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KNOCK-OUT OF THE BRADYZOITE MARKER P18 IN TOXOPLASMA GONDII: INSIGHTS TOWARDS A FUNCTIONAL CHARACTERIZATION DURING NEUROTOXOPLASMOSIS

by
NADIM M. TAWIL

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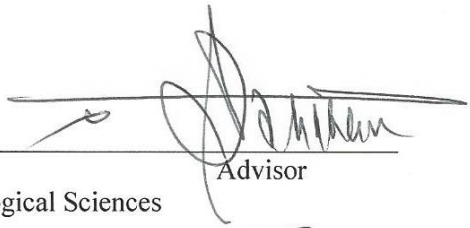
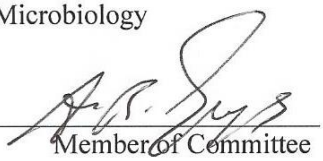


Dr. Marwan El-Sabban, Professor — Advisor
Department of Anatomy, Cell Biology and Physiological Sciences

Dr. Hiba El-Hajj, Assistant Professor — Co-Advisor
Department of Internal Medicine
Department of Experimental Pathology, Immunology and Microbiology



Dr. Abdo Jurjus, Professor — Member of Committee
Department of Anatomy, Cell Biology and Physiological Sciences

Dr. Ghassan Awar, Assistant Professor — Member of Committee
Department of Internal Medicine
Department of Experimental Pathology, Immunology and Microbiology

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Research in the field of *Toxoplasma gondii* is a new branch of research at the American University of Beirut. It was a long and strenuous path that demanded lots of efforts and patience, yet gladly we could achieve our goals and managed to complete this work on time. Nothing of this would have been possible without the tremendous efforts that Dr. Hiba El-Hajj put, the time she invested and the guidance she provided me with while working with me side by side always being more of a caring sister rather than just an observing supervisor or boss. Nothing of this would have been possible without the help, care and support of my family who were always there to say "it's alright" or "be patient, you'll get there". All that while embracing me with the warmest atmosphere possible to help focus and stay on route. Last but by far not least, all what has been achieved would have been out of reach without the hands-on help of my lab colleagues and the support, love and care of my dearest and closest friends who never got tired "over-nighting" by my side, always being there through harsh times, catching me whenever I slip, and providing me with the help and moral support one can only wish for at times of hardship. I am grateful to be surrounded by amazing exemplary people like Jessica Babikian, Martin Karam, Samer Monzer and Harout Arakilian, who walked this road with me and whose shoulder I could always find next to me, with every single step and at any time of day and night! I am really lucky!

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AN ABSTRACT OF THE THESIS OF

Nadim M. Tawil for Master of Science
Major: Human Morphology

Title: Knock-out of the bradyzoite marker P18 in Toxoplasma gondii: insights towards a functional characterization during neurotoxoplasmosis.

Toxoplasma gondii is an apicomplexan protozoan parasite that infects all warm blooded animals including humans. *T. gondii* causes a severely morbid or fatal disease in fetus and immunocompromised patients. During its life cycle, *T. gondii* exhibits three morphologically infectious stages: tachyzoites, bradyzoites, and sporozoites. Tachyzoites are rapidly multiplying and responsible for the acute toxoplasmosis leading to tissue damage. Bradyzoites are slow-growing and responsible for the chronic neurotoxoplasmosis that often reactivates in immunocompromized patients. Lastly, sporozoites are the infective forms found in oocysts. The back and forth switch between tachyzoite and bradyzoite stages is a key modulator of the progression of toxoplasmosis between acute and chronic phases. However, this switch remains very poorly understood. Here, we are investigating the role of the bradyzoite marker p18 for which the gene sequence is annotated on www.toxoDB.org. Specifically, we have used the vector (P2854) containing the selectable marker cassette hypoxanthine-xanthine-guanine-phosphoribosyl-transferase (HXGPRT) and cloned the 5' and 3' flanking regions of *p18*. This vector was introduced by

electroporation to the Pru *Δku80* strain which favors its integration by double crossing over and homologous recombination. We have successfully generated and cloned the Pru *Δku80*Δ*p18* knock-out parasites and investigated their phenotype *in vivo*. Our results have shown that deleting *p18* leads to the formation of more bradyzoite cysts in the brains of mice. However, these bradyzoites reactivate much later than the wild type strain. This result drove us to investigate the phenotype of the Pru *Δku80*Δ*p18* knock out during the acute phase of infection. We could clearly see a better survival rate of mice infected with the knock-out strain as compared to the wild type strain, furthermore, we have seen less parasites in all tested organs except in the brain where the amount of tachyzoites from both strains was similar. These results may suggest a role of p18 in the reactivation process and require further investigation on the immune profile of the knock-out parasites.

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CHAPTER ONE

INTRODUCTION

A. *Toxoplasma gondii*: Origins

In 1908, Nicolle and Manceaux were the first to describe *Toxoplasma gondii (T. gondii)* in an African hamster-like rodent called *Ctenoductylus gundi* used for *Leishmania* research at the Pasteur Institute in Tunis (Nicolle & Manceaux, 1908). In parallel, Splendore discovered the same parasite in a rabbit in Brazil. The name *Toxoplasma* is based on the morphological appearance of the parasite whereby in Greek "Toxo" is equivalent to "arc" or "bow" and "plasma" means "Life". The first viable specimen of *T. gondii* was isolated by Sabin and Olitsky in 1937 (Sabin & Olitsky, 1937), and the first case in humans was described in a neonate girl in 1938 who died at one month of age from an acute encephalomyelitis (Wolf, Cowen, & Paige, 1939a; Wolf, Cowen, & Paige, 1939b). Later, many cases of acquired and congenital toxoplasmosis were described, and the development of a serological test, the "Dye" test, by Sabin and Feldman in 1948 further showed that *T.gondii* is widespread among humans and mammals (Sabin & Feldman, 1948). Over the next fifty years, more attention was given to immune response against *T.gondii*, with the major discovery being the lymphoid cell mediated-immunogenicity against it. A crucial role of T-cells was confirmed when athymic nude mice infected with *T.gondii* failed to develop protective immunity against this parasite (Frenkel, 1988). In 1991, Gazzinelli showed that $CD8^+$ T lymphocytes producing interferon gamma (IFN-γ) are key players in the immune response to toxoplasmosis *in vivo* (Gazzinelli, Hakim, Hieny, Shearer, & Sher, 1991). Furthermore, the 80's and 90's witnessed attempts to determine genetic

differences between various *T. gondii* isolates from humans and animals (J. P. Dubey, 2008). The complete sequencing of the parasite's genome was accomplished in 2005 (Khan *et al.*, 2005), aiming to set a basis for better understanding of disease mechanisms and subsequently for designing better treatment or even prevention (J. P. Dubey, 2008).

B. *Toxoplasma gondii*: Taxonomy

The systematic classification of *T. gondii* goes back to 1980 (Levine, 1980) (Figure 1). Discoveries at the level of life cycle and advancements in electron microscopy have yielded to the identification of a complex structure and a number of unique organelles. Similarities were shown between the previously considered unrelated parasites such as *Plasmodium* species and *Emeria* species (Scholtyseck & Mehlhorn, 1970). All these resulted in a need for a major change in the classification of Protozoa, and eventually a new Phylum, the Apicomplexa, accounting for all this relatedness emerged encompassing the parasites of the genera *Babesia, Theileria, Plasmodium, Emeria, Toxoplasma, Neospora,* and *Cryptosporidium* (D. J. Ferguson, 2009).

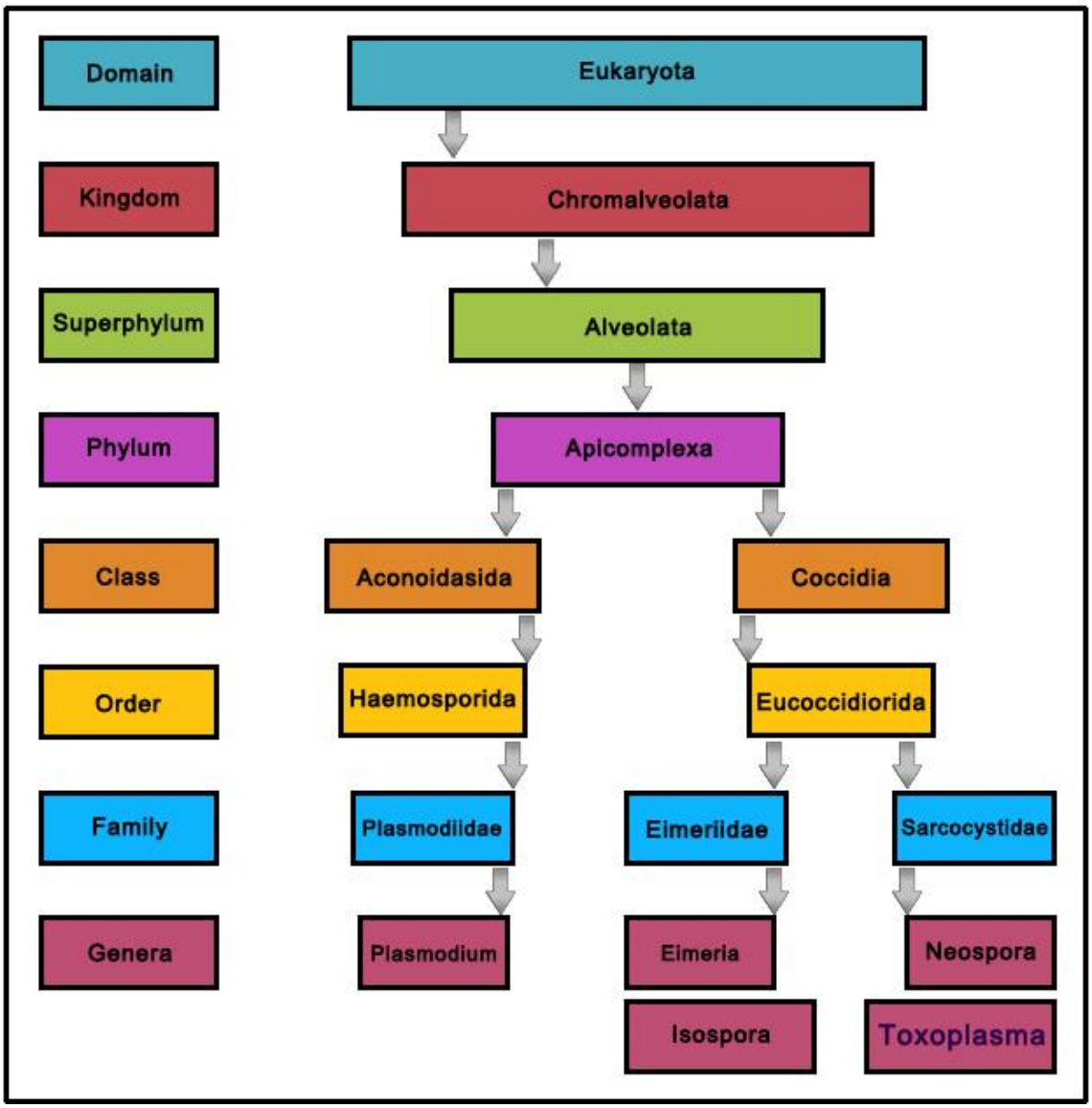

Figure1. Scientific classification of Apicomplexan parasites. Retrieved from http://www.ncbi.nlm.nih.gov/Taxonomy.

1. Toxoplasma gondii and the Apicomplexan phylum

The phylum Apicomplexa includes the malaria-causing parasite (*Plasmodium* spp.), several major animal pathogens (e.g. *Eimeria* spp., *Theileria* spp., *Babesia* spp., *Neospora caninum*) and the causative agents of toxoplasmosis (*Toxoplasma gondii*) and cryptosporidiosis (*Cryptosporidium* spp.). These parasites are responsible for some of the most deadly parasitic diseases afflicting humans, including malaria and

toxoplasmosis. They are also responsible for many diseases of veterinary and economic importance, and exhibit a complex life cycle and a coordinated cell cycle-dependent expression program. Among these parasites, *T. gondii* is an obligate intracellular protozoan parasite for which the need of a living host cell is detrimentally essential to support its life cycle's continuity.

Class	Order	Species	Parasitosis
Hematozoea	Haemosporida	*Plasmodium*	Malaria
	Piroplasmida	*Babesia*	Animal and human Babesiosis
		Theileria	Animal and human Theileriosis
Coccidea		*Eimeria Tenella*	Animal Coccidiosis
		Sarcocystis	Animal and Human Infection Cyst forms
		Cryptospridium	Animal and Human Infection Diarrhea
		Toxoplasma	Animal and Human Toxoplasmosis. Infection by cysts/Oocysts
Perkinsidea		*Perkinsus sp.*	Oyster Parasite

Table 1. Some members of the apicomplexan phylum, their classification, diseases and infectious stages pertaining to infection.

A general similarity among all the members of the phylum Apicomplexa is the complexity of their life cycles involving multiple host organisms and the fact that they are all essentially intracellular parasites. Having multiple hosts, these parasites undergo several stages of development restricted to one host or another. Being essentially intracellular, these parasites majorly benefit by gaining a considerably safe route to

evade the immune system of the host (Blader & Saeij, 2009). In case of *T. gondii*, this is primarily achieved by infecting macrophages and dendritic cells mainly, altering and down regulating the secretion of pro inflammatory cytokines such as IL-12 and activating anti-apoptotic machinery, hence securing a safe low profile vehicle to spread to various tissues (Blader & Saeij, 2009; Melo, Jensen, & Saeij, 2011).

C. Invasion of target host cell

Although the target-host cell type to be invaded is different according to the member of the phylum and its related disease, all these parasites share a common and conserved mode of invasion involving the apical complex; this later gives the phylum its name. As for the other members, successful invasion by *T. gondii* involves sequential discharge of the apical complex organelles termed micronemes and rhoptries (Figure 2) (Besteiro, Dubremetz, & Lebrun, 2011; Carruthers & Sibley, 1997), allowing the motility of the parasite, its attachment to the host cell by micronemal proteins, and finally penetration and establishment and maturation of the parasitophorous vacuole (Rhoptry Neck proteins, Rhoptry bulb Proteins and dense granules) (Figure 2) (Alexander, Mital, Ward, Bradley, & Boothroyd, 2005; Besteiro, Michelin, Poncet, Dubremetz, & Lebrun, 2009; Besteiro *et al.*, 2011; Bradley *et al.*, 2005; El Hajj *et al.*, 2006; El Hajj, Lebrun, Fourmaux, Vial, & Dubremetz, 2007; Lamarque *et al.*, 2011; Lebrun *et al.*, 2005; Mordue, Desai, Dustin, & Sibley, 1999).

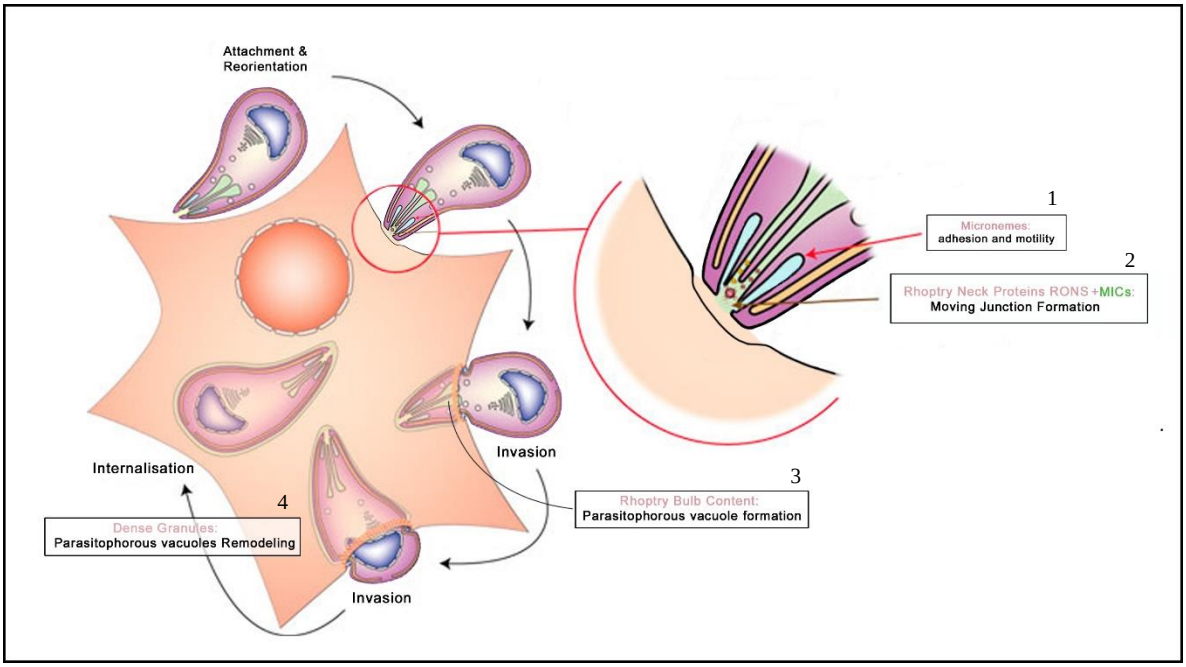

Figure 2. Schematic representation of *Toxoplasma* invasion. (1) The micronemes release their content in order to ensure host cell attachment. (2) Formation of the Moving Junction (MJ) by the release of RONs and MIC proteins. (3) Thereafter, ROPs are injected into the host cytoplasm at the invasion site and lead to the formation of the PV. (4) Exocytosis of the dense granules into the PV leading to its maturation. (Gilson & Crabb, 2009).

D. Stages of *Toxoplasma gondii*

Although *T. gondii* was discovered in 1908, the full life cycle was not uncovered until 1970 whereby only the asexual section of the life cycle and its stages were known, namely the tachyzoites and bradyzoites (J. Dubey, 2009). The uncovering of sexual part of the life cycle, occurring in a feline obligate host, was initiated by the work of Dr. J.K. Frenkel who investigated the screening on felids and several potential host species for shedding of oocysts (Miller, Frenkel, & Dubey, 1972). During its life cycle, *T. gondii* exhibits three morphologically distinct infectious stages: tachyzoites, bradyzoites, and sporozoites.

1. Tachyzoites

In Greek ‘tacos’ stands for ‘speed’. Tachyzoites, named so by Frenkel (Frenkel, 1973), and also known as trophozoites, are the 2 µm lunate/crescent forms initially discovered in the *gundi* rodent by Nicolle and Manceaux in 1909. Tachyzoites are the anteriorly pointed and posteriorly rounded proliferative intracellular forms, also called endozoites. As a result of a process known as endodyogeny, each tachyzoite, following the invasion of a host cell, divides into two daughter tachyzoites (Goldman, Carver, & Sulzer, 1958) (Figure 3a). The process continues until the host cell can no longer embrace the huge number of proliferating tachyzoites and simply bursts releasing them all to infect neighboring cells (Sheffield & Melton, 1968).

At the nano-structural level, the tachyzoite is an assembly of several organelles, inclusions, an outer covering (pellicle), sub pellicular microtubules, polar rings, apical rings, rough and smooth endoplasmic reticula, mitochondrion, conoid, rhoptries, micronemes, dense granules, micropore, Golgi complex, a plant inherited plastid-like organelle surrounded by 4 membranes called apicoplast, amylopectin granules (rare to absent), in addition to a centrally localized nucleus mainly featuring a central nucleolus surrounded by chromatin clumps (Figure 3B, C, D) (Ajioka, Fitzpatrick, & Reitter, 2001; J. P. Dubey, Lindsay, & Speer, 1998). Three membranes constitute the pellicle, an outer plasmalemma and an inner discontinuous membrane complex formed of two tightly adjacent membranes established by a “patchwork of flattened vesicles” (Figure 3D) (D. J. Ferguson, 2009). Anteriorly, a cylindrical truncated cone called “conoid” is formed by an average of seven microtubular structures twisted into a compressed coil shape (figure 3D) (D. J. Ferguson, 2009).

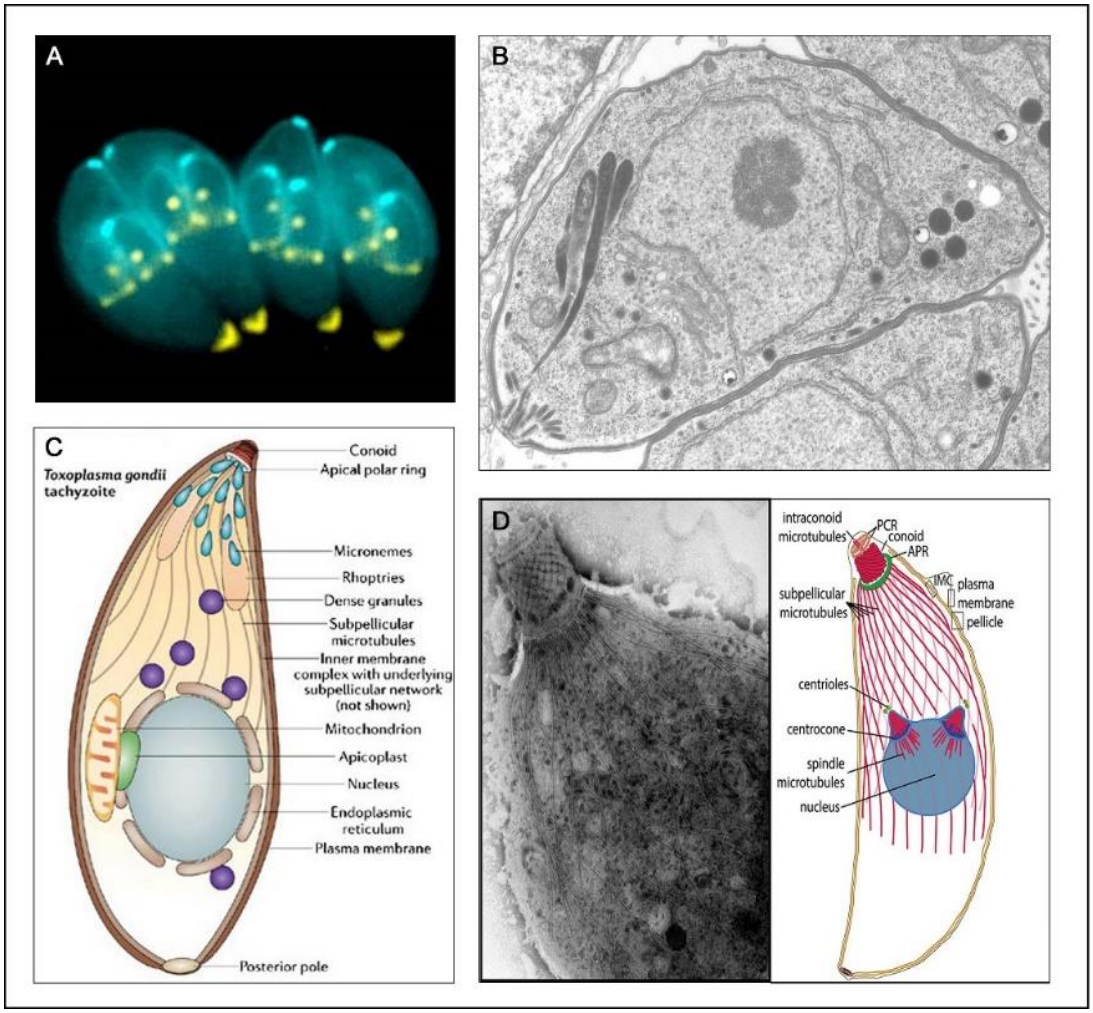

Figure 3. Toxoplasma *gondii* Tachyzoites. A. Endodyogeny process. The daughter Toxoplasma *gondii* parasites form inside the mother parasite (PLoS Pathogens Issue Image, 2006). B. Clicher of Jean-François Dubremetz: An intracellular *T. gondii* tachyzoite inside a parasitophorous vacuole membrane (PVM), showing the apical cytoskeleton (AC) and neighboring micronemes (M), rhoptry bulbs (ROP) and rhoptry necks (RON). Other components of the parasite, such as the nucleus (N), the Golgi apparatus (G) and the plastid that is specific to the Apicomplexa phylum, the 'Apicoplast' (A), are shown. The scale bar represents 0.5 μm. C. Schematic drawing showing the ultrastructure of Toxoplasma *gondii* tachyzoite (Expert Reviews in Molecular Medicine-2001 Cambridge University Press). D. Toxoplasma pellicle consists of the plasma membrane and an underlying patchwork of associated vesicular alveoli termed the IMC. The apical polar ring (APR) marks the site where the IMC begins, leaving the extreme apical region of the parasite only enclosed by plasma membrane. The conoid is a small motile organelle that can be extended from or retracted into the APR (Hammond, 1973; Nichols and Chiappino, 1987). A Detergent-extracted, negative stained Toxoplasma tachyzoite cytoskeleton showing the conoid, preconoidal rings, APR and twenty-two subpellicular microtubules (Morrissette, Murray, & Roos, 1997).

2. Bradyzoites

In 1973, Frenkel proposed the term “Bradyzoite”, with “brady” meaning “slow” in Greek, to describe the slow growing/dormant or non-proliferative developmental stage of *T. gondii*, encysting in tissues. Dubey and Beattie (1988) proposed that cysts should be called tissue cysts to avoid confusion with oocysts. The first characterization of these cysts appeared when they were found resistant to digestion by gastric enzymes in contrast to tachyzoites which were readily destroyed, hence highlighting the importance of encysted forms of *T. gondii* as key part of the life cycle’s continuity (Jacobs, Remington, & Melton, 1960a). In the same year, isolation of viable bradyzoites from *T. gondii* cysts from tissues of chronically infected animals was performed using pepsin digestion (Jacobs, Remington, & Melton, 1960b). When *T. gondii* oocysts were discovered in cat feces in 1970, oocyst shedding was added to the biological description of the cyst (J. Dubey & Frenkel, 1976). Therefore, Dubey and Frenkel (1976) performed the first in-depth study of the development of tissue cysts and bradyzoites and described their ontogeny and morphology (Figure 4). They found that tissue cysts formed in mice as early as three days after their inoculation with tachyzoites. Cats shed oocysts with a short prepatent period (3 to 10 days) after ingesting tissue cysts or bradyzoites, whereas after they ingested tachyzoites or oocysts the prepatent period was longer (~18 days), irrespective of the number of organisms in the inoculate (J. Dubey & Frenkel, 1976; J. Dubey, 1996; J. Dubey, 2001; J. Dubey, 2006).

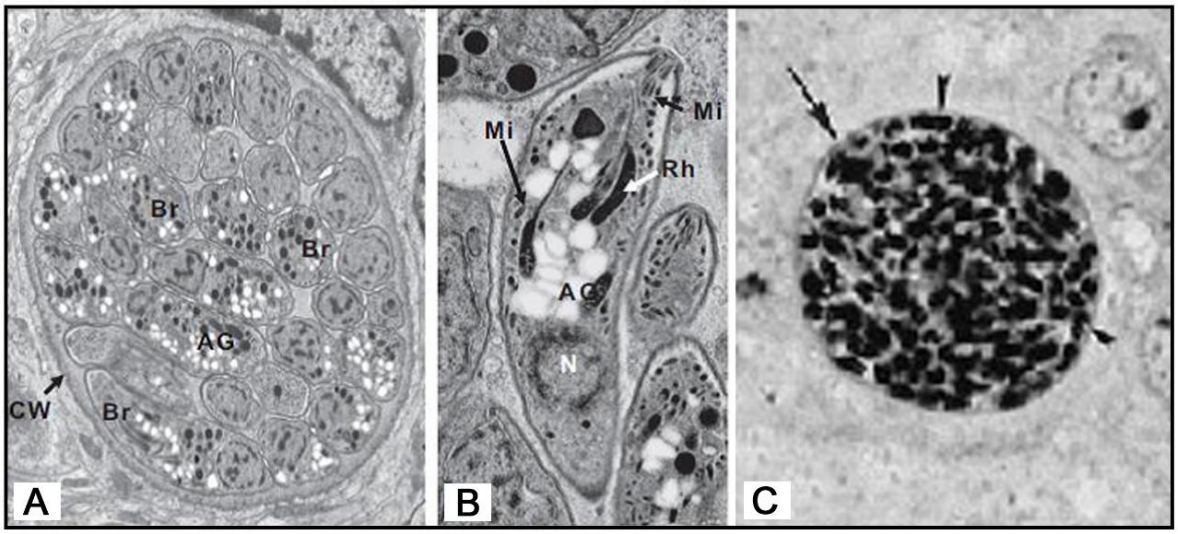

Figure 4. Toxoplasma *gondii* Bradyzoites. A. Transmission electron micrographs of bradyzoites (Br) within a tissue cyst. Note the presence of cyst wall (CW) and numerous amylopectin granules (AG) in the cytoplasm of bradyzoites. B. Higher magnification of ultrastructural morphology of bradyzoites. (C) Tissue cysts of *T. gondii* in mouse brains with numerous bradyzoites (arrow heads) enclosed in a cyst wall (arrow) (J. Dubey, 1998).

Investigations of ultrastructure further showed that bradyzoites differ from tachyzoites in several features (table 2; Figure 5) including the posterior position of the nucleus in bradyzoites in comparison to its central location in tachyzoites, and the abundance of amylopectin granules in these latent forms as compared to their rarity or absence in tachyzoites (J. P. Dubey, 2008). Furthermore, in comparison to the labyrinthine rhoptries in tachyzoites, these organelles are electron dense in mature bradyzoites and often appear convoluted and looping back on themselves (J. Dubey, 1998). Micronemes are rather numerous in bradyzoites and more slender than those of tachyzoites. Lipid bodies that are occasionally present in tachyzoites are virtually completely absent in Bradyzoites. The cyst wall which is in fact a modified parasitophorous vacuole membrane is thin with an average thickness of less than 0.5 µm (L. J. Knoll, Tomita, & Weiss, 2014).

Tachyzoites	Bradyzoites
Fast replicating forms	Slowly replicating forms
Intracellular and divide by endodyogeny	
More centrally located nucleus	Posteriorly located nucleus
Labyrinthine rhoptries	Solid rhoptries
Absent or few amylopectin granules	Numerous amylopectin granules
Occasionally presence of lipid bodies	Absence of lipid bodies
More susceptible to destruction by proteolytic enzymes	Less susceptible to destruction by proteolytic enzymes

Table 2. Comparison between tachyzoites and bradyzoites (J. Dubey, 1998).

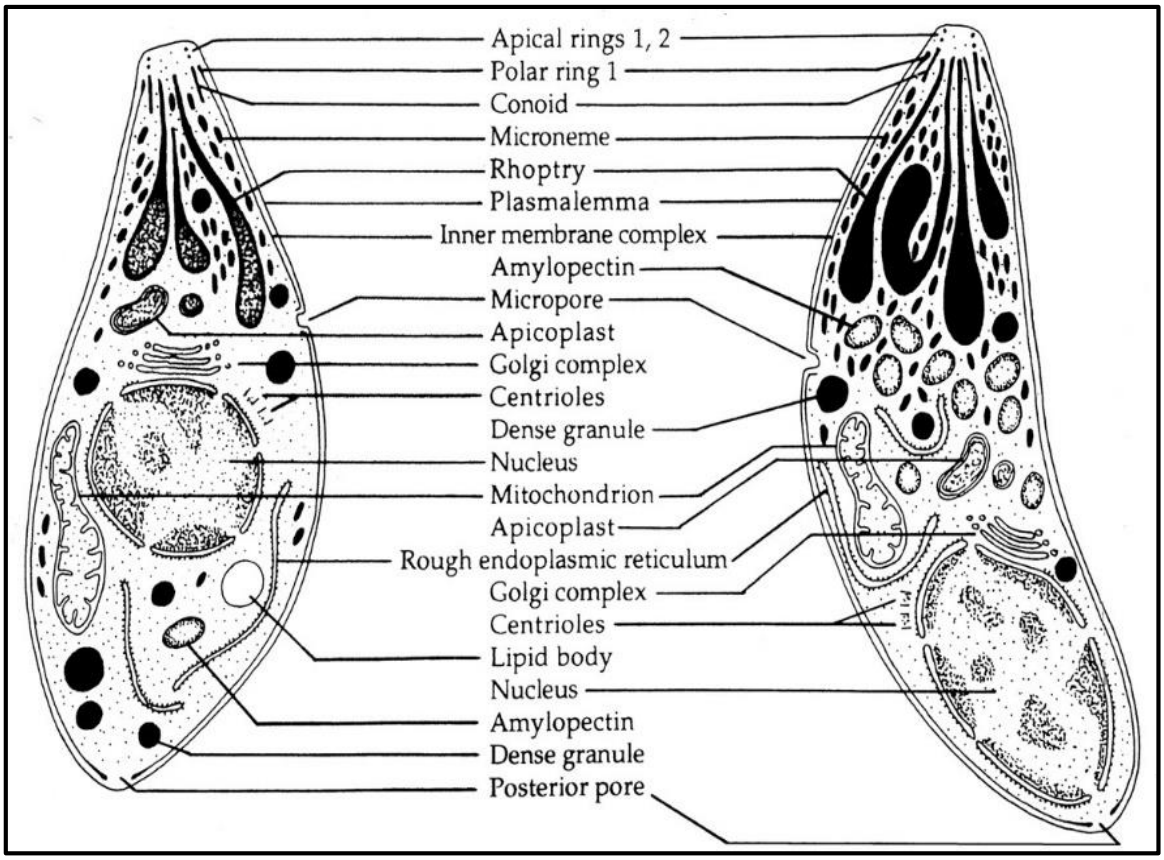

Figure 5. Tachyzoite versus Bradyzoite ultrastructure. (J.P. Dubey, D.S. Lindsay, and C.A. Speer, 1998)

Tissue cysts vary in size; young tissue cysts may be as small as 5 µm in diameter and contain only two bradyzoites while older ones may reach an average of 50 to 70 µm

in diameter, with a 1000 bradyzoites in mature cysts formed in the brain, and up to 100 µm with a more elongated form and definitely larger bradyzoite content when cysts in muscle tissues are addressed (L. J. Knoll *et al.*, 2014). Bradyzoites are responsible for the chronic phase of toxoplasmosis. They are formed after the immune system reacts against the virulent tachyzoite stage (J. P. Dubey, 2007). However, in immunocompromised patients, bradyzoites may become life threatening when they reactivate back into tachyzoites (Y.Sukthana *et al.*, 2008).

3. ***Sporozoites***

Felids are the only possible definitive hosts for *T. gondii*; sexual reproduction occurs in their intestine and culminates in the production of oocysts that are subsequently shed to the environment in feces (J. Dubey & Frenkel, 1972; Frenkel, 1970). An oocyst is essentially a cytoplasm containing a nucleoplasm, surrounded and protected by a double layered wall that makes it resistant to environmental stress; the inside content of a cyst is known as a "sporont".

At first, oocysts are unsporulated upon excretion (Figure 6). Sporulation occurs outside the cat within 1 to 5 days of excretion depending upon aeration and temperature conditions (J. Dubey, 1998). During the initial phases of the sporulation process, the nucleus divides twice and gives rise to 4 nuclei. Then the cytoplasm divides and 2 spherical sporoblasts are formed, each harboring 2 nuclei (Figure 6). As the sporulation continues, the sporoblasts elongate and sporocysts are formed. Later, each nucleus divides into 2 and following cytoplasmic cleavage, eventually four sporozoites are formed within each sporocyst. As a result, a sporulated oocyst containing two sporocysts, each with four infective sporozoites is formed, representing the infective

package ready to infect an intermediate host. Once ingested in contaminated food, these sporulated infective oocysts rupture by the action of the digestive enzymes of the gastro-intestinal tract and liberate the sporozoites which will rapidly transform into tachyzoites (Furtado, Winthrop, Butler, & Smith, 2013). It is worth noting that an infected cat may shed as many as one billion oocysts which are resistant to chemical and physical methods of inactivation used to treat waste-water and sewage (H. M. Fritz, Bowyer, Bogyo, Conrad, & Boothroyd, 2012). They can survive and remain infective for years in fresh water (Dumètre & Dardé, 2003) and for at least 24 months in salt water (Lindsay & Dubey, 2009).

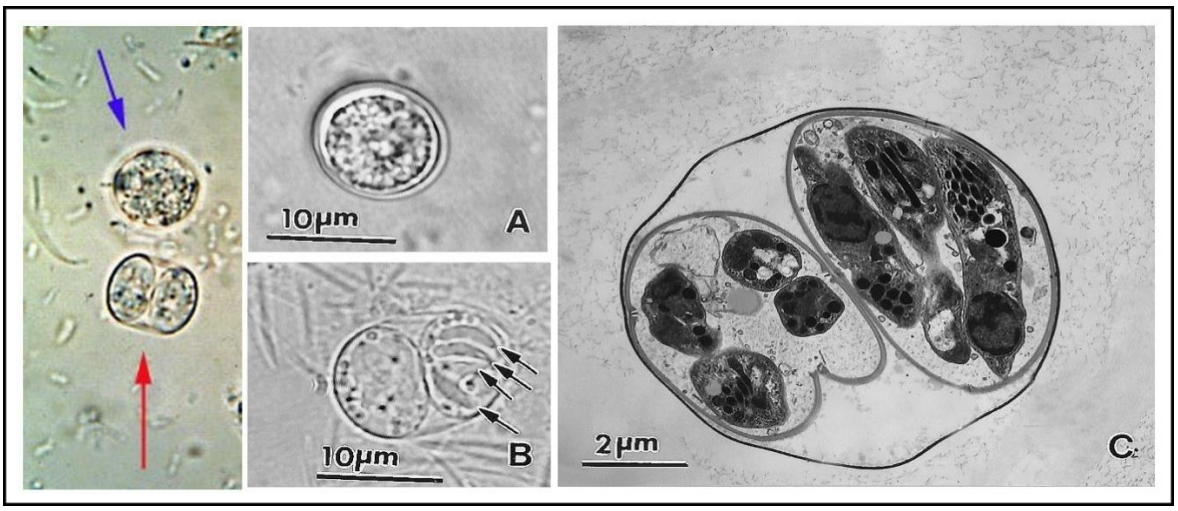

Figure 6. *Toxoplasma gondii* oocysts. An unsporulated oocyst (blue arrow) and a sporulated oocyst (red arrow) seen at high power (40 X) (University of Pennsylvania School of Veterinary Medicine, 2008). (A) Unsporulated oocyst. Note the central mass (sporont) occupying most of the oocyst. (B) Sporulated oocyst with two sporocysts. Four sporozoites (arrows) are visible in one of the sporocysts. (C) Transmission electron micrograph of a sporulated oocyst. Note the thin oocyst wall (large arrow), two sporocysts (arrowheads), and sporozoites, one of which is cut longitudinally (small arrows) (J. P. Dubey, Lindsay, & Speer, 1998).

Regarding its ultrastructure, the sporozoite is more similar to the tachyzoite than to the bradyzoite (Table 3), except the fact that it features an abundance of micronemes,

rhoptries, and amylopectin granules and absence of crystalloid bodies or any refractile bodies (J. Dubey, 1998; H. Fritz, Barr, Packham, Melli, & Conrad, 2012).

	Tachyzoites	bradyzoites	sporozoites
Number of rhoptries	similar numbers		
Appearance of rhoptries	uniformly labyrinthine	uniformly dense, some of which are folded back on themselves	both labrynthine and uniformly dense
Number of micronemes	Few	Many	intermediate
Dense granules	More numerous	Less numerous	more numerous
Amylopectin granules	few and small or absent	numerous and relatively large	numerous and relatively large
Lipid bodies	Rare	Absent	numerous

Table 3. Ultrastructural comparison of tachyzoites, bradyzoites and sporozoites (J. Dubey, 1998).

E. Life Cycle

T. gondii is an obligate intracellular parasite that infects all warm-blooded animals, including approximately 30% of the human population worldwide (Tenter, Heckeroth, & Weiss, 2000). Its complete life cycle requires a sexual life cycle restricted to the feline intestinal epithelium and an asexual life cycle possible within all warm-blooded animals (Figure 7) (Hutchison, Dunachie, Siim, & Work, 1970; Sheffield & Melton, 1970). Intermediate hosts such as humans can acquire *T. gondii* by ingestion of meat contaminated with cysts. Ingestion of sporulated oocysts in contaminated salads or

uncooked meat resulting in an infection with *T. gondii* (Fox *et al.*, 2011; Weiss & Kim, 2000).

1. Sexual life cycle

Oocysts are the result of the sexual cycle of *T. gondii* that begins when a domestic cat or any other member of the *Felidae* family ingests any of the infectious stages (tachyzoites, bradyzoites and sporozoites). This usually happens after feeding on an infected prey containing the latent stage of the parasite. The encysted bradyzoites within the prey are released upon digestion leading to the dissociation of the cyst wall by proteolytic enzymes in the stomach and the small intestine. This results in the initiation of the sexual development in the enterocytes of the cat's ileum.
Approximately 2 days post infection, gametogony begins and gamonts appear throughout the ileum inside enterocytes within a span of 3 to 15 days (J. Dubey, 2009). Following gametogenesis, microgametes and macrogametes develop as the male and female gametes respectively. The female macrogamete has numerous organelles while the male microgamont harbors up to 21 microgametes which possess a top end perforatorium organelle as well as flagella used to swim; these penetrate and fertilize mature female macrogametes (Speer & Dubey, 2005).

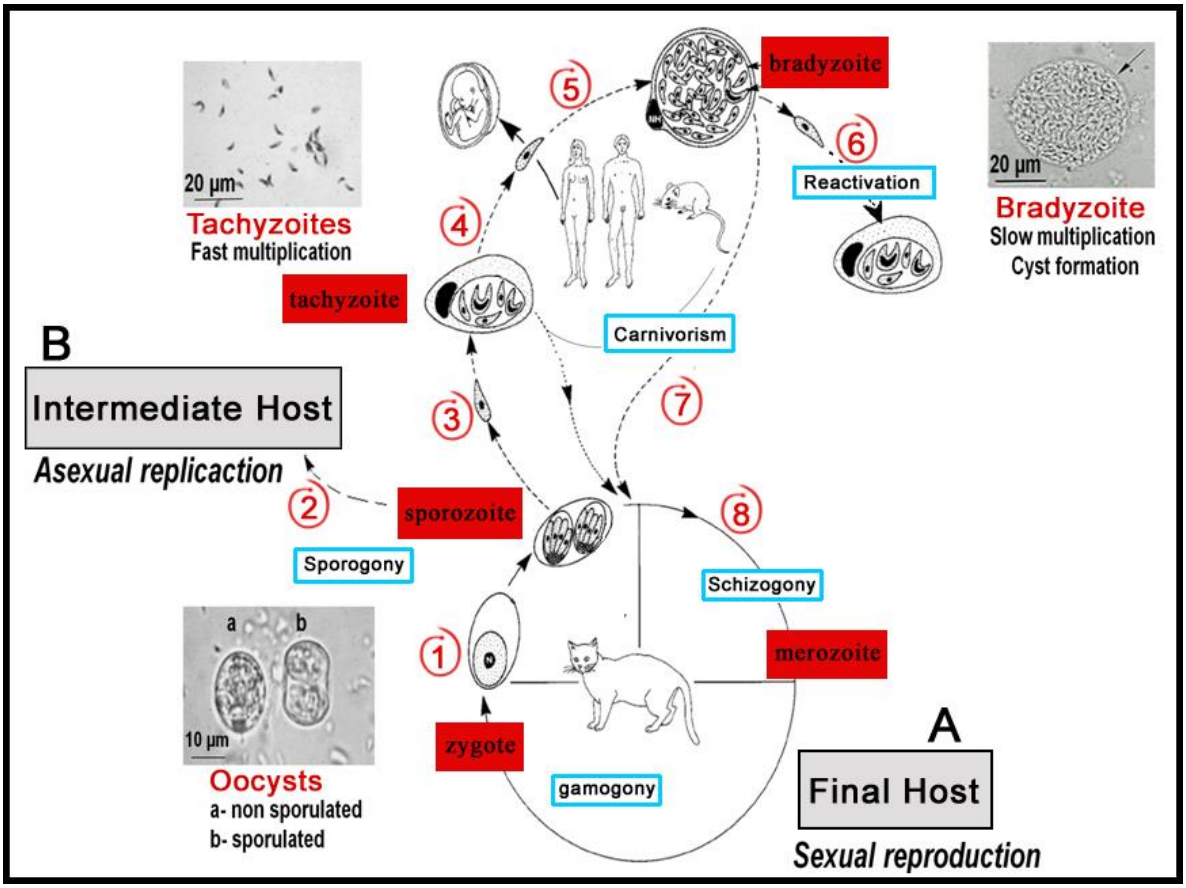

Figure 7**.** Life cycle of *T. gondii*. **A**. The sexual reproduction in feline (Final hosts). **B.** Asexual replication in intermediate hosts (including birds, rodents and humans). Unsporulated oocysts are shed in the cat's feces ①. Oocysts sporulate within 1-5 days in the environment and become infective. Intermediate hosts in nature become infected after ingesting any organic or inorganic material contaminated with sporulated oocysts ②. Oocysts rupture and liberate sporozoites which transform into tachyzoites shortly after ingestion ③. These tachyzoites are capable of infecting all types of nucleated cells and even cross the placental barrier and infect the fetus ④. Under the control of the immune system, these tachyzoites will transform into encysted bradyzoites in the brain, and the skeletal muscles ⑤. In immunocompromised patients, these bradyzoites can reactivate into tachyzoites ⑥. Cats become infected after predation of intermediate hosts harboring tissue cysts ⑦. Cats may also become infected directly by ingestion of sporulated oocysts ⑧.

After fertilization of a macrogamete by a microgamete, a zygote is formed in the intestinal lining cells of the infected feline definite host. Intestinal epithelial cells then rupture and oocysts are shed into the intestinal lumen as immature oocysts about 3-7 days following ingestion of the infected prey. Upon defecation, unsporulated oocysts

are released in nature. Sporulation occurs in the outside environment. This process starts with a single, relatively amorphous zygote and ends after 1 to 5 days post excretion and under appropriate environmental conditions with a sporulated oocyst containing 2 sporocysts harboring 4 sporozoites each and ready to start a new cycle (H. Dabritz & Conrad, 2010; H. A. Dabritz *et al.*, 2007; J. Dubey, 1998).

2. Asexual life cycle

Upon oral ingestion of contaminated food with sporulated oocysts by any warm-blooded animal, the asexual or intermediate life cycle begins (Figure 7). The digestive enzymes of the gastro-intestinal tract lead to the liberation of sporozoites which will rapidly transform into tachyzoites in the blood. These tachyzoites are responsible for the acute phase of infection and are capable of invading any nucleated cell as well as crossing the blood-placental barrier. Approximately 5 days after ingestion, and under the immune response, these tachyzoites transformed into bradyzoites that encyst in the brain and skeletal muscles. These formed tissue cysts may still dormant for years until the host is eaten by a predator and a new cycle starts (J. P. Dubey *et al.*, 1998; J. P. Dubey, 2007). These bradyzoites may become life threatening if they reactivate back into tachyzoites. This occurs in immunocompromised individuals (HIV patients or patients with organ or bone marrow transplantation) (Mele, Paterson, Prentice, Leoni, & Kibbler, 2002).

F. Modes of Transmission

The mechanism of transmission of *T. gondii* remained a mystery until its life cycle was unraveled in 1970. Despite the cat being its definitive host, it is found

virtually in all warm blooded animals (marsupials, birds and even marine mammals); however, these comprise facultative hosts which do not support the parasites sexual life cycle (Tenter *et al.*, 2000). This parasite is capable of infecting approximately 30% of the world's human population in addition to warm-blooded vertebrates and avian species (Skariah, McIntyre, & Mordue, 2010). At the American University of Beirut, Bouhamdan *et al.* realized a retrospective study targeting information related to IgG and IgM anti-*T. gondii* antibodies. They found that the overall *Toxoplasma* seropositivity was 62.2% for IgG and 6.8% for IgM (Bouhamdan, Bitar, Saghir, Bayan, & Araj, 2010). Based on their study, exposure to *Toxoplasma* infection is considered high among the Lebanese population. This high prevalence along with quite a range of bad clinical conditions that *T. gondii* can cause under certain circumstances, make it majorly important to know its modes of transmission.

Intermediate as well as definitive hosts can contract an infection by *T. gondii* mainly *via* one of two main modes of transmission:

(1) Horizontal mode of transmission either following oral ingestion of infectious sporulated oocysts in food contaminated by cats feces, or upon and ingestion of tissue cysts that can be potentially present in raw or partially raw meat.

(2) Vertical mode of transmission *via* the placental spread of tachyzoites from a pregnant primo-infected mother to her fetus/baby.

These routes of transmission allow a high level of sustainability of *T.gondii* in the environment. Indeed, transmission is not limited by any means; infection can move back and forth between definitive and intermediate hosts freely with all the needed routes available. Thus, carnivores can acquire *T. gondii* infection *via* carnivorism, herbivores *via* ingestion of infectious oocysts, and humans, being omnivores, are prone

to infection *via* both means. Furthermore, the asexual life cycle can sustain the parasites existence indefinitely *via* the transmission of tissue cysts in-between intermediate hosts. Same applies for the infectious oocysts able to support a continuity through the sexual life cycle in-between definitive hosts (Tenter *et al.*, 2000).

G. Diagnosis:

The greatest challenge in diagnosing toxoplasmosis is to identify the acute (primary) infection and distinguish it from chronic infection. *T. gondii* infection can be diagnosed using serologic tests, ultrasound scans, and amniocentesis (Chaudhry, Gad & Koren, 2014). Results of serologic tests measuring immunoglobulin IgM and IgG are often difficult to interpret when differentiating between acute and chronic infections. Following acute infection, IgM antibody titers rise starting on day 5 and reach the maximum level at 1 to 2 months. However, in many cases, the IgM antibodies persist for years following acute infection. In contrast, IgG antibodies are usually detectable within 1 to 2 weeks after acute infection, reach their peak within 12 weeks to 6 months, and usually remain detectable throughout life (Liesenfeld *et al.*, 1997). However, if test results are positive for both IgG and IgM, interpretation is difficult, as the positive results might be owing to either a recent infection or low levels of IgM antibodies from a previous infection (Iqbal & Khalid, 2007).

H. Pathogenesis of *Toxoplasma gondii*

More and more associations are being made between various medical conditions and *T. gondii* infections. Some claim that elevated levels of immunoglobulin G in pregnant ‘toxo-positive’ women are linked to prenatal anxiety and depression

(Groër *et al.*, 2011), others associate infection with *T. gondii* to behavioral disorders (Fekadu, Shibre, & Cleare, 2010), and yet others correlate various mental health disorders like schizophrenia, depression and even suicide attempts with seropositivity to *T. gondii* (Hsu, Groer, & Beckie, 2014). Three general areas of active medical and basic research on the pathogenesis of *T.gondii* regroup congenital toxoplasmosis, neurotoxoplasmosis and reactivation, and psychiatric disorders associated with *T.gondii.*

1. Congenital Toxoplasmosis

Congenital toxoplasmosis entitles the spectrum of complications resulting from the placental passage of *T. gondii* from a mother to her unborn child. Congenital *T. gondii* infection in a human child was initially described by Wolf *et al.* in 1939 (Wolf *et al.*, 1939a; Wolf *et al.*, 1939b) and later found to occur in many species of animals, particularly sheep, goats, and rodents. Severity of associated conditions depends on the time of acquisition of the parasitic infection by the mother in reference to her pregnancy progression. As early as 1974, Desmonts and Couvreur showed that out of 183 pregnancies where *T.gondii* infection was acquired during pregnancy, there were 11 abortions, 7 cases of still birth and postnatal death. Additionally among the 59 non-aborted infants in which toxoplasmosis was identified, there were 2 cases of death and 7 cases of severe disease involving cerebral and ocular conditions (Desmonts & Couvreur, 1974). In primo-infected mothers, congenital infection with *T. gondii* occurs and leads to a different spectrum of toxoplasmosis according to the semester of pregnancy (Desmonts & Couvreur, 1974). If infection occurs in the first trimester, the result is spontaneous abortion, stillbirth, or severe disease. Infants may be asymptomatic at birth only to develop disease months to years later. Most often these children develop

chorioretinitis with or without blindness or other neurologic problems, including retardation, seizures, microcephaly, and hearing loss (Murray, Rosenthal, & Pfaller, 2013). Many studies followed to confirm and support the work of Desmonts and Couvreur, among these Wong and Remington who confirmed that upon infection of serologically naïve pregnant women, transmission can occur to the fetus and result in fetopathy and relapsing chorioretinitis (L. J. Knoll *et al.*, 2014).

2. Toxoplasmosis in adults

Pinkerton and Weinman (1940) identified *T. gondii* in the heart, spleen and other tissues of a 22 year old patient who died in 1937 in Lima, Peru. The patient exhibited fever and concomitant *Bartonella* spp. infection. Pinkerton and Henderson (1941) isolated *T. gondii* from blood and tissues of two individuals (aged 50 and 43 years) who died in St. Louis, Missouri. Recorded symptoms included rash, fever and malaise. These were the first reports of acute toxoplasmosis in adults without neurological signs.

a. Lymphadenopathy

Siim (1956) drew attention to the fact that lymphadenopathy is a frequent sign of acquired toxoplasmosis in adults and these findings were confirmed by Beverley and Beattie (1958) who reported similar cases of 30 patients. A full appreciation of the clinical symptoms of acquired toxoplasmosis was achieved when outbreaks of acute toxoplasmosis were reported in adults in USA (Teutsch, Juranek, Sulzer, Dubey, & Sikes, 1979) and Canada (Bowie *et al.*, 1997).

b. Ocular disease

Before 1950, virtually all cases of ocular toxoplasmosis were considered to result from congenital transmission (Perkins, 1961). Wilder (1952) identified *T. gondii* in the retinas of eyes that had been enucleated. Then Jacobs *et al.* (1954) made the first isolation of *T. gondii* from an eye of a 30 year old male hospitalized at the Walter Reed Army Hospital. Additionally, a group of ophthalmologists from southern Brazil initially discovered ocular toxoplasmosis in siblings. Among patients with postnatally acquired toxoplasmosis who did not have retinochoidal scars before, 8.3% developed retinal lesions during a seven year follow-up (Silveira *et al.*, 2001; Silveira, Belfort, Burnier, & Nussenblatt, 1988). Ocular toxoplasmosis was diagnosed in 20 of 95 patients with acute toxoplasmosis associated with the Canadian waterborne outbreak of toxoplasmosis in 1995 (Burnett *et al.*, 1998; Holland, 2003).

c. Neurotoxoplasmosis

Immunodeficiency can be the result of malignancies, Human Immunodeficiency Virus (HIV) or oncology patients undergoing chemotherapy (J. P. Dubey, 2010). This makes latent or dormant toxoplasmosis a vicious opportunistic-like killer disease in such scenarios. Before the epidemic of the acquired immunodeficiency syndrome (AIDS) in adults in the 1980s, neurological toxoplasmosis in adults was rarely reported and limited essentially to patients treated for tumors or those given transplants. Luft and Remington (1983) reported acute toxoplasmosis induced encephalitis that was fatal if not treated. In almost all cases clinical disease occurred as a result of reactivation of chronic infection initiated by the depression of intracellular immunity due to HIV infection.

Furthermore, a positive correlation between seropositivity for *T. gondii* and incidence of brain cancer has led to the emergence of deeper knowledge of *Toxoplasma*-host interaction whereby, it has been reported that *Toxoplasma* increases the potential for brain cancer occurrence by interfering with the miRNAome in brain cells (Zeiner, Norman, Thomson, Hammond, & Boothroyd, 2010). However, molecular events are not well understood and the miRNAs specifically associated with brain cancers that are caused by *Toxoplasma* infection are yet to be identified. Finally, if this hypothesis is true, the outcome of this research would lead to the development of novel biomarkers and therapeutic tools against *Toxoplasma* driven brain cancers (Thirugnanam, Rout, & Gnanasekar, 2013).

3. Psychiatric Disorders Associated with T.gondii

Recent studies have been implicating *T. gondii* chronic infections in several psychiatric disorders. Although tentative, yet several results draw suspicions concerning a correlation between the parasite and schizophrenia, whereby *Toxoplasma* infection correlates with elevated levels of dopamine, a condition widely observed in schizophrenics (Webster & McConkey, 2010). Additionally a survey approach revealed that individuals with schizophrenia had a more profound exposure to cats in comparison to control non-schizophrenic groups (Yolken, Dickerson, & Fuller Torrey, 2009). Altered dopamine levels have been associated with several neurological conditions and imbalances; these are related to mood control, sleep patterns, Parkinson's disease, and even attention deficit disorder, in addition to several others (Gaskell, Smith, Pinney, Westhead, & McConkey, 2009). Ingram *et al.* showed that *Toxoplasma* is implicated in

potentially permanent loss of fear in mice reporting that mice which contracted *T. gondii* lost the instinct to be frightened by the smell of a cat. This persisted even after the parasites' presence in the mouse brain sunk below the detectable levels (Ingram, Goodrich, Robey, & Eisen, 2013). All this only highlights the potential depth of the immense extent of interference and host machinery overriding that *T. gondii* can attain once it successfully invades.

I. Strains Genetic overview and virulence of *Toxoplasma gondii* Strains

T.gondii genome consists of 3 DNA components, located in the nucleus, mitochondrion and apicoplast. Genetic analyses have revealed 87 Mbp nuclear genome haploid for most of the parasite's life cycle except for a brief diploid phase in the cat intestine before meiosis and consisting of 11 chromosomes designed by Roman numerals and ranging in size from approximately 1.8 Mbp to > 10 Mbp. This parasite also contains a 6 Kbp mitochondrial genome and an extra-chromosomal 35 Kbp circular DNA within the apicoplast: an organelle with plastid-like properties (Mcfadden, Waller, Reith, & Lang-Unnasch, 1997). The organellar genome has a limited coding capacity suggesting that proteins responsible for organelle function(s) must be encoded by the nucleus (Ajioka *et al.*, 2001). Later, an Affymetrix microarray with probe sets representing all predicted genes (based on ToxoDB release 4) was developed and made commercially available (Bahl *et al.*, 2010; Roos, 2005). Furthermore, *T. gondii* genome was fully sequenced by the J. Craig Venter Institute and is available on www.ToxoDB.org.

1. Virulence

T. gondii is an old parasite with ever growing interest. At a first glance, *T. gondii* appears to be a parasite of the immunocompromised where it shows signs of severe infections most, unlike in healthy and non-pregnant humans where the infection goes unnoticed. Not until strains of *T. gondii* capable of infecting and killing healthy human beings were discovered that this idea of opportunistic parasite has been questioned (Dubremetz & Lebrun, 2012).

Many parameters of virulence are associated with the success of the parasite to invade the host cells. The host cells have immunity related signaling factors and transcription of these signaling factors can be modulated by the parasite (Hunter & Sibley, 2012; Rosowski, 2013). Moreover, the parasite can act on the host immune response by acting on the intracellular effectors of the primary immune response and this is another aspect of the parasite virulence (Melo *et al.*, 2011).

Virulence of *T. gondii* has always been associated with the amount of tachyzoites needed to infect and kill a mouse (Saeij, Boyle, & Boothroyd, 2005). However, when it comes to humans the case is different since it is not possible to know the initial count of parasites infecting a human host and thus the virulence measure is partly based on the organ location and parasitemia (Dubremetz & Lebrun, 2012). In addition, virulence of *T. gondii* is not only related to the parasite itself but also to factors pertaining to the host it is infecting (Dubremetz & Lebrun, 2012), and the nature of the immune responses it triggers (Dubremetz & Lebrun, 2012; Melo *et al.*, 2011). In addition to that, virulence is nowadays looked at from a different angle of which SAG1 protein is a player but not indispensable (Dubremetz & Lebrun, 2012). More

importantly, virulence is still shifting towards the crucial genes responsible for drawing the final image of the severity of the infection (Dubremetz & Lebrun, 2012).

T. gondii population is a highly clonal one. It is true that a sexual life cycle exists but most of the isolated strains fall in one of the three lineages known as type I, II and III (Weilhammer & Rasley, 2011) (Table 4) which differ by less than 1 % at the DNA level. While these 3 types dominate the *Toxoplasma* population categorization, type II strains are the most commonly isolated from patients (Ajzenberg *et al.*, 2002). Although strains of *T. gondii* are genetically quite similar, they show strong phenotypic differences in the laboratory mouse whereby virulence in the mouse model is associated with the parasite genotype. Infection by type I strains is fatal ($LD_{50} = 1$) in all strains of laboratory mice, whereas infection with type II ($LD_{50} \sim 10^2$) or type III ($LD_{50} \sim 10^3$) strains generally result in controlled infections that persist for life (Sibley & Boothroyd, 1992).

Strains	Type I	Type II	Type III
Genetic	98 % similar		
Virulence	High	Intermediate	low
Examples	RH	ME49, Pru (Prugniaud), Pru KU80	NED
Characteristics	● unable to make cysts of bradyzoites because it lyses cells before making cysts ● grows faster than types II & III ● completely lyses a flask of cultured cells much faster than types II & III ● Extracellular parasites remain infectious for a longer time compared with the types II & III	● able to make bradyzoites cysts ● grow slower than type I ● completely lyses a flask of cultured cells much slower than types I ● Extracellular parasites remain infectious for a shorter time compared with the types I	● able to make bradyzoites cysts ● grow slower than type I ● completely lyses a flask of cultured cells much slower than types I ● Extracellular parasites remain infectious for a shorter time compared with the types I

Table 4. Comparison between the 3 different strains of *T. gondii* (Saeij *et al.*, 2005).

Unlike type II and III, type I lineage parasites are neither common in humans nor in wild animals yet several documented severe cases of human immunocompetent patients infected with *T. gondii* were found to be associated with type I isolates (Weilhammer & Rasley, 2011).

2. New genetic model of type II: PRU KU80

Double Strand Break (DSB) repair in most eukaryotes occurs primarily *via* two different recombination pathways (Haber, 2000). The homologous recombination

pathways repair a DSB using mechanisms that recognize highly homologous DNA sequences, while the non-homologous end-joining (NHEJ) pathway does not rely on DNA sequence homology. Instead, NHEJ involves direct ligation of the ends of broken DNA strands. KU70 and KU80 proteins form a heterodimer that tightly binds the DNA ends at the DSB, an early and essential step of NHEJ (Walker, Corpina, & Goldberg, 2001; Wu *et al.*, 2008). Many eukaryotes preferentially use the NHEJ pathway to repair a DSB, and exogenous targeting DNA can be integrated anywhere into the genome independent of DNA sequence homology (Haber, 2000). The NHEJ pathway also appears to be preferentially used by *T. gondii* based on the high rates of non-homologous recombination and low gene targeting frequencies observed experimentally (Donald & Roos, 1998; Fox & Bzik, 2002; Gubbels *et al.*, 2008; Morrissette *et al.*, 1997). Fox *et al.* relied on this NHEJ pathway and developed *KU80* knockouts (Δ*ku80*) in type II *T. gondii* (Fox, Ristuccia, Gigley, & Bzik, 2009). These Δ*ku80* are void of the dominant pathway mediating random integration of targeting episomes and hence allow for increased efficiency of double-crossover homologous recombination at targeted loci (Fox *et al.*, 2009). Fox *et al.* targeted the deletion of four parasite antigen genes (*GRA4, GRA6, ROP7,* and *tgd057*) that encode characterized $CD8^+$ T cell epitopes that elicit corresponding antigen-specific $CD8^+$ T cell populations associated with control of infection (Fox *et al.*, 2011), thus highlighting the importance and value of such knock-outs.

J. Significance of *T.gondii* as an Experimental Model

T. gondii is the major and tractable experimental model of choice for the study of apicomplexan biology and parasitism because of many advantages: its different

pathogenic stages are easily cultured, propagated and quantitated *in vitro* and the manipulation of the *Toxoplasma* genome is well established as well as the mouse model for studying host/parasite interactions during infection (Hager, Striepen, Tilney, & Roos, 1999; K. Kim & Weiss, 2004). In addition, *T. gondii* can be easily crossed and transfected both transiently and stably *in vitro* and in diverse animal models.

K. Bradyzoite markers: Overview of the literature

Studies have shown that the various *T. gondii* developmental stages can be quite different in their molecular marker expression profiles; such molecular markers can be those pertaining to metabolic iso-enzymes, secreted proteins, or surface and cytoplasmic molecules (D. Ferguson, 2004). Along with stage conversion that *T.gondii* undergoes, changes in morphology and biomolecular changes mainly represented by stage-specific antigen expression occur (Lyons, McLeod, & Roberts, 2002). Studies in this area have yielded the identification of several stage-specific surface antigens, enzymes and even heat shock proteins specific to either one of the developmental stages.

1. SAG1-related sequences superfamily of proteins (SRS)

The surface of *T. gondii* is coated with developmentally expressed, glycosylphosphatidylinositol (GPI)-linked proteins. Collectively, these surface antigens are known as the SRS (SAG1-related sequences) superfamily of proteins. A list of SRS known proteins along with their functions is summarized in table 5. In brief, SRS proteins are thought to mediate attachment to host cells and activate host immunity to regulate the parasite's virulence. The SRS superfamily of sequences phylogenetically

bifurcates into two subfamilies, the prototypic members being SAG1 (SRS29B or P30) and SAG2A (SRS34A), respectively (Nagel & Boothroyd, 1989; Tomavo, Schwarz, & Dubremetz, 1989). These families are structurally related and share conserved cysteine residues forming disulfide bonds (He, Grigg, Boothroyd, & Garcia, 2002), yet remain antigenically distinct (Jung, Lee, & Grigg, 2004). SRS proteins typically share between 24–99% amino acid sequence homology. These members modulate the subversion of stages and the status of the disease progression (Boothroyd, Hehl, Knoll, & Manger, 1998; He *et al.*, 2002). SAG3 (SRS57) is capable of binding sulfated proteoglycans (SPGs) with high affinity (Jacquet *et al.*, 2001) suggesting that the SRS superfamily exists to function as a set of quasi-redundant receptors that facilitate parasite entry into the broad spectrum of cell types and hosts that *Toxoplasma* infects. Importantly, *Toxoplasma* tachyzoïtes and bradyzoïtes apparently express distinct, largely non-overlapping sets of SRS protein. Expression of SAG1 and SAG2A happens only on tachyzoïtes, whereas SAG2C/D, BSR4, and SRS9 are found only on bradyzoïtes (Cleary, Singh, Blader, Brewer, & Boothroyd, 2002; L. Knoll & Boothroyd, 1998; L. J. Knoll *et al.*, 2014; Lekutis, Ferguson, & Boothroyd, 2000).

A pellicular surface antigen, P36 was extensively studied, and belongs to the SRS family and was used as a bradyzoïte marker. Indeed, Knoll and Boothroyd found that the BSR4 protein, a member of the SAG1 family, encodes the P36-reactive protein (L. Knoll & Boothroyd, 1998). Surprisingly, the BSR4 transcript was found to be equally abundant in tachyzoïtes and bradyzoïtes, suggesting post-transcriptional regulation of this gene (L. Knoll & Boothroyd, 1998). Investigation of other proteins detected by this antibody revealed SRS9 as the primary target of the P36 mAb. SRS9 encodes a bradyzoïte-specific protein with high similarity to members of the SRS family

of surface antigens and is located immediately downstream of BSR4 (Van, Kim, Camps, Boothroyd, & Knoll, 2007). Kim *et al.* created a bioluminescent strain lacking the *SRS9* gene and revealed that during an acute infection, wild-type and Δ*srs9* strains replicated at similar rates and could disseminate systemically following similar kinetics, and initially yielded similar brain cyst numbers. However, during a chronic infection, Δ*srs9* cyst loads substantially decreased compared to those of the wild type, suggesting that SRS9 plays a role in maintaining parasite persistence in the brain. When chronically infected mice were treated with the immunosuppressant dexamethasone, however, the Δ*srs9* strain reactivated in the intestinal tissue after only 8 to 9 days, versus 2 weeks for the wild-type strain. Thus, SRS9 appears to play an important role in both persistence in the brain and reactivation in the intestine (S. K. Kim, Karasov, & Boothroyd, 2007; L. J. Knoll *et al.*, 2014).

Finally, a bradyzoite specific cyst wall, CST1, a 250 kDa protein localized to the granular material in the cyst wall was identified as a key contributor to cyst wall robustness and hence cyst persistence (Tomita *et al.*, 2013; Weiss, LaPlace, Tanowitz, & Wittner, 1992; Zhang, Halonen, Ma, Wittner, & Weiss, 2001). Parasites deficient of CST1 were shown to result in reduced cyst number and fragility of brain cyst wall along with thinning and easy disruption of the cyst wall underlying granular region (Tomita *et al.*, 2013).

2. Other bradyzoite markers

Stage specific markers also comprise a number of metabolically affiliated molecules and heat shock proteins. A core difference in morphology between tachyzoites and bradyzoites dictates different modes of metabolic activity and hence

different expression of metabolically active molecules (L. J. Knoll *et al.*, 2014). Along this line of reasoning, Lactate Dehydrogenase 2 (LDH2) was identified as a 35 kDa cytoplasmic antigen transcriptionally regulated and expressed solely in bradyzoites (Yang & Parmley, 1995; Yang & Parmley, 1997). The evidence for the crucial role of LDH2 as a key metabolically affiliated molecule comes from the fact that down-regulation of LDH2 impaired bradyzoite growth and differentiation (Al-Anouti, Tomavo, Parmley, & Ananvoranich, 2004).

Enolase 1 (ENO-1), a bradyzoite-specific isoform of enolase (Kibe *et al.*, 2005), known as hsp48 in yeast, is a stress related heat shock protein (Iida & Yahara, 1985). It is mainly expressed in bradyzoites after exposing tachyzoites to stress (Kibe *et al.*, 2005). ENO-1, responsible for catalysis of 2-phosphoglycerate to phosphoenolpyruvate conversion, was identified as bradyzoite specific marker (D. J. Ferguson, Parmley, & Tomavo, 2002).

Finally, bradyzoite specific heat shock proteins were characterized, among which is BAG-1. BAG1 has homology to small heat shock proteins and therefore has also been called hsp30 (Bohne, Gross, Ferguson, & Heesemann, 1995; Parmley, Weiss, & Yang, 1995). Both BAG1 mRNA and protein (a 28 kDa cytoplasmic antigen) are up-regulated during bradyzoite formation, suggesting transcriptional regulation of its expression. In fact, BAG-1 is one of the most abundant bradyzoite specific genes found in the *T. gondii* bradyzoite. BAG-1 expression is detectable within 24 hours post stress induction on *T. gondii* parasites and hence its characterization as a stress respondent would naturally accompany the transition from tachyzoites to bradyzoites under stress conditions (L. J. Knoll *et al.*, 2014).

L. *SAG-4* or *p18*: discovery and cloning

For quite a long while, research in the field of toxoplasmosis remained centered around the readily available tachyzoite developmental stage. However with the emerging interest in stage conversion, many studies were directed towards the characterization of stage specific developmentally regulated molecules. Antibodies against stage-specific markers for bradyzoïtes and tachyzoïtes were developed (Tomavo *et al.*, 1991). These antibodies were selected by differential immunofluorescence assays aiming to isolate them from hybridomas produced against these organisms. Antigenically reactive markers to these antibodies were found on human isolates of *T. gondii*, as well as on laboratory strain bradyzoïtes obtained from either brain cysts or *in vitro*-grown parasites (Tomavo *et al.*, 1991). These monoclonal antibodies are T_8 $4A_{12}$ recognizing the previously mentioned 36 kDa protein called P36, T_8 $2C_2$ recognizing a 34 kDa protein called P34, T_8 $4G_{10}$ recognizing a 21 kDa protein called P21 and T_8 $3B_1$ recognizing an 18 kDa protein called P18 (Tomavo *et al.*, 1991) (table 5). These proteins represent four pellicular antigens, three of which are exposed on the surface of the organism (Tomavo *et al.*, 1991). Among the three bradyzoite markers (P34, P21 and P18), only the gene encoding for P18 was sequenced and published (Ödberg-Ferragut *et al.*, 1996). Real time using P18-specific primers demonstrated the stage specific expression of this gene only in the bradyzoites transcripts. The sequenced gene showed no substantial homology to any of the known genes, hence it was recognized as novel and in accordance to the nomenclature proposed by Sibley, the *P18* gene was given the name *SAG4* (Ödberg-Ferragut *et al.*, 1996). Insights towards a functional characterization of P18 are still absent. This work focuses on establishing the Knockout of *p18* in the Pru *Δku80* type II strain (PRU *Δku80ΔP18*) and trying to understand its

function in a murine model of toxoplasmosis. Our work highlights some aspects of the role of *P18* along the path towards brain cyst formation and the stage conversion of neurotoxoplasmosis following reactivation.

Name of Antigen	Monoclonal Antibody	Size of Immunoblot (kDa)	Location by IFA	Comments	Cloned Toxodb Number
BAG1 (hsp30, BAG5)	7E5 74.1.8	28	Cytoplasm	Small heat-shock protein	TGME49_259020
BSR4 (p36, SRS 16C)	T84A12	36	Surface	SRS family antigen. Also in sporozoites	TGME49_320180
SAG4A (p18, SRS35A)	T83B1	18	Surface	SRS family	TGME49_280570
p21	T84G11	21	Surface	Surface Antigen	No
p34	T82C2	34	Surface	Surface Antigen	No
SRS-9 (SRS 16B)	Murine Polyclonal only	43	Surface	SRS family	TGME49_320190
MAG-1	None	65	Matrix	Studies Indicate presence also in tachyzoites	No
CST-1	73.18	250	Cyst Wall	Structural protein	TGME49_064660
LDH-2	Polyclonal sera weakly cross reacts with LDH1	35	Cytosolic	Tachyzoite isoform LDH1	TGME49_291040
ENO-1	Polyclonal sera to ENO1 and ENO2 do not cross react	48	Nuclear and Cytosolic	Tachyzoite isoform ENO2	TGME49_268860

Table 5. Common bradyzoite markers (Knoll, Tomita & Weiss, 2014).

CHAPTER TWO

MATERIALS AND METHODS

A. Genomic DNA isolation

Genomic DNA purification from a pellet of $3x10^8$ parasites of PRU *Δku80* strain was performed using wizard Genomic DNA purification system Promega (Ref A 2361 0000035819).

B. Primers

The designed oligonucleotide primers were named as follows: ML 1514 & ML 1515 recognizing the 5'flanking region of *p18*, ML1516 & ML1517 recognizing the 3'flanking region of *p18* (table 6).

Primers' names	Primers sequences (5'→3')	Restriction Enzymes
ML1514	gcgcGGGCCCCGATCCGCAGACATCTGGGGGTCTC TTGGCGTTCGTCCCCGCCAACAAAGCG	Apa1
ML1515	cccAAGCTTGGTTGAAGACAGACGAAAGCAGTTGC AGTATGCTGCGACGCGTCTTCCGAG	HindIII
ML1516	ggACTAGTGAGTTCATTGCCAGTGAAGAAGGTGAC TGGTAGTGTCACATTTGGCAACTGG	Spe1
ML1517	ataagaatGCGGCCGCTTGTTACCTGGCACACGTCACT TGCAACATTGTAAACTTGTTTGTTGTCTGG	Not1

Table 6: Summary of PCR primers used in this study. Restriction site specific for each enzyme is highlighted in yellow and the rest of the primer complementary to the 3' or 5' regions is highlighted in green.

C. Polymerase chain reaction (PCR)

PCR amplifications were performed on genomic DNA using PrimeSTAR HS DNA/ Phusion® High-Fidelity DNA Polymerases that provide high fidelity, accuracy and robust performance during amplification of large genomic DNA products. PCR was performed as follows: a denaturation step at 98°C for 30 seconds (1 cycle) followed by 30 cycles of : 98°C for 5 seconds, a gradient of annealing temperature from 52°C→60°C (Phusion Polymerase) and 54°C→62°C (PrimeStar Polymerase) for 30 seconds and an elongation at 72°C for 2.5 minutes. The last step is a final elongation at 72°C for 10 minutes. PCR products were analyzed by agarose gel electrophoresis and then purified from gel using Qiagen gel extraction PCR kit (Cat # 28704).

D. Cloning of 5' and 3' flanking regions of *p18*

After purification of the PCR products, we have used the zero blunt Topo PCR cloning kit (K2820-40, Invitrogen) which allows the cloning of blunt-end PCR products (figure 8). Briefly, 4 µl of PCR products (5' flanking region or 3' flanking region of *p18*), were incubated with 1µl of salt solution and 1 µl of pCRII-blunt-TOPO for 5 minutes at room temperature according to the manufacturer (Invitrogen, K2820-40). Afterwards, 2µl of the mixtures of 5'*p18* pCRII-blunt-TOPO or 3'*p18* pCRII-blunt-TOPO were transformed into one vial of OneShot Chemically competent *Esherichia coli* each, incubated for 30 minutes on ice, heat-shock for 30 seconds at 42ºC without shaking and immediately transferred to ice where 250 µl of LB were added. Both tubes were then shaken for 1 hour at 37°C at 200 rpm then plated overnight on 2 agar plates containing Kanamycin as selective antibiotic.

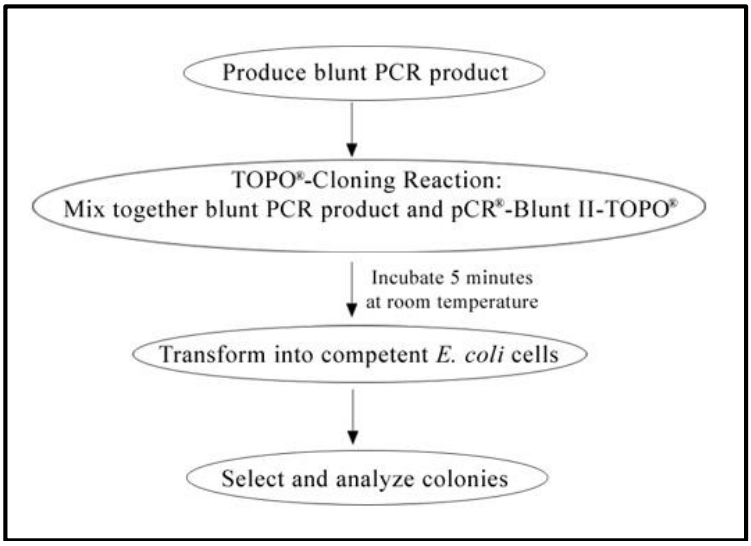

Figure 8. Flow chart of cloning blunt-end PCR products in TOPO vectors

E. Selection and analysis of positive colonies

10 colonies from each Petri dish were picked and numbered from 1 to 10 for analysis. PCR using appropriate primers (ML1514-ML1515 for the 5' and ML1516-ML1517 for the 3') were done on the bacterial colonies directly for verification of the clones. At the same time, and in order to save the original colonies that are verified by PCR, we patched them to a fresh Agar plate containing kanamycin selection, by taking one drop from each PCR tube and loading it on the appropriate corresponding number (Figure 9). This new plate was incubated overnight at 37°C. As for the PCR, Taq DNA polymerase (Qiagen) was used and it was performed as follows: a denaturation step at 95°C for 30 seconds (1 cycle) followed by 30 cycles of: 95°C for 30 seconds and 58°C for 30 seconds and an elongation step at 68°C for 2.5 minutes. The last step is a final elongation at 68°C for 10 seconds. After selection of positive colonies, a trace from each one was incubated in 3 ml LB and 1.8 µl Kanamycin (1.2 µl/2 ml) and was shaken at 37°C at 200 rpm overnight. Subsequently, DNA was extracted using the miniprep kit (Thermo Scientific Gene JET Plasmid Miniprep Kit № K0502, K0503).

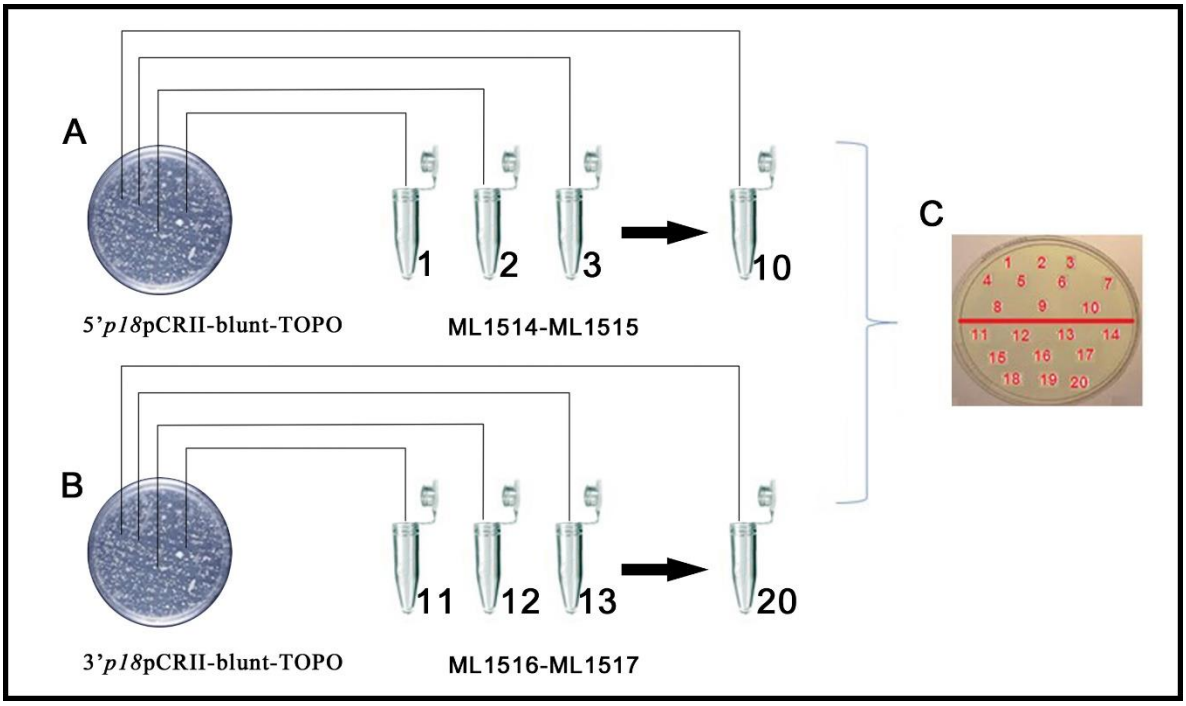

Figure 9. Scheme of the procedure followed in order to perform the selection of positive colonies after PCR. (A): Kanamycin plate harboring 5'p18 pCRII-blunt-TOPO. (B): Kanamycin plate harboring 3'p18 pCRII-blunt-TOPO. (C) Fresh Agar plate, each number corresponds to the number of the tube from which we have taken the drop. Each time, a colony was picked from the plate and resuspended with the content of PCR tube. Then, a drop was taken and transferred to appropriate number on the kanamycin agar plate.

F. Construction of 5' *p18*-P2854 HXPRT- 3' *p18*

We have used the vector (P2854) containing the selectable marker cassette hypoxanthine-xanthine-guanine-phosphoribosyl-transferase (HXGPRT) and having in its multi-cloning sites, the unique enzymatic restriction sites HIND III, Apa I, Not I and Spe I which are absent from the 5'*p18* and the 3' *p18* respectively. These restriction sites were added to our designed primers (table 6) to ensure the proper ligation of the flanking areas of *p18* to the selection vector.

1. 5' and 3' digestion

To insert the 5'flanking region of *p18* between HIND III and Apa I of P2854 (figure10), 2 µg of clone 9 (5'*p18* pCRII-blunt-TOPO) or P2854 were digested each

with 1 µl HIND III (Fermentas) in the appropriate buffer (Bf2) for 2 hours at 37°C followed by an inactivation step at 65°C for 20 minutes. This inactivation step is required in order to stop the random fragmentation that may occur because of the star activity resulting from cutting with this enzyme after a long incubation period. After migration on an agarose gel, the digested DNA was visualized over UV transilluminator and the desired bands were excised and correspond respectively to 5'*p18* pCRII-blunt-TOPO HINDIII and P2854-HIND III, using a sterile scalpel. Cut DNA was purified using the gel DNA extraction kit according to the manufacturer (Cat# 28704).

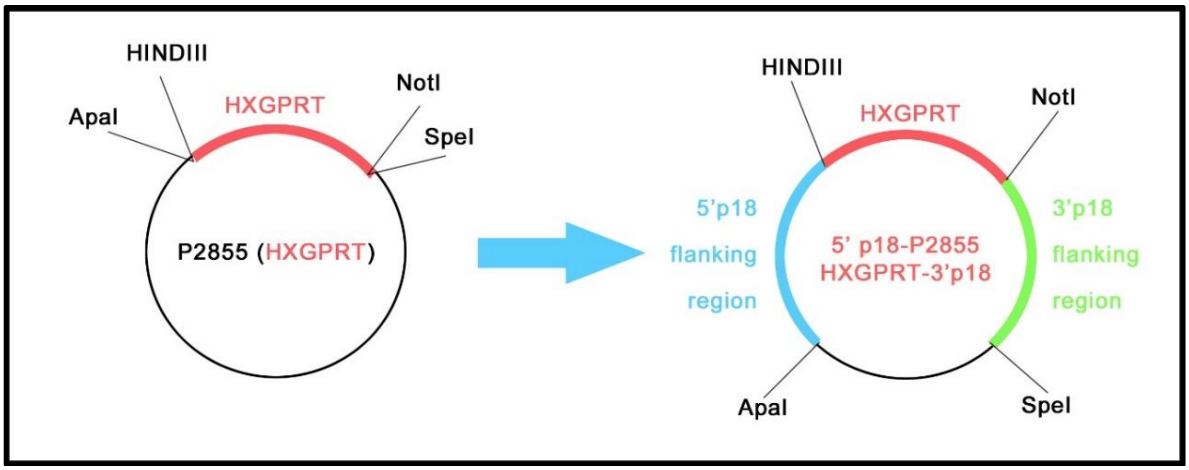

Figure 10. Design of the desired vector.

Eluted DNA was used later to perform the 2nd 5'digestion with Apa I (Fermentas) but using buffer 4 and BSA. The double digested DNA (P2854-HINDIII-ApaI and 5'*p18* pCRII-blunt-TOPO HINDIII-ApaI) were then migrated on Agarose gel and DNA was purified and eluted as described above.

In order to clone the 3'flanking region of *p18* in P2854.5'*p18*, the 1st 3' digestion with NotI (Thermo scientific, #FD0504, lot: 00092970) was performed on the vector and the insert proceeding exactly like for the 5' digestion. Then,

P2854.5'*p18*.NotI and 3'*p18* PCRII-blunt-TOPO Not1 were digested with Spe1 (Thermo scientific, #ER1251, lot: 00132724) as per above.

2. Ligation

Ligation was performed using the T4 DNA ligase (Thermo scientific, #EL0011, lot: 00129006) at RT for 30 minutes followed later by heat shock transformation in DH5α bacteria and an overnight plating Ampicillin LB agar plate.

3. Verification of insertion of the 5'p18 and 3' p18 flanking regions in P2854

After ligation of 5' *p18* in P2854, we performed PCR using the following primers: ML1514-ML1515. As for the PCR reaction, we used Taq DNA Polymerase (REDTaq ReadyMix^{TM} PCR Reaction Mix with $MgCl_2$, Catalog number R2523). 20 colonies were picked from the ligation plates as described previously (Figure 9). The PCR consisted of a denaturation step of 94˚C for 1 min (1 cycle), followed by 30 cycles of (94˚C for1 min, 58˚C for 2 min and 72˚C for 3 min) and a final elongation of 72˚C for 10 min (1 cycle). As for the verification of insertion of the 3'flanking region of *p18,* a double digestion with NotI and SpeI was performed.

G. Electroporation

The construct (5'*P18*-P2854-3'*P18*) was introduced by electroporation to the Pru *Δku80* strain which favors its integration and facilitates the double crossing over as described in Fox *et al.*, 2009. This would allow the homologous recombination between the 5' and 3' flanking regions of *p18,* and helped introduce the selectable marker HXGPRT, which eventually replaced the *p18* gene (figure 11). Technically this was

achieved using by electroporating 5x10^7/ml Pru *Δku80* with 50 µg of plasmid in a cytomix buffer [120 mM KCl, 0.15 mM CaCl2, 10 mM K2HPO4–KH2PO4, 25 mM (Hepes), 2 mM (EDTA), and 5 mM MgCl2 at pH 7.6] in a 0.4-cm gap cuvette (Bio-Rad Laboratories, Hercules, CA, USA) with with 1500 V at 25 µF and 50 Ω with a Bio-Rad Gene Pulser Xcell™ Electroporation System.

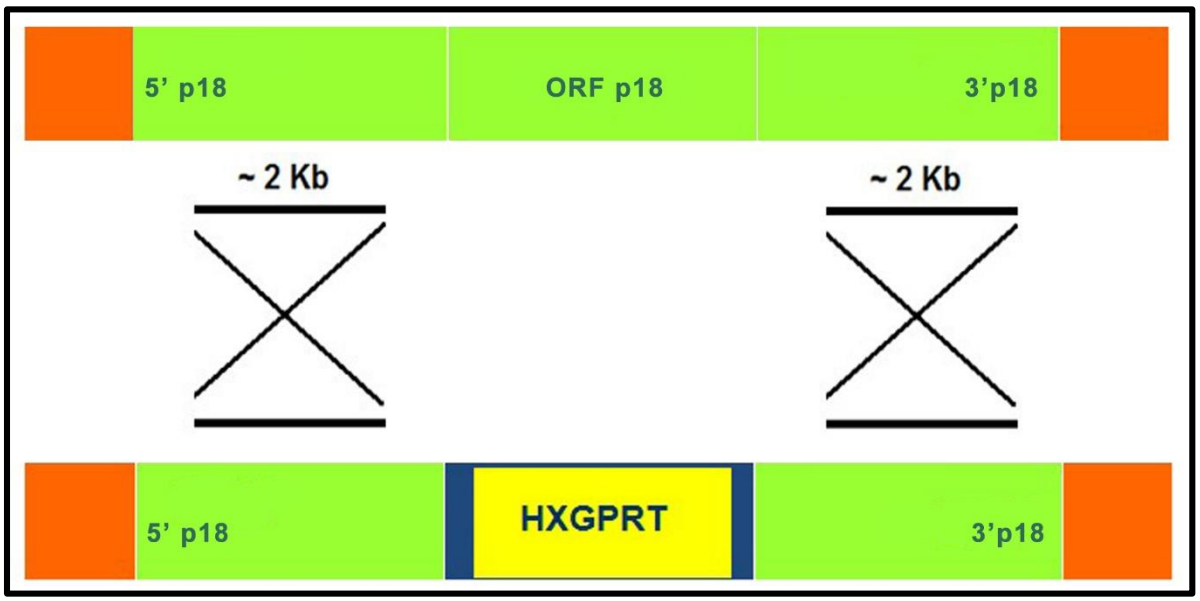

Figure 11**.** Process of knock-out of *p18* in Pru *Δku80* strains. By double crossing-over between the 5' and 3' flanking regions of *p18*, *HXGPRT* will replace the *p18* gene and form the KO strain.

H. Selection

Following electroporation and the potentially successful introduction of *HXGPRT* in place of the *p18* gene, the total population of the genetically modified parasites was subjected to 4 weeks of selection by Xanthine and Mycophenolic acid at 25 µg/ml and 50 µg/ml respectively (as described by Donald, 1996) (Donald, Carter, Ullman, & Roos, 1996).

I. Cloning

Following a month of selection by Xanthine and Mycophenolic acid, total transfected population of parasites was collected, counted and serial dilutions prepared. A volume corresponding to 1 parasite was introduced to each well of a 96-well plate in which HFF were cultured and grown to reach confluency. Infected wells were closely monitored throughout the few days following infection and those showing only one plaque were identified. Upon consumption of HFF in identified wells containing single clones, parasites were passaged into a 24-well plate then into a T25 flasks containing confluent HFF to obtain a higher number of potentially knock-out parasites. After lysis of HFF, parasites were collected and pelleted for further verification of knock-out status; all clones were maintained in culture until verification and only one clone was then selected to perform the current study.

J. Verification of *p18* Knock-out Clones

13 clones were verified by PCR using *ROP18* as a positive control, *p18* and *HXGPRT* primers for knock out verification and for selection cassette integration respectively (table 7). PCR on the 13 screened clones was performed (35 cycles) using the BioRad PCR machine (T100 Thermal Cycler).

Primer	Target gene	Size (Kbp)	Sequences
HH7-8	*ROP 18*	1.7	HH-7: 5' gTg ATg TTT TCg gTA CAg Cgg CCA 3' (Fwd) HH-8: 5' CTT TTA TTC TgT gTg gAg ATg TTC 3' (Rev)
HH9-10	*p18*	3	HH-9: gcgcGGGCCCCGATCCGCAGACATCTGGGGGTCT CTTGGCGTTCGTCCCCGCCAACAAAGCG (Fwd) HH-10: cccAAGCTTGGTTGAAGACAGACGAAAGCAGTTG CAGTATGCTGCGACGCGTCTTCCGAG (Rev)
HH11-12	*p18*	4.5	HH-11: ggACTAGTGAGTTCATTGCCAGTGAAGAAGGTGA CTGGTAGTGTCACATTTGGCAACTGG (Fwd) HH-12: ataagaatGCGGCCGCTTGTTACCTGGCACACGTCAC TTGCAACATTGTAAACTTGTTTGTTGTCTGG (Rev)
HH1-2	*HXGP RT*	2.3	HH-1: CACCAACgCTCCCgATAC (Fwd) HH-2: TgTgAggACgACTCACgg (Rev)
HH5-2	*HXGP RT*	1	HH-5: AggggAATAggCTgATCC (Fwd) HH-2: TgTgAggACgACTCACgg (Rev)
HH3-4	*HXGP RT*	2.8	HH-3: CTATgCACTTgCAggATg (Fwd) HH-4: ACAACgTAgACAgCAgTg (Rev)
HH3-6	*HXGP RT*	1	HH-3: CTATgCACTTgCAggATg (Fwd) HH-6: CTTCCACCgAACgTTTCC (Rev)

Table 7. Primers used to verify potential KO clones.

K. Host cell and Parasite culture

Toxoplasma gondii type II strains (Pru *Δku80*) (kindly provided by Drs Maryse Lebrun and Jean Francois Dubremetz, France) and Pru *Δku80*Δ*p18*) tachyzoites were maintained by serial passage in Human Foreskin Fibroblasts (HFFs) grown in Dulbecco's modified Eagle medium (DMEM) (Invitrogen) supplemented with 10% fetal calf serum (FCS) and 2 mM glutamine 1% penicillin –streptomycin and kanamycin (Lonza).

L. *In vivo* study of the Pru *Δku80Δp18*

1. Timeline for the in vivo experiments

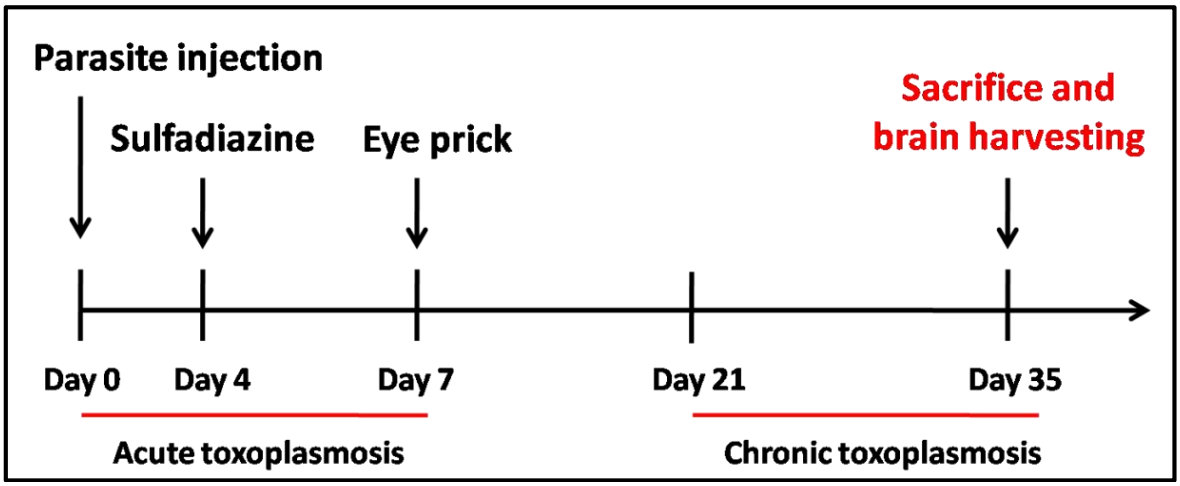

Figure 12. Time line of the *in vivo* manipulations

2. Ethic statement

All murine protocols were approved by the Institutional Animal Care and Utilization Committee (IACUC number: #1312273) of the American University of Beirut. All animals were housed in specific facility with a 12h ON/OFF light cycle. Animals were sacrificed by cervical dislocation after deep anesthesia with isoflurane.

3. In vivo capacity of the Pru Δku80Δp18 to switch into bradyzoites

Swiss mice of 10 to 12 weeks were intraperitoneally injected with 100 parasites either from Pru *Δku80* and Pru *Δku80Δp18.* Mice were treated with sulfadiazine (300 mg/liter of drinking water) on day 4 dpi as previously published by Jost (2007) (figure.12) (Jost, Reiter-Owona, & Liesenfeld, 2007). On d7 pi, acute phase was verified as described in sections (a, b, and c) (Figure 12). Upon week 5 post infection,

brains were homogenized before total RNA extraction, cDNA preparation and bradyzoite quantification by quantitative real time PCR.

a. Verification of the number of injected parasites: “Plage de Lyse” or plaque assay

After rupture of the HFFs infected with Pru *Δku80* and Pru *Δku80Δp18*, tachyzoites were collected, centrifuged at 1500 r.p.m for 5 minutes, washed and resuspended in free serum medium at the concentration of 100 parasites in 200 µl per mouse.

To ensure that the same amount of tachyzoites is injected to mice, the “plages de lyse” or plaque assay was applied. Briefly, a volume corresponding to a known number of parasites is taken of the former mentioned suspension. HFF cells were cultured in a 24 well plate until confluency. After counting 100 parasites per injection per mouse, and after injection, a 5 fold dilution was performed and was used to infect a well of confluent HFF, As a result shores/plaques of lysis (appearance of numerous tachyzoites in distinct regions) will appear, where actually each patch/plaque corresponds to a an initial invasion by a single parasite. Staring day 4 HFF post-infection, the number of patches per well was counted. The number of visualized patches multiplied by 5 corresponds to the number of injected parasites per mouse.

b. Verification of the acute phase in mice

At day 7 p.i., blood sample using retro-orbital puncture was performed and approximately, 100 µl of blood were obtained from the Medial Cathenus of each infected mouse. This blood collection served for verifying the successful infection (as described in the following section).

c. Protein gel electrophoresis and western blotting

A sample of freshly collected tachyzoites of Pru *Δku80* obtained from a T25 flask were boiled in Laemmli SDS-PAGE sample buffer and separated on 12% polyachrylamide gels. Proteins were transferred to nitrocellulose membranes (BIO RAD Cat# 162-0112) at 30V overnight using a BioRad transfer unit. Nitrocellulose membranes were tested for successful transfer using Ponceau Red and cut into strips that were saturated/blocked for 1 h in 5% non-fat dry milk in wash buffer (15 mM Tris-HCl (pH 8), 150 mM NaCl, and 0.05% Tween 20). On average 100µl of blood collected from eye pricks were centrifuged at 13000 r.p.m for 15 minutes. Then strips were incubated with sera (10µl in 1ml of 5 % non-fat milk in wash buffer) from different mice O/N at 4ºC, to check for seropositivity. After washing, the strips were then incubated with HRP (Horseradish peroxidase)-conjugated secondary antibodies (anti-mouse, 1:5000 dilution, Santa cruz, sc-2031, lot: B2212) and revealed with luminol-based chemiluminescent substrate (Santa cruz, sc-048) which binds to the secondary antibody and produces light detected by autoradiography.

d. RNA Extraction

At week 5, brains of mice were harvested, smashed smoothly with a tissue homogenizer and ready for RNA extraction. Total RNA was extracted using Trizol extraction method and experiments were performed starting from 5µg of RNA in a total of 20µl. Briefly, 1ml of Trizol was added to homogenized brain. Mixture was left for 5min at room temperature and followed by a 15000g spin for 5 min at 4°C. Next, 200 µl of chloroform were added, mixture was vortexed for 1min, and then incubated for 1 min at room temperature. Mixture was then centrifuged at 15000g for 5min at 4°C. After

obtaining a clear layer separation, the upper phase containing the total RNA was gently taken, transferred into a new Eppendorf tube and precipitated by adding 0.7V isopropanol. After centrifugation at 15000g for 5min at 4°C, RNA pellet was allowed to air-dry for few minutes to remove any excess of isopropanol, and then was resuspended in 30 µl of RNAse/DNAse Free water. RNA concentration was then quantified using the nano drop machine (ND-1000 Spectrophotometer).

e. cDNA Preparation

For cDNA synthesis, components were added in the same order indicated in table 8. The mixture was gently mixed and incubated for 5 min at 25ºC followed by 60 min at 42ºC, then for 5 min at 70ºC and the reaction was stopped at 4ºC.

RNase/DNase free water containing 5 µg of template RNA	11 µl
random primers	1 µl
5X Reaction Buffer	4µl
Ribolock RNase Inhibitor(20U/µl)	1µl
10 Mm dNTP Mix	2µl
RevertAid M-MuLV RT (200U/µl)	1µl
Total Volume	20µl

Table 8. Components of cDNA Preparation process and volumes involved.

f. Quantitative Real Time PCR (q RT PCR)

Syber green qRT PCR was performed using the BIORAD machine (CFX96 Optics Module, Serial No. 785BR04788). ENO1 forward primer (HH-18: 5´-TggCTTggCTCTTCAAgAAT-3´) and reverse (HH-20: 5´-gCgTCCAAgCCATACTTgTT-3´) primers were chosen as bradyzoite specific marker and were used to detect and quantify the bradyzoites in both wild type Pru *Δku80* and Pru *Δku80Δp18* strains as described by Walker *et al.* (2013). Primers for the

housekeeping gene are directed against Glyceraldehyde-3-Phosphate dehydrogenase GAPDH, they correspond to HH36 (5'-CATggCCTTCCgTgTTCCTA-3') and HH37 (5'-CCTgCTTCACCACCTTCTTgAT-3'). In qRT-PCR, individual reactions were prepared with 0.25 µM of each primer, 150 ng of cDNA and SYBR Green PCR Master Mix to a final volume of 10 µl. PCR reaction consisted of a DNA denaturation step at 95°C for 3min, followed by 40 cycles (denaturation at 95°C for 15 sec, annealing at 56°C for 60 sec, extension at 72°C for 30 sec). For each experiment, reactions were performed in duplicates and expression of individual genes was normalized to GAPDH Ct values. The Threshold cycle (Ct) corresponds to the cycle at which there is a significant detectable increase in fluorescence. So, comparing the CT values of a target gene (Eno-1 in this case) to that of an endogenous reference gene (GAPDH), allows the gene expression level of the target gene to be normalized to the amount of input RNA or cDNA. This is mainly done by calculating ΔCt.($Ct_{(BAG-1)} - Ct_{(GAPDH)}$). Thereafter, ΔΔCt is calculated according to the Livak method: $2^{-\Delta\Delta Ct}$ to obtain the percentage of expression. This method is widely used for relative gene expression analysis (Schmittgen and Livak, 2008).

4. Reactivation ability of the Pru Δku80Δp18 knock-out parasites

In order to test the reactivation ability of the Pru *Δku80*Δ*p18* knock out parasites, 10 to 12 weeks old swiss mice were injected with the with 100 parasites from both Pru *Δku80* and Pru *Δku80Δp18*. Mice were monitored as described in (figure.12) and were verified for their seropositivity for *T. gondii* as described (sections 3- a, b, and c). On day 42 pi, when cysts are already formed in the brain (Djurković-Djaković *et al.*, 2012), the chronically infected mice were treated with Dexamethasone (16mg/L) in

order to immune-suppress them and allow bradyzoite cysts to reactivate. The reactivated mice were left for survival to compare the capacity of the Pru *Δku80*Δ*p18* and the wild type Pru *Δku80* strain to reactivate.

5. Pru Δku80Δp18 knock out phenotype during the acute phase of toxoplasmosis: survival

In order to understand the role of *p18* in the bradyzoite stage conversion biology of the parasite, the Pru *Δku80Δp18* knock out parasites generated in the previous sections were tested *in vivo* during the acute phase of toxoplasmosis. Swiss mice of 10 to 12 weeks were intraperitoneally injected with 100, 250, 500, or 10^6 parasites from either the wild type Pru *Δku80* or the Knock out Pru *Δku80Δp18* and monitored for survival.

6. Kinetics of invasion of Pru Δku80Δp18 knock-out in Different Organs

Syber green qRT PCR was performed using the BIORAD machine (CFX96 Optics Module, Serial No. 785BR04788). SAG-1 forward primer (HH-21: 5'-ACT CAC CCA ACA ggC AAA TC 3') and reverse (HH-235'- gAg ACT AgC AgA ATC CCC Cg-3') primers were chosen as tachyzoite specific marker and were used to detect and quantify the tachyzoites in both wild type Pru *Δku80* and Pru *Δku80Δp18* strains in spleen, liver, lung, brain and peritoneal lavage, as described by Walker *et al.* (2013). Primers for the housekeeping gene are directed against Glyceraldehyde-3-Phosphate dehydrogenase GAPDH, they correspond to HH36 (5'-CATggCCTTCCgTgTTCCTA-3') and HH37 (5'-CCTgCTTCACCACCTTCTTgAT-3'). qRT PCR was performed as described in section 3-f.

7. Statistics

Survival results are presented as mean values +/- SEM. Two-tailed Student's *t*-tests were used using GraphPad to determine the statistical significance of differences observed between indicated groups for parametric comparisons. For *in vivo* experiments, the level of significance was determined with the Logrank test using GraphPad.

CHAPTER THREE

RESULTS

A. Generation of 3’ and 5’ flanking regions of p18

After extraction of genomic DNA from the PRU *Δku80* strain, we performed a gradient PCR using temperatures (56 °C→62°C) for both the 3’ and 5’ flanking regions of *p18*, each with its specific primers (ML1516-ML1517 for the 3’ region and ML1514-ML1515 for the 5’ one) and the Prime star HSDNA polymerase. 5 tubes for each flanking region were tested. Then, PCR products were loaded on a 1% agarose gel supplemented with 2.5 µg of Ethidium Bromide. We could successfully amplify only the 3’ flanking region of *p18* which is a 2719 bp segment successfully appearing (Lanes 6 to 10) irrespective of the annealing temperature (figure 13).

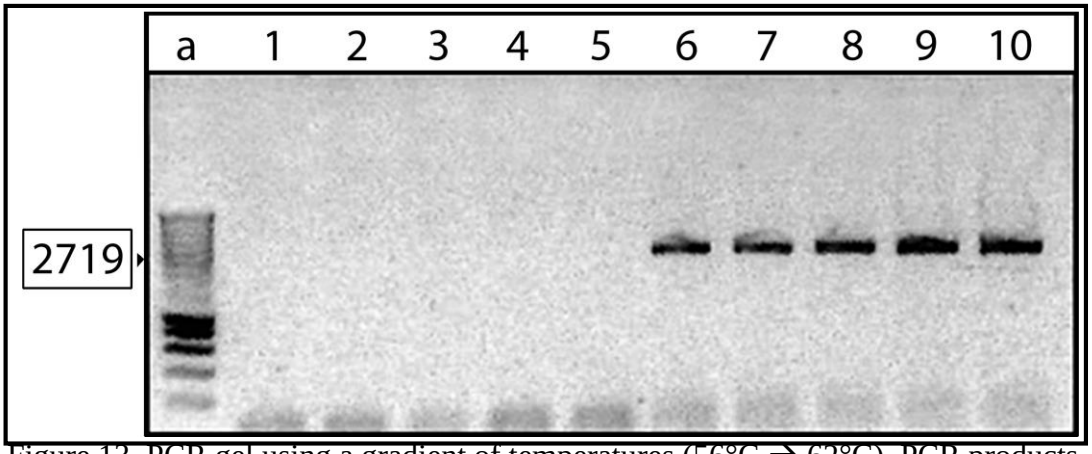

Figure 13. PCR gel using a gradient of temperatures (56°C → 62°C). PCR products were obtained using ML1514-ML1515 primers (lanes 1 to 5) and ML1516-ML1517 (lanes 6 to 10).

Since the PCR for the 5’flanking region of *p18* did not work, we tried to reduce the annealing temperature and to use another high fidelity PCR polymerase, Phusion Polymerase along with re-testing the PrimeSTAR polymerase reagent. PCR products

corresponding to the 5' flanking region of *p18* (2274 bp) were only obtained when we used the Phusion Polymerase (Lanes 1 to 5) but the DNA was much more amplified in lanes (3→5) corresponding to annealing temperatures 56, 58 and 60°C respectively (figure 14).

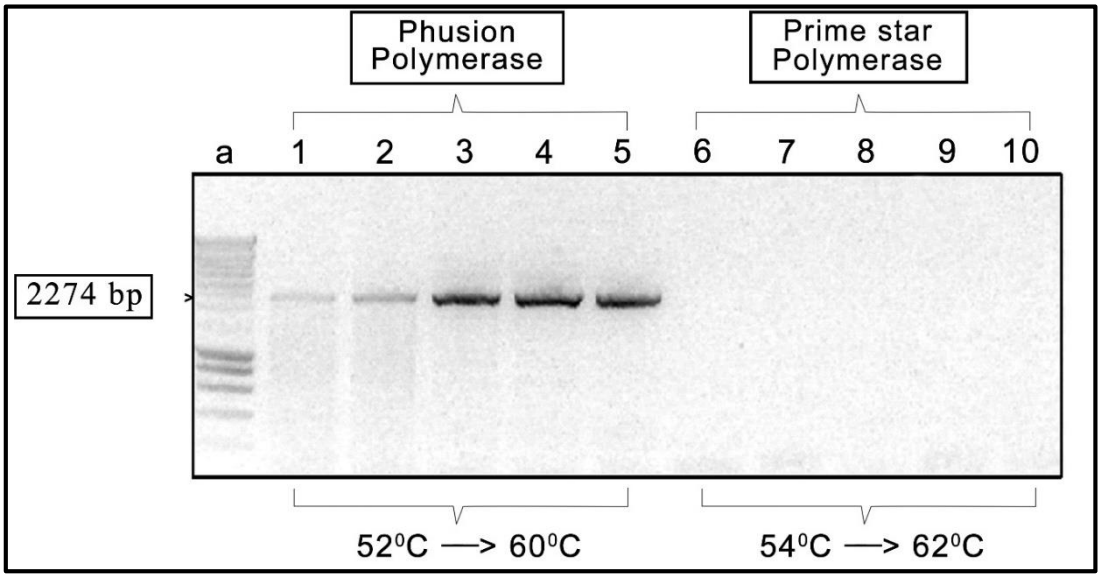

Figure 14. PCR gel using a gradient of temperatures (52°C → 60°C). PCR products were obtained using ML1514-ML1515 primers in presence of Phusion Polymerase (lanes 1 to 5) and PrimeSTAR Polymerase (lanes 6 to 10).

PCR products were then purified from agarose gel after pooling all lanes from 6-10 for the 3' flanking region and lanes 3, 4 and 5 for the 5'flanking region. Since both polymerases (Phusion and PrimeStar) create Blunt end PCR produts, the 3' and 5' *p18* flanking regions were then cloned in the Zero Blunt TOPO PCR cloning kit and transformed in the chemically competent Top10 bacteria. For verification, PCR was performed directly on 10 colonies for each flanking region, and bands at 2274 bp (lanes 1 to 10) and 2719 bp (lanes 11 to 20)corresponding to the appropriate sizes of the 5' and 3' flanking regions respectively, were detected in all verified clones (figure 15).

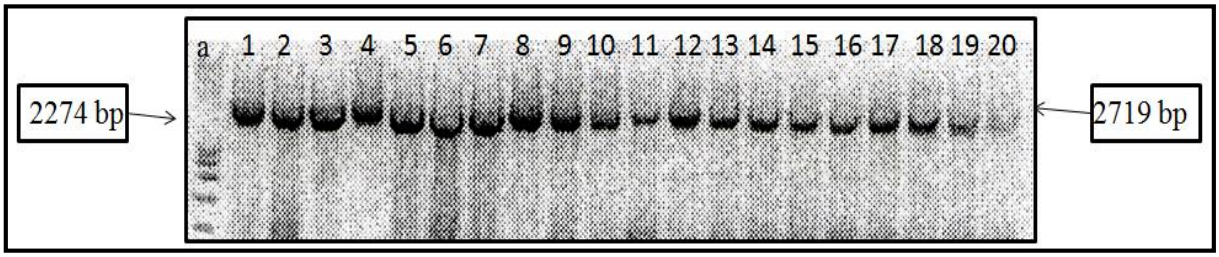

Figure 15. PCR result on 20 colonies. Lanes 1→10 correspond to colonies which contain the 5' flanking region of *p18* whereas lanes 11→20 correspond to colonies containing the 3'flanking region of *p18.*

Collectively, these results show that we could successfully amplify the 3' and 5' flanking regions of *p18* from the PRU *Δku80* genomic DNA.

1. Construction of P2854.5'p18.3'p18 vector

a. 5' flanking region introduction: Digestion with HIND III and Apa I

In order to introduce the 5'*p18* in the selection vector P2854, both vectors, P2854 and 5' *p18* TOPO clone 9 were cut by the restriction enzyme, HIND III. Non-digested P2854 and 5' vector TOPO were used as control to allow the comparison (figure 16).

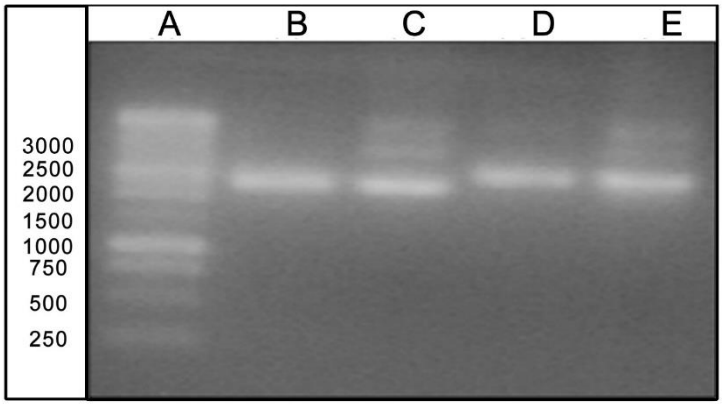

Figure 16. DNA gel analysis after 5'digestion with HIND III.(A) DNA ladder. none digestedP2854 (B), P2854 cut by HIND III (C), none digested p18 TOPO clone 9 (D) and p18 TOPO clone 9 cut by HIND III (E) were separated on 1 % agarose gel. Numbers on the left represent the DNA ladder in bp.

Prior to their digestion by ApaI, DNA products from lanes C and E were purified. Then digestion was performed using Apa I (lanes C and C'), We could successfully detect the insert at 2274 bp from the *p18* TOPO clone 9 as compared to the linearized P2854 (lanes B and B') (figure 17).

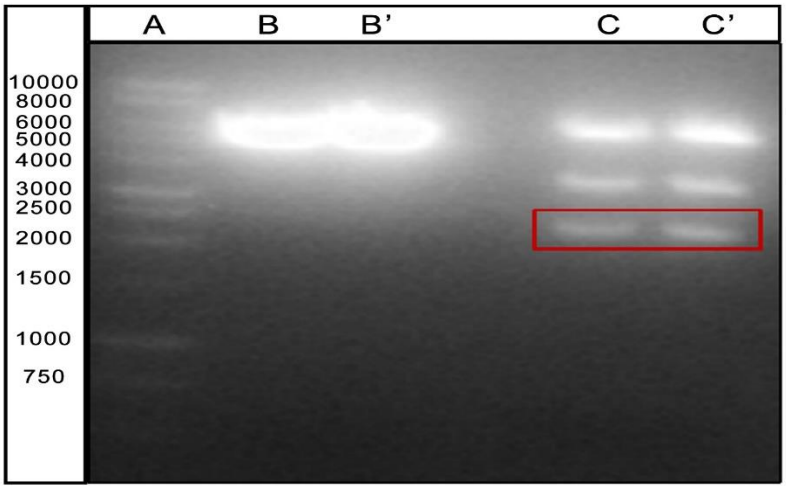

Figure 17. DNA gel electrophoresis after 5'digestion with Apa I-HINDIII.(A) DNA ladder. P2854 double digested with HIND III and Apa I (B and B') and 5' p18 TOPO clone 9 double digested with with HIND III and Apa I (C and C'). Numbers on the left represent the ladder in bps. Bands framed in red are the bands of interest.

b. Verification of P2854.5'*p18*

After ligation of the double cut P2854 and 5'*p18* TOPO clone 9 with HIND III and Apa I, we transformed the ligated vector in DH5α at the ratio of 1:3 vector to insert and plated on agar plates supplemented with ampicillin. After overnight incubation at 37°C, we picked up 20 colonies and performed a PCR using the 5' specific primers (ML1514-ML1515) and the Taq polymerase. Among the positive clones, we are showing one verified clone in figure 18.

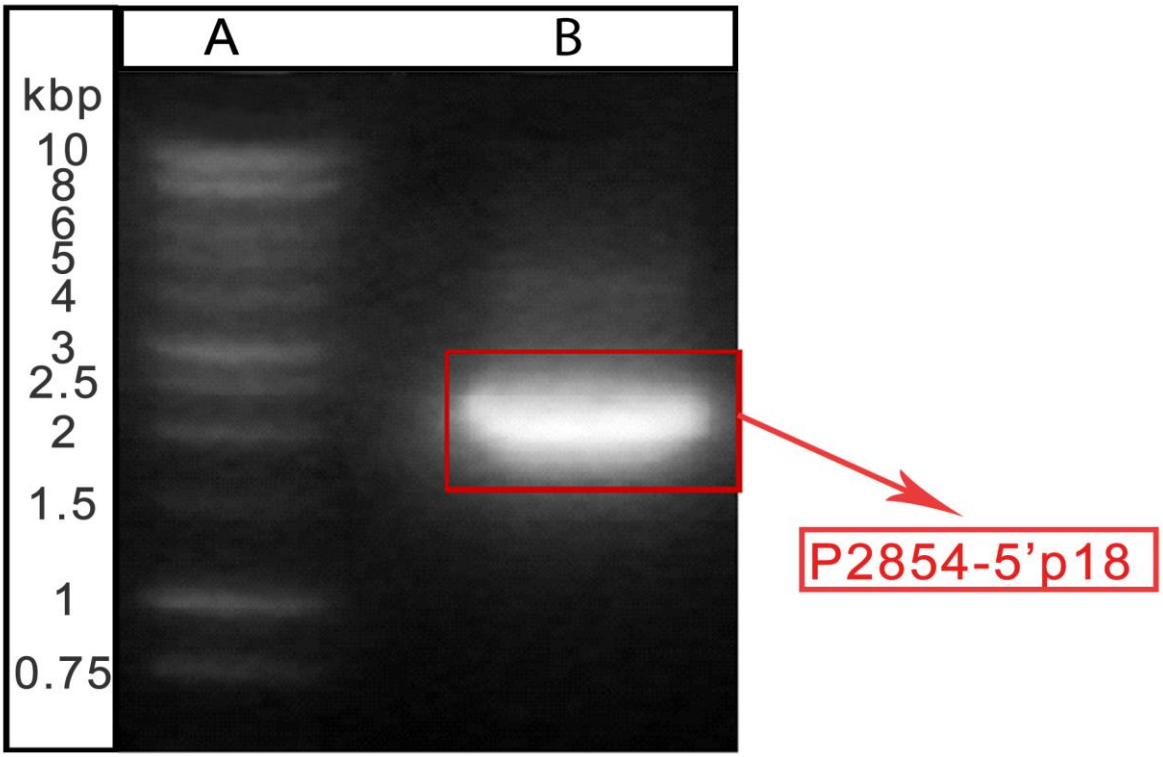

Figure 18. PCR result on one positive colony after ligation (A) DNA ladder. (B) Corresponds to colony 12 with a band showing at the appropriate molecular weight (2274 bp). Numbers on the left correspond to the DNA ladder in kbp.

c. 3' *p18* Flanking region introduction: Digestion with Not-1 and Spe-1

In the interest of introducing the 3'*p18* in the selection vector P2854.5'*p18*, the same procedure was followed using the appriapriate resctriction enzymes. Both vectors, P2854.5' *p18* and 3'*p18* TOPO clone 18 were fisrt cut and successfully linearized by the restriction enzyme, Not-1 (Figure 19).

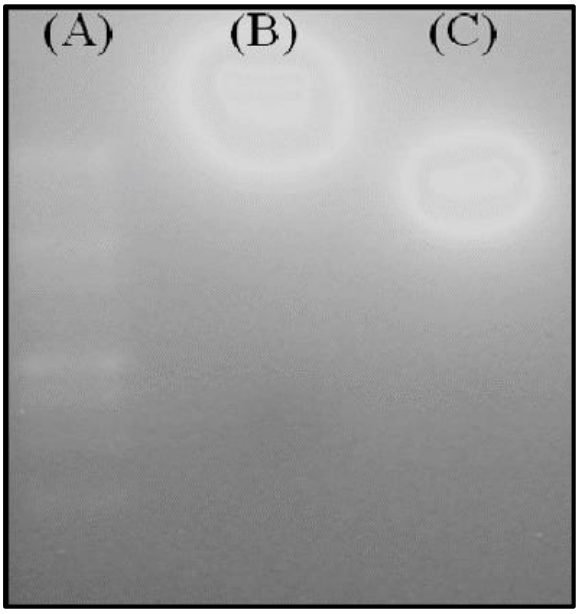

Figure 19. DNA gel analysis after 3'digestion with NotI. (A) DNA ladder. (B) Linearized P2854.5'p18 and (C) linearized 3'p18 TOPO clone 18.

After gel DNA purification from lanes B and C, digestion with Spe-1 was performed. Double digestion was then verified and the P2854.5' *p18* was linearized (lanes B and C, figure 20). A band at the appropriate molecular weight of 2719 bp (lanes D and E, figure 20) was obeserved.

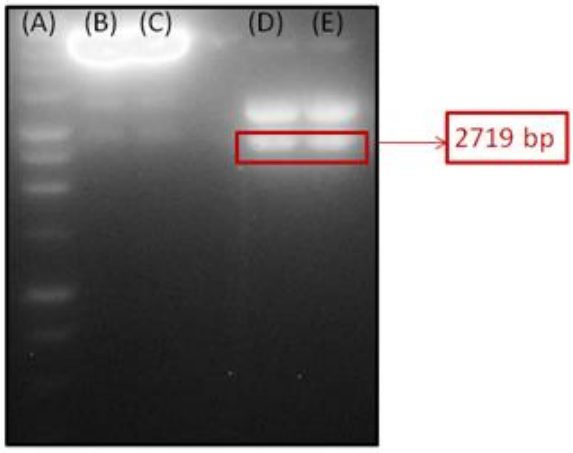

Figure 20. DNA gel electrophoresis following the 3'digestion with Spe-I. (A) DNA ladder. (B-C) double digested P2854.5' p18 with Not-1 and Spe-1. (C-D) double digested 3'p18 TOPO clone 18 with Not-1and Spe-1 (D and E). Bands framed in red are the bands of interest.

2. Verification of P2854.5'p18.3'p18 vector

Gel purification of DNA products was followed by the ligation of the double cut of Not-1/ Spe-I P2854 vector and 3'*p18* clone 18 insert . The ligation vector was then transformed in DH5α at the ration 1:3 vector to insert and plated on agar plates supplemented with ampicillin. After overnight incubation at 37°C, we picked up few colonies, extracted the plasmid and verified the ligation by a double digestion with Not-I and Spe-I, followed by gel migration on 1% agarose gel. After double digestion, we could successfully obtain the 3' flanking *p18* insert at the appropriate 2719 bp (figure 21).

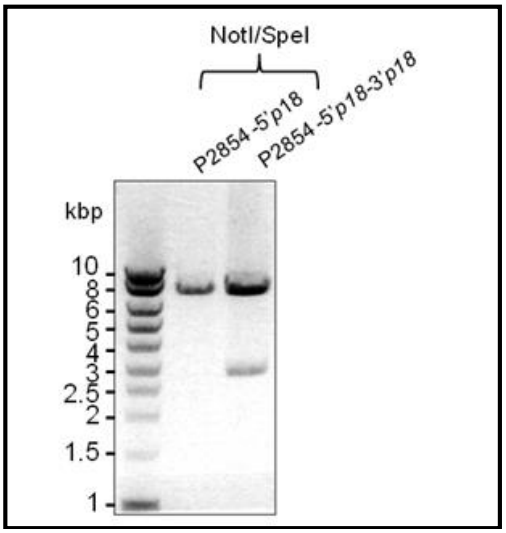

Figure 21. DNA gel electrophoresis after double digestion by Not-I and Spe-I. (A) DNA ladder (B) P2854.5'p18 and (C) P2854.5'p18.3'p18 after double digestion with Not-1 and Spe-1.

3. Generation of Knock-out Strain PRU ΔKU80Δp18

After successfully obtaining of the selection cassette containing the 3' and 5' flanking regions, in addition to the selection marker hypoxanthine-xanthine-guanine phosphoribosyl transferase (*HXGPRT)*, the linearized plasmid was introduced to PRU *Δ*

KU80 tachyzoites by electroporation as described in (Donald and Roos, 1998; Nakaar *et al.*, 2000).

4. Selection and cloning of the stable Pru Δku80Δp18 knock out parasites

The selection for such the knock out Pru *Δku80Δp18* was done by adding Xanthine and Mycophenolic acid to the medium. This selection is used because *T. gondii* strains synthesize their purines by transforming the Adenosine TriPhosphate (ATP) into Xanthine MonoPhosphate (XMP) then into Guanine MonoPhosphate (GMP). Note that this process can happen in presence of Xanthine and HXGPRT. Since the mycophenolic acid will block the normal general pathway of Guanine synthesis, adding Xanthine will allow the survival and proliferation of only the parasites that are capable to make the GMP through their HXGPRT. Therefore, The HXGPRT parasites will be the only ones that will survive the selection (Donald and Roos, 1998; Nakaar *et al.*, 2000) (Figure. 22).

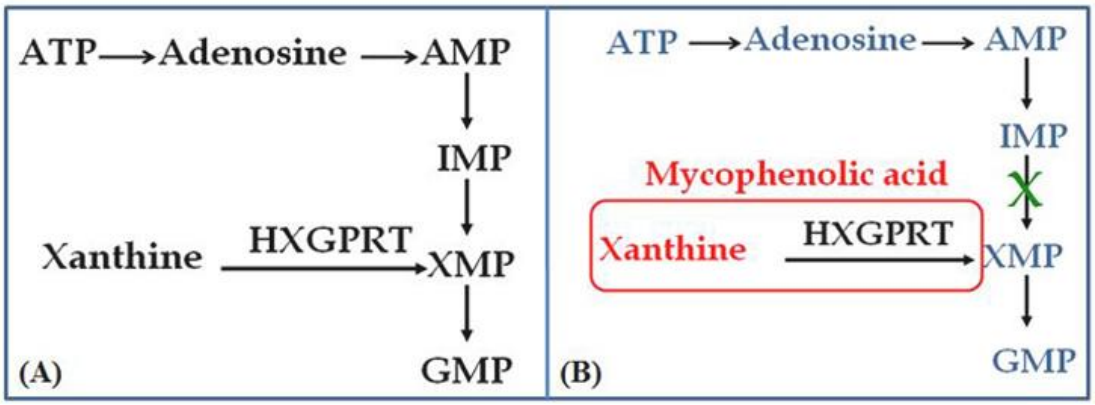

Figure 22. Purine synthesis in *T.gondii*. (A): wild type of *T.gondii*. (B): PRU *Δku80Δp18* strain of *T.gondii*. In presence of mycophenolic acid, the general pathway of purine synthesis is blocked in the KO strain and only the strain who integrates *HXGPRT* can survive after adding the Xanthine.

Stable clones that had successfully integrated the P2854.5'*p18*.3'*p18* were identified after mycophenolic acid-xanthine selection for one month and clones were isolated by limiting dilution. The loss of *p18* and the appropriate integration of the selection cassette were confirmed by genomic PCR analysis on 13 screened clones. The PCR result confirmed the replacement of the open reading frame of *p18* with the *HXGPRT* resistance cassette in all of them. For simplicity reasons, we are showing only 2 clones (Figure 23).

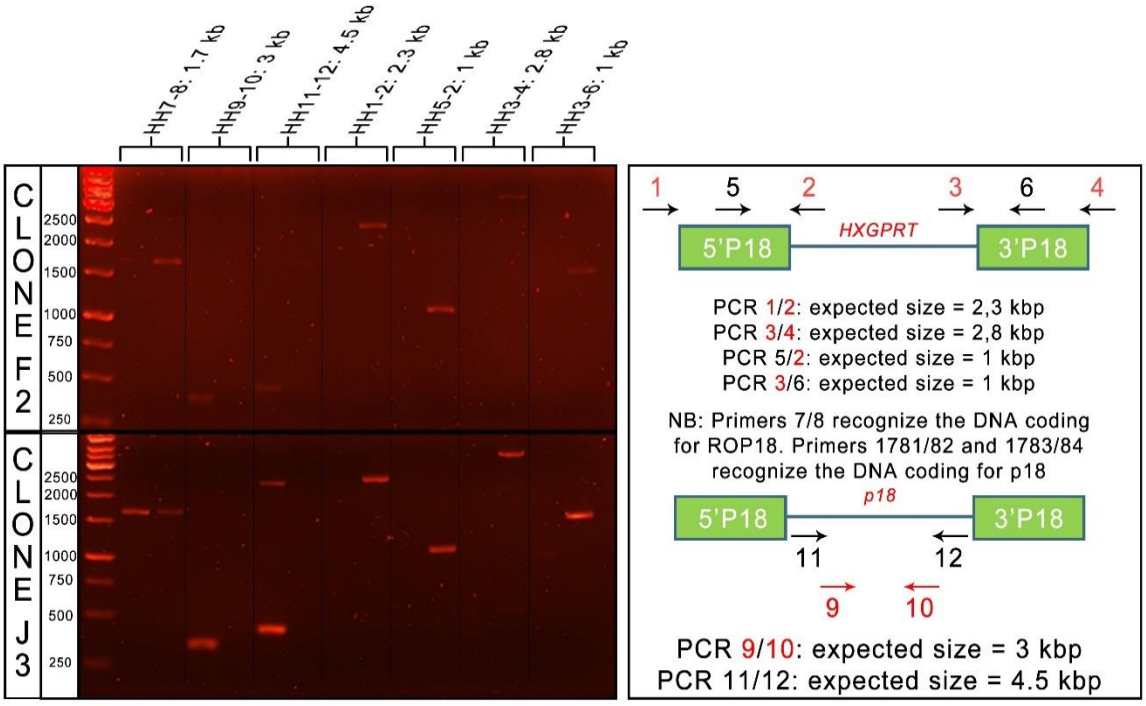

Figure 23. Verification of two potential knock out clones, F2 and J3

B. Pru Δ*ku80*Δ*p18* parasites form more brain bradyzoite cysts than the Wild Type Pru Δ*ku80* parasites

In order to examine if the disruption of *p18* gene can affect the bradyzoites formation and/or their number, an *in vivo* murine model of Swiss mice was used. Mice were injected with 100 tachyzoites of either the PRU*Δku80* or PRU*Δku80Δp18* strains

on day 0. On day 4 pi, both groups of mice were treated with sulfadiazine over the period of two weeks (Figure 12). This treatment helps mice to overcome the acute phase of toxoplasmosis and start forming cysts in their brains. On day 7 pi, blood sample collection using retro-orbital puncture from the Medial Cathenus of each infected mouse was performed, and served for verifying the successful acute infection (figure 24).

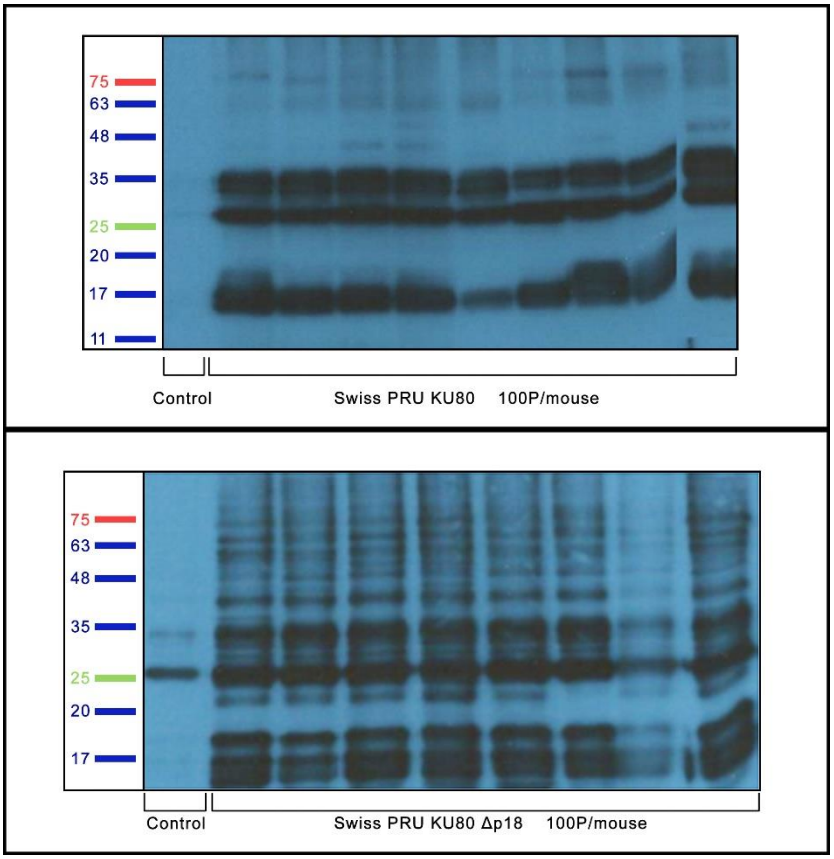

Figure 24. Western blots verifying the sero-positivity of mice. Infected with (A) Pru *Δku80* and (B) Pru *Δku80Δp18*.

On week 5, brains from both groups of mice were harvested, total RNA was extracted, qRT PCR was performed using bradyzoite specific primers for Eno1. (Ferguson, D. J., Parmley, S. F., & Tomavo, S., 2002). Brains of mice injected with the knock-out PRU Δ*KU80*Δ*p18* strain were found to harbor much more bradyzoite cysts than those injected with the wild type PRUΔ*KU80* strain with an increase of 41.93-fold of ENO-1 levels in the knock out strain as compared to the wild type strain (Figure 25).

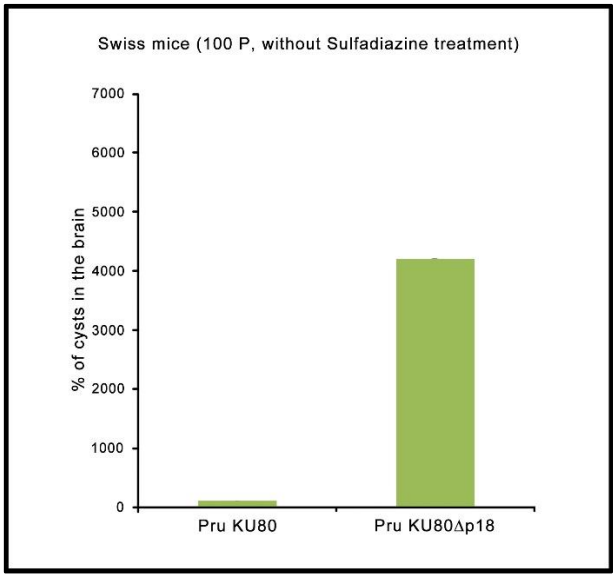

Figure 25. Levels of ENO 1 expression in brains of Swiss mice injected with Pru *Δku80* and Pru Δ*ku80*Δ*p18*.

C. Survival Assay Assessing Reactivation Capacity of PRU Δ*ku80*Δ*p18*

In a separate experiment, 2 groups of mice were injected with 100 parasites of either Pru Δ*ku80* and Pru Δ*ku80*Δ*P18*. Subsequent treatment with sulfadiazine ensured the survival of mice in both experimental groups and hence the development of chronic toxoplasmosis. Following induction of reactivation using Dexametasone, a significant difference was observed. Fifty days post-reactivation induction the first death was recorded in the group of mice originally infected with 100 wild type parasites and by day 80 post reactivation induction 100% of these mice were dead (figure 26). On the contrary, with the reactivation induction of chronic toxoplasmosis induced by Pru Δ*ku8* Δ*p18* parasites, no mortality was observed until day 120 post-reactivation induction and 75% survival rate was observed up to 135 days after initial administration of Dexamethasone (Figure 26).

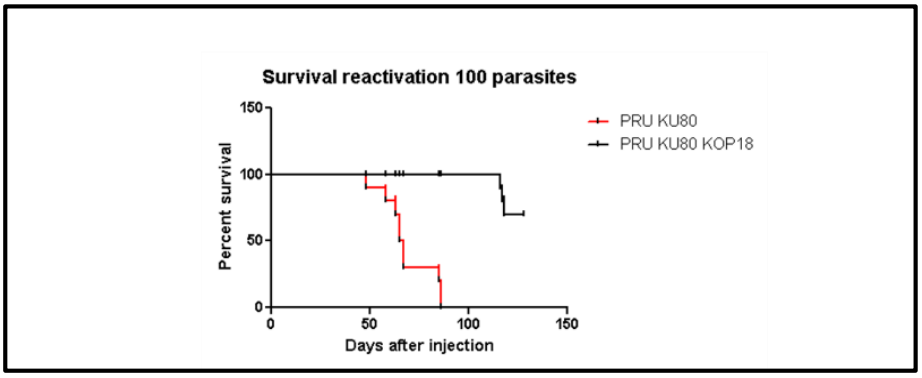

Figure 26. Survival of Pru Δ*ku80*Δ*p18* infected with 100 parasites mice after reactivation with Dexamethasone.

D. Pru Δ*ku80*Δ*p18* parasites are less virulent than the wild type ones even during the acute phase of toxoplasmosis

During the acute phase of toxoplasmosis, tachyzoite multiplication is inhibited by the immune response. The result of this immunologic response is the development of bradyzoites, the hallmark of chronic infection. Although p18 is a bradyzoite marker (Ferragut, 1996), and since our results showed that infection with PRU *Δku80ΔP18* is responsible for the formation of a higher number of cysts in the brain at the level of chronic toxoplasmosis, yet, this higher number of cysts failed to reactivate at the same time than the wild time and had a much delayed reactivated phenotype, we investigated in a retrograde manner, the effect of this knock out on the acute phase of infection. Therfore, 100 parasites either from PRU*Δku80* or PRU*Δku80ΔP18* were intraperitoneally injected and mice survival was assessed without any sulfadiazine treatment. Unexpectedly, the knock strain injected mice remained all alive whereas all wild type injected mice died within 7 days (Figure 27A). This result led us to test the effect of higher numbers of injected parasites (250, 500 and 1 million parasites) in 3 other groups of mice (Figure 27 B, C, D). The same result was obtained for mice injected with 250 or 500 parasites of the PRU*Δku80ΔP18* strain where all mice were

still alive whereas all mice injected with the wild type strain died by 18 and 16 days respectively (Figure 27 B and C). The highest injected dose of 1 million parasites resulted in 100% death rates of mice infected the PRU*Δku80ΔP18* knock out strain but after a significant increase of survival as compared to mice infected with the wild type PRU*Δku80* strain (Figure 27 D).

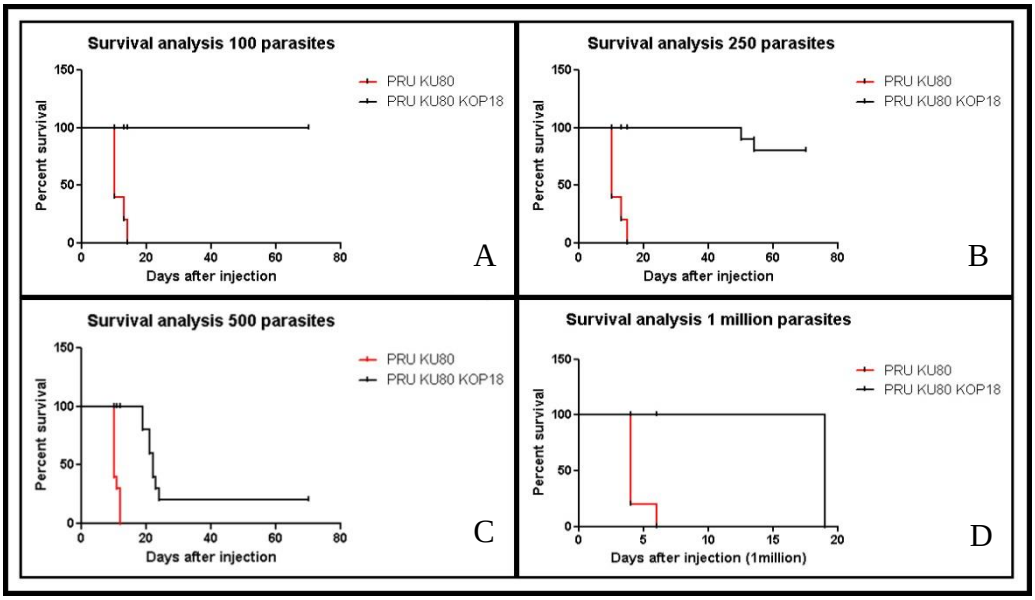

Figure 27. Graphs showing the survival of mice in response to infection with Knock out versus wild type strain at different injection loads.

E. Pru Δ*ku80*Δ*p18* parasites have a reduced ability to invade organs during the acute phase of toxoplasmosis

The pattern observed with the survival in the experiment of 1 million parasites injected drove us investigate the extent of invasiveness of the Pru Δ*ku80*Δ*p18* parasites. Brains, livers, lungs, kidneys, and peritoneal cavity lavage samples collected from mice injcected with 1 million of either PRU Δ*ku80* or PRU Δ*ku80*Δ*p18* were analysed for detection of tachyzoite load. In all organs and peritoneal cavity with the exception of the brain, the wild type parasites proved to be much more capable of invading and

persisting with levels of SAG 1 in liver, lungs, spleen and peritoneal cavity in case of infection with KO parasites, not exceeding 10% of that in the case of infection with the wild type strain Figure 26 A, B, C, D. However in the case of the brain, expression level of SAG 1 seemed to be comparable in both cases of infection senario figure 26 E.

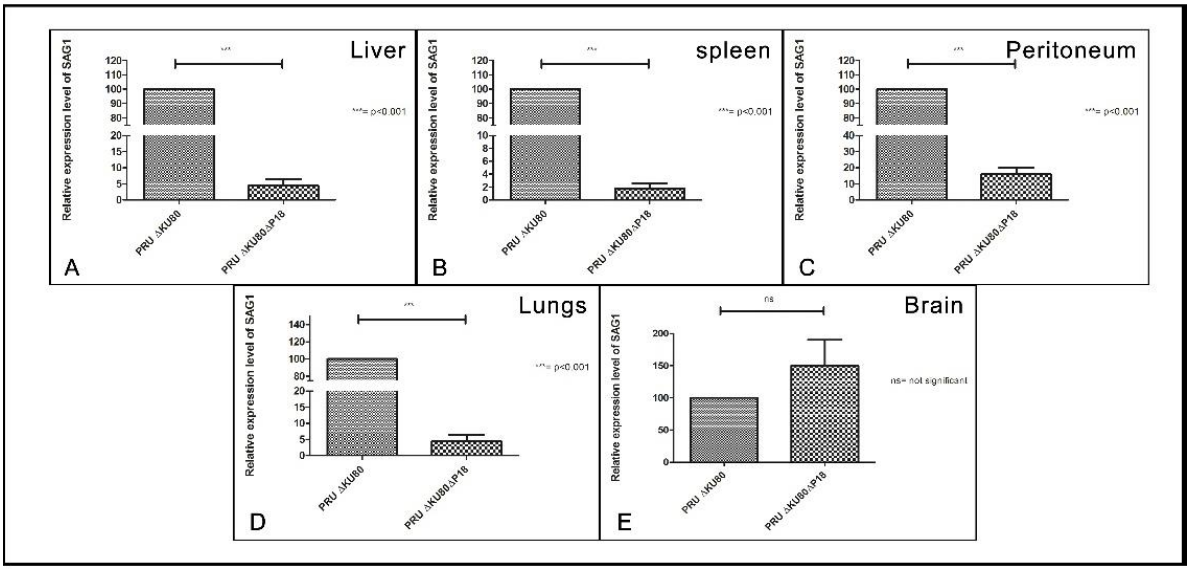

Figure 28. Real Time PCR analysis showing expression of SAG 1 in (A) Liver, (B) Spleen, (C) Peritoneum, (D)Lung, (E) Brain. of mice infected with 1 million parasites of PRU Δ*ku80* or PRU Δ*ku80*Δ*p18* and sacrificed at day 4 pi

CHAPTER FOUR

DISCUSSION AND FUTURE PERSPECTIVES

For decades, tachyzoite-bradyzoite interconversion has been a crucial event in the life cycle of *T. gondii* and a major challenge in immunocompromised individuals. Reactivation of chronic toxoplasmosis can have severe and potentially lethal impacts upon immunosuppression. Hence there is a growing need to fill in the large number of gaps in the area pertaining to the mechanisms of cyst formation in terms of initial prevention and the mechanism of cyst reactivation in the scope of post infection control of toxoplasmosis. In this work, we aimed at unraveling any potential role of the bradyzoite marker p18 in the cyst formation and the conversion from the chronic to the acute phase of toxoplasmosis. Fox *et al.* developed the PRU *Δku80* in type II *T. gondii* and showed that this strain allows an increased efficiency of double-crossover homologous recombination at targeted loci (Fox *et al.*, 2009). We have used this strain to suppress the gene encoding for p18 and examined the phenotype of this knock-out in a murine model.

Our results showed that the knock-out parasites Pru *Δku80Δp18* resulted in the formation of more cysts in the brain of infected mice when compared to mice infected with the wild type Pru *Δku80*. However, paradoxical results were obtained upon suppressing the immune system of infected mice with the knock out strain with dexamethasone, we have obtained a better survival when *p18* was deleted. Our results demand further in-depth study. Several potential reasons need to be further investigated to understand this phenotype. This delay in reactivation even with an initial higher number of cysts being formed when *p18* is deleted, led us to investigate in a

retrospective manner the acute phase of infection by the KO strain versus the wild type strain. Our results shed light on another prospective aspect potentially playing an important role in explaining our observation. Although a bradyzoite marker not expressed in tachyzoite forms, p18 might have implications on the overall virulence of the parasite. Real time PCR analysis showed that in the case of acute infection, tachyzoite burden in organs 4 days pi is vastly different between mice infected with the wild type strain and those with the KO strain. Considerably lower amounts were detected in all organs of mice infected with the KO strain compared to burden in organs infected with wild type strain, except in the case of the brain where burdens were comparable. This may suggest a potential role of p18 as an immunogenic trigger, the absence of which may result in a parasite that is more successful in evading the host immune system and reaching the brain. Indeed, there is a vast number of factors that interplay all together determining the parasite's virulence and contributing to its efficiency in terms of cyst formation and thus posing a serious threat of reactivation in case of immunologic drop-down. Some examples on genetically identified players affecting parasite virulence are Type II GRA15/TRAF6/NF-κB, Type I ROP 16/STAT3/STAT6, Type III ROP38 and Map p38 kinase, Type I GRA10/Taf1 B, ROP5/ROP18/ murine IRGs and ATF6b, among other interactions of ligands with specific host cell receptors or molecules (Yamamoto *et al.*, 2011). A recent study suggested that the host immune response itself may play a very important role in determining the virulence characteristics of the parasite (Eric Y. Denkers, 2014). Infection of mice with a type II strain was shown to lead to less apoptosis and to lower amounts of INF-γ (Denkers *et al.*, 2014). In addition, nitric oxide (NO) levels were elevated. Indeed, type II parasites are very effective in activating an early response

(Figure 28). These parasites express the active form of the dense granule protein GRA15, which activates NF-κB in the infected cells, and a less functional form of the rhoptry protein ROP16, which leads to a transitory activation of STAT3/6 (Saeij *et al.*, 2007). As a consequence, there is a massive production of pro-inflammatory cytokines early after infection. The environment induced by the parasite modulates activation of several T cell subtypes, mainly directing the response towards a Th-1 type (Denkers and Gazzinelli *et al.*, 1998). Aspects of the Th-17 response to *Toxoplasma* seem to have opposite effects on host survival, mainly an IL-23 driven IL-22 response by $CD4^+$ T cells has a negative effect while signaling through the IL-17 receptor can have a beneficial effect by lowering parasite burden (M. Munoz *et al.*, 2009). Therefore, intracellular parasite growth is controlled due to the expression of an avirulent form of ROP18 (Fentress, *et al.* and Steinfeldt *et al.* 2010). Furthermore, type II parasites also express ROP5 alleles associated with low virulence (Behnke *et al.* and Reese *et al.* 2011).

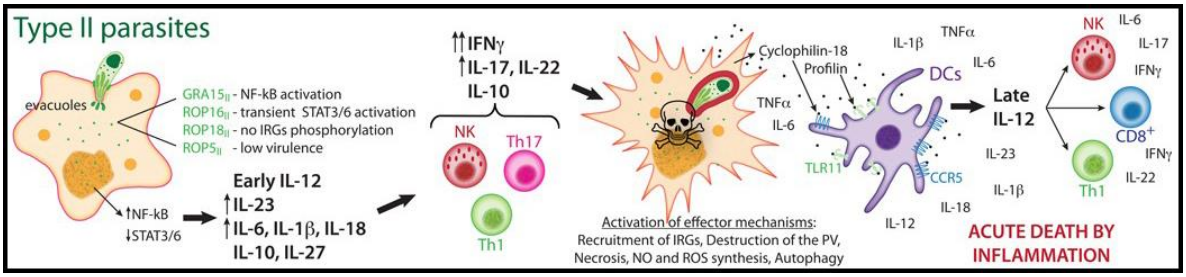

Figure 29. Overview of Toxoplasma strains that modulate host immune pathways in type II parasites (Melo *et al.*, 2011)

We have shown that the KO parasites were more successful in cyst formation capacity than the wild type strain. This result may be due to a presumably less immunogenic Δ*p18* parasites would evade clearance by nitric oxide secreted after activation of microglia by IFN-γ (Chao *et al.*, 1993a, c, 1994b). Hence, more

tachyzoites would survive in the brain and would be capable of switching to bradyzoites and thus resulted in a larger bradyzoite burden in the brain after *p18* was deleted. In an attempt, to validate such a hypothesis of decreased virulence of our KO strain, measuring IFN-$\boldsymbol{\gamma}$, IL-10, IL-12 secreted levels in sera of mice infected with either the wild type or knock out strain 4 days p.i. may reveal if deletion of p18 may modulate the immune response in mice. Furthermore, nitric oxide is one of the main IFN-γ induced anti-toxoplasmacidal mechanisms known to mediate resistance to *T. gondii* in mice (Hayashi *et al.*, 1996b). IFN-γ induces synthesis of the enzyme inducible nitric oxide synthase (iNOS) that produces nitric oxide (NO) from L-arginine (Adams *et al.*, 1991). The L-arginine dependent production of NO and subsequent conversion to reactive nitrogen species (RNS) has direct antimicrobial activity and results in parasite killing (James, 1995). It would be also very important to compare the transcript levels of iNOS in the brains of mice infected with the KO strain versus the wild type strain 4 days p.i. This would reflect any potential difference in immunogenicity of the two strains.

Finally, complementation of *p18* in the KO strain is required in order to check if we can revert the two observed phenotypes of the increased cyst formation and the delay of reactivation. This would be the clear cut linking p18 to such phenotypes, hence providing ultimate validation to our results.

BIBLIOGRAPHY

Ajioka, J. W., Fitzpatrick, J. M., & Reitter, C. P. (2001). Toxoplasma gondii genomics: Shedding light on pathogenesis and chemotherapy. Expert Reviews in Molecular Medicine, 3(01), 1-19.

Ajioka, J. W., Boothroyd, J. C., Brunk, B. P., Hehl, A., Hillier, L., Manger, I. D., . . . Sibley, L. D. (1998). Gene discovery by EST sequencing in toxoplasma gondii reveals sequences restricted to the apicomplexa. Genome Research, 8(1), 18-28.

Ajzenberg, D., Cogne, N., Paris, L., Bessieres, M. H., Thulliez, P., Filisetti, D., . . . Darde, M. L. (2002). Genotype of 86 toxoplasma gondii isolates associated with human congenital toxoplasmosis, and correlation with clinical findings. The Journal of Infectious Diseases, 186(5), 684-689.

Al-Anouti, F., Tomavo, S., Parmley, S., & Ananvoranich, S. (2004). The expression of lactate dehydrogenase is important for the cell cycle of toxoplasma gondii. The Journal of Biological Chemistry, 279(50), 52300-52311.

Alexander, D. L., Mital, J., Ward, G. E., Bradley, P., & Boothroyd, J. C. (2005). Identification of the moving junction complex of toxoplasma gondii: A collaboration between distinct secretory organelles. PLoS Pathogens, 1(2), e17.

Bahl, A., Davis, P. H., Behnke, M., Dzierszinski, F., Jagalur, M., Chen, F., . . . Roos, D. S. (2010). A novel multifunctional oligonucleotide microarray for toxoplasma gondii. BMC Genomics, 11, 603-2164-11-603.

Besteiro, S., Dubremetz, J., & Lebrun, M. (2011). The moving junction of apicomplexan parasites: A key structure for invasion. Cellular Microbiology, 13(6), 797-805.

Besteiro, S., Michelin, A., Poncet, J., Dubremetz, J., & Lebrun, M. (2009). Export of a toxoplasma gondii rhoptry neck protein complex at the host cell membrane to form the moving junction during invasion. PLoS Pathogens, 5(2), e1000309.

Beverley, J., & Beattie, C. (1958). Glandular toxoplasmosis a survey of 30 cases. The Lancet, 272(7043), 379-384.

Blader, I. J., & Saeij, J. P. (2009). Communication between toxoplasma gondii and its host: Impact on parasite growth, development, immune evasion, and virulence. Apmis, 117(5-6), 458-476.

Bohne, W., Gross, U., Ferguson, D. J., & Heesemann, J. (1995). Cloning and characterization of a bradyzoite-specifically expressed gene (hsp30/bag1) of toxoplasma gondii, related to genes encoding small heat-shock proteins of plants. Molecular Microbiology, 16(6), 1221-1230.

Boothroyd, J. C., Hehl, A., Knoll, L. J., & Manger, I. D. (1998). The surface of Toxoplasma: More and less. International Journal for Parasitology, 28(1), 3-9.

Bouhamdan, S. F., Bitar, L. K., Saghir, H. J., Bayan, A., & Araj, G. F. (2010). Seroprevalence of toxoplasma antibodies among individuals tested at hospitals and private laboratories in beirut. Le Journal Medical Libanais.the Lebanese Medical Journal, 58(1), 8-11.

Bowie, W. R., King, A. S., Werker, D. H., Isaac-Renton, J. L., Bell, A., Eng, S. B., & Marion, S. A. (1997). Outbreak of toxoplasmosis associated with municipal drinking water. The Lancet, 350(9072), 173-177.

Bradley, P. J., Ward, C., Cheng, S. J., Alexander, D. L., Coller, S., Coombs, G. H., . . . Boothroyd, J. C. (2005). Proteomic analysis of rhoptry organelles reveals many novel constituents for host-parasite interactions in toxoplasma gondii. The Journal of Biological Chemistry, 280(40), 34245-34258.

Burnett, A. J., Shortt, S. G., Isaac-Renton, J., King, A., Werker, D., & Bowie, W. R. (1998). Multiple cases of acquired toxoplasmosis retinitis presenting in an outbreak. Ophthalmology, 105(6), 1032-1037.

Carruthers, V. B., & Sibley, L. (1997). Sequential protein secretion from three distinct organelles of toxoplasma gondii accompanies invasion of human fibroblasts. European Journal of Cell Biology, 73(2), 114-123.

Chaudhry, S. A., Gad, N., & Koren, G. (2014). Toxoplasmosis and pregnancy. Canadian Family Physician Medecin De Famille Canadien, 60(4), 334-336.

Cleary, M. D., Singh, U., Blader, I. J., Brewer, J. L., & Boothroyd, J. C. (2002). Toxoplasma gondii asexual development: Identification of developmentally regulated genes and distinct patterns of gene expression. Eukaryotic Cell, 1(3), 329-340.

Dabritz, H., & Conrad, P. (2010). Cats and toxoplasma: Implications for public health. Zoonoses and Public Health, 57(1), 34-52.

Dabritz, H. A., Miller, M. A., Atwill, E. R., Gardner, I. A., Leutenegger, C. M., Melli, A. C., & Conrad, P. A. (2007). Detection of toxoplasma gondii-like oocysts in cat feces and estimates of the environmental oocyst burden. Journal of the American Veterinary Medical Association, 231(11), 1676-1684.

Desmonts, G., & Couvreur, J. (1974). Congenital toxoplasmosis: A prospective study of 378 pregnancies. New England Journal of Medicine, 290(20), 1110-1116.

Djurković-Djaković, O., Djokić, V., Vujanić, M., Živković, T., Bobić, B., Nikolić, A., . . . Ivović, V. (2012). Kinetics of parasite burdens in blood and tissues during murine toxoplasmosis. Experimental Parasitology, 131(3), 372-376.

Donald, R. G., & Roos, D. S. (1998). Gene knock-outs and allelic replacements in toxoplasma gondii: HXGPRT as a selectable marker for hit-and-run mutagenesis. Molecular and Biochemical Parasitology, 91(2), 295-305.

Donald, R. G., Carter, D., Ullman, B., & Roos, D. S. (1996). Insertional tagging, cloning, and expression of the toxoplasma gondii hypoxanthine-xanthine-guanine phosphoribosyltransferase gene. use as a selectable marker for stable transformation. The Journal of Biological Chemistry, 271(24), 14010-14019.

Dubey, J. P. (2008). The history of toxoplasma gondii—the first 100 years. Journal of Eukaryotic Microbiology, 55(6), 467-475.

Dubey, J. P. (2010). Toxoplasmosis of animals and humans CRC press.

Dubey, J. P., & Beattie, C. (1988). Toxoplasmosis of animals and man. CRC Press, Inc.

Dubey, J. (1996). Infectivity and pathogenicity of toxoplasma gondii oocysts for cats. The Journal of Parasitology, , 957-961.

Dubey, J. (1998). Advances in the life cycle of toxoplasma gondii. International Journal for Parasitology, 28(7), 1019-1024.

Dubey, J. (2001). Oocyst shedding by cats fed isolated bradyzoites and comparison of infectivity of bradyzoites of the VEG strain toxoplasma gondii to cats and mice. Journal of Parasitology, 87(1), 215-219.
Dubey, J. (2006). Comparative infectivity of oocysts and bradyzoites of toxoplasma gondii for intermediate (mice) and definitive (cats) hosts. Veterinary Parasitology, 140(1), 69-75.
Dubey, J. (2009). History of the discovery of the life cycle of toxoplasma gondii. International Journal for Parasitology, 39(8), 877-882.
Dubey, J., & Frenkel, J. (1972). Cyst-Induced toxoplasmosis in cats*. The Journal of Protozoology, 19(1), 155-177.
Dubey, J., & Frenkel, J. (1976). Feline toxoplasmosis from acutely infected mice and the development of toxoplasma cysts*. The Journal of Protozoology, 23(4), 537-546.
Dubey, J. P. (2007). 1 - the history and life cycle of toxoplasma gondii. In L. M. Weiss, & K. Kim (Eds.), Toxoplasma gondii (pp. 1-III). London: Academic Press.
Dubey, J. P., Lindsay, D. S., & Speer, C. A. (1998). Structures of toxoplasma gondii tachyzoites, bradyzoites, and sporozoites and biology and development of tissue cysts. Clinical Microbiology Reviews, 11(2), 267-299.
Dubremetz, J. F., & Lebrun, M. (2012). Virulence factors of toxoplasma gondii. Microbes and Infection, 14(15), 1403-1410.
Dumètre, A., & Dardé, M. (2003). How to detect toxoplasma gondii oocysts in environmental samples? FEMS Microbiology Reviews, 27(5), 651-661.
El Hajj, H., Demey, E., Poncet, J., Lebrun, M., Wu, B., Galéotti, N., . . . Labesse, G. (2006). The ROP2 family of toxoplasma gondii rhoptry proteins: Proteomic and genomic characterization and molecular modeling. Proteomics, 6(21), 5773-5784.
El Hajj, H., Lebrun, M., Fourmaux, M. N., Vial, H., & Dubremetz, J. F. (2007). Inverted topology of the toxoplasma gondii ROP5 rhoptry protein provides new insights into the association of the ROP2 protein family with the parasitophorous vacuole membrane. Cellular Microbiology, 9(1), 54-64.
Fekadu, A., Shibre, T., & Cleare, A. J. (2010). Toxoplasmosis as a cause for behaviour disorders--overview of evidence and mechanisms. Folia Parasitologica, 57(2), 105-113.
Ferguson, D. J. (2009). Toxoplasma gondii: 1908-2008, homage to nicolle, manceaux and splendore. Memórias do Instituto Oswaldo Cruz, 104(2), 133-148.
Ferguson, D. J., Parmley, S. F., & Tomavo, S. (2002). Evidence for nuclear localisation of two stage-specific isoenzymes of enolase in toxoplasma gondii correlates with active parasite replication. International Journal for Parasitology, 32(11), 1399-1410.
Ferguson, D. (2004). Use of molecular and ultrastructural markers to evaluate stage conversion of toxoplasma gondii in both the intermediate and definitive host. International Journal for Parasitology, 34(3), 347-360.
Fichera, M. E., & Roos, D. S. (1997). A plastid organelle as a drug target in apicomplexan parasites. Nature, 390(6658), 407-409.

Fox, B. A., & Bzik, D. J. (2002). De novo pyrimidine biosynthesis is required for virulence of toxoplasma gondii. Nature, 415(6874), 926-929.
Fox, B. A., Falla, A., Rommereim, L. M., Tomita, T., Gigley, J. P., Mercier, C., . . . Bzik, D. J. (2011). Type II toxoplasma gondii KU80 knockout strains enable functional analysis of genes required for cyst development and latent infection. Eukaryotic Cell, 10(9), 1193-1206.
Fox, B. A., Ristuccia, J. G., Gigley, J. P., & Bzik, D. J. (2009). Efficient gene replacements in toxoplasma gondii strains deficient for nonhomologous end joining. Eukaryotic Cell, 8(4), 520-529.
Frenkel, J. (1970). Pursuing toxoplasma. The Journal of Infectious Diseases, , 553-559.
Frenkel, J. (1973). Toxoplasma in and around us. Bioscience, 23(6), 343-352.
Frenkel, J. (1988). Pathophysiology of toxoplasmosis. Parasitology Today, 4(10), 273-278.
Fritz, H., Barr, B., Packham, A., Melli, A., & Conrad, P. (2012). Methods to produce and safely work with large numbers of toxoplasma gondii oocysts and bradyzoite cysts. Journal of Microbiological Methods, 88(1), 47-52.
Fritz, H. M., Bowyer, P. W., Bogyo, M., Conrad, P. A., & Boothroyd, J. C. (2012). Proteomic analysis of fractionated toxoplasma oocysts reveals clues to their environmental resistance. PloS One, 7(1), e29955.
Furtado, J. M., Winthrop, K. L., Butler, N. J., & Smith, J. R. (2013). Ocular toxoplasmosis I: Parasitology, epidemiology and public health. Clinical & Experimental Ophthalmology, 41(1), 82-94.
Gaskell, E. A., Smith, J. E., Pinney, J. W., Westhead, D. R., & McConkey, G. A. (2009). A unique dual activity amino acid hydroxylase in toxoplasma gondii. PLoS One, 4(3), e4801.
Gazzinelli, R. T., Hakim, F. T., Hieny, S., Shearer, G. M., & Sher, A. (1991). Synergistic role of CD4+ and CD8+ T lymphocytes in IFN-gamma production and protective immunity induced by an attenuated toxoplasma gondii vaccine. Journal of Immunology (Baltimore, Md.: 1950), 146(1), 286-292.
Gilson, P. R., & Crabb, B. S. (2009). Do apicomplexan parasite-encoded proteins act as both ligands and receptors during host cell invasion? F1000 Biology Reports, 1, 64-64. Epub 2009 Aug 26.
Goldman, M., Carver, R. K., & Sulzer, A. J. (1958). Reproduction of toxoplasma gondii by internal budding. The Journal of Parasitology, , 161-171.
Groër, M. W., Yolken, R. H., Xiao, J., Beckstead, J. W., Fuchs, D., Mohapatra, S. S., . . . Postolache, T. T. (2011). Prenatal depression and anxiety in toxoplasma gondii–positive women. American Journal of Obstetrics and Gynecology, 204(5), 433. e1-433. e7.
Gubbels, M., Lehmann, M., Muthalagi, M., Jerome, M. E., Brooks, C. F., Szatanek, T., . . . Striepen, B. (2008). Forward genetic analysis of the apicomplexan cell division cycle in toxoplasma gondii. PLoS Pathogens, 4(2), e36.
Haber, J. E. (2000). Partners and pathways: Repairing a double-strand break. Trends in Genetics, 16(6), 259-264.
Hager, K. M., Striepen, B., Tilney, L. G., & Roos, D. S. (1999). The nuclear envelope serves as an intermediary between the ER and golgi complex in the intracellular

parasite toxoplasma gondii. Journal of Cell Science, 112 (Pt 16)(Pt 16), 2631-2638.
Hammond, D. (1973). Life cycles and development of coccidia. The Coccidia: Eimeria, Isospora, Toxoplasma, and Related Genera.University Park Press, Baltimore, Md, , 45-79.
He, X., Grigg, M. E., Boothroyd, J. C., & Garcia, K. C. (2002). Structure of the immunodominant surface antigen from the toxoplasma gondii SRS superfamily. Nature Structural & Molecular Biology, 9(8), 606-611.
Holland, G. N. (2003). Ocular toxoplasmosis: A global reassessment:. part I: Epidemiology and course of disease. American Journal of Ophthalmology, 136(6), 973-988.
Hsu, P., Groer, M., & Beckie, T. (2014). New findings: Depression, suicide, and toxoplasma gondii infection. Journal of the American Association of Nurse Practitioners.
Hunter, C. A., & Sibley, L. D. (2012). Modulation of innate immunity by toxoplasma gondii virulence effectors. Nature Reviews Microbiology, 10(11), 766-778.
Hutchison, W. M., Dunachie, J. F., Siim, J. C., & Work, K. (1970). Coccidian-like nature of toxoplasma gondii. British Medical Journal, 1(5689), 142-144.
Iida, H., & Yahara, I. (1985). Yeast heat-shock protein of mr 48,000 is an isoprotein of enolase.
Ingram, W. M., Goodrich, L. M., Robey, E. A., & Eisen, M. B. (2013). Mice infected with low-virulence strains of toxoplasma gondii lose their innate aversion to cat urine, even after extensive parasite clearance. PloS One, 8(9), e75246.
Iqbal, J., & Khalid, N. (2007). Detection of acute toxoplasma gondii infection in early pregnancy by IgG avidity and PCR analysis. Journal of Medical Microbiology, 56(Pt 11), 1495-1499.
Jacobs, L., Fair, J. R., & Bickerton, J. H. (1954). Adult ocular toxoplasmosis: Report of a parasitologically proved case. AMA Archives of Ophthalmology, 52(1), 63-71.
Jacobs, L., Remington, J. S., & Melton, M. L. (1960). The resistance of the encysted form of toxoplasma gondii. The Journal of Parasitology, , 11-21.
Jacobs, L., Remington, J. S., & Melton, M. L. (1960). A survey of meat samples from swine, cattle, and sheep for the presence of encysted toxoplasma. The Journal of Parasitology, , 23-28.
Jacquet, A., Coulon, L., De Nève, J., Daminet, V., Haumont, M., Garcia, L., . . . Biemans, R. (2001). The surface antigen SAG3 mediates the attachment of toxoplasma gondii to cell-surface proteoglycans. Molecular and Biochemical Parasitology, 116(1), 35-44.
Jost, C., Reiter-Owona, I., & Liesenfeld, O. (2007). The timing of sulfadiazine therapy impacts the reactivation of latent toxoplasma infection in IRF-8−/− mice. Parasitology Research, 101(6), 1603-1609.
Jung, C., Lee, C. Y., & Grigg, M. E. (2004). The SRS superfamily of Toxoplasma surface proteins. International Journal for Parasitology, 34(3), 285-296.
Khan, A., Taylor, S., Su, C., Mackey, A. J., Boyle, J., Cole, R., . . . Sibley, L. D. (2005). Composite genome map and recombination parameters derived from three

archetypal lineages of toxoplasma gondii. Nucleic Acids Research, 33(9), 2980-2992.
Kibe, M. K., Coppin, A., Dendouga, N., Oria, G., Meurice, E., Mortuaire, M., . . . Tomavo, S. (2005). Transcriptional regulation of two stage-specifically expressed genes in the protozoan parasite toxoplasma gondii. Nucleic Acids Research, 33(5), 1722-1736.
Kim, K., & Weiss, L. M. (2004). Toxoplasma gondii: The model apicomplexan. International Journal for Parasitology, 34(3), 423-432.
Kim, S. K., Karasov, A., & Boothroyd, J. C. (2007). Bradyzoite-specific surface antigen SRS9 plays a role in maintaining toxoplasma gondii persistence in the brain and in host control of parasite replication in the intestine. Infection and Immunity, 75(4), 1626-1634.
Knoll, L., & Boothroyd, J. (1998). Molecular biology's lessons about Toxoplasma development: Stage-specific homologs. Parasitology Today, 14(12), 490-493.
Knoll, L. J., Tomita, T., & Weiss, L. M. (2014). Chapter 15 - bradyzoite development. In L. M. Weiss, & K. Kim (Eds.), Toxoplasma gondii (second edition) (pp. 521-549). Boston: Academic Press.
Lamarque, M., Besteiro, S., Papoin, J., Roques, M., Vulliez-Le Normand, B., Morlon-Guyot, J., . . . Faber, B. W. (2011). The RON2-AMA1 interaction is a critical step in moving junction-dependent invasion by apicomplexan parasites. PLoS Pathogens, 7(2), e1001276.
Lebrun, M., Michelin, A., El Hajj, H., Poncet, J., Bradley, P. J., Vial, H., & Dubremetz, J. F. (2005). The rhoptry neck protein RON4 relocalizes at the moving junction during toxoplasma gondii invasion. Cellular Microbiology, 7(12), 1823-1833.
Lekutis, C., Ferguson, D. J., & Boothroyd, J. C. (2000). Toxoplasma gondii: identification of a developmentally regulated family of genes related to SAG2. Experimental Parasitology, 96(2), 89-96.
Levine, N. D. (1980). Some corrections of coccidian (apicomplexa: Protozoa) nomenclature. The Journal of Parasitology, , 830-834.
Liesenfeld, O., Press, C., Montoya, J. G., Gill, R., Isaac-Renton, J. L., Hedman, K., & Remington, J. S. (1997). False-positive results in immunoglobulin M (IgM) toxoplasma antibody tests and importance of confirmatory testing: The platelia toxo IgM test. Journal of Clinical Microbiology, 35(1), 174-178.
Lindsay, D. S., & Dubey, J. (2009). Long-term survival of toxoplasma gondii sporulated oocysts in seawater. Journal of Parasitology, 95(4), 1019-1020.
Luft, B., Conley, F., Remington, J., Laverdiere, M., Levine, J., Strandberg, D., . . . Rice, N. (1983). Outbreak of central-nervous-system toxoplasmosis in western europe and north america. The Lancet, 321(8328), 781-784.
Lyons, R. E., McLeod, R., & Roberts, C. W. (2002). Toxoplasma gondii tachyzoite–bradyzoite interconversion. Trends in Parasitology, 18(5), 198-201.
Mcfadden, G. I., Waller, R. F., Reith, M. E., & Lang-Unnasch, N. (1997). Plastids in apicomplexan parasites Springer.
Mele, A., Paterson, P. J., Prentice, H. G., Leoni, P., & Kibbler, C. C. (2002). Toxoplasmosis in bone marrow transplantation: A report of two cases and

systematic review of the literature. Bone Marrow Transplantation, 29(8), 691-698.
Melo, M. B., Jensen, K. D., & Saeij, J. P. (2011). Toxoplasma gondii effectors are master regulators of the inflammatory response. Trends in Parasitology, 27(11), 487-495.
Miller, N. L., Frenkel, J., & Dubey, J. (1972). Oral infections with toxoplasma cysts and oocysts in felines, other mammals, and in birds. The Journal of Parasitology, , 928-937.
Mordue, D. G., Desai, N., Dustin, M., & Sibley, L. D. (1999). Invasion by toxoplasma gondii establishes a moving junction that selectively excludes host cell plasma membrane proteins on the basis of their membrane anchoring. The Journal of Experimental Medicine, 190(12), 1783-1792.
Morrissette, N. S., Murray, J. M., & Roos, D. S. (1997). Subpellicular microtubules associate with an intramembranous particle lattice in the protozoan parasite toxoplasma gondii. Journal of Cell Science, 110 (Pt 1)(Pt 1), 35-42.
Munoz, M., Heimesaat, M. M., Danker, K., Struck, D., Lohmann, U., Plickert, R., . . . Liesenfeld, O. (2009). Interleukin (IL)-23 mediates toxoplasma gondii-induced immunopathology in the gut via matrixmetalloproteinase-2 and IL-22 but independent of IL-17. The Journal of Experimental Medicine, 206(13), 3047-3059.
Murray, P. R., Rosenthal, K. S., & Pfaller, M. A. (2013). Medical microbiology, with STUDENT CONSULT online access, 7: Medical microbiology Elsevier Health Sciences.
Nagel, S. D., & Boothroyd, J. C. (1989). The major surface antigen, P30, of toxoplasma gondii is anchored by a glycolipid. The Journal of Biological Chemistry, 264(10), 5569-5574.
NICHOLS, B. A., & CHIAPPINO, M. L. (1987). Cytoskeleton of toxoplasma gondii1. The Journal of Protozoology, 34(2), 217-226.
Nicolle, C., & Manceaux, L. (1908). Sur une infection à corps de leishman (ou organismes voisins) du gondi. CR Acad Sci, 147(763).
Ödberg-Ferragut, C., Soête, M., Engels, A., Samyn, B., Loyens, A., Van Beeumen, J., . . . Dubremetz, J. (1996). Molecular cloning of the toxoplasma gondii sag4 gene encoding an 18 kDa bradyzoite specific surface protein. Molecular and Biochemical Parasitology, 82(2), 237-244.
Parmley, S. F., Weiss, L. M., & Yang, S. (1995). Cloning of a bradyzoite-specific gene of toxoplasma gondii encoding a cytoplasmic antigen. Molecular and Biochemical Parasitology, 73(1), 253-257.
Perkins, E. S. (1961). Uveitis and toxoplasmosis. Uveitis and Toxoplasmosis.,
Pinkerton, H., & HENDERSON, R. G. (1941). Adult toxoplasmosis: Previously unrecognized disease entity simulating the typhus-spotted fever group. Journal of the American Medical Association, 116(9), 807-814.
Pinkerton, H., & Weinman, D. (1940). Toxoplasma infection in man. Arch.Pathol., 30(1), 374-392.
Roos, D. S. (2005). Themes and variations in apicomplexan parasite biology. Science (New York, N.Y.), 309(5731), 72-73.

Rosowski, E. E. (2013). Modulation of Innate Immune Signaling Pathways by the Intracellular Pathogen Toxoplasma Gondii, (Doctoral dissertation, Massachusetts Institute of Technology).
Sabin, A. B., & Feldman, H. A. (1948). Dyes as microchemical indicators of a new immunity phenomenon affecting a protozoon parasite (toxoplasma). Science (New York, N.Y.), 108(2815), 660-663.
Sabin, A. B., & Olitsky, P. K. (1937). Toxoplasma and obligate intracellular parasitism. Science (New York, N.Y.), 85(2205), 336-338.
Saeij, J. P., Boyle, J. P., & Boothroyd, J. C. (2005). Differences among the three major strains of toxoplasma gondii and their specific interactions with the infected host. Trends in Parasitology, 21(10), 476-481.
Schmittgen, T. D., & Livak, K. J. (2008). Analyzing real-time PCR data by the comparative CT method. Nature Protocols, 3(6), 1101-1108.
Scholtyseck, E., & Mehlhorn, H. (1970). Ultrastructural study of characteristic organelles (paired organelles, micronemes, micropores) of sporozoa and related organisms. Zeitschrift Für Parasitenkunde, 34(2), 97-127.
Sheffield, H. G., & Melton, M. L. (1968). The fine structure and reproduction of toxoplasma gondii. The Journal of Parasitology, , 209-226.
Sheffield, H. G., & Melton, M. L. (1970). Toxoplasma gondii: The oocyst, sporozoite, and infection of cultured cells. Science (New York, N.Y.), 167(3919), 892-893.
Sibley, L. D., & Boothroyd, J. C. (1992). Virulent strains of toxoplasma gondii comprise a single clonal lineage. Nature, 359(6390), 82-85.
Siim, J. C. (1956). Toxoplasmosis acquisita lymphonodosa: Clinical and pathological aspects. Annals of the New York Academy of Sciences, 64(2), 185-206.
Silveira, C., Belfort Jr, R., Muccioli, C., Abreu, M. T., Martins, M. C., Victora, C., . . . Holland, G. N. (2001). A follow-up study of toxoplasma gondii infection in southern brazil. American Journal of Ophthalmology, 131(3), 351-354.
Silveira, C., Belfort, R.,Jr, Burnier, M.,Jr, & Nussenblatt, R. (1988). Acquired toxoplasmic infection as the cause of toxoplasmic retinochoroiditis in families. American Journal of Ophthalmology, 106(3), 362-364.
Skariah, S., McIntyre, M. K., & Mordue, D. G. (2010). Toxoplasma gondii: Determinants of tachyzoite to bradyzoite conversion. Parasitology Research, 107(2), 253-260.
Speer, C., & Dubey, J. (2005). Ultrastructural differentiation of toxoplasma gondii schizonts (types B to E) and gamonts in the intestines of cats fed bradyzoites. International Journal for Parasitology, 35(2), 193-206.
Sukthana, Y., Waree, P., Pongponratn, E., Chaisri, U., & Riganti, M. (2003). Pathologic study of acute toxoplasmosis in experimental animals.
Tenter, A. M., Heckeroth, A. R., & Weiss, L. M. (2000). Toxoplasma gondii: From animals to humans. International Journal for Parasitology, 30(12), 1217-1258.
Teutsch, S. M., Juranek, D. D., Sulzer, A., Dubey, J., & Sikes, R. K. (1979). Epidemic toxoplasmosis associated with infected cats. New England Journal of Medicine, 300(13), 695-699.

Thirugnanam, S., Rout, N., & Gnanasekar, M. (2013). Possible role of toxoplasma gondii in brain cancer through modulation of host microRNAs. Infect Agent Cancer, 8(8).

Tomavo, S., Fortier, B., Soete, M., Ansel, C., Camus, D., & Dubremetz, J. F. (1991). Characterization of bradyzoite-specific antigens of toxoplasma gondii. Infection and Immunity, 59(10), 3750-3753.

Tomavo, S., Schwarz, R. T., & Dubremetz, J. F. (1989). Evidence for glycosyl-phosphatidylinositol anchoring of toxoplasma gondii major surface antigens. Molecular and Cellular Biology, 9(10), 4576-4580.

Tomita, T., Bzik, D. J., Ma, Y. F., Fox, B. A., Markillie, L. M., Taylor, R. C., . . . Weiss, L. M. (2013). The toxoplasma gondii cyst wall protein CST1 is critical for cyst wall integrity and promotes bradyzoite persistence. PLoS Pathogens, 9(12), e1003823.

Van, T. T., Kim, S., Camps, M., Boothroyd, J. C., & Knoll, L. J. (2007). The BSR4 protein is up-regulated in toxoplasma gondii bradyzoites, however the dominant surface antigen recognised by the P36 monoclonal antibody is SRS9. International Journal for Parasitology, 37(8), 877-885.

Walker, J. R., Corpina, R. A., & Goldberg, J. (2001). Structure of the ku heterodimer bound to DNA and its implications for double-strand break repair. Nature, 412(6847), 607-614.

Walker, R., Gissot, M., Croken, M. M., Huot, L., Hot, D., Kim, K., & Tomavo, S. (2013). The toxoplasma nuclear factor TgAP2XI-4 controls bradyzoite gene expression and cyst formation. Molecular Microbiology, 87(3), 641-655.

Webster, J. P., & McConkey, G. A. (2010). Toxoplasma gondii-altered host behaviour: Clues as to mechanism of action. Folia Parasitologica, 57(2), 95-104.

Weilhammer, D. R., & Rasley, A. (2011). Genetic approaches for understanding virulence in toxoplasma gondii. Briefings in Functional Genomics, 10(6), 365-373.

Weiss, L. M., & Dubey, J. P. (2009). Toxoplasmosis: A history of clinical observations. International Journal for Parasitology, 39(8), 895-901.

Weiss, L. M., & Kim, K. (2000). The development and biology of bradyzoites of toxoplasma gondii. Frontiers in Bioscience: A Journal and Virtual Library, 5, D391-405.

Wilder, H. C. (1952). Toxoplasma chorioretinitis in adults. AMA Archives of Ophthalmology, 48(2), 127-136.

Wolf, A., Cowen, D., & Paige, B. (1939). Human toxoplasmosis: Occurrence in infants as an encephalomyelitis verification by transmission to animals. Science (New York, N.Y.), 89(2306), 226-227.

Wolf, A., Cowen, D., & Paige, B. H. (1939). Toxoplasmic encephalomyelitis: III. A new case of granulomatous encephalomyelitis due to a protozoon. The American Journal of Pathology, 15(6), 657-694.11.

Wu, W., Wang, M., Wu, W., Singh, S. K., Mussfeldt, T., & Iliakis, G. (2008). Repair of radiation induced DNA double strand breaks by backup NHEJ is enhanced in G2. DNA Repair, 7(2), 329-338.

Yang, S., & Parmley, S. F. (1995). A bradyzoite stage-specifically expressed gene of toxoplasma gondii encodes a polypeptide homologous to lactate dehydrogenase. Molecular and Biochemical Parasitology, 73(1), 291-294.

Yang, S., & Parmley, S. F. (1997). Toxoplasma gondii expresses two distinct lactate dehydrogenase homologous genes during its life cycle in intermediate hosts. Gene, 184(1), 1-12.

Yolken, R., Dickerson, F., & Fuller Torrey, E. (2009). Toxoplasma and schizophrenia. Parasite Immunology, 31(11), 706-715.

Zeiner, G. M., Norman, K. L., Thomson, J. M., Hammond, S. M., & Boothroyd, J. C. (2010). Toxoplasma gondii infection specifically increases the levels of key host microRNAs. Plos One, 5(1), e8742.

Zhang, Y. W., Halonen, S. K., Ma, Y. F., Wittner, M., & Weiss, L. M. (2001). Initial characterization of CST1, a toxoplasma gondii cyst wall glycoprotein. Infection and Immunity, 69(1), 501-507.