ISOLATION AND EVALUATION OF cDNA CLONES SPECIFIC TO POLYCLONAL ANTIBODY AGAINST 12 kDa EXCRETORY SECRETORY ANTIGEN (ESA) OF *Toxoplasma gondii*

by

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This thesis is dedicated to my parents, Abdul Aziz and Jameela, for their endless support and encouragement throughout.

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ABBREVIATIONS

T. gondii	*Toxoplasma gondii*
ESA	Excretory Secretory Antigen
RPMI	Roswell Park Memorial Institute medium
RPMI-PS	RPMI containing Penicillin/Streptomycin
SDS	Sodium Dodecyl Sulphate
PAGE	Polyacrylamide Gel Electrophoresis
ELISA	Enzyme-Linked Immunosorbent Assay
PBS	Phosphate Buffered Saline
Ig	Immunoglobulin
BSA	Bovine Serum Albumin
NCP	Nitrocellulose Membrane
ECL	Enhanced Chemiluminescence
CFA	Complete Freund's Adjuvant
IFA	Incomplete Freund's Adjuvant
OD	Optical Density
RF	Rheumatoid Factor
HRP	Horseradish Peroxidase
cDNA	Complementary Deoxyribonucleic Acid
BLAST	Basic Local Alignment System Tool
MW	Molecular Weight
DMSO	Dimethyl sulfoxide

APS	Ammonium persulfate
AI	Avidity Index
IU	International Unit
IPTG	Isopropyl-beta-D-thiogalactopyranoside
pfu	Plaque Forming Unit
PVM	Parasitopotous vacuole membrane
TEMED	Tetramethylethylenediamine
M	Molar
mM	milimolar
L	Liter
ml	mililiter
g	gram
mg	miligram
µg	microgram
rpm	Revolutions per minute

PENGASINGAN DAN PENILAIAN KLON cDNA SPESIFIK KEPADA ANTIBODI POLIKLONAL TERHADAP 12 kDa ANTIGEN REMBESAN PERKUMUHAN (ESA) *Toxoplasma gondii*

ABSTRAK

Toxoplasmosis adalah jangkitan zoonotik yang disebabkan oleh parasit *Toxoplasma gondii*. Ia merupakan parasit protozoa intraselular yang boleh didapati di seluruh dunia, dengan kekerapan yang lebih tinggi di negara-negara tropika. Walaupun risiko penyakit pada individu imunokompeten adalah rendah, gejala yang serius boleh berlaku apabila individu yang sistem imunnya terkompromi dan wanita hamil dijangkiti. Ia sangat mencabar lagi penting untuk menentukan sama ada jangkitan akut telah berlaku di kalangan wanita hamil kerana risiko jangkitan pada janin boleh menyebabkan kematian atau kerosakan organ yang kekal. Potensi diagnostik pelbagai antigen telah dikaji untuk menangani masalah ini kerana ujian diagnostik yang efisyen adalah penting untuk menentukan jangkitan peringkat akut. Rembesan antigen secara aktif oleh *T. gondii* yang telah didokumenkan sebagai antigen rembesan perkumuhan (ESA) didapati imunogenik pada kedua-dua manusia dan haiwan. Ia telah dilaporkan dapat merangsang tindak balas imun yang lebih baik daripada antigen parasit larut, oleh itu ESA merupakan antigen yang berpotensi untuk penyelidikan.

Sebelum ini, jalur 12 kDa dari ESA *T. gondii* telah dilaporkan sebagai penanda jangkitan akut dengan potensi diagnostik yang baik. ESA dihasilkan dari takizoit yang dituai daripada peritoneum tikus dan SDS-PAGE telah dilakukan menggunakan ESA tersebut. Jalur protein 12 kDa telah dikenal pasti dan dipotong, seterusnya digunakan

untuk menghasilkan antibodi poliklon dalam arnab. Dua jenis strategi imunisasi telah digunakan dalam kajian ini iaitu imunisasi menggunakan protein daripada kepingan gel akrilamid dan imunisasi menggunakan protein yang telah di 'elektro-elute' dan dicampurkan dengan adjuvan. Antibodi yang dihasilkan kemudiannya melalui proses penulenan dan dijerap dengan protein perumah *E. Coli* terlebih dahulu. Perpustakaan cDNA *T. gondii* Lambda ZAP II telah disaring dengan antibodi poliklon yang dijana dengan anti-rabbit IgG-HRP yang merupakan antibodi sekunder. Klon reaktif / positif telah diasingkan, diamplifikasikan dan prosedur di atas diulangi untuk langkah saringan yang kedua. Untuk saringan pusingan ketiga, klon yang dipilih dari saringan pusingan kedua telah disaring menggunakan tiga sampel serum individu yang dijangkiti toxoplasma dan tiga sampel serum individu yang negatif / kawalan. Antibodi anti-manusia IgM-HRP telah digunakan sebagai antibodi sekunder. Dua klon yang memberikan hasil yang terbaik dari segi sensitiviti dan spesifisiti telah disaring seterusnya menggunakan 43 sampel serum daripada pesakit and individu sihat, dan antibodi sekunder yang sama digunakan. Akhirnya, dua klon terbaik diasingkan dan dihantar untuk penjujukan bagi menentukan rantau lengkap pengekodan DNA.

Imunisasi menggunakan protein 'elektro-eluted' yang dicampurkan dengan adjuvan menghasilkan antisera bertiter tinggi iaitu 1:35, 000 manakala arnab yang diimunisasi dengan kepingan gel akrilamid gagal untuk menghasilkan antibodi terhadap 12 kDa ESA daripada *T. gondii*. Dua klon telah diasingkan, iaitu klon 5c dan klon 23. Sensitiviti dan spesifisiti klon 5c adalah 79% manakala bagi klon 23, sensitiviti adalah 75% dan spesifisiti adalah 79%. Jika kedua-dua klon ini digabung, sensitiviti dan spesifisiti sebanyak 79% diperoleh. Klon 5c telah dikenal pasti sebagai protein hipotesis dan klon 23 telah dikenal pasti sebagai protein hipotesis konserv.

Sebagai kesimpulan, saringan perpustakaan cDNA dengan antisera poliklon arnab dan sera manusia telah menghasilkan dua klon cDNA yang menunjukkan sensitiviti dan spesifisiti yang baik untuk mengesan penyakit toksoplasmosis akut bila diuji menggunakan serum pesakit yang dijangkiti dengan *T.gondii* dan serum individu yang tidak dijangkiti.

ISOLATION AND EVALUATION OF cDNA CLONES SPECIFIC TO POLYCLONAL ANTIBODY AGAINST 12 kDa EXCRETORY SECRETORY ANTIGEN (ESA) OF *Toxoplasma gondii*

ABSTRACT

Toxoplasmosis is a zoonotic infection caused by *Toxoplasma gondii*. It is an obligate intracellular protozoan parasite distributed worldwide, with higher prevalence in the tropical countries. Although the risk of an overt disease in immunocompetent individuals is not high, severe manifestation may result when immunocompromised individuals and pregnant women are infected. It is very challenging yet crucial to establish whether acute infection has occurred in pregnant women as the fetus could be at risk of infection which can cause death or permanent organ damage. The diagnostic potential of various antigens have been studied to address this issue as a good diagnostic tool is essential to determine the acute stage of the infection. The active secretion of antigens by *T. gondii* i.e. excreted-secreted antigens (ESA) has been found to stimulate a better immune response than the parasite soluble antigen. Thus, ESA is a potential antigen for research.

Previously 12 kDa band from *T. gondii* ESA has been determined as an acute infection marker with good diagnostic potential. In this study, ESA was produced from tachyzoites harvested from mice peritoneal exudates and subjected to SDS-PAGE. The 12 kDa protein band was excised and used to produce polyclonal antibody in rabbits. Two types of immunization strategies were employed in this study namely immunization using protein from acrylamide gel pieces, and immunization using

electro-eluted protein mixed with adjuvant. The antibody produced was then purified and pre-adsorbed with the *E. coli* host proteins. The *T. gondii* Lambda Zap II cDNA library was screened with the polyclonal antibody generated with anti-rabbit IgG-HRP as the secondary antibody. The reactive/positive clones were cored out, amplified and the above procedure was repeated for secondary screening step. For tertiary screening step, the selected clones from secondary screening were subjected to immunoscreening using three toxoplasma positive serum samples and three negative/normal control human serum samples. Anti-human IgM-HRP was used as the secondary antibody. Two clones that exhibited the best results were subjected to sensitivity and specificity test using 43 serum samples from toxoplasma patient sera and healthy individuals, and the same secondary antibody. Finally, the best two clones were isolated and sent for sequencing to determine the complete coding region.

Immunization using electro-eluted protein mixed with adjuvant produced antisera which produced a high titer of 1:35 000 while the rabbit immunized with acrylamide gel pieces failed to produce antibody against the 12 kDa ESA of *T. gondii*. Immunoscreening of the *T. gondii* cDNA library finally resulted in the isolation of two clones which showed the best immunoreactivity, namely clones 5c and 23. The sensitivity and specificity of clone 5c were both 79%; while for clone 23 the sensitivity was 75% and the specificity was 79%. If the results of these two clones were combined, sensitivity and specificity of 79% was obtained. DNA sequencing identified clone 5c as hypothetical protein and clone 23 as conserved hypothetical protein.

In conclusion, immunoscreening of the *T. gondii* cDNA library with rabbit polyclonal antisera to 12 kDa *T. gondii* ESA had produced two cDNA clones which showed good sensitivity and specificity for detection of acute toxoplasmosis when tested with patient and healthy sera samples.

CHAPTER ONE

INTRODUCTION

1.1. Toxoplasmosis: Overview

Toxoplasmosis is a cosmopolitan zoonotic infection with higher prevalence in the tropical countries. It is a disease caused by *Toxoplasma gondii*, an obligate intracellular protozoan parasite distributed worldwide and they have become highly adapted in order to invade and develop within host cells. This apicomplexan parasite is capable of infecting warm-blooded animals and other mammals including humans, who act as the intermediate hosts of the parasite. Cats and other members of the Felidae are the definitive hosts of this parasite. This infection which is transmitted by cats has a high prevalence especially at places where raw meat or undercooked meats are widely consumed.

Toxoplasmosis can be divided into three categories; acquired toxoplasmosis, congenital toxoplasmosis and recrudescent/ reactivated toxoplasmosis. Acquired toxoplasmosis is the result of accidentally consuming food or water contaminated with cat faeces while congenital toxoplasmosis is when infected pregnant women pass the disease to her unborn child. It is assumed that one third of the world populations are infected with this parasite (Indra, 2003). Recurrence or reactivation of *T. gondii* infection is one of the clinical problems in congenitally infected individuals, immunocompromised hosts and AIDS patients (Ferguson and Dubremetz, 2007; Jackson and Hutchison, 1989). According to Bhopale (2003a), recrudescence of active infection could be influenced by host factors such as genetic predisposition or variation in virulence among different strains, resulting in stage conversion back to actively proliferating tachyzoites.

There are four groups of individuals in whom the diagnosis of toxoplasmosis is most critical namely pregnant women who acquire primary infection during gestation, fetuses and newborns who are congenitally infected, immunocompromised patients, and those with chorioretinitis. Most infections in healthy individuals are asymptomatic and self-limiting, but these individuals remain chronically infected with presence of cysts in various organs especially brain. Rupture of the cysts and reactivation into serious disease may occur when the individuals are immunosuppressed (Dubey and Beattie, 1988).

It is apparent that toxoplasmosis is still very much a challenging subject including the aspect of diagnosis of the infection. Besides that, further understanding of *T. gondii* antigens can help researchers to come up with new vaccines, better prevention strategies and more effective treatment regimes.

1.2. *Toxoplasma gondii*

1.2.1. Historical perspective

Charles Nicolle and Louis Manceaux in 1908 discovered the parasite in the lymph and liver of *Ctenodactylus gundi*, a North African rodent. It marks the beginning of the discovery of *Toxoplasma gondii*. Later in 1910, Mello reported about the same parasite found in sanguineous exudates and in nodules in the lung of a dog in Turin, Italy. Acute toxoplasmosis described by Mello made it the first canine toxoplasmosis case to be reported (Miro *et al*., 2008). The first case of human toxoplasmosis was described by Janku in Czechoslovakia in 1923, who found the protozoan parasite in patients' with chorioretinitis while in 1939, Wolf had successfully isolated the parasite from neonates with encephalitis and reported that the parasite caused congenital infection to the fetus (Wolf *et al*., 1939).

First feline toxoplasmosis was not reported until 1942, although the transplacental transmission of the parasite was long known before that. In the 1942 study by Olafson and Monlux, *T. gondii* was identified in the lymph nodes and lungs of a cat. Only in 1970 that the life cyle of the parasite was clearly understood and according to Levine (1990), several researchers from different countries found that *T. gondii* produces oocysts in the cat, and the oocysts contain two sporocysts and four sporozoites were found in each sporocysts. Dubey (2008) stated that in the 1980s and 1990s, several attempts were made to develop methods to recognize genetic differences among *T. gondii* isolates from humans and animals but only in 2005 that mapping of *T. gondii* genes was achieved. The first in-depth study of genetic variability among *T. gondii* isolates obtained worldwide by Lehmann *et al*. (2006) who found geographic differences, with some isolates being confined to Brazil whereas others were distributed worldwide. Until today, the search for better antigens for diagnosis and protection, and mechanism of disease are still ongoing.

1.2.2. Morphology

The infectious stages of *T. gondii* can be divided into three namely the tachyzoites, bradyzoites, and oocysts.

1.2.2.1. Tachyzoites

The tachyzoite (figure 1.1a) is the proliferative form of the parasite that Nicolle and Manceaux (1908) found in the gundi. This rapidly multiplying stage of the parasite has also been referred to as trophozoite, the feeding form, and endozoite. They are lunate or crescent-shaped, measure 7 x 3 µm and can infect virtually every kind of host cell in the body (Ferguson and Dubremetz, 2007)**,** phagocytic and non-phagocytic, and also nucleated cells by active penetration but not the non-nucleated red blood cells (Waree, 2008). Multiplication occurs through a specialized process that was first described by Goldman *et al*., (1958) called endodyogeny. Endodyogeny is a process during which two progeny form within the parent parasite (Wong and Remington, 1993). The disrupted host cells will disseminate tachyzoites to the bloodstream and this causes a strong inflammatory response and infection of the tissues including eye, skeletal and heart muscle as a result of its repeated replication. The rapid invasion of the parasite into the host cells and neighbouring cells suggest that they probably have a secretory function that helps them to enter the host cell (Nichols *et al*., 1983). As the immune response of the host cell take charge as a result of an infection, the tachyzoites are transformed into bradyzoites to form cysts (Montoya and Liesenfeld, 2004).

1.2.2.2. Bradyzoites

Bradyzoites (figure 1.1b) or life-long tissue cysts are slow dividing form of the parasite and they persist in latent infection for the life of the host. Cysts are the infective stage for intermediate and definitive hosts. The 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 contain thousands of organisms, and this could explain why the bradyzoite is the only stage that can initiate the sexual phase (Dubey, 2010). This tissue cysts containing hundreds and thousands of bradyzoites form may develop in visceral organs, including the lungs, liver, and kidneys, they are more prevalent in the neural and muscular tissues, including the brain, eyes, and skeletal and cardiac muscles (Dubey, 2010). The encysted bradyzoites do not cause any harm but if the cysts rupture and bradyzoites are released from the cysts, they can transform back into tachyzoites and cause recrudescence of infection especially in immunocompromised patients (Montoya and Liesenfeld, 2004).

1.2.2.3. Oocysts

Cats are the definitive host for *T. gondii* and oocysts are normally shed in cat faeces after the completion of sexual phase in the feline gut epithelium. The oocysts (figure 1.1c) excreted are unsporulated and non-infectious, but it can become infectious after 48 hours or more of environmental incubation (Miro *et al.*, 2008). As a result of environmental incubation, sporulation of the oocysts take place which give rise to sporocyst containing sporozoites, and when ingested by mammals (including man), they will become infected (Montoya and Liesenfeld, 2004).

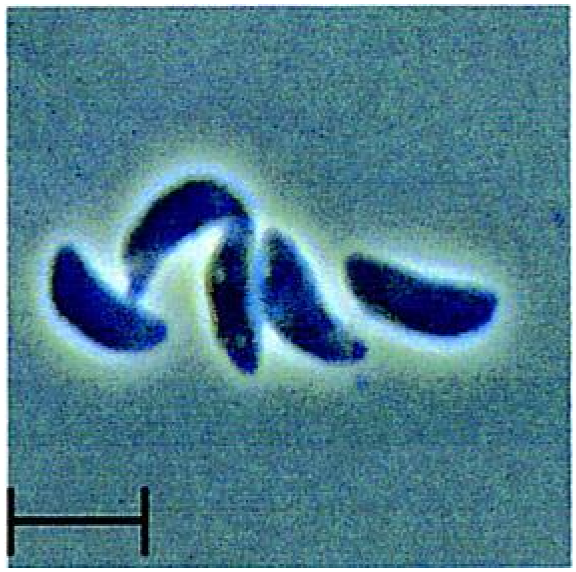

Figure 1.1a: The tachyzoites, which are the invasive form of the *T. gondii* parasite. Scale bar = 10 µm. (http://www.sciencedirect.com/science/article/pii/S0166685101003140)

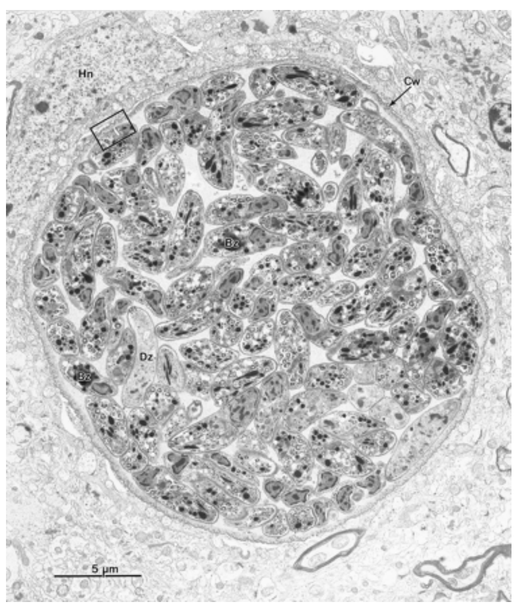

Figure 1.1b: The bradyzoite stage or the encysted form of *T. gondii* (http://parasitology1000.blog.com/educationalimages/)

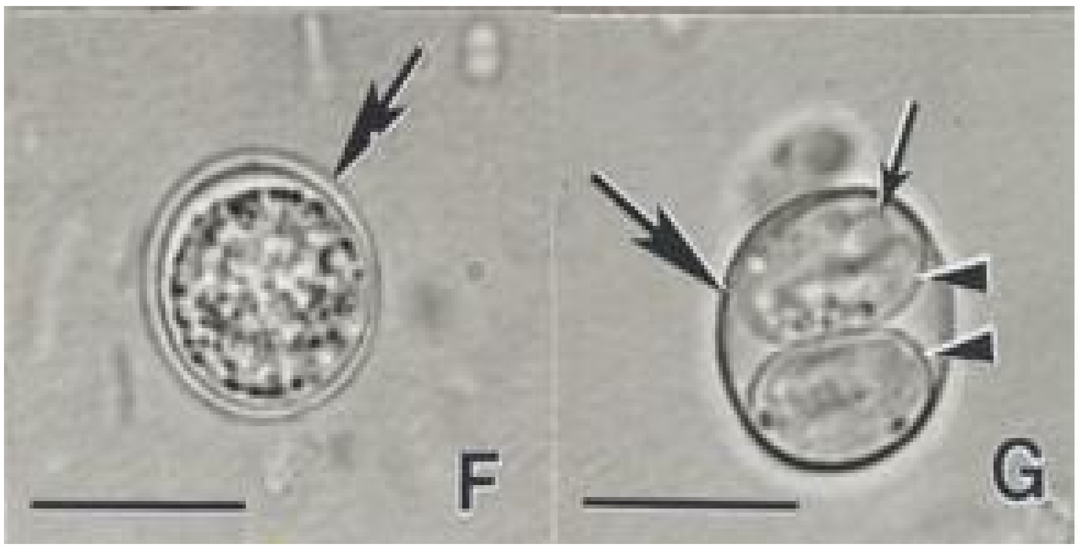

Figure 1.1c shows **F.** Unsporulated oocyst in fecal float of cat feces. **G.** Sporulated oocyst with a thin oocyst wall (large arrow), 2 sporocysts (arrowheads). Each sporocyst has 4 sporozoites (small arrow) which are not in complete focus. Scale bar = 10 µm. (http://www.ars.usda.gov/main/docs.htm?docid=11013)

1.2.3. Life cycle

The life cycle of *T. gondii* can be divided into two phases; the sexual phase that occurs exclusively in feline, the definitive host and the asexual phase that takes place in mammals or intermediate hosts. The sexual phase or entero-epithelial cycle is initiated after ingestion of an infected prey or faeces contaminated with infected sporocysts (figure 1.2). A typical process of gamete formation and fusion will then take place in the cat gut epithelium giving rise to a zygote. The zygote will eventually develop into immature oocysts and will be shed in cat faeces. Depending on the environmental temperature and humidity, the oocysts will mature into resistant sporulated oocysts (sporocysts) containing sporozoites. At this stage, the oocysts have become highly infectious and accidental consumption by other cats will result in initiation of a new sexual cycle. But if ingested by any warm blooded animal, asexual cycle will then take place.

The asexual cycle consists of two stages, depending on whether the infection is in acute or chronic phase. Once in the intermediate hosts, the sporozoites will be released in the intestinal lumen and undergo endodyogeny. They will differentiate into the rapidly dividing tachyzoite and invade different cells during the acute phase. They replicate inside a cell, and when tachyzoites are accumulated in the cell, the cell rupture and they exit the cell to infect the neighboring cells. As the host's immune response rises to the challenge, tachyzoites differentiate into bradyzoites and form tissue cysts that are very stable and infectious if tissue from the animal is eaten. The cysts are predominantly

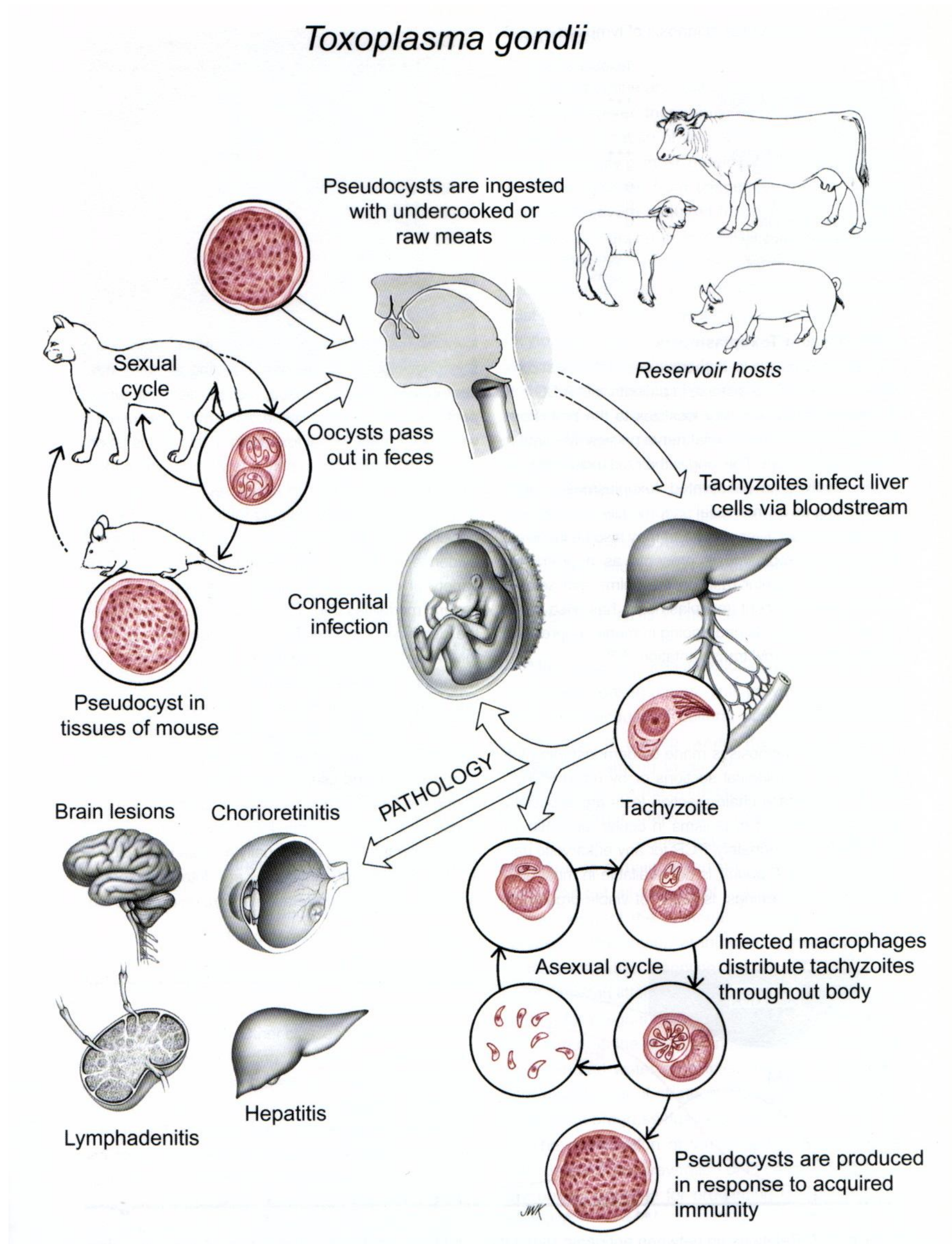

Figure 1.2: Summary of complete life cycle of *Toxoplasma gondii*.
(http://www.microbeworld.org/images/stories/twip/t_gondii_cycle.jpg)

found in the central nervous system and muscle tissue, and may reside for life of the host (Black and Boothroyd, 2000).

As they pass through the digestive tract, the ingested cysts are ruptured causing the release of the bradyzoites. These bradyzoites are capable of differentiating back to the proliferative tachyzoite stage and will be dispersed throughout the body, thereby completing the asexual phase (Black and Boothroyd, 2000). More often than not the immune response will effectively prevent the spreading of the tachyzoites. However, such reactivation may go unimpeded in immunocompromised patients, resulting in a massive and potentially fatal infection.

1.2.4. *T. gondii* strains

There is more than one strain of *T. gondii* and there are evidences of marked differences between the strains (Bhopale, 2003b). Virulence of a strain is determined on the basis of its ability to kill the white mice (Thomas, 1979) and different strains of Toxoplasma vary in their virulence. The strains have three clonal lineages designated type I, II and III which differ in virulence and epidemiological pattern of occurrence. Type I and type II are virulent strains while type III is avirulent. Strains isolated from animals are mostly type III while type I and type II strains have been recorded in patients with congenital disease and AIDS (Montoya and Liesenfeld, 2004). Various techniques like immunoprecipitation, Western blot, isoenzyme analysis as well as molecular genetic techniques have been employed to demonstrate the strain-specific differences of *T.*

gondii isolates and all the informations revealed that there is more than one *T. gondii* strain among isolates in the nature with difference in virulence (Bhopale, 2003b).

1.3. Epidemiology

T. gondii is distributed world-wide but the frequency of infection in man and animals varies from country to country. The disease is one of the most important infectious diseases causing epidemiological and clinical impacts in humans. The prevalence rate of infection is greater in the tropics and subtropics than in colder regions. Predisposing factors like direct contact with cats and consumption of undercooked meat play a crucial role in *Toxoplasma* infection. Lones *et al*. (2001) reported that the United States has low *T. gondii* seroprevalence compared to France and Latin America or sub-Saharan Africa. They indicated that a significant proportion of toxoplasmosis in the United States may be due to ingestion of raw or undercooked infected meat or cross-contamination from such meat. Ancelle *et al*. (1996) also related the higher seroprevalence of *T. gondii* in France to the common consumption of undercooked meat.

Evidence of *Toxoplasma* infection in man and in a number of domestic and wild animals had been shown in serological surveys in Malaysia and Singapore (Thomas, 1979). High prevalence was recorded among the Malaysian population and the highest seroprevalence of toxoplasmosis in most of the studies was in Malays, followed by Indians, Orang Asli (aborigines) and Chinese (Nissapatorn and Abdullah, 2004). The highest prevalence rate among Malays may indicate that oocysts from cats are the main source of infection. This study is supported by other studies in this region (Partono and

Cross, 1975; Gandahusada, 1978; Wong *et al*, 2000). Various groups of population have been studied and the presence of specific antibodies were demonstrated in people of both sexes in all age groups including neonatals, infants, children and adults of all races. However, the prevalence rate was highest among children below 10 years of age indicating possible infection with oocysts from contaminated soil rather than from contaminated meat (Thomas, 1979).

1.4. Transmission

There are various ways that *T. gondii* can be transmitted since cats are everywhere except the frozen arctic. It could be transmitted at any stage of its life cycle and to humans and animals. The transmission could occur congenitally, from mother to the fetus, carnivorism, through fecal oral route and other less common route of infection like transplantation and laboratory accidents (figure 1.3).

1.4.1. Congenital

Wolf *et al*. (1939) first described congenital *T. gondii* infection in a human child and later found that it occur in many species of animals as well, particularly sheep, goats, and rodents (Dubey, 2008). The differing risk factors and modes of transmission could probably explain the significant divergence on the prevalence and incidence of toxoplasma infection among pregnant women between countries (Hall *et al*., 2001). The birth prevalence of congenital toxoplasmosis ranges from one to ten per 10, 000 live births (Lebech *et al*., 1999) where in the majority of cases the infection is either

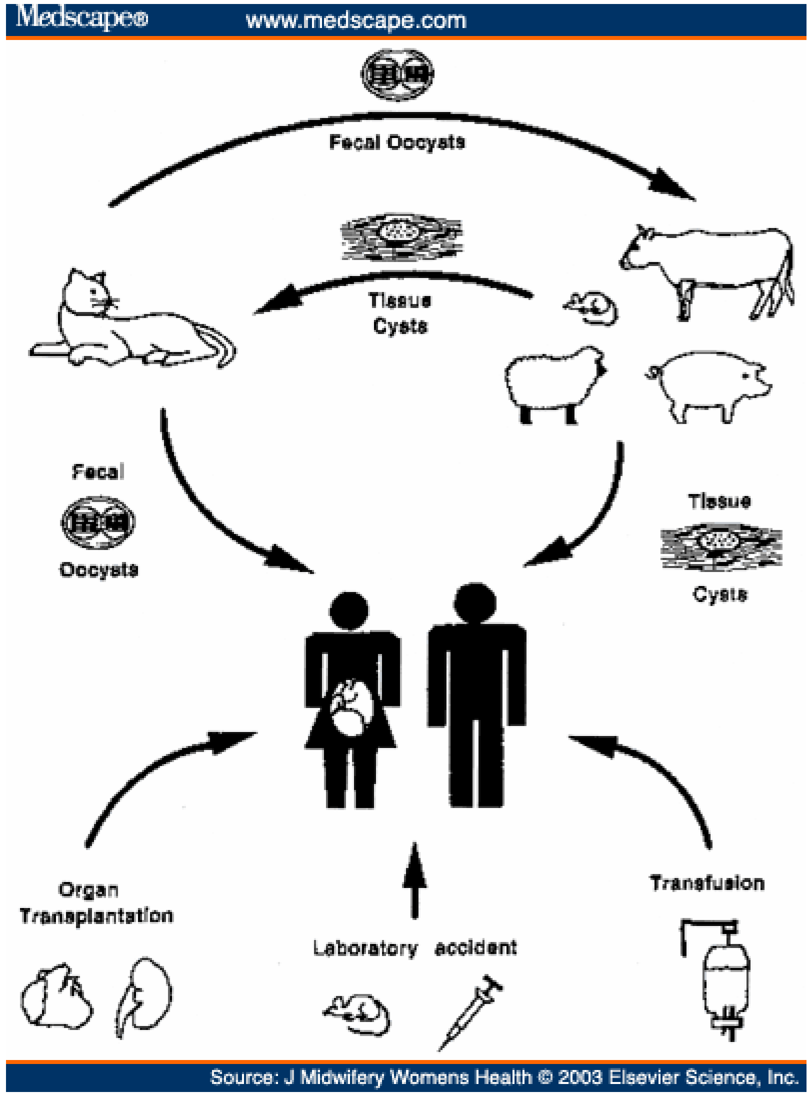

Figure 1.3: Transmission of *T.gondii* to humans and animals. (http://www.medscape.com/viewarticle/462440_2)

asymptomatic or symptoms may pass unnoticed. As can be seen from figure 1.4, the frequency of transmission varies considerably according to the time during gestation that the mother became infected and is inversely related to severity of the disease (Montoya and Lisenfeld, 2004).

The parasite enters the fetal circulation by infection of the placenta and if it happens in early pregnancies, it may result in death and spontaneous abortion. However, infection during late pregnancies usually results in normal appearing newborns where infection initially goes unnoticed but some complications of the disease may develop later in life (Remington *et al.*, 2001). The rate of transmission rises to 30–50 percent in women infected in the second trimester, and 60–70 percent for those infected during the third trimester. It is therefore generally accepted that there is no significant risk of congenital infection in the fetus when infection occurs before conception (Health protection agency, 2010).

1.4.2. Carnivorism

There is increasing evidence that the ingestion of cysts in meat is a common method of transmission of *T. gondii*. In 1954, Weinman and Chandler suggested that transmission might occur through the ingestion of undercooked meat while Jacobs *et al.* (1960) provided evidence to support this idea. They found out that the cysts wall was immediately dissolved by proteolytic enzymes of *T. gondii* but the released bradyzoites remain intact. Desmonts *et al.* (1965) illustrated the importance of carnivorism in

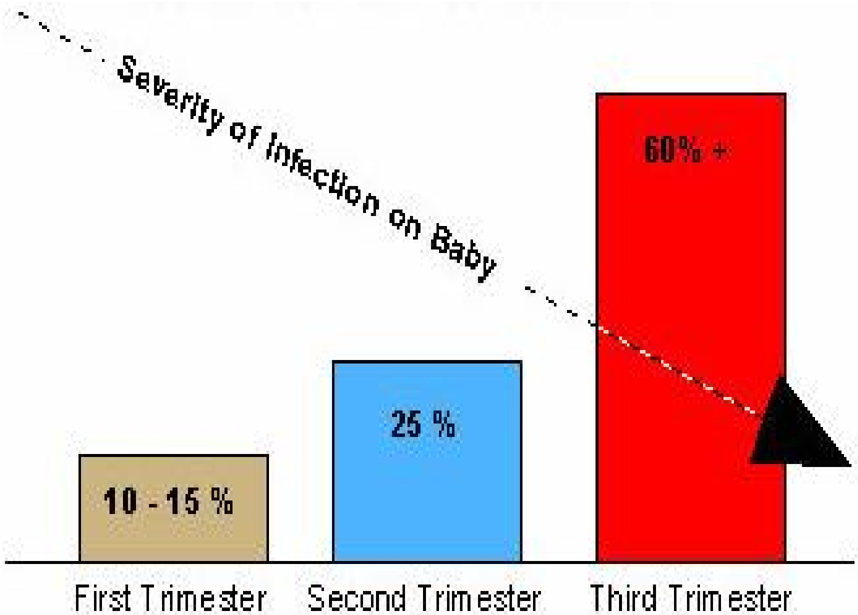

Figure 1.4: The frequency of transplacental transmission of toxoplasmosis from mother to fetus by trimester and its relation to the severity of infection on fetus. (http://www.obgyn.net/english/pubs/features/presentations/OB_toxoplasmosis_pregnancy-Agrawal/OB_toxoplasmosis_pregnancy.ppt)

transmission of *T. gondii* by proving that the prevalence of *T. gondii* is much higher in sheep than in horses or cattle. Dubey (2008) highlighted that it is common in humans in some vicinities where raw meat is consumed regularly. The ability of cysts to survive gastric juice and incidence of toxoplasma which is common in swine, sheep, cattle and other animals (Jacobs *et al.*, 1960) appear to illustrate the carnivorism transmission of the parasite. Durfee and Chien (1971) reported the serial transmission of toxoplasma from mouse to pig to cat, and back to mouse.

1.4.3. Fecal oral

The high incidence of toxoplasma infection in vegetarians and herbivores cannot be explained by congenital and carnivorism transmission. *T. gondii* infectivity associated with cat feces was initially discovered by Hutchinson (1965) and was considered as a breakthrough because, until then, both known forms of *T. gondii* (tachyzoites and bradyzoites) were killed by water. Cats can shed millions of oocysts after ingesting only one bradyzoite, indicating the important role of oocysts excreted by infected cats that attributes to epidemics of toxoplasmosis in human beings and sheep (Teutsch *et al.*, 1979). Sheep could readily acquire infection because they are known to graze closely to the ground (Quinn and McGraw, 1972). *T. gondii* oocysts could also contaminate water as several outbreaks of toxoplasmosis in humans have been linked epidemiologically to drinking of unfiltered water (Bowie *et al.*, 1997).

1.4.4. Other routes

Congenital, carnivorism and fecal-oral transmission are the most common routes of transmission of *T. gondii*. There are some other ways that toxoplasma can be transmitted, though they are less common or very rare. Organ transplantation and blood transfusion are two extraordinary methods of spread in man. Reynolds *et al*. (1966) reported that a patient with chronic renal disease who was placed in an immunosuppressive regimen died of acute disseminated toxoplasmosis one month after receiving a renal transplant. The cause of death was identified from serologic studies and it suggested that primary infection with *T. gondii* occurred at the time of transplantation. Raisanen (1977) studied the transmission of *Toxoplasma* via blood transfusion using laboratory rabbits. Human blood was inoculated with toxoplasma organisms and stored for 28 days before being injected into the ear vein of the rabbits. The test rabbits developed toxoplasmosis and similar result were obtained by transfusing rabbits with blood obtained from rabbits subcutaneously injected with toxoplasma organisms. Herwaldt and Juranek in their review of laboratory-acquired parasitic infections in 1993 reported that needlestick injuries due to carelessness were the most common laboratory accident that contributes to the infection.

1.5. Toxoplasma infection

1.5.1. Infection in animals

T. gondii is one of the most ubiquitous organisms and several reports of fatal toxoplamosis infection in animals are available in the literature. Exposure to the organism and infection by it are common but the clinical signs of toxoplasmosis vary considerably with the species affected and very likely with the strain of organism involved (Quinn and McGraw, 1972). According to Lindsay *et al*. (1997), there is no toxoplasmosis in areas where there are no cats until Prestud *et al*. (2007) reported other possible sources of infection due to *T. gondii* in the Arctic. The first animal toxoplasmosis reported was canine toxoplasmosis in Italy in 1910 and over the next 30 years, canine toxoplasmosis was reported in a few other countries (Dubey and Beattie, 1988).

Toxoplasmosis in farm animals like sheep and goats deserves special attention because of their economic impact. According to Dubey and Beattie (1988), *T. gondii* infected goats acquire the disease through contaminated food and water from infected cat faeces and concluded that toxoplasmosis in these animals was significantly associated with the presence of cats roaming in the farms. Fetal infection, abortion and neonatal loss can occur if the parasite is encountered during pregnancy thus explaining its major role in economical impact upon their farming. Levine (1990) described that toxoplasma appear to be less common in cattle than sheep and Dubey (2003) supported the fact by proving that cattle and horses are resistant to clinical *T. gondi*. It is even capable of infecting marine lives as Cole *et al*. (2000) isolated viable *T. gondii* from sea otters in the United States and since then, many reports of fatal toxoplasmosis in marine mammals have been published.

1.5.2. Entry into host cell

Since *T. gondii* is an obligate intracellular parasite, it requires an appropriate host cell for its replication. *T. gondii* is capable of invading and establishing productive infection in both phagocytic and non-phagocytic cells through active invasion. It is termed the active invasion because the entry mechanism is largely driven by the parasite itself and has been observed in many different cell types (Sibley *et al.*, 1985). In order to invade the cell, the parasite will recognize an attachment site (Figure 1.5) and attach to the target cell. Upon entry into the host cell, the parasite will look for an appropriate point of attachment and forcibly penetrates the host cell through an anular junction formed between them (Bhopale, 2003a). Modifications of the cell membrane as a result of the parasite penetration give rise to the parasitophorous vacuole, a specialized compartment in which the parasite resides. Replication of the parasite will not take place in the vacuole, instead new vacuole will be formed and the parasite will exit into the new vacuole where replication takes place (Speer *et al.*, 1995). In the presence of complement, extracellular tachyzoites will be killed by specific antibody.

1.5.3. Host parasite interaction

T. gondii may possess organelles that are required for independent existence; however, intracellular environment of a host is required for its growth and replication (Wong and Remington, 1993). *T. gondii* usually parasitizes both definitive and intermediate hosts but severe clinical manifestations occur very rarely. The spread of *T. gondii* to mesenteric lymph nodes and to distant organs could be fatal to the host as they are at high risk of intestinal necrosis and severe organ damage (Schreiner and Liesenfeld, 2009). Necrosis is caused by the intracellular growth of tachyzoites since all stages of the parasite contain functional mitochondria. According to Pfefferkorn (1990), this is how the parasite produces energy to support its growth. Inflammation usually follows the initial necrosis. By about the third week after infection, tachyzoites may localize as

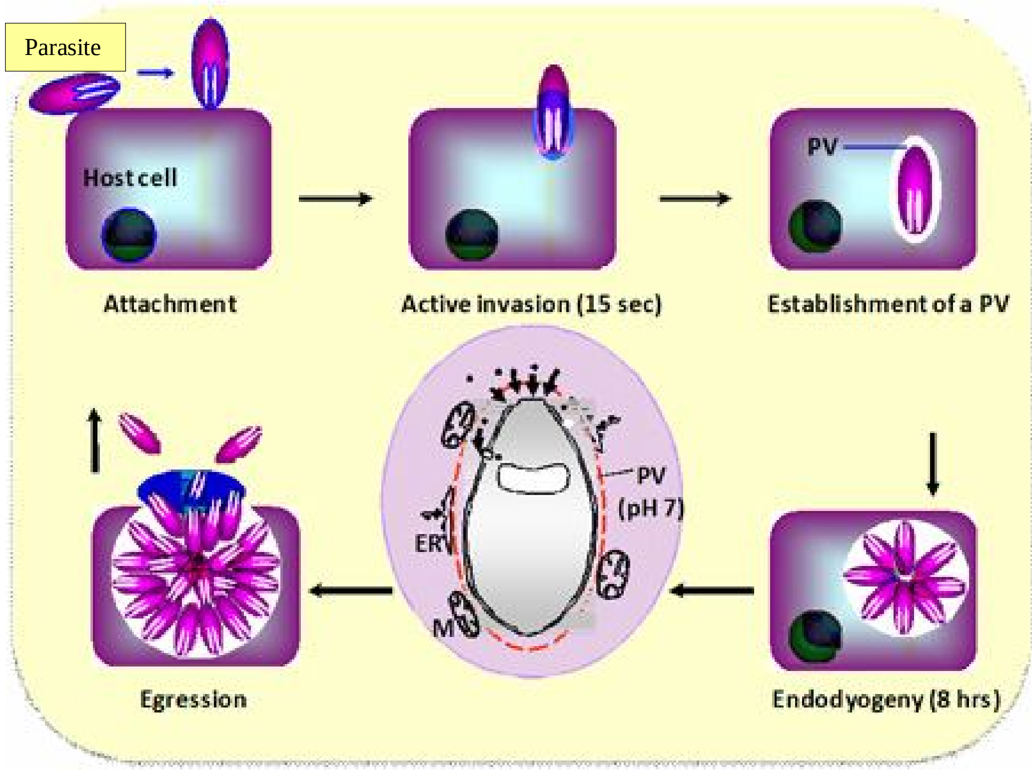

Figure 1.5: The process of parasitophorous vacuole formation and the endodyogeny that take place during asexual cycle of the parasite. (http://www.charite.de/sysbio/research/toxoplasma/)

PV: Parasitophorous vacuole

ER: Endoplasmic reticulum

M: Mitochondrion

tissue cysts in neural and muscular tissues as it begin to disappear from visceral tissues. Since immunity is less effective in neural organs, tachyzoites persist longer in the spinal cord and brain than in visceral tissues, although this varies with the strain of *T. gondii* and the host species (Dubey, 2010).

1.5.4. Mode of infection and pathogenesis

Humans, who are the intermediate host for *T. gondii* are generally infected by ingestion of oocysts released in cat faeces or by consuming undercooked meat from infected animals. Depending on factors like age of the host and the organs involved, the response of the host to the infection varies (Quinn and McGraw, 1972). Following ingestion, enzymatic degradation of the outer wall of the cysts will occur and bradyzoites and sporozoites are released into intestinal lumen. There they undergo rapid invasion and multiplication where they become tachyzoites, disseminating through the lymphatic system to different organs inside the host. Intracellular growth of the parasite will lead to apoptosis and cell necrosis surrounded by an acute inflammatory response (Miro *et al*., 2008). Often, necrosis is the only response observed in tissues of organisms, especially for acute cases. Virulent parasites can destroy host cells in 24 to 48 hours (Bommer, 1969) **.** At this point, the parasite can be excreted through different biological fluids but these tachyzoites are very labile and are easily destroyed which makes transmission to other hosts very unlikely.

According to Feldmen (1968), the rapid multiplication of the parasite in fetus and young children can cause severe outcomes. If the parasite invades the liver and spleen, areas of focal necrosis may be formed and could cause hepatosplenomegaly and sometimes jaundice in the neonate. Retinochoroiditis, hydrocephaly and microcephaly as well as cerebral calcification may be present. Both fatal and self-limited encephalitis with lymphadenopathy have been described in young children. However, severe inflammatory reactions are less common in adults (Christie, 1969) and the spread of the infection is rarely fatal (Jacobs, 1970).

Appearance of IgA antibodies indicate the sub-acute phase of the disease. This IgA which is specific for *T. gondii* enteroepithelial stages eliminates tachyzoite replication in the intestinal phase but not those localized in the nervous system. The chronic form of the disease is characterized by the disappearance of tachyzoites from visceral tissues whereby bradyzoites persist within cysts. This phase, which inhibits tachyzoites proliferation in blood and tissues, is long-lasting and is associated with a systemic immune response (Montoya and Liesenfeld, 2004). One of the most common causes of CNS complications is reactivation of latent infection in immunocompromised patients such as AIDS (Luft and Remington, 1992).

1.5.5. Immune response

T. gondii is a highly successful pathogen as it is able to evade the immune system mechanism of the host. When tachyzoites are disseminated to the organs in the human body, both cellular and the humoral immune responses control the infection, but the type I cell-mediated immunity plays a key role in the host resistance to *T. gondii* infection (Hou *et al*., 2011). The antibodies produced during humoral immune response are important for diagnosis of toxoplasmosis in human as they have been found to be able to kill the extracellular *T. gondii* by restricting the multiplication of the parasite. The parasite will be lysed in the presence of the complement. The main cells of defense against the parasite are the macrophages and NK cells, in which the macrophages produce cytokine together with dendritic cells during the acute phase of infection (Waree, 2008).

The cytokines that are involved in the immune process against *T. gondii* infection include IL-2, IL-12, IFN-γ, TNF-α, and IL-6. IFN-γ activates the macrophages and hydrogen peroxide that kills the parasites will be released. However, a recent finding suggested that they inhibit the function of macrophage by inducing rapid activation of transcription factors (Buzoni-Gatel and Werts, 2006). By blocking the nuclear translocation of both factors and preventing macrophages from producing IL-12 or TNF-α, the parasite is able to evade or subvert the immune response of its host. In immunocompetent hosts, the tissue cysts are able to persist for several years after infection, most of the time as long as the life of the host and the immunity does not eliminate an established infection.

1.6. Clinical Presentation/ Characteristics

The clinical manifestation of *T. gondii* infection can go unnoticed or asymptomatic. Depending on the immune status of the patient and the clinical setting, the signs and symptoms of infection vary. For instance, different clinical presentations have been observed in immunocompetent adults and children, immunocompromised patients with and without AIDS, congenital toxoplasmosis and ocular toxoplasmosis.

1.6.1. Immunocompetent adults and children

Aquired acute toxoplasmosis infection in immunocompetent adults (including pregnant women) and children often manifests as asymptomatic disease and only about 10% of individuals acutely infected with *T. gondii* present with a short, self-limiting and non-specific illnesses that rarely need treatment (Montoya and Liesenfeld, 2004). The most common clinical presentation is cervical lymphadenopathy, where the lymph nodes measure only a few centimeters in diameter (McCabe *et al*., 1987). Toxoplasmosis may account for 5 percent of clinically significant lymphadenopathy cases (McCabe *et al*., 1987) and very infrequently, pneumonitis, myocarditis, hepatitis, polymyositis, encephalitis and fever of unknown origin can arise in apparently immunocompetent individuals (Montoya and Liesenfeld, 2004). Acute toxoplasma infection in pregnant women does not differ from *T. gondii* infection in other immunocompetent individuals. Although cervical lymphadenopathy may seem likely, the infection is most commonly asymptomatic. Due to the asymptomatic course, the disease may be transmitted to the fetus unknowingly (Petersen and Liesenfeld, 2007).

1.6.2. Immunocompromised patients with and without AIDS

Immunocompromised patients are not as fortunate as immunocompetent individuals when it comes to *T. gondii* infection as the disease can be life-threatening in immunocompromised individuals (Liesenfeld *et al*., 1999). This is because these individuals are at high risk of reactivation of chronic or latent infection following rupture of cysts in the central nervous system (Porter and Sande, 1992). Liesenfeld *et al*. (1999) reported that patients with reactivated toxoplasmosis often present with signs