IN-VIVO INDUCED ANTIGENS OF *Toxoplasma gondii* AND THEIR APPLICATION IN DIAGNOSIS OF HUMAN TOXOPLASMOSIS

BY

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To my parents

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APS	ammonium persulphate
BLAST	Basic Local Alignment Search Tool
bp	base pair
BSA	bovine serum albumin
cDNA	Complementary DNA
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
ESTs	expressed sequence tags
EtBr	Ethidium bromide
HRP	horse-radish peroxidase
i.e	id est (that is)
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
INFORMM	Institute for Research in Molecular Medicine
INF-γ	Interferon γ
IPTG	Isopropyl-beta-D-thiogalactopyranoside
IVIAT	*in-vivo* induced antigen technology
ivi	*in-vivo* induced
kDa	kilo Dalton
LB	Luria-Bertani
MW	molecular weight
NCP	nitrocellulose membrane
Ni-NTA	Nickel-nitrilotriacetic acid
OD	Optical Density
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
pfu	plaque forming units
qPCR	Quantitative PCR
RT-PCR	Reverse Transcriptase PCR
RNA	Ribonucleic acid
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TB	Terrific-Broth
TBE	Tris/Borate/EDTA
TBS	tris buffered saline
T. gondii	*Toxoplasma gondii*
UV	ultraviolet

ANTIGEN ARUHAN *IN-VIVO Toxoplasma gondii* DAN APLIKASINYA DALAM DIAGNOSIS TOKSOPLASMOSIS PADA MANUSIA

ABSTRAK

Toxoplasma gondii bertaburan secara meluas di seluruh dunia, dengan prevalens yang tinggi di kawasan tropika. Jangkitan primer oleh *T.gondii* dalam wanita hamil mungkin mengakibatkan jangkitan kongenital terhadap fetus, manakala pesakit jangkitan kronik dengan system imun terkompromi adalah berisiko tinggi untuk mengalami pengaktifan kembali penyakit tersebut di sepanjang kehidupan mereka. Diagnosis bagi toksoplasmosis biasanya dijalankan melalui pengesanan antibodi IgM dan IgG yang spesifik terhadap *T.gondii*. Kekurangan sensitiviti dalam kebanyakan ujian IgM (khususnya bagi jangkitan kongenital) dan isu spesifisiti dalam ujian-ujian IgG dan IgM adalah masih wujud. Oleh itu, kajian perlu dilakukan untuk mengenalpasti antigen *T. gondii* yang boleh meningkatkan ketepatan diagnosis bagi toksoplasmosis.

Teknologi antigen aruhan *in-vivo* (IVIAT) adalah kaedah yang sesuai untuk mengesan antigen-antigen baru dengan nilai diagnostik, memandangkan antigen aruhan *in-vivo* dianggap berkait secara langsung dengan jangkitan manusia yang sebenar. Dalam kajian ini, IVIAT menggunakan saringan imun perpustakaan cDNA faj *T. gondii* dan prob IgG/IgM dijalankan menggunakan serum terjerap daripada pesakit yang mempunyai bukti serologi bagi jangkitan kronik dan akut. Penjerapan serum dilakukan menggunakan tiga persediaan antigen yang berbeza daripada setiap sel *E.coli* XL-1 Blue MRF' dan *T. gondii* yang ditumbuhkan secara *in-vitro*; seterusnya serum terjerap tadi digunakan untuk menyaring perpustakaan cDNA faj

ekspresi *T.gondii*. Klon yang mempamerkan reaktiviti yang tinggi kemudiannya dijujuki, dan klon yang mempunyai homologi yang tinggi dengan *T.gondii* seterusnya dianalisis kadar pengekspresannya menggunakan *PCR masa-nyata* kuantitatif.

Melalui IVIAT yang menggunakan serum kronik dan prob IgG,8 klon reaktif telah ditemui memiliki homologi yang tinggi terhadap gen *T.gondii.* Analisis pengekspresan melalui *PCR masa-nyata* kuantitatif. menunjukkan 'SAG-1 related sequence 3'(*SRS3*) dan dua gen hipotetikal adalah 'up-regulated' secara *in-vivo* relatif kepada tahap pengekspresannya secara *in-vitro*. Melalui IVIAT menggunakan serum akut, 29 klon reaktif dari setiap saringan imun IgM dan IgG telah ditemui mempunyai homologi yang tinggi dengan gen *T. gondii*. Analisis pengekspresan *PCR masa-nyata* kuantitatif menunjukkan bahawa 21 gen yang dikesan oleh IgM dan 11 gen yang dikesan oleh IgG adalah 'up-regulated' secara *in-vivo* relatif kepada tahap pengekspresannya secara *in-vitro*. Empat belas klon menunjukkan lebih daripada 10 kali ganda tahap pengekspresan secara *in-vivo* berbanding secara *in-vitro*. Satu klon yang dikesan oleh IgM (AM15) telah ditemui secara ekslusifnya diekspreskan secara *in-vivo* tetapi bukan *in-vitro,* dengan kadar 'fold change' sebanyak 1217. Dengan menggunakan sampel serum individu, klon ini menunjukkan sensitiviti yang tinggi (100%, n=18) dan spesifisiti yang tinggi (100%, n=10) bagi mengesan antibodi *T. gondii* spesifik IgM. Ia dikenalpasti sebagai gen yang mengekspreskan RAP 'domain-containing protein' (TGME49_269830).

Jujukan sisipan DNA bagi klon AM15 telah di 'custom-cloned' ke dalam vektor ekspresi (pET28), diikuti oleh pengekspresan dan penulenan protein rekombinan (TgRA15); dan pengesahan identity oleh MALDI-TOF-TOF. Protein rekombinan TgRA15 menunjukkan 100% (15/15) sensitiviti diagnostik dan 97% (29/30)

spesifisiti diagnostik untuk mengesan antibodi IgM anti-*Toxoplasma*. Satu 'lateral flow dipstick dot test' menggunakan protein rekombinan TgRA15 telah dibangunkan dan ia menunjukkan 100% (27/27) sensitiviti diagnostik dan 97% (29/30) spesifisiti diagnostik untuk pengesanan IgM *Toxoplasma.*

Secara kesimpulannya, kajian ini telah mengenalpasti beberapa antigen aruhan *in-vivo* menggunakan sampel serum daripada pesakit akut dan kronik yang dijangkiti oleh *Toxoplasma*, dan prob IgM dan IgG. Antaranya, klon AM15 yang dikenalpasti menggunakan prob IgM, secara eksklusif diekspresi secara *in-vivo.* Protein rekombinan tulen TgRA15, menunjukkan sensitiviti dan spesifisiti diagnostik yang tinggi, dan ujian 'dipstik dot' yang dibangunkan menggunakannya menunjukkan potensi yang baik untuk diaplikasikan sebagai ujian pantas IgM bagi mengesan penyakit toksoplasmosis pada manusia.

IN-VIVO INDUCED ANTIGENS OF *Toxoplasma gondii* AND THEIR APPLICATION IN DIAGNOSIS OF HUMAN TOXOPLASMOSIS

ABSTRACT

Toxoplasma gondii is widely distributed throughout the world, with higher prevalence in tropical areas. Primary infection with *T. gondii* during pregnancy may result in congenital infection of the fetus while chronically-infected patients with compromised immune system are at higher risk for reactivation of the disease later in their life. Diagnosis of *T. gondii* infection is usually performed by detection of IgM and IgG antibodies against *T. gondii.* The lack of sensitivity in many IgM tests (particularly for congenital infection) and issues of specificities in IgG and IgM tests still exist. Thus research is still needed to identify *T. gondii* antigens that can improve the accuracy of diagnosis of toxoplasmosis.

In-vivo induced antigen technology (IVIAT) is a promising method for identification of new antigens of diagnostic value, since *in-vivo* expressed antigens are thought to be directly related to the actual human infection. In this study, IVIAT using *T. gondii* cDNA phage library immunoscreening and IgG/IgM probes were performed using pre-absorbed sera from patients with serological evidence of chronic and acute infection. Sera pre-adsorption was performed against three different preparations of antigens from *in-vitro*-grown cells of each *E. coli* XL1-Blue MRF' and *T. gondii*; subsequently the adsorbed sera was used to screen *T. gondii* cDNA phage expression library. The strongly reactive clones were sequenced, and those with high homology to *T. gondii* were subjected to expression analysis using quantitative real-time PCR.

With IVIAT using chronic sera and IgG probe, 8 reactive clones were found to have high homology to *T. gondii* genes. Expression analysis using real-time PCR showed that SAG-1 related sequence 3 (*SRS3*) and two hypothetical genes were up-regulated

in-vivo relative to their expression levels *in-vitro*. With IVIAT using acute sera, 29 reactive clones from each IgM and IgG immunoscreenings were found to have high homology to *T. gondii* genes. Real-time PCR expression analysis showed that 21 IgM-detected genes and 11 IgG-detected genes were up-regulated *in-vivo* relative to their expression levels *in-vitro*. Fourteen clones showed more than 10 times fold expression levels *in-vivo* as compared to *in-vitro*. An IgM detected clone (AM15) was found to be almost exclusively expressed *in-vivo* but not *in-vitro,* with 1217 fold change. Using individual serum samples, this clone showed high sensitivity (100%, n=18) and specificity (100%, n=10) for detection of *T. gondii*-specific IgM antibody. It was identified as a gene that expresses RAP (RNA associated protein) domain-containing protein (TGME49_269830).

The DNA sequence insert of AM15 clone was custom-cloned into an expression vector (pET 28), followed by expression and purification of the recombinant protein (TgRA15); and confirmation by MALDI-TOF-TOF. The TgRA15 recombinant protein demonstrated 100% diagnostic sensitivity (n=15) and 97% specificity (29/30) for detection of anti-*Toxoplasma* IgM antibody. A lateral flow dipstick dot test using TgRA15 recombinant protein was developed and it showed 100% diagnostic sensitivity (n=27) and 97% specificity (29/30) for *Toxoplasma* IgM detection.

In summary, this study has identified a panel of *in-vivo* induced antigens using serum samples from acute and chronic *Toxoplasma*-infected patients, and IgM and IgG probes. Among them, clone AM15 identified using IgM probe, was found to be exclusively and highly expressed *in-vivo*. The purified recombinant protein TgRA15, showed high diagnostic sensitivity and specificity; and the dipstick dot test developed using this recombinant antigen showed good potential for application as an IgM rapid test to diagnose human toxoplasmosis.

CHAPTER ONE

INTRODUCTION

1.1 Overview of *Toxoplasma gondii* and toxoplasmosis

Toxoplasma gondii is an obligate intracellular parasite from the the phylum Apicomplexa. It is capable of producing life-long chronic infection in humans and warm blooded animals especially in the tropical countries. Approximately one third of the world's population is infected with this protozoan parasite. Environmental, cultural factors and eating habits are thought to be key contributing factors in transmission of this infection. The word *Toxoplasma* comes from the Greek world toxon that mean "bow" and plasmid which mean "form". Cats and other Felidae members are the definitive hosts for this parasite where the sexual phase occurs (Dubey, 2008; Thompson *et al*., 2009).

Toxoplasmosis is divided into three groups; congenital toxoplasmosis, acquired toxoplasmosis and reactivated/ recrudescent toxoplasmosis. Acquired toxoplasmosis is caused by consumption of food or water contaminated with the parasite from faeces of infected cats. Congenital toxoplasmosis is the result of transmission of the parasite from infected mother to her unborn child. In immunocompetent individuals infection is usually asymptomatic and these people remain chronically infected with presence of cysts in the body especially brain. However during immunosuppression, reactivations of the disease can occur due to the conversion of slowly dividing bradyzoites into rapidly proliferating tachyzoites which may be influenced by genetic predisposition or diversity in virulence between different parasite strains (Kopecna, 2006; Dubey, 2010; Feustel *et al*., 2012).

1.2 Historical background and taxonomy

T. gondii was initially found in the liver and lymph of the gundi (*Ctenodactylus gundi)*, a small rodent from North Africa by Charles Nicolle and Louis Manceaux in 1908 and independently by Splendore in Brazil. Then the same parasite was found by Mello in the lung of a dog in Turin Italy. He was also the first to report on acute canine toxoplasmosis (Miro *et al*., 2008; Ferguson, 2009). However the first case of human toxoplasmosis with the description of *Toxoplasma*-like organisms was identified in human retinal tissue by the ophthalmologist Josef Jankú from Czechoslovakia in 1923. The parasite was isolated from retinal tissue of neonates followed by additional reports on congenital infection caused by this parasite by Wolf in 1939. The life cycle of this parasite remained unknown until 1970, when several scientists found that the definitive host is the cat. Genetic diversity among *T. gondii* strains from animals and humans was first performed by Lehman in 2006 who reported geographic differences; including some strains which were found to be limited to Brazil only while others were found to be worldwide. Mapping of *T. gondii* genome was performed by Dubey in 2005 (Miro *et al*., 2008; Dubey 2008; Ferguson, 2009). According to the classical taxonomy, the parasite was placed in the domain Eukarya, kingdom Alveolata, phylum Apicomplexa, class Coccidia, order Eucoccidiorida, family Sarcocystidae, genus *Toxoplasma* and species *T. gondii* (Pereira *et al*., 2010). Figure 1.1 shows the taxonomical classification of Apicomplexan parasites including *T. gondii*.

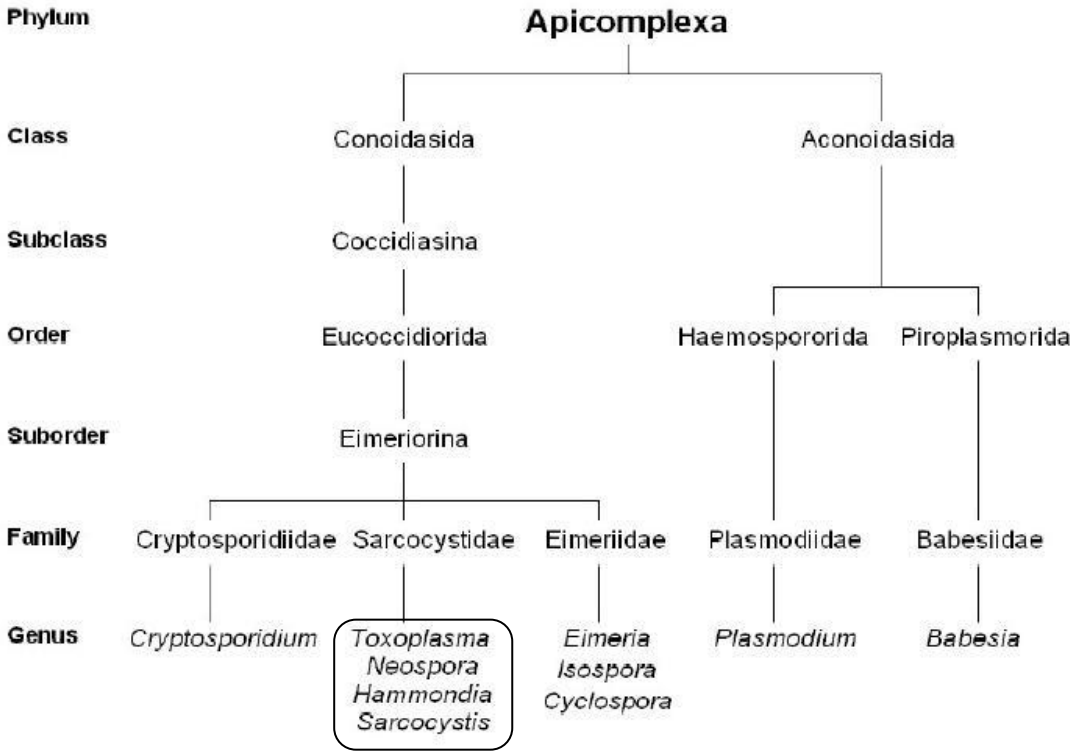


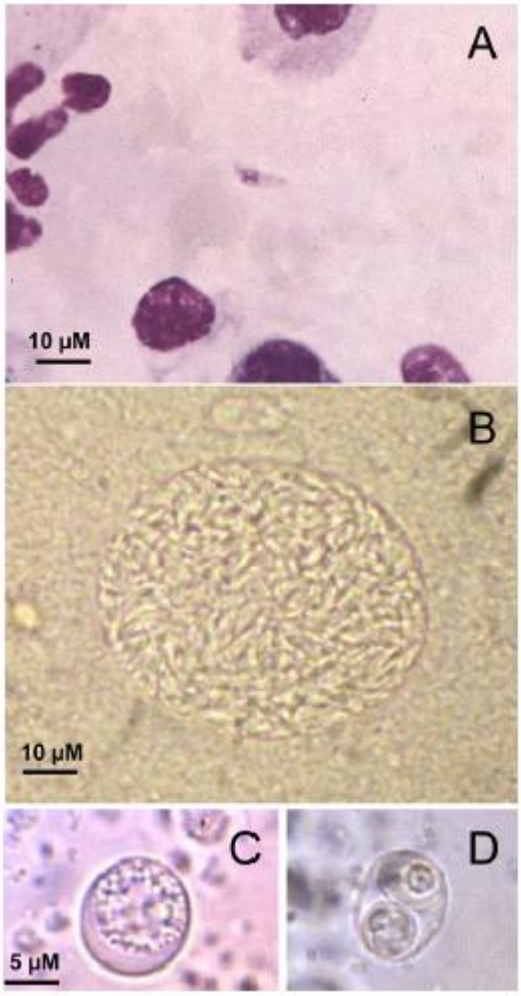
Figure 1.1 Taxonomical classifications of Apicomplexan parasites including *Toxoplasma* (Roberts and Janovy, 2005).

1.3 Morphology

There are three infectious stages of *T. gondii*; i.e tachyzoites (in groups or clones), bradyzoites (in tissue cysts), and sporozoites (in oocysts) (Dubey, 2008) as shown in Figure 1.2.

1.3.1 Tachyzoites

Frenkel in 1973 for the first time assigned the term "tachyzoite" (tachos = speed in Greek) to describe the rapidly multiplying stage in an intermediate host cell and in non-intestinal epithelial cells of definitive host. The term "tachyzoite" replaces the term "trophozoite" (trophicos = feeding in Greek) which was previously used. Tachyzoites have also been termed endozoites or endodyozoites. Aggregates of multiple tachyzoites are called terminal colonies, clones, or groups. The tachyzoite is a crescent shaped structure, approximately 2 by 6 µm (Figure 1.2a), with a pointed anterior (conoidal) end and a rounded posterior end. Ultrastructurally, the tachyzoite is composed of various organelles and inclusion bodies such as a pellicle (outer covering), polar rings, apical rings, micronemes, conoid, micropore, rhoptries, mitochondrion, golgi complex, endoplasmic reticulum, subpellicular microtubules, rough and smooth endoplasmic reticula, micropore, ribosomes, dense granules, nucleus, amylopectin granules (which might be absent), and a multiple-membrane-bound plastid- like organelle which has also been called a golgi adjunct or apicoplast. The nucleus is usually situated toward the central area of the cell and contains clumps of chromatin and a centrally-located nucleolus (Dubey *et al*., 1998; Dubey, 2010).



A) Tachyzoites stained with Giemsa

B) Cyst in the brain containing bradyzoites

C) Unsporulated oocyt

D) Sporulated oocyt

Figure 1.2 Images of tachyzoites, bradyzoites and oocysts of *T. gondii* (Robert-Gangneuxa and Dardéc, 2012)

This proliferative form can infect every kind of nucleated cells including phagocytic and non-phagositic cells by active transportation (Waree, 2008). Goldman in 1958 introduced endodyogeny as a specialized kind of multiplication which occurs when two progeny form inside the parent parasite. The replication of tachyzoites inside the host cells leads to damage and rupture of the cells. The release of tachyzoites into the blood stream causes intense inflammatory response and tissue infection especially for some organs such as eyes, skeletal and heart muscle. When the host immune response system takes control of infection process, the tachyzoites which are responsible for acute stage of infection transform to slowly dividing bradyzoites that are related to chronic stage of infection (Montoya and Liesenfeld, 2004; Waree, 2008; Dubey, 2010). Figure 1.3 shows the schematic image of structures of tachyzoites.

1.3.2 Bradyzoites

Frenkel in 1973 coined the term "bradyzoite" (brady = slow in Greek) to describe the slowly multiplying organism within a tissue cyst responsible for producing life-long chronic infection of the host. Bradyzoites are also called cystozoites (Figure 1.2b). A young cyst is small; 5 μm diameter with two bradyzoite inside it while others which are not so young contains thousands or hundreds of bradyzoites (Dubey, 2010). Tissue cysts with bradyzoites mostly form in visceral organs such as liver, lung and has predilection for muscular and neural tissues such as cardic and skeletal muscles, brain and eyes (Dubey, 2010). These cysts become important in the case of immunosuppressed people in whom the cyst may rupture and release the bradyzoites which transform back to rapidly dividing tachyzoites that lead to the reactivated infection (Montoya and Liesenfeld, 2004; Lindsay and Dubey, 2009). As shown in

Figure 1.3, the internal structures of bradyzoite are similar to tachyzoite, however nucleus of the bradyzoite is located toward the posterior end; whereas in the nucleus of a tachyzoite is more centrally situated. In addition the content of the rhoptry in bradyzoite is electron dense, while those in tachyzoites are complex (labyrinthine) (Dubey, 2010).

1.3.3 Oocysts

Oocyst is the sexual form of *T. gondii,* with subspherical to spherical shape, 10 by 12 µm in diameter. Unsporulated oocyts (Figure 1.2c) can be found in the faeces of cats following completion of sexual stage in the epithelium of the feline gut; and sporulation occurs outside the definitive host within 1 to 5 days after excretion, depending upon temperature and aeration (Miro *et al*., 2008). Sporulated oocysts (Figure 1.2d) have sub-spherical to ellipsoidal shapes and 11 by 13 µm diameter. Each oocyst contains two ellipsoidal sporocysts, each measuring 6 by 8 µm in diameter and contains four sporozoites. When the sporocysts are digested by mammals including humans, they become infected (Montoya and Liesenfeld, 2004).

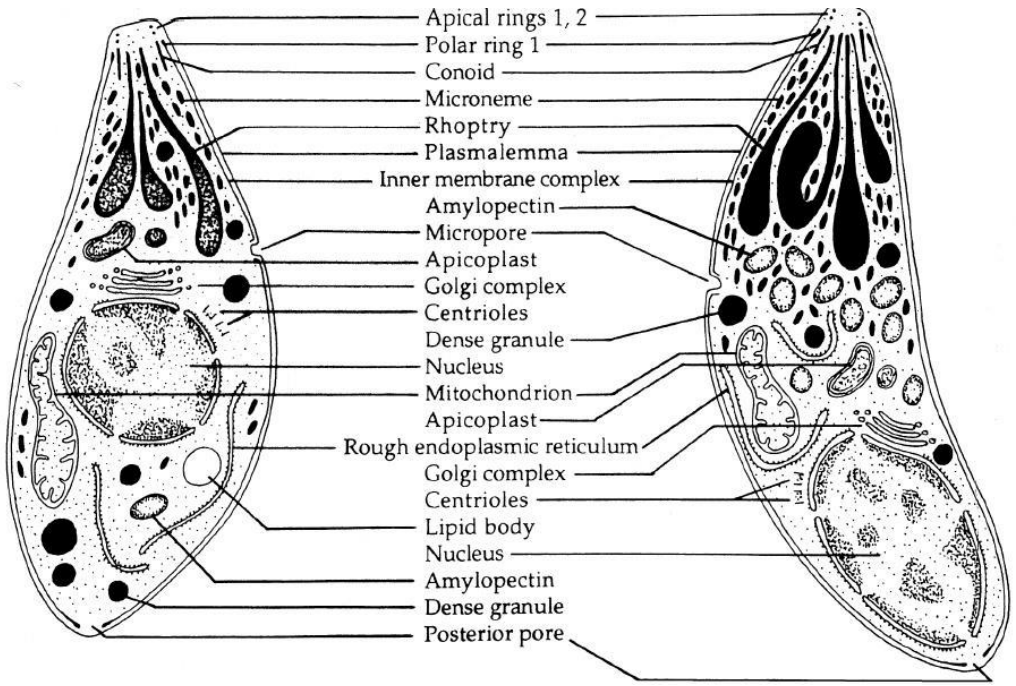


Figure 1.3 Schematic drawings of tachyzoite (left) and bradyzoite (right) of *T. gondii*. Drawings are composites of electron micrograohs (Dubey *et al*., 1998)

1.4 Life cycle of *T. gondii*

T. gondii is a parasite with a heteroxenous life cycle, requiring different animal hosts to complete its life cycle. The definitive hosts for this parasite are family of Felidae, either domestic cats or other felids such as ocelots, jaguarundi, margays, bobcats, Bengal tiger and Pallas cats. Invertebrates such as flies, earthworms and cockroaches can spread oocysts mechanically (Torda, 2001, Nutter *et al*., 2004). The sexual phase of *T. gondii* takes place in the gut intestinal epithelium of the cat, starting with ingestion of tissue cysts containing the bradyzoite (Figure 1.4). After that the cyst wall is digested by the gastric acid, lytic enzymes and bile of the upper digestive tract. The bradyzoites are released and invade the gut intestinal epithelium followed by bradyzoites conversion into invasive tachyzoites stage with systemic dissemination.

Meanwhile some parasites inside the epithelium pass five different stages gradually where one tachyzoite produce two daughter cells by schizogeny, together with formation of multiple merozoite around a previously divided nucleus (Dubey *et al*., 1998). The sexual stages (or gamete) are produced three to fifteen days after cyst ingestion. Several bi-flagellated microgametes formed through mature male gamete division, and in parallel with development of a female gamete. The sexual stage takes place only in the microwilli of the small intestine of the definitive host cell microvilli, especially the ileum. Aided by its flagella, the male microgamete fertilizes the female gamete and forms a zygote within a thick walled oocyst. Oocysts are released from the ruptured epithelial cells into the intestinal lumen of the definitive host and excreted as unsporulated oocysts into the environment *via* the cat faeces.

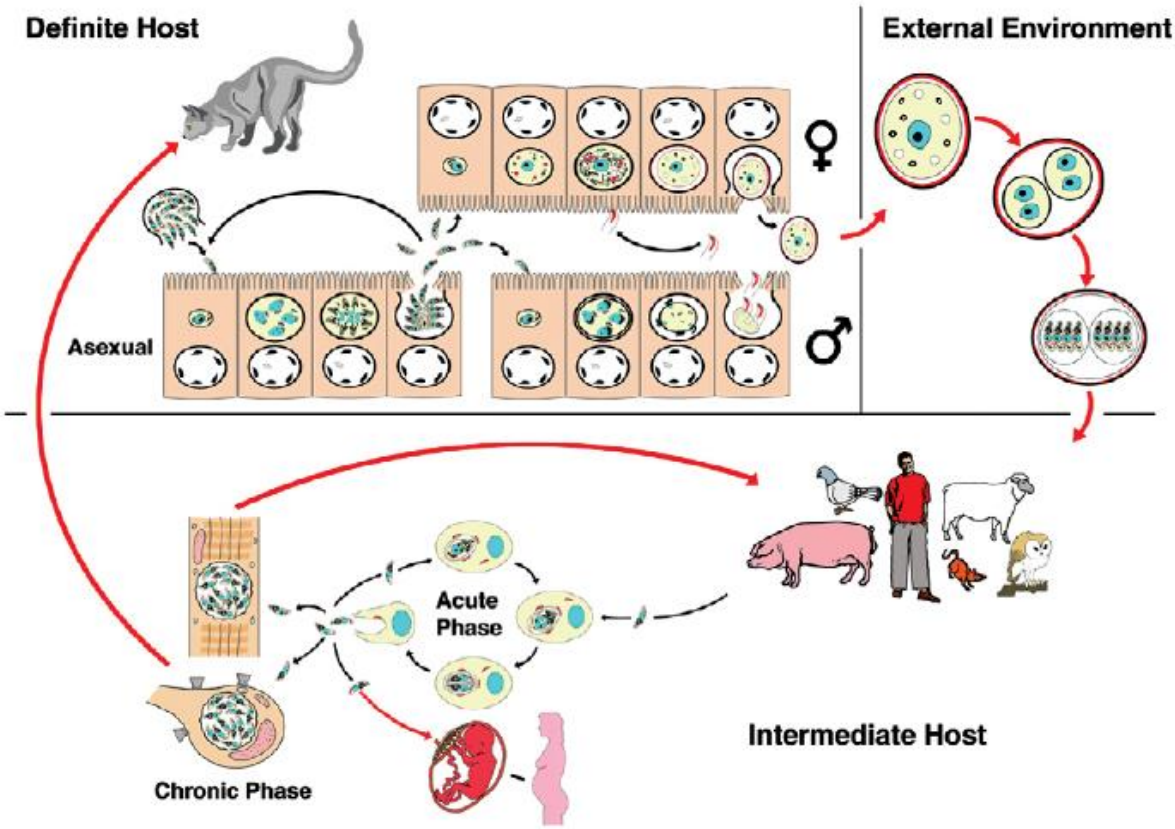


Figure 1.4 Summary of the life cycle of *T. gondii*
(Ferguson, 2009)

After 1-20 days, two internal sporocysts containing four sporozoites are produced inside the oocysts.Since oocyts are very resistant to variations of environmental conditions; they can maintain their infectivity for a long time until ingested by a new host (Dubey, 2004; Jones and Dubey 2010).

Ingestion of oocysts by intermediate (non-feline) hosts including humans will result in systemic infection; while ingestion of oocyts by other felines leads to formation of sexual stages as well as systemic infection. After primary invasion of epithelial cells in a new host, the sporozoites convert to the actively proliferative tachyzoite form. Reproduction *via* asexual proliferation (endodyogeny) takes places in the intestinal epithelium followed by crossing of the lamina propria. The tachyzoites rapidly move from the intestinal tract to muscles and internal organs, especially the brain, to establish a latent infection which occurs by conversion of tacyzoites to the slowly replicating bradyzoites. Enclosed in the tissue cysts, the slowly replicating bradyzoites are viable for many months to many years, if not for the whole life of the host. Persistent chronic infection in tissues helps at some time point to ensure that the parasite will be transferred to a new host through consumption of raw or undercooked meat from infected animals (Dubey, 2004; Dubey, 2008; Jones and Dubey 2010). Humans are susceptible to *Toxoplasma* infection either through tissue cysts in undercooked meat from other intermediate hosts such as sheep, goat, cow or pigs or from ingestion of oocysts containing sporozoites in environment. The widespread prevalence of felines ensures worldwide dissemination of this parasite *via* environmental oocyst contamination (Dubey, 2004; Behnke *et al*., 2014).

1.5 Epidemiology

Toxoplasmosis is a widespread infectious disease in animals and men, with variable prevalence from country to country and also different regions of a country depending on socio-economic habits and climates. The seroprevalence of *T. gondii* infection in various regions throughout the world is up to 90% in some populations; it is often higher in areas with hot, humid climates and lower altitudes which favour survival of oocysts and appears to be lower in colder areas. Under suitable circumstances (i.e. in moist, warm soil), oocysts can survive for approximately 1 year while they do not survive well in cold, arid climates (Sukthana, 2006; Pappas *et al*., 2009; Innes 2010).

In the United State, the prevalence of this infection appears to be declining. During 1988–1994 a seroprevalence of 14.1% was found in 12–49 years persons, whereas a seroprevalence of 9.0% was found for the same age group during 1999–2004 (Nutter *et al*., 2004, Pappas *et al*., 2009). According to a report by Tenter *et al.* (2000) on *T. gondii* prevalence in women at child-bearing age (1990–2000), the rates of positive seroprevalence, were 51–72% in several Latin-American countries, 58% in Central European countries, and 54–77% in West African countries. Low seroprevalence (4–39%) was reported in southwest Asia, Korea and China as well as in cold and arid climate areas such as Scandinavian countries (11–28%). The seroprevalence of this disease was reported to be 28.3% for women in southern Thailand (Nissapatorn *et al*., 2011). Seropositive prevalence of *T. gondii* in the same country may differ among geographical regions or populations and world-wide prevalence in older populations is higher (Tenter *et al*., 2000, Pappas *et al*., 2009). It has also been reported that the seroprevalence of *T. gondii* infection in USA is less compared with that in Latin America, central Europe and sub-Saharan Africa; however the

seroprevalences in Scandinavian countries and in England are lower than in USA (Lones *et al*., 2001, Pappas *et al*., 2009). It has also been reported that toxoplasmosis is widely prevalent in animals and humans in Brazil (Dubey *et al*., 2012). Data for *Toxoplasma* infection in Malaysia demonstrated seroprevalence of about 30% with highest prevalence of this disease in Malays followed by Indian (Nissapatorn and Abdullah, 2004). Another study reported that chronic toxoplasmosis in Malaysia was estimated to vary from 10-50% (Nissapatorn *et al*, 2003).

1.6 Transmission

Infection caused by *T. gondii* commonly could be seen in many animals used for food such as, sheep, goats, rabbits, and pigs. The probable source of *T. gondii* infection, either oocysts or tissue cysts, is affected mostly by the eating habits and presence of infected domestic cats in the near environment. *Toxoplasma* infection is not passed from person-to-person, except in the case of mother-to-child (congenital) transmission, organ transplantation or blood transfusion. Human become infected in one of three main ways: foodborne by ingesting *T. gondii* tissue cysts in the undercooked meat of infected food animals; animal-to-human (zoonotic) by ingesting infectious oocysts in soil, water and other materials contaminated by infected cat faeces; or mother-to-child (congenital) through vertical transplacental transmission of tachyzoites from mothers to the fetus (Figure 1.5).

1.6.1 Congenital transmission

Congenital toxoplasmosis is caused by vertical transmission of *T. gondii* from a seronegative acutely infected pregnant woman to her fetus. The disease was reported for the first time by Wolf, Cowen and Page in a human child in 1939 (Dubey, 2008).

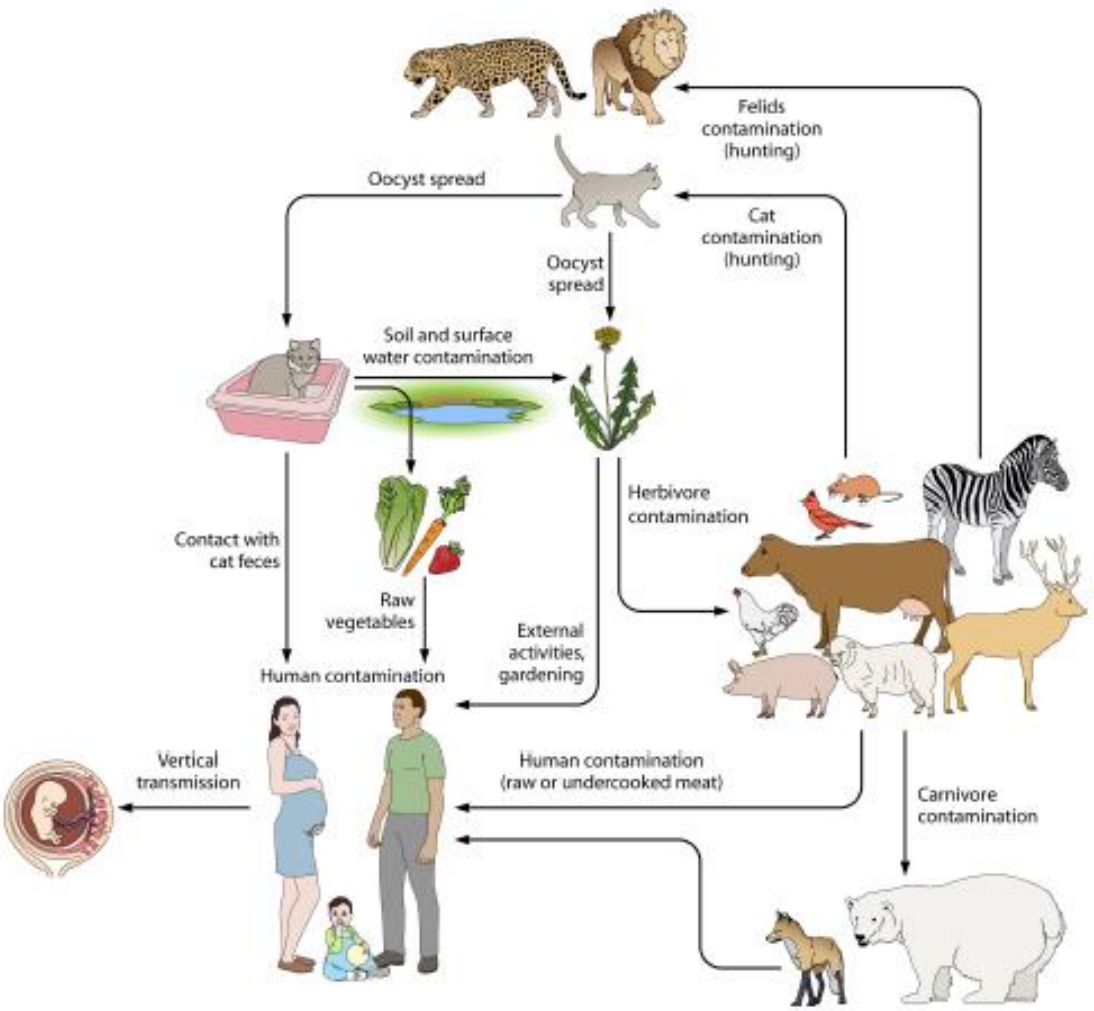


Figure 1.5 Transmission of *T. gondii* to humans and animals (Robert-Gangneuxa and Dardéc, 2012)

Different factors are associated with congenital toxoplasmosis, including climate, route of transmission, eating habits, cultural behavior, and hygienic standards. This association results in marked differences among nations and populations. For example, the prevalence of congenital infection in France and Belgium is 2–3 cases per 1000 live births, significantly higher compared with US prevalence of 1 in 10,000 to 1 in 1000 per live births. Reports from England indicates that congenital toxoplasmosis occur approximately 1 in 10,000 live births (Lebech *et al*., 1999; Pappas *et al*., 2009). Clinical manifestations of this infection in newborns are varied and can be developed at different times before and after birth. Majority of newborns infected with *T. gondii in utero* are asymptomatic at birth (70–90%) or may be unnoticed (Lebech *et al*., 1999). The classic triad of intracranial calcifications, chorioretinitis and hydrocephalus occurred in fewer than 10% of infected newborns.

Microcephaly or/and hydrocephalus may occur when intra-uterine infection results in meningoencepahlitis (Lebech *et al*., 1999; Zhou *et al.,* 2011). There may also be a relationship between toxoplasmosis and function of the brain, with asymptomatic infected infants exhibiting lower intelligence quotients (IQs) later in their adulthood than do their healthy counterparts (Torrey and Yolken, 2003; Jones *et al*., 2014). The risk of fetal infection and sequelae produced during the infant's life is multifactorial, depending on the immunological response of the mother during parasitemia, time of maternal infection, parasite load and strain virulence (Ayi *et al*. 2009; Jones *et al*., 2014).

The possibility of fetal infection is approximately 1% when primary maternal infection happens just prior to the preconception period but ascends as pregnancy progresses. During the first trimester of pregnancy, not treated infection acquired by women results in congenital infection in 10 to 25% of infants. During the second and third trimesters of pregnancy the incidences of fetal infection are 30–55% and 60–65%, respectively (Lynfield and Guerina, 1997; Ayi *et al*., 2009; McLeod *et al*, 2009). However the consequences and sequelae are more severe when infection occurs in first trimester of pregnancy, such as miscarriage and abortion (Jones *et al*., 2014) (Figure 1.6). Manifestations of ocular toxoplasmosis at birth are less severe, and recurrences are fewer in those teenagers who were treated promptly early in the first year of life and *in utero* (Delair *et al*., 2011).

It has been reported that uninfected babies were born later than infected babies. In addition congenital infection was associated with increased risk of preterm delivery if seroconversion happens before 20 weeks of gestation. Latent chronic *T. gondii* infection may be reactivated in immunodeficient patients (such as HIV-infected pregnant women) and may result in congenital transmission of the parasite to the fetus. A critical step in congenital toxoplasmosis diagnosis and evaluation of the time of infection is achieved *via* applying laboratory techniques and monitoring the immune response (Petersen, 2007, McLeod *et al*, 2009).

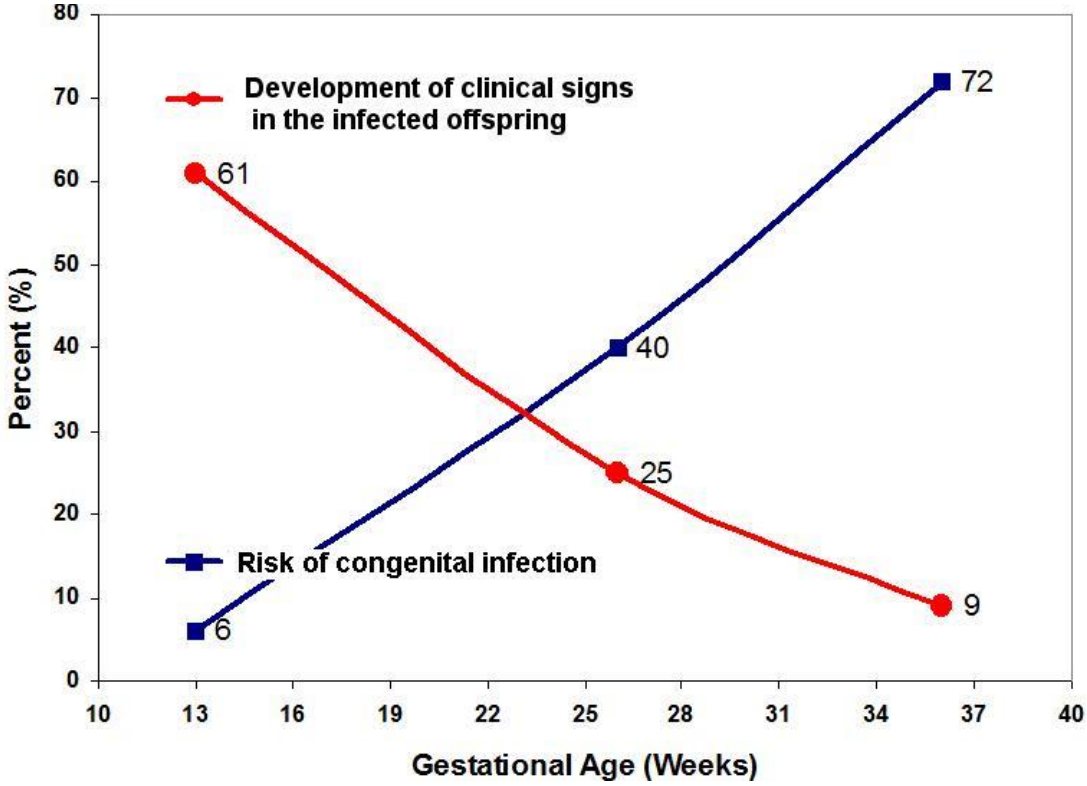


Figure 1.6 The frequency of congenital toxoplasmosis and its relation to the severity of consequences on fetus and gestational age (www.perinatology.com/exposures/infectionlist.ht)

1.6.2 Foodborne transmission

Toxoplasmosis is considered as one of the infectious diseases associated with foodborne hospitalizations and sometimes, deaths. Undercooked meat, especially goat, lamb and pork; and raw vegetables and fruits with oocysts from soil contaminated with cat faeces are the major ways of foodborne transmission in humans. In a recent assessment regarding foodborne diseases in the United States, toxoplasmosis was reported as the second major cause of foodborne disease–related deaths and fourth main cause of foodborne disease–related hospitalizations (approximately, 4428 hospitalizations and 327 deaths annually) (Scallan *et al*., 2011, Jones *et al*., 2014). Recently researches have shown that *T. gondii* is considered as one of the five most important emerging pathogens involved in foodborne disease in the world; while in Greek toxoplasmosis was found to be one of the contributor of foodborne diseases to lost years of life, lived years with disability, and disability-adjusted lived years per million persons (9.7, 14, and 23 years, respectively) (Schlundt *et al*., 2004; Gkogka *et al*., 2011).

Foodborne transmission of toxoplasmosis can be prevented by reducing *T. gondii* in meat *via* improving meat production, sufficient cooking of meat, adequate washing of raw vegetables and fruits, prevention of any cross contamination in the kitchen, and decreasing the spread of oocysts in the environment. However, based on retail meat samples parasitologists cannot provide a true measurement of risk for fresh meat since nearly half of the pork meat and a substantial portion of chicken meat are injected with salt and water before cooking, which can kill *T. gondii* tissue cysts and the treated products are labeled as “enhanced” meat (Schlundt *et al*., 2004; Dubey *et*

al., 2005). Furthermore, in the United States most of the retail chickens are sold frozen; another way which can kill *T. gondii* cysts (Schlundt *et al*., 2004; Jones and Dubey, 2012).

A live vaccine for sheep which produces protective immunity for approximately 18 months is available to protect lambs. An oral live vaccine can also prevent felines from shedding oocysts. Unfortunately, commercial cat vaccine production was discontinued because of its high cost, short shelf life, the need for a facility to keep the vaccine frozen and lack of concern among cat owners (Jones *et al*., 2014). Thus far vaccination for human is not available.

1.6.3 Animal-to-human (zoonotic) transmission

Human contact with infected feral cats can be considered as one the main routes for transmission of toxoplasmosis. Feral cats and other members of the family Felidae may become infected either by ingesting infectious oocysts in the environment or via ingesting tissue cysts containing bradyzoites from intermediate hosts. Cats which are allowed to hunt may acquire the infection by feeding on corpse of birds or small mammals infected with *T. gondii.* After primary infection of feral cats with tissue cysts, the bradyzoites turn into tachyzoites and initiate an asexual proliferation phase which consists of several cycles of enteric endopolygeny. The terminal stages of asexual proliferation commence the phase of sexual proliferation which results in the foundation of oocysts (Sukthana *et al*., 2003; Dubey, 2008; Torrey and Yolken, 2013).

Cats which are kept inside the houses may shed large numbers of oocysts and thereby putting their owners at a serious risk of toxoplasmosis infection. Stray cats or cats which are roaming on farms may shed their oocysts in the environmental soil or water and cause contamination which may infect livestock need for human consumption such as sheep. However, freshly passed oocysts by cats are unsporulated and, therefore, direct contact with cats usually does not lead to *T. gondii* infection. If cat faeces are removed daily from the household by the owner, then keeping of cats inside houses or flats does not provide risk of *T. gondii* infection generally (Tenter *et al*., 2000; Schlundt *et al*., 2004; Jones and Dubey, 2012).

1.6.4 Other routes of transmission

Congenital, foodborne and zoonotic transmission are the most common ways of *T. gondii* infection transmission. There are some other routes which *Toxoplasma* can be transmitted but they are not very common; for example blood transfusion and organ transplantation. Organ transplant recipients can develop toxoplasmosis due to transmission of the parasite with the transplanted organ from a *Toxoplasma*-seropositive donor to a *Toxoplasma*-seronegative recipient. Heart transplantation is the most common type of organ transplantation procedure that is at risk, as cysts are commonly found in the cardiac muscle (Martina *et al*. 2011; Derouin and Pelloux 2012). Reynolds *et al*. (1996) published a case report regarding a patient who received a renal transplant and died one month later after transplantation. Serologic studies identified that primary infection with *T. gondii* was the main reason for his death. It has also been reported that *Toxoplasma* can enter the human body by several other ways, including respiratory system, conjunctiva, and skin. Moreover, accidental laboratory events due to carelessness in laboratories were found as the

most common way of laboratory-acquired infections, where the personnel are in contact with contaminated glassware, needles, or particularly infected animals. There was also a case report of toxoplasmosis infection in a breast-fed infant whose mother acquired the infection (Renolds *et al*., 1966; Derouin and Pelloux 2012).

1.7 Pathogenesis of toxoplasmosis

Toxoplasmosis is classified into chronic and acute phases. The acute phase (early stage) is mostly associated with the rapidly dividing form (tachyzoite), while the tissue cyst is the predominant form of *T. gondii* during latent chronic infection. During early acute infection, the proliferative tachyzoites can invade every kind of nucleated host cells except non-nucleated red blood cells. Invasion of parasite into host cell is a main step in its pathogenesis.

After attachment to host cell, tachyzoite enter by active phagocytosis through the host cell's plasmalemma. The sequential release of proteins from the apical secretary organelles of parasite (micronemes, rhoptries and dense granules) assist in attachment and invasion into host cell and in parasitophorous vacuole generation. The micronemes are responsible for recognition and adhesion to the target cell while the rhoptry enzymes (ROP proteins) are released through a slender duct to produce parasitophorous vacuole; and the enzyme discharged by dense granules result in maturing of the vacuole as a metabolically active compartment (Zhou *et al*., 2005; Dubey, 2008). Formation of parasitophorous vacuole, which is resistant to acidification, gives the parasite a chance to be in a safe environment for proliferation inside the cell. Their intracellular proliferation inside host cells result in necrosis and rupture of the cells. The liberated parasites subsequently invade and destroy

neighboring cells, producing larger focal lesions. Lesions or tissue necrosis could be found in many organs during acute toxoplasmosis, but mainly observed in liver, intestine, spleen, lung, pancreas and heart (Waree, 2008). If the initial *Toxopasma* infection occurs in a host during pregnancy, the tachyzoites can move throughout the placenta and infect the fetus, which can result in congenital infection.

Liver is usually the first organ affected, it appears grossly swollen, with white foci randomly distributed throughout the organ. Hepatic lesions consist of necrosis and sometimes infiltration of heterophils. Large collections of tachyzoites are present in hepatocytes, resulting in the host cells degeneration (Waree *et al*., 2008; Bottari *et al*., 2014). The spleen may become swollen with pale yellow diffused necrotic foci. General necrosis is seen and fills with groups of parasites (Waree 2008). The brain of an acutely infected host is congested with microscopic hemorrhages. There is inflammatory reaction at the telencephalone and mesencephalon and *T. gondii* tissue cysts are seen near the lesions. Numerous mononuclear cells invade into the meninges and some mononuclear cells are seen around the vessels. With tissue cysts, the most notable feature is the complete absence of inflammatory cells (Kamerkar and Davis, 2012; Chew *et al*., 2012).

Around three weeks after infection, as the host mount an immune response, tachyzoites disappear from the visceral tissues and tissue cysts are formed in neural and muscular tissues (Waree, 2008). The bradyzoites in the tissue cysts multiply slowly and can persist for the life of the host (Dubey and Frenkel, 1972; Speer and Dubey, 1998).

1.8 Strains of *T. gondii*

T. gondii has an unusual population structure with three clonal lineages (I, II and III) which differ in epidemiological pattern of occurrence and virulence (Montoya and Liesenfeld, 2004). These three clonal lineages have emerged within the past 10,000 years (Su *et al*., 2003). Studies show that the type I strain is highly virulent; as a single parasite has a lethal dose regardless of the genotype of the host. Type II and III strains have a 50% lethal dose of more than 10^3 parasites and the outcome is dependent on the genetic background of the host (Mordue *et al.*, 2001). Type I and II have been reported in human while type I often is associated with severe ocular and congenital disease, suggesting that it might be more pathogenic in humans. Type III has a common prevalence in animals (Montoya and Liesenfeld, 2004).

Studies on *T. gondii* isolates from Brazil showed atypical genotype that they are both genetically and biologically different from those in the USA and Europe (Velmurugan *et al*., 2008). A recent study on *T. gondii* isolates from chickens in six different African countries (Nigeria, Congo, Egypt, Burkina Faso, Kenya and Mali) revealed four genotypes. Most isolates belonged to the clonal type II and III strains with one Nigerian isolate having an atypical genotype (Velmurugan *et al*., 2008). In Malaysia, a study on wild boars of Peninsular Malaysia showed that the predominant strain of *T. gondii* is type I (Puvanesuaran *et al*, 2013a). In another local study, type I and II strains of *T. gondii* were the main isolates from free-range farm ducks (Puvanesuaran *et al*, 2013b). In Central and South America, types I, III, strains of *T. gondii* parasites are more prevalent (Stillwaggon *et al*., 2011).

1.9 Immune response in toxoplasmosis

T. gondii is one of the most successful parasites in the world which can produce asymptomatic life-long chronic infection inside the host. The immune response to *Toxoplasma* infection is unique, complicated and compartmented. It has the ability to acquire a balance between the immune strategies for evasion and the immune response of the host, with the aim of not only maximizing the parasite proliferation, but also at minimizing host immunopathology (Nissapatorn and Khairul, 2004). A balanced interaction between neutrophils, enterocytes, dendritic cells, and macrophages create the immune response to *T. gondii*. These interactions occur through a complicated group of molecular signaling pathways that bring about regulation and activation of cytokine responses as well as production of effecter molecules.

The high level of genetic heterogeneity background may cause individual variations in immune response. In addition, *Toxoplasma* infection has the ability to spread in all the organs and tissues. Each tissue compartment possesses its own specific immune response, especially in the placenta and in the central nervous system. An additional level of complexity occurred due to the possibility of reactivation of infection which may vary with strain virulence (Waree, 2008).

During the earliest stages of infection, *T. gondii* is able to trigger non-specific response of natural killer (NK) cells, macrophage, and some other cells such as fibroblasts, endothelial or epithelial cells. This activation is necessary to limit parasite proliferation because of its cytotoxic action and to activate a specific