IDENTIFICATION OF POTENTIAL *Toxoplasma gondii* cDNA PHAGE CLONES AND PRODUCTION OF THE CORRESPONDING RECOMBINANT PROTEINS IN THE DEVELOPMENT OF IgG AVIDITY ASSAY FOR SERODIAGNOSIS OF TOXOPLASMOSIS

TEH AI YING

UNIVERSITI SAINS MALAYSIA
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by

TEH AI YING

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LIST OF ABBREVIATIONS

ABTS	2, 2'-azino-bis 3-ethylbenzthiazoline-6- sulfonic acid
APS	ammonium persulphate
BLAST	Basic Local Alignment Search Tool
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
DALY	disability-adjusted life year
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
EtBr	ethidium bromide
His	histidine
HRP	Horseradish Peroxidase
i.e.	id est (that is)
Ig	Immunoglobulin
INFORMM	Institute for Research in Molecular Medicine
IPTG	Isopropyl-beta-D-thiogalactopyranoside
IVIAT	*in-vivo* induced antigen technology
Kb	kilo base pair
kDa	kilo Dalton
LB	Luria-Bertani
MALDI-TOF/TOF	Matrix-Assisted Laser Desorption/ Ionization-Time of Flight/ Time of Flight
MWCO	molecular weight cut-off
NCC	nitrocellulose membrane circle
NCP	nitrocellulose membrane
Ni-NTA	nickel-nitrilotriacetic acid
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RC DC™	reducing agent and detergent compatible
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TB	terrific broth
TBE	Tris/ Borate/ EDTA
TBS	Tris buffered saline
TEMED	tetramethylethylenediamine
T. gondii	*Toxoplasma gondii*
UV	ultraviolet

PENGENALPASTIAN KLON FAJ cDNA *Toxoplasma gondii* YANG BERPOTENSI DAN PENGHASILAN PROTEIN REKOMBINAN BAGI PEMBANGUNAN ASAI IgG AVIDITI UNTUK SERODIAGNOSIS PENYAKIT TOKSOPLASMOSIS

ABSTRAK

Toksoplasmosis merupakan jangkitan berleluasa di seluruh dunia yang disebabkan oleh *Toxoplasma gondii*. Jangkitan akut (primer) di dalam kalangan wanita hamil boleh menyebabkan toksoplasmosis kongenital yang mengakibatkan kecacatan neonatal, kerosakan saraf dan mata fetus, atau kematian janin. Oleh sebab itu, penilaian peringkat toksoplasmosis (kronik atau akut) di dalam kalangan wanita hamil adalah penting untuk pengurusan pesakit yang baik. Ujian serologi kekal sebagai pendekatan yang paling biasa untuk diagnosis makmal dan juga penilaian peringkat jangkitan bagi penyakit *Toxoplasma*. Pengunaan kaedah IgG aviditi telah dibuktikan berguna dalam perbezaan jangkitan toksoplasmosis akut dan kronik. Aviditi IgG adalah rendah pada awal jangkitan dan meningkat secara beransur-ansur dari masa ke masa. Aviditi IgG yang tinggi mengesahkan jangkitan kronik, manakala aviditi IgG yang rendah menunjukkan kemungkinan jangkitan yang baru. Kebanyakan asai serologi *Toxoplasma* di pasaran adalah berdasarkan ekstrak asli antigen yang berasas *T. gondii* yang dikultur secara *in-vivo* atau *in-vitro*, dan asai-asai ini menunjukkan keputusan yang bervariasi. Antigen *T. gondii* yang dihasikan secara rekombinan merupakan alternatif yang menarik dalam pembangunan asai IgG aviditi yang lebih baik. Oleh itu, kajian ini dijalankan untuk memenuhi keperluan tersebut. Dalam kajian ini, 30 klon faj cDNA *T. gondii* telah diuji dengan imunosaringan menggunakan sampel serum yang negatif terhadap *Toxoplasma*-

khusus IgG dan IgM (IgG-, IgM-) dan keputusan ujian ini telah menunjukkan sembilan klon adalah ≥ 70% tidak-kereaktifan. Klon-klon ini telah diuji seterusnya dengan sampel serum yang positif terhadap *Toxoplasma*-khusus IgG dan IgM (IgG+, IgM+) dan tujuh klon dengan ≥ 70% kereaktifan didapati. Selanjutnya, klon-klon ini diuji dengan imunosaringan IgG aviditi menggunakan sampel serum yang IgG aviditi rendah (LGA) dan IgG avidity tinggi (HGA). Dua klon cDNA yang menunjukkan prestasi tertinggi telah dikenal pasti dan dijujuk. Klon AG12b mengekodkan protein *T. gondii* apical complex lysine methyltransferase (AKMT) manakala klon AG18 mengekodkan *T. gondii* forkhead-associated (FHA) domain-containing protein. Jujukan sisipan DNA telah diklonkan ke dalam vektor ekspresi pET32. Protein rekombinan gabungan-His telah diekspres dan dinilai dengan blot western dan ELISA IgG aviditi. Dengan blot western IgG aviditi, rAG12b mengenal pasti 86.4% sampel serum LGA (n=22) dan 90.9% HGA (n=22); manakala rAG18 mengenal pasti 81.8% sampel serum dari setiap kumpulan LGA and HGA. Dengan ELISA IgG aviditi, rAG12b mengenal pasti 86.4% sampel serum dari setiap kumpulan LGA (n=22) and HGA (n=22); manakala rAG18 mengenal pasti 77.3% sampel serum LGA dan 86.4% HGA. Secara umum, keputusan yang ditunjukkan adalah lebih baik atau setanding dengan asai IgG avidity lain yang dilaporkan. Secara kesimpulan, kajian ini telah mengenalpastikan dua klon faj cDNA *T. gondii* dengan potensi diagnostik untuk diguna dalam asai IgG aviditi dan berjaya mengekspreskan dan menulenkan protein rekombinan yang berkenaan sebagai rAG12b dan rAG18. Nilai diagnostik tinggi terutamanya oleh rAG12b, membuat kedua-dua protein rekombinan ini berpotensi untuk diguna dalam pembangunan asai IgG aviditi *Toxoplasma* untuk serodiagnosis penyakit toksoplasmosis.

IDENTIFICATION OF POTENTIAL *Toxoplasma gondii* cDNA PHAGE CLONES AND PRODUCTION OF THE CORRESPONDING RECOMBINANT PROTEINS IN THE DEVELOPMENT OF IgG AVIDITY ASSAY FOR SERODIAGNOSIS OF TOXOPLASMOSIS

ABSTRACT

Toxoplasmosis is a worldwide infection caused by *Toxoplasma gondii*. Acute (primary) infection in pregnant women can lead to congenital toxoplasmosis which causes neonatal malformation, neurological damage, eye lesions or fetal death. Therefore, assessment of the stage (chronic or acute) of toxoplasmosis in pregnant women is crucial for appropriate patient management. Serodiagnosis remains the most common approach for laboratory diagnosis of *Toxoplasma* infection as well as to assess the stage of the infection. Determination of IgG avidity has been proven to be useful in differentiating acute from chronic *Toxoplasma* infection. The IgG avidity is low at the beginning of the infection and gradually increases over time. High IgG avidity confirms chronic infection while low IgG avidity indicates a probable recent infection. Most of the commercially available *Toxoplasma* serological assays uses native antigen isolated from *in-vivo* or *in-vitro* culture of *T. gondii*, and they show variability in performance. The recombinantly produced *T. gondii* antigens are attractive alternatives in the development of an improved IgG avidity assay. Therefore, the present study was conducted to address this need. In this study, 30 *T. gondii* cDNA phage clones were immunoscreened using serum samples negative for *Toxoplasma*-specific IgG and IgM (IgG-, IgM-), and the results showed 9 clones with non-reactivity of ≥ 70%. These clones were then tested with serum samples positive for *Toxoplasma*-specific IgG and IgM (IgG+, IgM+) and showed 7

clones with the reactivity of ≥ 70%. They were further tested with IgG avidity immunoscreening using low IgG avidity (LGA) and high IgG avidity (HGA) serum samples. Two cDNA clones with the highest performance were identified and sequenced. Clone AG12b encoded *T. gondii* apical complex lysine methyltransferase (AKMT) protein while AG18 encoded *T. gondii* forkhead-associated (FHA) domain-containing protein. The DNA sequences were cloned into pET32 expression vector; and the His-tagged recombinant proteins rAG12b and rAG18 were expressed and evaluated with IgG avidity western blot and ELISA. With IgG avidity western blot, rAG12b identified 86.4% LGA (n=22) and 90.9% HGA serum samples (n=22); while rAG18 identified 81.8% of each LGA and HGA serum group. With IgG avidity ELISA, rAG12b identified 86.4% of each LGA (n=22) and HGA (n=22) serum group; while rAG18 identified 77.3% LGA and 86.4% HGA serum samples. In general, these results were either better or comparable to the reported IgG avidity assays. In conclusion, this study has identified two *T. gondii* cDNA phage clones with diagnostic potential for use in IgG avidity assay and successfully expressed and purified the corresponding recombinant proteins as rAG12b and rAG18. The good diagnostic potential demonstrated by the recombinant proteins, in particular, rAG12b, make them potentially useful in the development *Toxoplasm*a IgG avidity assay for serodiagnosis of toxoplasmosis.

CHAPTER 1

INTRODUCTION

1.1 *Toxoplasma gondii* and toxoplasmosis

Toxoplasma gondii (T. gondii) is an obligate intracellular protozoan parasite that causes over 6 billion cases of human infection worldwide (Furtado *et al.*, 2011). The high prevalence of this infection makes it one of the most common parasitic infections in human. *T. gondii* can infect and grow in many cell types of various animal species, which covers all warm-blooded animals including human (Schwartzman, 2001). The definitive host of this parasite is Felidae members including domestic cats. *T. gondii* infection, or toxoplasmosis, is commonly asymptomatic or shows only mild symptoms in immunocompetent adults. However, it can cause severe clinical symptoms in immunocompromised persons or congenitally infected infants.

Toxoplasmosis in human is generally categorized into acquired toxoplasmosis, congenital toxoplasmosis, and reactivated toxoplasmosis. Acquired toxoplasmosis may be transmitted through *T. gondii* oocysts, usually by direct transmission, or ingestion of tissue cysts found in the intermediate host. Organ transplantation from a *Toxoplasma*-positive donor may also contribute to this. Congenital toxoplasmosis occurs when a pregnant woman gets primary *T. gondii* infection during her pregnancy and vertically transmitted the infection to her fetus. The frequency of the cross-placenta transmission increases with the gestational age but the risk for severe clinical symptoms in the infant is higher if the congenital infection occurs at the early pregnancy stage. Congenital toxoplasmosis may result in fetal death, miscarriage and various debilitating effects in the infected children. On

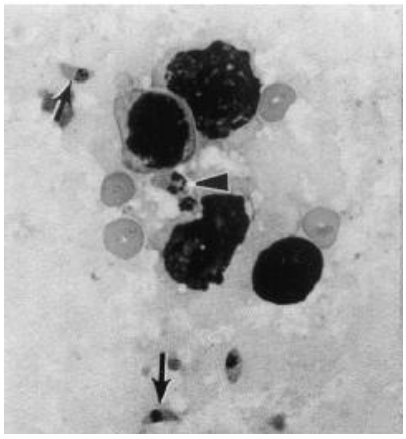
the other hand, reactivated toxoplasmosis occurs in an immunocompromised person when a chronically infected person becomes immunosuppression, and this condition can be life-threatening.

1.2 Taxonomy of *T. gondii*

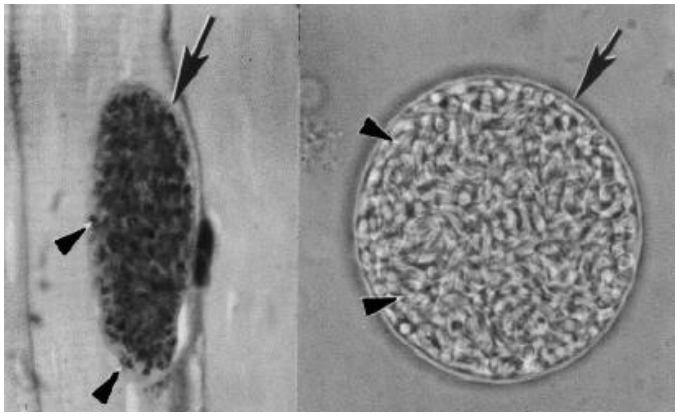
T. gondii is classified in domain Eukarya as it is a single cell organism that contains a nucleus and has membrane-bound organelles. It is a member of Kingdom Alveolata, which is one major group of Protists. This organism is categorized under Phylum Apicomplexa and Class Coccidia, where all members of this group are obligate intracellular parasites that must live and reproduce within an animal cell. The parasite is a member of Order Eucoccidiorida, Family Sarcocystidae, and Genus *Toxoplasma* since it carries out life cycle in more than one host while the definitive host is a member of Felidae. *Toxoplasma gondii* is the sole member of the genus *Toxoplasma*.

1.3 Stages and morphology of *T. gondii*

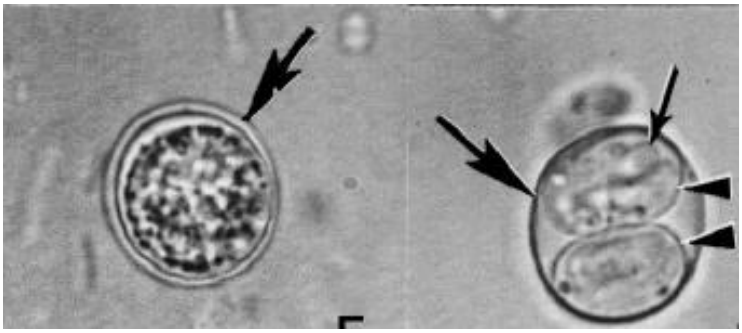
T. gondii has three distinct infectious stages, i.e. the tachyzoite, bradyzoite, and sporozoite. The microscopic examinations and the schematic drawings of the three different forms of *T. gondii* are shown in Figures 1.1 and 1.2, respectively.



(A) Crescent-shaped (arrows) and dividing (arrowhead) tachyzoites observed in impression smear of lung.



(B) Tissue cysts found in muscle (left) and brain (right). Tissue cyst wall is thin (arrows) and it contains hundreds of bradyzoites (arrowheads).



(C) Unsporulated (left) and sporulated (right) oocysts with oocysts wall (big arrows) enclosing two sporocysts (arrowheads) and sporozoites (small arrow).

Figure 1.1 Microscopic examinations of (A) tachyzoites, (B) bradyzoites and (C) oocysts of *T. gondii* (Hill and Dubey, 2002).

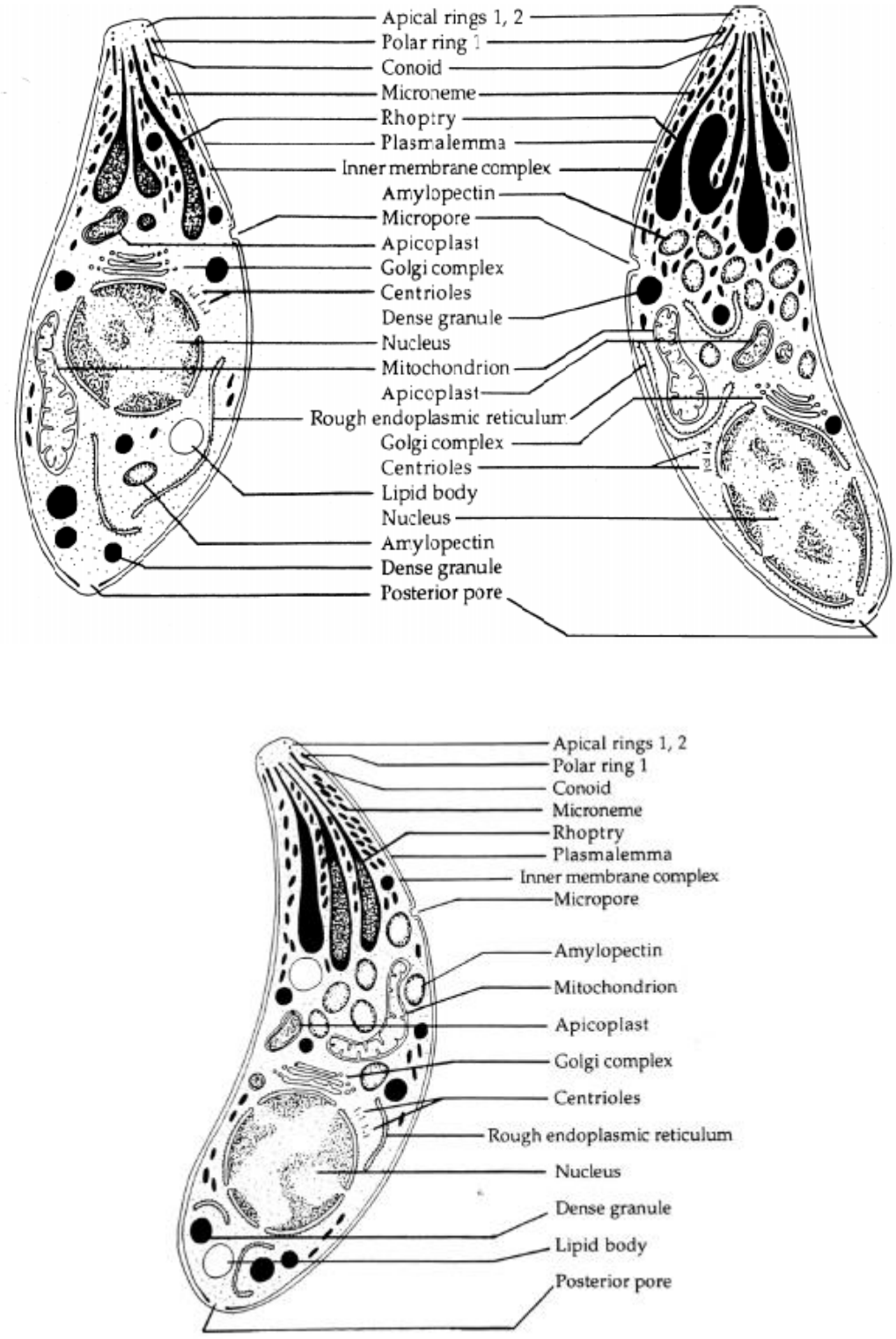


Figure 1.2 Schematic drawings of a tachyzoite (top left), a bradyzoite (top right) and a sporozoite (bottom) of *T. gondii* (Dubey et al., 1998).

1.3.1 Tachyzoite

T. gondii tachyzoite is the rapidly multiplying form of the parasite which is also known as the trophozoite, the proliferative form, the feeding form, and endozoite (Dubey, 2008). Nicolle and Manceaux in the year 1909 found this form of the parasite from the tissue of the gundii rodent. It is oval or crescent-shaped with a conoidal anterior end and a rounded posterior end (Figure 1.1A) with approximately 2-4 μm of width and 6-8 μm of length. The tachyzoite contains various organelles and inclusion bodies (Frenkel, 1973). A few structural and secretory elements are located within the anterior region of the tachyzoite, including apical ring, polar ring, conoid and inner membrane complex (Figure 1.2), these make up the apical complex that is responsible for the mobility and host invasion of the parasite. Apicoplast, an organelle that is typical for the members of Apicomplexa, is also present in the tachyzoite (Figure 1.2). This is a non-photosynthetic organelle homologous to the chloroplast in the plant that is responsible for protein synthesis and may be involved in fatty acid synthesis and lipid metabolism (Lim and McFadden, 2010).

T. gondii tachyzoites invade all types of cells and the invasion process is by active penetration mediated by proteins secreted from the microneme, rhoptries, and dense granules (Arrizabalaga and Boothroyd, 2004; Boothroyd and Dubremetz, 2008; Besteiro *et al.*, 2011). The tachyzoites multiply asexually within the host cell by a process called endodyogeny (Goldman *et al.*, 1958), in which two daughter cells are formed within the parent parasite. During the asexual stage, the *T. gondii* tachyzoite grows rapidly intracellular.

1.3.2 Bradyzoite

Bradyzoite (brady in Greek = slow) was proposed by Frenkel in the year 1973 to describe the stage of *T. gondii* where it replicates slowly within a host cell and surrounded by a thin, elastic wall (Figure 1.1B) to form cysts in tissues. Encysted bradyzoites vary in size, are less susceptible to destruction by proteolytic enzymes and are found mostly in neural and muscular tissues including the brain, eye, skeletal, cardiac muscle and the central nervous system. The tissue cysts site may vary depending on the host species (Ferguson and Dubremetz, 2007). Encysted bradyzoites do not cause inflammation and thus have little effect on the surrounding cellular function and host immune response. However, the bradyzoites released from a breakdown cysts can be converted to the active tachyzoites causing necrosis and inflammation or acute encephalitis in the immunocompromised patient (Schwartzman, 2001; Dubey, 2007). The internal structure of bradyzoite is similar to tachyzoite with the most distinct difference is the position of the nucleus. The nucleus in bradyzoite is located towards the posterior end while the nucleus in tachyzoite is more centrally located (Figure 1.2).

1.3.3 Sporozoite

Oocysts formation takes place in the small intestine of a definitive host (usually a cat) after the fertilization of *T. gondii* female gametes by the male gametes. The non-infective parasite oocysts are then released into the intestinal lumen after the rupture of the intestinal epithelial cells. These oocysts are resistant to the environment and sporulate under the appropriate environmental condition, i.e. good aeration, humidity, and temperature. Each sporulated oocysts contain two sporocysts which later divided into four sporozoites (Figure 1.1C). The sporulated oocysts are

infectious (Dubey and Frenkel, 1972). The ultrastructure of sporozoite is similar to that of tachyzoite, except that there is abundance of micronemes, rhoptries, and amylopectin granules in the former (Figure 1.2).

Unlike the other two forms of *T. gondii*, sporozoite can contaminate the water source as it survives in water (Dubey, 2007). Sporulated oocysts are infectious in water with a temperature between 10^{o}C to 20^{o}C, but the infectivity decreases dramatically above 30^{o}C, and are killed at 60^{o}C (Dubey, 1998b). A toxoplasmosis outbreak in Brazil in November 2001 to January 2002 infecting 155 persons was caused by *T. gondii* oocysts that contaminated the reservoir (de Moura *et al.*, 2006).

1.4 Life cycle of *T. gondii*

The complete *T. gondii* life cycle was resolved by Dubey and Frenkel (1972) through the description of the asexual and sexual phases of *T. gondii* that takes place in the small intestine of a cat. The sexual cycle occurs in the definitive hosts (the Felidae family) while the asexual cycle occurs in the intermediate hosts (all warm-blooded mammals) (Figure 1.3).

A cat gets infected by *T. gondii* either by ingestion of tachyzoites, encysted bradyzoites, and sporozoites of the parasite (Dubey, 2001; Dubey, 2006). It has been reported that the *T. gondii* is transmitted most efficiently by bradyzoites to the cat and by the oocysts to other hosts. As such, cats may shed millions of oocysts after ingestion of a bradyzoite while mice may not get infected even after ingestion of 100 bradyzoites. Meanwhile, pigs and mice can be infected by ingestion of single oocyst although cat may only get infected after ingestion of more than 100 oocysts (Dubey, 2008).

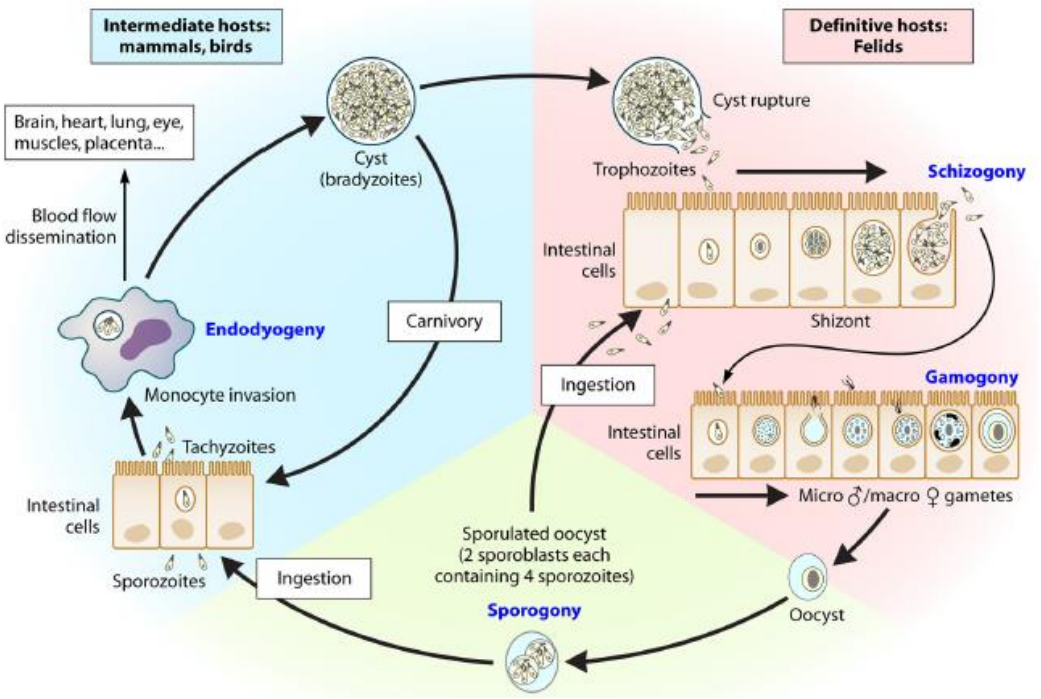


Figure 1.3 Life cycle of *T. gondii* (Robert-Gangneux and Darde, 2012).

The sexual cycle takes place in the definitive hosts while asexual cycle occurs in intermediate hosts.

Upon ingestion of encysted bradyzoites, the cyst wall of the bradyzoite is dissolved by proteolytic enzymes in the cat's intestine and stomach. The bradyzoites are then release and penetrate the epithelial cells of the small intestine. This is followed by multiplication of the bradyzoites asexually in the epithelial cells, forming a stage known as schizonts. Schizonts produce merozoites and the merozoites form male and female gametes through gametogonia. The male gamete uses its flagella to reach the female gamete and fertilizes the female gamete. The oocyst wall is then formed, in the epithelium cells of the small intestine of the cat.

Oocysts are released into the intestinal lumen following the rupture of the intestinal cells, usually 3-10 days after the cat consumes the bradyzoites. If the cat ingests oocysts or tachyzoites, the prepatent period is 18 days or more (Dubey, 2001; Dubey, 2006). The oocysts are passed into the feces of the cat and get disseminated to the environment through the feces. Several millions of the unsporulated oocysts may be released in the feces of an infected cat. Since the sexual cycle of *T. gondii* occurs solely in the definitive host, only felids shed the oocysts of *T. gondii* (Miller *et al.*, 1972). The excreted oocysts get sporulated under suitable environment condition within 1-5 days and then infect its hosts, e.g. cow, sheep or human, through contaminated water or soil.

Human and other intermediate hosts may accidentally ingest infectious *T. gondii* sporulated oocysts, which initiate the asexual stage of *T. gondii*. Once ingested, the sporozoites escape from the oocysts and invade the epithelial cells lining the internal surfaces of the small intestine. The sporozoites multiply asexually to form tachyzoites which invaded other host cells by active penetration. Inside the host cells, the tachyzoites form vacuoles surrounding them to protect themselves from host immune system and then multiply asexually within the host cells. Two

daughter cells are formed within the parent parasite, which then replace the parent parasite. The process is repeated continuously until the host cell is filled with the parasites (usually 8-32 tachyzoites in a host cell) that leads to host cell death and subsequently breakdown of the cell. The tachyzoites are then released into the blood stream and disseminated in the body. The same invasion and replication processes are initiated in a new host cell. The multiplication and accumulation of the tachyzoites in the host cause acute *Toxoplasma* infection. The infection of circulating monocytes is thought to be the medium of spreading the infection to other tissues (Dubey, 1998a; Schwartzman, 2001).

Meanwhile, the large number of the tachyzoites found in the host cells also triggers the activation of body's immune system. Following this, the tachyzoites transform into slower multiplying form known as bradyzoites and establish chronic infection. Bradyzoites accumulate in large number within a host cell and are surrounded by a thin wall to form a tissue cyst. A tissue cyst can have hundreds of bradyzoites within the enclosed area.

1.5 Epidemiology

Toxoplasmosis is a widespread infection in animals and humans worldwide. It is estimated that one-third of the world's human population have this infection (Montoya and Liesenfeld, 2004). Prevalence studies of this infection have shown variable results from country to country and place to place, ranging from 10 – 80%. Many factors determine the seroprevalence of *T. gondii*. Higher seroprevalence is often seen in tropical countries with humid and warm climates which encourage the sporulation of *T. gondii* oocysts. Dietary habit of human is also a factor that affects the seroprevalence since a person can get infected through ingestion of *T. gondii*

bradyzoites in non-properly cooked meat. Economic, social or cultural habits including water quality, sanitation level, and education also play a role. Seroprevalence is often categorized into low, moderate and high where low indicate seroprevalence of below 30%, moderate indicate seroprevalence of 30-50% and high indicate the seroprevalence above 50%. In general, low seroprevalences were observed in North America, South East Asia, Northern Europe, and in Sahelian countries of Africa. Meanwhile, moderate seroprevalences were observed in Central and Southern Europe, and high prevalences were observed in Latin America and tropical African countries (Robert-Gangneux and Darde, 2012).

In the United States, *T. gondii* seroprevalence has decreased over the years. The seroprevalence among people born in the United States of age 12-49 was reported to decrease from 14% in 1988-1994 to 9% in 1999-2004, and 6.7% in the year 2009-2010 by using samples from the population-based National Health and Nutrition Examination Survey (NHANES). The women borned in USA of age 15-44 were reported to have a prevalence of 11% in the year 1999-2004 and the number decreased to 9.1% in 2009-2010 (Smith *et al.*, 1996; Jones *et al.*, 2007; Jones *et al.*, 2014). Besides, the seroprevalence among military recruits at the year 1962 and 1989 showed seroprevalence of 14.4% and 9.5%, respectively, clearly indicating a decreasing trend (Smith *et al.*, 1996).

In Colombia, a national study in the year 1980 found an overall 47% of *T. gondii* seroprevalence with similar proportions in men and women. The seroprevalence among pregnant women in this South America country ranged from 47% to 73%, throughout 1992 – 2007 (Canon-Franco *et al.*, 2014). Another study in the year 2008 found 53% seroprevalence among healthy individuals (Pordeus *et al.*, 2008).

A study in Poland showed mean seroprevalence of 41.3% among 4916 pregnant women aged 19 – 46 years old from year 1998 to 2003 (Nowakowska *et al.*, 2006). In another study, the mean prevalence of *T. gondii* was reported to be 40.6% among 8281 Polish pregnant women aged 18 – 47 years old between the years 2004 and 2012 (Nowakowska *et al.*, 2014). The prevalence obtained in the year 2004 – 2012 was similar but slightly lower compared to the prevalence obtained in the year 1998 – 2003, showing a consistent rate of *T. gondii* infection in the Poland.

The mean *T. gondii* seroprevalence reported among general Mexican population is 50%. Since Mexico is a large country which covers areas of subtropical, arid and temperate regions, the prevalence varies depending on the climate and humidity of the area (Galvan-Ramirez *et al.*, 2012). High prevalence was reported in wet coastal regions of the Gulf while arid regions have low prevalence (Hernandez-Cortazar *et al.*, 2015).

Brazil has a very high *T. gondii* seroprevalence. It is estimated that 50-80% of women of childbearing age and 50% of children at school age are infected by *T. gondii* (Dubey *et al.*, 2012). A survey conducted in the year 1964 using serum samples collected from males age 18-21 reported *T. gondii* seroprevalence of 56% in samples from Brazil compared with 13% in the United States (Walls and Kagan, 1967; Walls *et al.*, 1967; Lamb and Feldman, 1968). Santos *et al.* (2009) showed 97.4% *T. gondii* seroprevalence among 116 farm workers in Brazil. Another study in the year 2008 found 67.7% *T. gondii* prevalence among pregnant women who were screened for toxoplasmosis in Goiania, Brazil (Sartori *et al.*, 2011).

In Malaysia, seroprevalence of *T. gondii* has shown an increasing trend among healthy individuals and pregnant women. The prevalence in healthy individuals was reported as 13.9 – 20% in 1971-1980, and increased to 25 – 30% in

1981-1990. Meanwhile, the prevalence among pregnant women was reported as 23 – 27.4 % in the year 1971-1980, 27.9 – 31.6% in the year 1991-2000, and 49% in the year of 2003 (Nissapatorn *et al.*, 2003; Nissapatorn and Abdullah, 2004). A recent study found 42.3% *T. gondii* seroprevalence among pregnant women in Malaysia (Andiappan *et al.*, 2014).

1.6 Transmission

Toxoplasmosis is transmitted to humans in three main ways: congenital, foodborne and animal-to-human (zoonotic). Organ transplantation and blood transfusion also transmit the infection although these are uncommon.

1.6.1 Congenital

In a recent study, the global annual incidence of congenital toxoplasmosis was estimated to be 190,100 cases, which were approximately 1.5 cases per 1000 livebirths (Torgerson and Mastroiacovo, 2013). A pregnant woman who acquires primary *T. gondii* infection during her pregnancy may vertically transmit the infection to the fetus through the infected placenta (Montoya and Liesenfeld, 2004). The maternal-fetal transmission rate is approximately 10% during the first trimester, 30% during the second trimester and 60 to 70% during the third trimester. Therefore, the risk of congenital infection increases from 6% at 13th week to 72% at the 36th week of gestation (Dunn *et al.*, 1999). However, the risk of congenital infection and the risk of developing clinical signs are inversely related. The risk of developing clinical signs has been estimated to be 61% at 13th week and dropped to 25% at 26th week followed with 9% at 36th week of gestation (Dunn *et al.*, 1999). A fetus who is congenitally infected at early gestational age usually suffers severe or life-

threatening consequences compared with those infected during late pregnancy. The latter often has mild clinical symptoms or sometimes asymptomatic. A pregnant woman who acquires primary infection between the 10th to 24th week of gestational age tends to have a child with high probability of severe clinical symptoms (Tenter *et al.*, 2000).

On the other hand, pregnant women with chronic *T. gondii* infection have little risk or no risk of transmitting the infection to their fetus. Nevertheless, chronically infected women who are immunosuppressed may transmit the infection to the fetus.

1.6.2 Foodborne

Weinman and Chandler (1954) suggested that the toxoplasmosis may be transmitted by ingestion of raw or undercooked meat. This was then supported by Jacobs *et al.* (1960) when they reported that the bradyzoites were resistant to proteolytic enzymes. In another study, Desmonts *et al.* (1965) found that the yearly acquisition rates of *T. gondii* infection rose from 10% to 50% or 100% when different barely cooked meats were added to the orphans' daily diets.

In a multicenter European study, ingestion of inadequately cooked meat among pregnant women was identified as the main risk for toxoplasmosis (Cook *et al.*, 2000). Pomares *et al.* (2011) reported that toxoplasmosis caused by atypical *T. gondii* strains in France were most probably acquired *via* ingestion of raw horse meat originated from Canada and Brazil.

On the other hand, the *T. gondii* oocysts which survive in water may contaminate soil and water sources. Drinking untreated water or unpasteurized milk was thought to increase the risk of toxoplasmosis transmission (Jones *et al.*, 2009).

Oocysts in the soil can mechanically spread by invertebrates such as flies, cockroaches, earthworms into other forms of food source. Therefore, inadequate washing of contaminated raw vegetables and fruits may result in *T. gondii* infection.

Consumption of raw oysters, mussels and clams may be a potential risk for toxoplasmosis (Jones *et al.*, 2009). The *T. gondii* oocysts are carried to the ocean by contaminated water sources and infect the wild oysters (Lindsay *et al.*, 2004).

In the United Sates, toxoplasmosis has been identified as the second leading cause of foodborne illness-related death with an estimated of 327 deaths, and fourth leading cause of foodborne illness-related hospitalizations with 4428 hospitalizations annually (Scallan *et al.*, 2011). In Greece, toxoplasmosis is ranked the top 5 contributors of foodborne illnesses, causing an estimated 9.7 years of live lost, 14 years lived with disability and disability-adjusted life year (DALY) of 23 years per million persons (Gkogka *et al.*, 2011).

1.6.3 Animal-to-human (zoonotic)

Human contact with cat fecal material containing *T. gondii* oocysts is one of the risk factors of animal-to-human transmission. Domestic cats are the main source of this transmission route as a cat may excrete millions of oocysts after ingestion of only one bradyzoite. The domestic cats or kittens may get infected by ingesting intermediate hosts such as rodents and birds; then they contaminate the environment with *T. gondii* oocysts shed in their feces. Sporulated oocysts are resistant to environment conditions up to 18 months at temperatures of -20^{o}C to 35^{o}C (Frenkel *et al.*, 1975). Improper handling of cat feces may cause the transmission to the cat owner. A study in Canada screened *T. gondii* antibody from 998 children and adolescents reported the infection chance of the rural children with more than one cat

were two times higher than those who had one cat; and three times higher than children with no cat (Pereira *et al.*, 1992). Similarly, Jones *et al.* (2009) found that there is an increased in *T. gondii* transmission risks with cat owners who had three or more kittens. Infected wild felids in zoos may transmit the infection to zoo workers and visitors through the shed oocysts. It was found that 83% of 131 bobcats, one of the main wild felids in the United States, were seropositive to *T. gondii* (Mucker et al., 2006).

1.6.4 Others

Solid organ transplantation (SOT) is a potential transmission route of *T. gondii* infection. *Toxoplasma*-seronegative recipient gets infected by *T. gondii* after receiving the transplanted organ from a *Toxoplasma*-seropositive donor. This transmission was found mainly in cases of heart transplantation as cardiac muscle is one of the common sites for encysted bradyzoites. Approximately 50% of seronegative heart transplantation recipients acquired toxoplasmosis from the seropositive donors when prophylactic medication was not given (Schaffner, 2001). The rates of transmission were reported as 20% and less than 1% for liver and kidney transplantations respectively (Schaffner, 2001).

The risk of *T. gondii* transmission through blood transfusion is extremely low. Serological testing of *T. gondii* antibodies in the blood of donors is unnecessary except for those that are to be delivered to immunocompromised individuals and pregnant women. These high-risk groups are advised to receive blood component without *T. gondii* antibody (Singh and Sehgal, 2010).

1.7 Pathogenesis of toxoplasmosis

Tachyzoites can invade and multiply in the nucleated cells of all warm-blooded animals. The sequential release of parasite proteins from three major secretory organelles, i.e. the micronemes, rhoptries and dense granules, facilitate host cell attachment, invasion, and generation of the parasitophorous vacuoles (Carruthers and Sibley, 1997; Coppens and Joiner, 2001). The microneme proteins assist in recognition and adhesion to the target host cell while the rhoptry proteins are secreted at the time of invasion to produce parasitophorous vacuoles. Dense granules secrete enzymes for maturation of the vacuole. The parasitophorous vacuole formed inside the host cell is resistant to acidification and lysosomal fusion thus this allow the tachyzoites to multiply (Mordue *et al.*, 1999). Extracellular tachyzoites are susceptible to environmental changes and likely to be killed by the antibodies.

The proliferation of tachyzoites intracellularly during acute toxoplasmosis causes host cells death. This results in necrosis with a vigorous acute inflammatory reaction. As the tachyzoites continue to replicate and burst more host cells, more lymphocytic infiltrations develop. Tissue necrosis or lesions may be found in many organs including intestines, liver, spleen, pancreas, lung and heart (Dubey, 1996). This is damaging to tissues in which the cells do not regenerate, such as brain, eye, and muscles (Frenkel, 1974).

A study by Waree *et al.* (2007) reported that the inflammatory lesions were observed mainly in the liver and spleen following acute toxoplasmosis in mice. The accumulation of tachyzoites in the cytoplasm of hepatocytes caused degeneration of host cells. As such, the liver appeared swollen with random pinpoint white foci caused by hepatocellular necrosis and mononuclear cell infiltration. Meanwhile, the spleen was swollen with pale yellow necrotic foci distributed all over the organ.

Besides, the brain was congested with microscopic hemorrhages and the mononuclear cells that invaded into the meninges also caused meningitis. Lesions in central nervous system indicate the process of brain tissue destruction (Waree, 2008).

The replication of tachyzoites falls under control with the trigger of host immune response. Tachyzoites disappear from the visceral tissue and tissue cysts containing bradyzoites are formed in neural and muscular tissues (Waree, 2008). Tissues are restored while the tissue cysts may appear silent to the host immune response and persist for the life of the host. In chronic toxoplasmosis, lesions are often observed in eye muscle and brain rather than in visceral tissues (Dubey and Beattie, 1988).

1.8 Immune response

T. gondii infection triggers both cellular and humoral immune responses in the immunocompetent host. The cell-mediated immune response is the main control factor for the infection compared to the effects of antibody (Schwartzman, 2001). Macrophages produce immunologic mediators which help in the regulation of cellular immune response with interleukin-12 (IL-12) as the main activator. IL-12 activates natural killer (NK) cells and T-cells to produce interferon-γ (IFN-γ). Macrophages act synergistically with IFN-γ and tumor necrotic factor (TNF) to kill the tachyzoites. The IFN-γ and TNF elevated the free radical and nitric oxide levels that also accelerate the killing of the parasite.

T-lymphocytes with CD8+ are the major effector cells that act against *T. gondii* with the help of T-lymphocytes with CD4+ (Figure 1.4). Mature CD4+ T-lymphocytes cells are divided into T-helper 1 (Th-1) and 2 (Th-2). CD8+ cells secreted IFN-γ while Th-1 CD4+ cells produced IFN-γ and IL-2. IL-2 induce

lymphokine to activate natural killer (NK) cells that are cytotoxic to *T. gondii*-infected host cells. Th-2 CD4+ cells produce IL-4, IL-5, and IL-10 which play a role in down-regulation of the protective cell-mediated immune response. The interleukins are able to cross-regulate each other's activity; IL-10 inhibits production of IFN-γ while IFN-γ inhibits multiplication of Th-2 cells. Cytokines produced by T-lymphocyte cells such as IL-18, IL-17 and IL-15 encourage the production of IFN-γ which plays a role during acute and chronic infection (Bhopale, 2003; Waree, 2008).

The antibodies in humoral immune response are thought to play a minor role in killing of *T. gondii* compared to cell-mediated response. The IgG, IgM, IgA and IgE antibodies are produced against the membrane antigens and the other proteins excreted by *T. gondii*. In the presence of complement, specific antibodies act against extracellular tachyzoite and lyse it. The specific IgA antibody plays a role in the disruption of parasite interaction with the host cells at the mucous membrane. Platelets are cytotoxic to tachyzoites in the absence of antibody. The level of thromboxane and other metabolites of arachidonic acid also increase simultaneously (Bhopale, 2003).

The immune responses from an immunocompetent individual often control the infection effectively, leaving little organ damage. The encysted bradyzoite caused little immune response and live long. The bradyzoite can be reactivated to become tachyzoites when there are changes in the host immune function and this may cause severe tissue and organ damage.

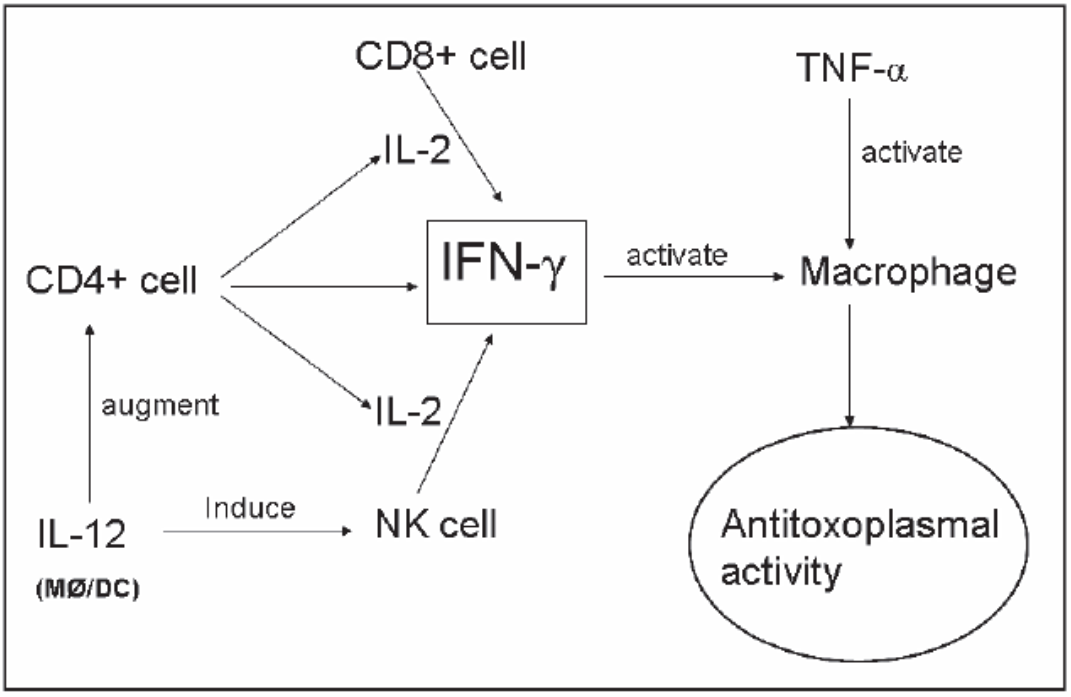


Figure 1.4 The major cytokines involved in cell-mediated immune response towards tachyzoites and their relationships (Waree, 2008).

1.9 Clinical presentations of toxoplasmosis

T. gondii infection causes variable clinical manifestations based on the immune status of the infected person. Acquired toxoplasmosis usually causes mild symptoms or is asymptomatic in an immunocompetent individual. However, congenital toxoplasmosis may cause debilitating effects in infected infants while reactivated toxoplasmosis that occurs in immunocompromised person can be life-threatening.

1.9.1 Immunocompetent individuals

Most cases of acquired toxoplasmosis in immunocompetent individuals are without clinical symptoms and are self-limited. It is reported that more than 80% of infected immunocompetent individuals in European countries or North America are asymptomatic (Montoya and Liesenfeld, 2004). In the symptomatic infections, enlarged lymph nodes are commonly observed. Lymphadenopathy is often associated with fever, fatigue, muscle pain, sore throat and headache (Hill and Dubey, 2002). Less commonly, the acute acquired infection causes toxoplasmic chorioretinitis with visual impairment (Delair *et al.*, 2008). Bowie *et al.* (1997) found that 51 out of 100 individuals infected with toxoplasmosis during an outbreak had lymphadenopathy while 19 of the remaining individuals had retinitis. Acquired toxoplasmosis was reported to be responsible for 23.5% of 425 cases of ocular toxoplasmosis (Delair *et al.*, 2008). Other common symptoms of acquired toxoplasmosis include stiff neck, poor appetite, joint pain, rash, confusion, nausea, eye pain and abdominal pain. Pneumonia and nephritis were also reported (Canon-Franco *et al.*, 2014). Table 1.1 shows the frequency of symptoms in immunocompetent people reported from several toxoplasmosis outbreaks.

Table 1.1 Frequency of symptoms in immunocompetent people with acquired toxoplasmosis (Dubey, 2009).

Symptoms	Patients with symptoms (%)		
	Atlanta, USA outbreak (35 patients)	Panama outbreak (35 patients)	Paráná outbreak (155 patients)
Fever	94	90	82
Lymphadenopathy	88	77	75
Headache	88	77	87
Myalgia	63	68	80
Stiff neck	57	55	NR
Anorexia	57	NR	69
Sore throat	46	NR	NR
Arthralgia	26	29	61
Rash	23	0	7
Confusion	20	NR	NR
Earache	17	NR	NR
Nausea	17	36	38
Eye pain	14	26	NR
Abdominal pain	11	55	NR

* NR: not reported

1.9.2 Congenital toxoplasmosis

Congenital toxoplasmosis from primary acquired maternal infection during the first trimester of gestation often causes severe consequences such as abortion, stillbirth or infant abnormalities compared to maternal-fetal transmission during the second and third trimesters. Fetal infection at the second trimester can be of variable severities while the infection at third trimester is usually less severe.

The multiplication of *T. gondii* induces necrosis foci and inflammation reactions, leading to infant abnormalities in brain and eye tissues. The most common symptoms of congenital toxoplasmosis among infants are retinochoroiditis and intracranial abnormalities with or without developmental delay (Gilbert *et al.*, 2006). Other severe clinical manifestations of congenital toxoplasmosis include mental retardation, seizures, microcephalus, hydrocephalus, intracranial calcification, encephalitis, deafness, extensive cerebral destruction, psychomotor deficiency, convulsion and eye lesions (Frenkel, 1974; McAuley *et al.*, 1994; Swisher *et al.*, 1994). Severe eye lesions are observed in congenital toxoplasmosis at early pregnancy, this included microphthalmia, cataract, increased intraocular pressure, strabismus, optic neuritis and retinal necrosis (Roberts *et al.*, 2001b; Delair *et al.*, 2011).

Infants with congenital toxoplasmosis may have obvious systemic manifestation such as fever, hypothermia, jaundice, hepatosplenomegaly, diarrhea, vomit, lymphadenopathy, pneumonitis, myocarditis and petechial or purpuric rash (McAuley *et al.*, 1994; Schwartzman, 2001). Meanwhile, the classical triad of chorioretinitis, hydrocephalus, and cerebral calcifications is uncommon as these symptoms are present in less than 10% of infected infants (Montoya and Liesenfeld, 2004).

Approximately 75% of infants born with toxoplasmosis are asymptomatic but subsequently, develop clinical symptoms within months to years (Schwartzman, 2001). Phan *et al.* (2008) reported that 72% of 25 congenitally infected infants developed eye lesions during a mean follow-up of 5.7 years. Freeman *et al.* (2008) also reported 50 ocular diseases and 17 recurrent retinochoroiditis developed out of 281 children with congenital toxoplasmosis in a median follow-up of 4.1 years. Table 1.2 shows clinical presentation in untreated congenital toxoplasmosis patients at birth and with follow-up of four years and more.

1.9.3 Immunocompromised patients

Acute or reactivated *T. gondii* infection causes serious life-threatening diseases in immunocompromised patients. The immunocompromised groups with increased risk of developing toxoplasmosis include transplantation patients who are treated with immunosuppressive agents, cancer patients with low immunity and acquired immunodeficiency syndrome (AIDS) patients. The most common manifestation of toxoplasmosis seen in immunocompromised patients is encephalitis (Hill and Dubey, 2002). Besides, the heart and lungs of an immunocompromised patient can also be infected.

The clinical symptoms of toxoplasmosis reported in organ transplantation include febrile myocarditis, encephalitis or pneumonitis. The symptoms can be observed as early as two weeks but are commonly seen within the first three months after the transplantation (Derouin *et al.*, 2008). The mortality rates of organ transplantation patients who developed toxoplasmosis are high.