. C57BL/6 mice presented high expression of TgHSP70 in the brain with the progression of infection and under DXM treatment. They conclude that these data suggest that the TgHSP70 release into the bloodstream depends on the death of the parasites mediated by the host immune response, whereas the increased TgHSP70 expression in the brain depends on the multiplication rate of the parasite.

More recent study with IgY and *T. gondii* was published by Cakir-Koc [111]. In this investigation, specific IgY antibodies were produced against the surface antigen 1 (SAG1) protein of *T. gondii* and the antibody activity was carried out by ELISA. Specific and higher amounts of IgY antibody against SAG1 were obtained. Regarding the advantages of IgY antibody and the importance of SAG1 for the diagnosis of toxoplasmosis, anti-SAG1 IgY would be a promising reagent in research, diagnostics, and immunotherapy against toxoplasmosis.

2.6. Future perspectives for the use of IgY for *Toxoplasma gondii* investigations

Although there are a reduced number of papers with IgY and *T. gondii*, the yolk IgY antibody has been demonstrated as an efficient tool for application for various kinds of different methods. Regarding phylogeny distance between chicken and mammals, which reflects differences in antigens profile recognition, IgY technology may be applied for the screening of new antigen libraries by random phage display method and the selection of candidate targets for therapy, diagnostics, or vaccine. In this context, polyclonal IgY and scFvs may be a promising alternative for proteomic studies. In the field of diagnostic methods, yolk IgY might be used as an alternative to mammalian IgG for rapid test to detect phase disease-associated circulating antigens, ELISA, flow cytometry test, and fluorescence techniques. Enzyme-labeled or fluorophore-labeled IgY as a primary antibody is employed to detect *T. gondii* antigens in fixed tissue samples or parasite-cultured cells. Speculating about a possible therapy for oral infection by *T. gondii*, IgY protecting the definitive and the intermediary hosts against infection may be an efficient tool for public health to control human and animal toxoplasmosis.

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Chapter 10

Microparticle Vaccines Against *Toxoplasma gondii*

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Additional information is available at the end of the chapter

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Abstract

Significant information indicates that future investigations on *Toxoplasma* vaccine development have to include adjuvants for enhancing protective immunity against *Toxoplasma gondii*. Especially, safe and effective adjuvants capable of fulfilling Th1-dependent cell-mediated immunity appear to be more likely to be allowed to use for anti *Toxoplasma* vaccine development. Recently, biodegradable and biocompatible polymers, such as poly (lactide-co-glycolide) (PLG) polymers, have been utilized as safe and efficacious adjuvants to encapsulate antigens for producing long-term release microparticle-based vaccines. PLG microencapsulation allows the sustained release of antigens and facilitates antigen uptake via antigen-presenting cells (APCs) to favorably generate Th1 cell-mediated immunity, which is required for the prevention of *T. gondii* infection. In our recent work, recombinant surface antigens (rSAGs), including rSAG1, rSAG2, and rSAG1/2, have been, respectively, encapsulated with the PLG polymer for production of PLG-encapsulated rSAG1 (PLG-rSAG1), PLG-encapsulated rSAG2 (PLG-rSAG2), or PLG-encapsulated rSAG1/2 (PLG-rSAG1/2) microparticles. This chapter describes adjuvant effect of PLG microparticles, controlled release of PLG microparticles, PLG microparticles-immune system interaction, *Toxoplasma* SAG-loaded PLG microparticles, protective immunity by *Toxoplasma* SAG-loaded PLG microparticles, and future prospects. PLG microparticle vaccines would be advantageous for their application in the development of long-lasting vaccines against *T. gondii* for future use in humans and animals.

Keywords: adjuvants, poly(lactide-co-glycolide) (PLG), antigen-presenting cells (APCs), PLG-rSAG1 microparticles, PLG-rSAG2 microparticles, PLG-rSAG1/2 microparticles

1. Introduction

Toxoplasma gondii is an intracellular protozoan parasite that uses felines as final hosts and various endothermic animals, including humans, as intermediate hosts [1]. Toxoplasmosis is of major clinical and veterinary importance. The infection in domestic animals such as sheep

or pigs usually generates adverse economic impact due to the induction of severe abortion and neonatal loss [2]. In addition, toxoplasmosis in pregnant women may result in severe congenital fetal disorders, including hydrocephaly, blindness, and mental retardation [3]. Toxoplasmosis in immunocompromised individuals such as AIDS patients often develops lethal toxoplasmic encephalitis as an important opportunistic infection [4]. Although prophylactic anti-*Toxoplasma* vaccines have been studied for a long time, only one commercial attenuated vaccine (Toxovax) has been licensed for use in sheep [5]. Most inactivated and recombinant vaccines developed in the past have produced only little to moderate protective efficacy against infections with a lethal challenge dose of the virulent strain of *T. gondii* [6].

Numerous earlier studies have demonstrated that cell-mediated immunity is considered as the 'appropriate' immune response in the prevention of *T. gondii* infection [7]. Therefore, potent adjuvants that can improve cell-mediated immunity have to include in *Toxoplasma* vaccine development in the future. In addition, gamma interferon (IFN-γ), one of Th1-type cytokines, mainly produced by $CD4^+$ Th1 cells is able to subsequently stimulate $CD8^+$ Tc cells to convert into cytotoxic effector cells for preventing acute and chronic *T. gondii* infection [6–8]. Thus, effective protection against *T. gondii* infection is critically dependent on the IFN-γ-associated Th1 cell-mediated immunity. Therefore, effective vaccines formulated with potent adjuvants that are promised to induce an IFN-γ-associated Th1 cell-mediated immune response seem more likely to be approved for use.

Most modern vaccines based on subunits of pathogens, such as purified proteins, are likely to be less immunogenic than traditional vaccine antigens and are often unable to initiate a strong immune response [9]. These subunit vaccines require effective adjuvants to aid them to elicit strong protective immune responses [10, 11]. Therefore, one of the current important issues in vaccinology is the urgent need for the development of new or improved adjuvants to enhance the immunogenicity or effectiveness of vaccines [9, 10, 12]. Different adjuvants capable of improving immunity and protection have been described in numerous studies [9, 10, 12]. However, the safety concern of an adjuvant is still a crucial issue to limit adjuvant development [13].

Adjuvants are special substances used in combination with an antigen to generate a more robust immune response than the antigen alone [11, 14, 15]. This broad definition encompasses a very wide range of materials for adjuvant development. Actually, adjuvants can be broadly separated into two classes, vaccine delivery systems and immunostimulatory agents, based on their main mechanisms of action [11]. Vaccine delivery systems are generally particulate, such as emulsions [16], micro/nano particles [17], iscoms, and liposomes [18], and their main function is to deliver antigens into antigen-presenting cells (APCs) [11]. In contrast, immunostimulatory agents are predominantly derived from pathogens and often represent pathogen-associated molecular patterns (PAMP), such as lipopolysaccharides (LPS) [19], monophosphoryl Lipid A (MPL-A) [20], and CpG oligodeoxynucleotides (CpG-ODN) [21], which activate cells of the innate immune system to induce the following acquired immune response [11].

This chapter will focus predominantly on microparticle adjuvant, especially poly(lactide-co-glycolide) (PLG) polymer, to be used in tachyzoite surface antigen (SAG)-based subunit vaccines against *T. gondii*, the etiological agent of toxoplasmosis. In our recent work, recombinant SAGs

(rSAGs), including rSAG1, rSAG2, and rSAG1/2, have been, respectively, encapsulated with the PLG polymer for production of PLG-encapsulated rSAG1 (PLG-rSAG1) [22], PLG-encapsulated rSAG2 (PLG-rSAG2) [23], or PLG-encapsulated rSAG1/2 (PLG-rSAG1/2) microparticles [24]. This chapter describes adjuvant effect of PLG microparticles, controlled release of PLG microparticles, PLG microparticles-immune system interaction, *Toxoplasma* SAG-loaded PLG microparticles, protective immunity by *Toxoplasma* SAG-loaded PLG microparticles, and future prospects. The capability of these PLG microparticle vaccines to control the stable release of antigenic rSAG1 and effectively induce and extend protective immunity would be advantageous for their application in the development of long-lasting vaccines against *T. gondii* for future use in humans and animals.

2. Adjuvants effects of PLG microparticles

Microparticles, one of the vaccine delivery systems, derived from different biodegradable and biocompatible polymers, including poly(lactide-co-glycolide), alginate, starch, and other carbohydrate polymers, can be designed as safe carriers for proteins or drugs to perform the main function of delivery systems [25]. Particularly, PLG polymers approved by the US Food and Drug Administration (FDA) have been extensively used as sutures [26] and drug carriers [27] for many years. Different forms of PLG polymers can be generated according to the ratio of lactide to glycolide used for the polymerization [28]. In the recent 10 years, PLG polymers have further become safe and potent adjuvants or delivery systems to encapsulate vaccine antigens for the development of controlled release microparticle vaccines [29]. The PLG microparticles are biodegradable through hydrolysis to break down into the biocompatible metabolites, lactic and glycolic acids, which produce little inflammatory activity and are excreted from the body via natural metabolic pathways [28]. PLG polymers provide a number of practical advantages in acting as vaccine adjuvants or delivery systems following PLG encapsulation. The PLG microencapsulation protects antigens from unfavorable degradation [30], allows the sustained and extended release of antigens for a long period [31], and enhances antigen uptake by APCs, such as macrophages and dendritic cells, in specific lymphoid regions [32]. These adjuvant effects strengthen antigen immunogenicity to favorably generate strong specific immunity, especially cell-mediated immunity [29], which is urgently required for eliminating intracellular pathogens, such as *T. gondii*.

3. Microparticles-immune system interaction

Earlier significant studies have shown that potent cell-mediated immunity induced by PLG microparticles following vaccination is likely to be due to the uptake of PLG microparticles into APCs and the effective delivery of microparticle-containing APCs to specific lymphoid compartments [25, 32, 33]. The size of PLG microparticles used for animal vaccination is a crucial parameter in facilitating the uptake of APCs [32]. Particles smaller than 10 μm in diameter are appropriate for direct uptake by APCs, such as macrophages and dendritic cells [25, 32].

The proper size range thus can stimulate APCs to facilitate the microparticle uptake. Following the uptake of microparticles, the APCs containing microparticles then migrate to other lymphoid compartments [25], such as the spleen and mesenteric lymph nodes, where they effectively present antigenic epitopes to T lymphocytes, especially Th1 and Tc, thereby inducing strong specific cell-mediated immunity [32, 33]. In other words, facilitation of uptake and delivery of PLG microparticles by APCs can lead to more effective antigen processing and presentation to T lymphocytes capable of inducting cell-mediated immune responses [25, 32–34]. Significant earlier studies have further demonstrated that the APCs containing microparticles can travel to specialized mucosal lymphoid compartments, including mucosal-associated lymphoid tissues (MALTs), the inductive sites for stimulating potent immunity following intranasal or oral vaccination [35, 36]. Thus, PLG-encapsulated antigens can be designed as effective mucosal vaccines that have potential to stimulate mucosal systems, such as intestinal and vaginal tracks via intranasal or oral administration [36].

4. Controlled release of PLG microparticles

The capability of PLG microparticles to regulate the extended release rate of PLG-encapsulated antigens can lead to long-term immunity in microparticle-vaccinated animals [29, 37]. Various studies have demonstrated that PLG microparticles may perform pulsed and/or slow release of encapsulated antigens to promote effective immune responses [38]. The sustained and extended antigen release appears to substantially enhance and prolong antigen-specific immunity for achieving long-term protection [29, 31]. The antigen release from PLG microparticles is controlled by the degradation rate of PLG copolymer, which is largely due to the ratio of lactide to glycolide of PLG polymer, the molecular weight, and hydrophilicity of PLG polymer as well as the characteristics of PLG microparticles such as the morphology, size, and encapsulation efficiency [38]. The sustained antigen release of antigen-loaded PLG microparticles have been applied in the development of various potent microparticle vaccines [29, 31]. In addition, antigen-loaded PLG microparticles capable of sustaining release of an antigen also show potential for being designed as a single-dose vaccine without the need for booster doses [37, 39]. However, as some sophisticated events, including enhancement of protein load in PLG microparticles as well as optimization and stabilization of protein release are involved in the design of a single-dose vaccine [40], the feasibility needs to be assessed in future studies.

5. Encapsulation methods

The microparticles based on biodegradable PLG polymers can be prepared by number of methods, such as spray drying, double emulsion, and phase separation-coacervation [30]. However, the most widely used technique for preparation of protein-loaded microparticles is the double emulsion method [30].

The protein is encapsulated in 50:50 poly(lactide-co-glycolide) microparticles using the double emulsion method as described previously [41, 42], with minor modifications [22–24]. In the process, PLG polymer is first dissolved in an organic solvent. The organic solvent dichloromethane is mainly used to dissolve PLG polymer. Protein in aqueous solvent is then emulsified with nonmiscible organic solution of PLG polymer by high speed homogenization or sonication to produce a water/oil emulsion. The resulting emulsion is further transferred to a solution of polyvinyl alcohol, which is used as a stabilizer. Again homogenization or intensive stirring is necessary to generate a double emulsion of water/oil/water. The water/oil/water emulsion is then stirred for 18 h at room temperature (RT) and pressurized to promote solvent evaporation and microparticle formation in a laboratory fume hood. Solvent extraction can also be undertaken yielding microparticles containing protein. The microparticles are collected by centrifugation and washed with distilled water to remove nonentrapped protein.

Based on previous studies, proteins used for PLG encapsulation can be scaled down by using the water/oil/water double emulsion method [30]. In addition, this method also results in high microparticle yields and encapsulation efficiencies [30]. However, there is still a potential concern of antigen denaturation due to organic solvent exposure during the encapsulation process [41, 42], although numerous proteins have been successfully entrapped in PLG microparticles without loss of structural integrity, immunogenicity, or bioactivity [25, 30]. Especially, antigenicity retention following the process of double emulsion method is a critical event to subsequently initiate effective immunity by vaccinating antigen-loaded microparticles [30].

6. Preparation of *Toxoplasma* SAG-loaded microparticles

Development efforts of subunit vaccines against *T. gondii* in our laboratory have been focused mainly on the major immunodominant SAGs of tachyzoites [22–24, 43, 44], the rapidly multiplying stage during the acute phase infection. Furthermore, SAG1 and SAG2 proteins have been identified as two major tachyzoite SAGs in the previous study [45]. These two proteins are involved in the process of host cell invasion [46] and can induce anti *Toxoplasma* immune responses [6]. Therefore, both SAG1 and SAG2 can be considered as potential candidate antigens for *Toxoplasma* vaccine development. SAG1 gene, SAG2 gene, and a hybrid gene consisting of SAG1 and SAG2 sequences had been, respectively, cloned in our previous work to produce recombinant SAG1 (rSAG1) protein [22, 43], recombinant SAG2 (rSAG2) protein [23, 43], and a recombinant chimeric protein, rSAG1/2 [24, 43]. Further animal studies in mice demonstrated that rSAG1, rSAG2, or rSAG1/2 emulsified with an oil adjuvant, Vet L-10, induced partial protection against a lethal subcutaneous challenge of *T. gondii* tachyzoites [43]. If alternative effective adjuvants, such as the PLG polymer are used to make these recombinant proteins more immunogenic, more protective immunity against *T. gondii* may be achieved in animals.

In our recent work, rSAG1, rSAG2, or rSAG1/2 was then, respectively, encapsulated with the PLG polymer by using the double emulsion method for production of PLG-encapsulated rSAG1 (PLG-rSAG1) [22], PLG-encapsulated rSAG2 (PLG-rSAG2) [23], or PLG-encapsulated

rSAG1/2 (PLG-rSAG1/2) microparticles [24]. Some microparticle characteristics, such as size (diameter), microparticle morphology, protein entrapment, and *in vitro* release were analyzed after PLG encapsulation (**Tables 1** and **2**). The morphological studies based on scanning electron microscopy showed that these microparticles are uniform population of spherical particles with a smooth surface. In addition, particle sizes of all three PLG microparticles in diameter were smaller than 10 μm (**Table 1**). Thus, the three PLG microparticles have an appropriate feature for direct uptake by APCs, such as macrophages and dendritic cells.

More importantly, the release of rSAG1, rSAG2, or rSAG1/2 from PLG microparticles was also analyzed (**Table 2**). We found that the *in vitro* cumulative release of rSAG1, rSAG2, and rSAG1/2 from PLG microparticles suspended in phosphate buffered saline (PBS) could be, respectively, sustained for 35, 33, and 56 days with three distinct phases consisting of an initial burst release, a very slow release, and a final rapid release (**Table 2**). Actually, based on previous critical investigations, such three-phase fluctuation in antigen release from PLG microparticles is due to the initial rapid diffusion of coated antigen on the PLG microparticle surface, the very slow and gradual diffusion of encapsulated antigen, and the final rapid diffusion of antigen because of the PLG microparticle degradation [47–49]. Furthermore, in the triphasic antigen release profile, both initial and final rapid release of entrapped antigen, respectively, look like priming and boosting doses usually employed in a conventional immunization procedure [49]. Thus, vaccination with a single dose of PLG-rSAG microparticles that are able to fulfill the triphasic rSAG release may be thought of as treating with two doses of rSAG protein. However, further improvements such as enhancement of protein load in PLG microparticles, as well as, optimization and stabilization of protein release are needed to evaluate the feasibility [40]. On the other hand, Western blotting assay with use of mouse monoclonal antibodies specific to tachyzoite SAGs demonstrated that released rSAG proteins from PLG microparticles still retained the original SAG antigenicity during the release from PLG microparticles [22–24]. These data indicate that both the encapsulation procedure and release from microparticles in our previous work are not detrimental to the antigenicity of

Microparticle	Mean particle size (μm)	Entrapment efficiency (%)	Reference
PLG-rSAG1	4.25–6.58	69–81	[22]
PLG-rSAG2	2.14–3.63	74–80	[23]
PLG-rSAG1/2	1.27–1.65	72–83	[24]

Table 1. Particle size and entrapment efficiency of PLG-rSAG microparticles.

Microparticle	Release protein	Release period	Release profile	Reference
PLG-rSAG1	87.8% rSAG1	35 days	Three phases	[22]
PLG-rSAG2	88.3% rSAG2	33 days	Three phases	[23]
PLG-rSAG1/2	88.5% rSAG1/2	56 days	Three phases	[24]

Table 2. Release of rSAG from PLG microparticles.

rSAG. Thus, rSAG proteins have been successfully encapsulated with PLG polymers by the double emulsion method and the resulting PLG-rSAG microparticles not only properly preserved the SAG's antigenicity, but also sustained the controlled, stable release of the antigenic rSAG proteins from PLG microparticles. Based on these data, therefore, released rSAG proteins from PLG microparticles have the potential to induce anti SAG immune responses.

7. Protective immunity by *Toxoplasma* SAG-loaded microparticles

Although different adjuvants capable of improving immune responses and protection against *T. gondii* have been studied [6, 50], the biodegradable and biocompatible PLG polymers are so far seldom used as potent adjuvants for *Toxoplasma* vaccine development. Stanley and his coauthors first employed the double emulsion method to produce PLG-encapsulated microparticle vaccine against *T. gondii* [51]. In the same study, the PLG microparticle vaccine containing a tachyzoite extract plus a mucosal adjuvant, cholera toxin, failed to provide protection in sheep [51]. The unexpected protection in sheep indicates that more effort is therefore needed to improve not only the stability of encapsulated *Toxoplasma* antigens but also the immune responses and protection they induce in animals.

On the other hand, the adjuvant effects of the PLG encapsulation had been exercised to, respectively, prepare PLG-rSAG1, PLG-rSAG2, and PLG-rSAG1/2 microparticles in our three previous studies [22–24]. The ability of these PLG-rSAG microparticles to trigger protective immunity against *T. gondii* was subsequently evaluated in BALB/c mice by vaccination through the intraperitoneal route. Results showed that both PLG-rSAG1 and PLG-rSAG1/2 microparticles effectively induced not only significant long-lasting (10 weeks) specific humoral and cell-mediated immune responses, accompanied by secretion of a large amount of IFN-γ, but also high protection (80% for PLG-rSAG1 microparticles and 83% for PLG-rSAG1/2 microparticles) against *T. gondii* tachyzoite infection [22, 24]. However, PLG-rSAG2 microparticles could induce sustained (10 weeks) lymphocyte proliferation and IFN-γ production [23]. Furthermore, after a lethal subcutaneous challenge of 1×10^4 *T. gondii* tachyzoites (RH strain), PLG-rSAG2 microparticles also improved anti *Toxoplasma* protection (87%) [23], which is higher, though not statistically significant, than either 80% of PLG-rSAG1 microparticles [22] or 83% of PLG-rSAG1/2 microparticles [24].

As *T. gondii* is an obligate intracellular parasite, protective immunity to *T. gondii* is largely mediated by Th1 cell-mediated immunity [6, 7]. Previous studies have shown that induction of both lymphocyte proliferation and IFN-γ production (one of Th1-type cytokines) positively correlates with protective Th1 cell-mediated immunity against *T. gondii* [43, 44, 51]. In addition, IFN-γ has been demonstrated to be a critical mediator that has to be secreted for as long as possible in order to maintain anti *Toxoplasma* immunity [52, 53]. We found that sustained lymphocyte proliferation and significant IFN-γ production readily detected in mice immunized with PLG-rSAG microparticles in our previous studies [22–24]. These findings indicate that immunization with PLG-rSAG microparticles really elicits the IFN-γ-associated Th1 cell-mediated immunity, which is the expected response that we aimed to induce in mice.

Based on previous studies, PLG microparticles appear to favorably facilitate a size-dependent interaction with APCs, such as macrophages and dendritic cells [32–34]. The particles, like PLG-rSAG microparticles prepared in our previous studies (**Table 1**), smaller than 10 μm in diameter are directly taken by APCs [32]. Based on our previous results, the proper size range thus could stimulate peritoneal macrophages to facilitate the uptake of these PLG-rSAG microparticles administered in the mouse peritoneal cavity [22–24]. Therefore, the microparticle-containing macrophages in the peritoneal cavity then traveled to other lymphoid compartments, including the spleen, and effectively presented SAG epitopes to Th1 and Tc; thereby, inducing strong SAG-specific Th1 cell-mediated immunity to protect mice from the tachyzoite challenge. Our previous studies [22–24] and those recorded by others [32–34] have shown that facilitation of uptake and delivery of PLG-rSAG microparticles by macrophages can lead to more effective antigen processing and presentation to T lymphocytes capable of inducting cell-mediated immunity. Thus, the high survival rates in mice have demonstrated that PLG-rSAG microparticles effectively elicit protective Th1 cell-mediated immunity to remove tachyzoite-infected cells for limiting parasite dissemination during the experimental tachyzoite challenge [7].

In addition to Th1-dependent cell-mediated immunity, in our previous studies, high titers of anti *Toxoplasma* IgG in mouse sera elicited by PLG-rSAG microparticles have indicated that systemic humoral immunity mediated by Th2 may participate in the prevention of *T. gondii* infection [54, 55]. However, further measurements by the dye test are still needed to assay these antibodies to elucidate their functional lytic activities. Therefore, peritoneal vaccination of mice with PLG-rSAG microparticles may generate mixed Th1/Th2 immunity against *T. gondii*.

8. Conclusions and future prospects

PLG polymers are the primary candidates for the development of microparticle vaccines [25]. The rSAG proteins (rSAG1, rSAG2, and rSAG1/2) prepared in our laboratory have been successfully encapsulated with PLG polymers to generate PLG-rSAG microparticles capable of sustaining long-term stable release of antigenic rSAG proteins. Moreover, following peritoneal immunization in mice, PLG-rSAG microparticles induce not only long-term (10 weeks) SAG-specific humoral and cell-mediated immune responses, but also high protection against a lethal challenge of *T. gondii* tachyzoites [22–24]. Our studies provide a valuable basis for developing long-lasting vaccines against *T. gondii* for future use in humans and animals. Our experimental data indicate that the encapsulation procedure we used for production of PLG-rSAG microparticles is feasible at the laboratory level. However, this procedure have never been used to try mass production of PLG-rSAG microparticles. More effort is therefore needed to evaluate the optimized encapsulation conditions used to fulfill the need for mass production [40].

The PLG-rSAG microparticles we prepared previously could allow the sustained and extended release of rSAG proteins over a long period. Such long-term release of rSAG proteins could

repeatedly stimulate the immune effector cells to maintain enhanced immunity following immunization with PLG-rSAG microparticles [29, 37]. However, the triphasic rSAG release detected in the cumulative release assay we carried out in the previous studies was done *in vitro* in PBS and; therefore, may not completely reflect *in vivo* release in mice [22–24]. Further studies are therefore needed to confirm the critical effect of triphasic rSAG release on *in vivo* anti *Toxoplasma* immune responses.

One adjuvant effect acted by PLG microparticle vaccines is to facilitate antigen uptake via APCs [25, 32, 33]. Different APCs populated in various administrating routes are able to perform the uptake of antigen-loaded PLG microparticles and then process and present the epitopes of PLG-encapsulated antigen on the major histocompatibility (MHC) molecules [32]. Therefore, different routes of delivery of antigen-loaded PLG microparticles give rise to different vaccine efficacy in animals [32]. The mouse protective immunity induced by intraperitoneally administered PLG-rSAG microparticles protected mice from a lethal subcutaneous challenge of *T. gondii* tachyzoites. However, such intraperitoneal administration of the microparticle vaccine appears to be inappropriate for use in large animals such as sheep or swine. In order to corroborate the conclusions drawn from the mouse model, more studies are needed to evaluate the proper route for administration of PLG-rSAG microparticles in target animals. In addition, due to the natural infection initiated by ingesting oocysts released in cat feces or consuming meat from infected animals containing the long-lived tissue cysts, future experiments will also be necessary to assess whether mucosal administration (oral or nasal route) of PLG-rSAG microparticles protects these animals from an oral challenge of oocysts or tissue cysts of *T. gondii*.

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Toxoplasma gondii was first identified more than 100 years ago in the tissues of birds and mammals. Although toxoplasmosis is important all over the world, its approaches to diagnostic strategies considerably differ among countries. Its wide distribution may be attributed to complex transmission patterns and parasite coevolution with multiple hosts. Although T. gondii infections of immunocompetent people are generally considered asymptomatic, infections in immunocompromised individuals, such as those with AIDS or organ transplant recipients, can result in severe consequences. This book, composed of a series of articles, including effective diagnosis of laboratory in toxoplasma infections, congenital toxoplasmosis, relationship between toxoplasmosis and public health genomics, prevalence, genetic diversity of toxoplasmosis, and microparticle vaccines against Toxoplasma gondii by authors from all over the world, presents a wide open point of view for toxoplasmosis.

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