DETECTION AND IDENTIFICATION OF CIRCULATING *Toxoplasma gondii* ANTIGENS AND HOST SPECIFIC PROTEINS IN TOXOPLASMOSIS VIA SERUM PROTEOMIC APPROACH

by

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Thesis submitted in fulfillment of the requirements

for the degree of

Doctor of Philosophy

June 2010

DEDICATIONS

This thesis is dedicated to my beloved children, Nafisa and Fahim Razin who have been a great source of motivation and inspiration throughout my study

ACKNOWLEDGEMENTS

I would like to express my deep sense of appreciation to my main supervisor, Prof. Rahmah Noordin for her guidance and support in both my academic and mental formation here in INFORMM, Universiti Sains Malaysia (USM). During my study, she has been training me in many ways to become a good researcher. A huge thank you also goes to my co-supervisor, Dr. Chen Yeng for being very helpful, understanding and a good listener to me. I am also very grateful to my other co-supervisors, Assoc. Prof. Dr. Gam Lay Harn and Assoc. Prof. Dr. Sulaiman Osman for all the co-operation and technical advice rendered.

Special thanks to all the collaborators involved in this study, namely Dr. Zeehaida and Prof. Rohela from Microbiology Department, USM, and Parasitology Department, University Malaya (UM) respectively, who providing *Toxoplasma* serum samples for this research. Many thanks also to Prof Asma, Assoc. Prof. Dr. Rusli, and INFORMM's lecturers for the advice and support. I would also like to appreciate all the administrative staffs at INFORMM Penang, especially to Cik Linda, Encik Irwan, Kammini, Kak Anum, Kak Siti, Nurul Jannah, Azam and Azizi who always being helpful to me. I wish to express my gratitude to the lab staffs at INFORMM, Penang namely Puan Ida, Encik Zul, Puan Maimunah, Puan Sabariah, Izan, Yana and Wani for their technical support during my study. Further, I am grateful to all my colleagues who direct or indirectly helped me throughout my study especially Geita, Hossein, Syahida, Zul, Nyambar,

Nurul Hasanah, Madihah, Anisah, Cik Ah, Kak Su and Kak Ana. I really had such a pleasure time with all of these good friends at the institute.

I may like to express my sincere thanks to my family members, especially my beloved parents for all their love, encouragement, help and support during my study. Finally, I would like to take this opportunity to place on record my hearty thanks to my husband and children for their greatest love, patience, inspiration and support. This is as much success for them as it is for me.

This research was financially funded by research grant from Ministry of Science and Technology (MOSTI) fund and USM Research University (RU) fund. Thank you also to University Kebangsaan Malaysia (UKM) for giving me the SLAB fellowship opportunity and has supported in funding my study.

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LIST OF ABBREVIATIONS

Number	Description	Abbreviation
1	Centimeter	cm
2	Circulating antigen	CA
3	Clusterin 2	CLU2
4	Cut off value	COV
5	Degree Celcius	^{o}C
6	Enzyme-linked immunosorbent assay	ELISA
7	Haptoglobin	HAP
8	Hours	hr
9	Immobiline pH gradient	IPG
10	Immunogobulin	Ig
11	Isoelectric focussing	IEF
12	Isoelectrion point	pI
13	Kilodalton	kDa
14	Litre	L
15	Mass spectrometry	MS
16	Matrix-assisted laser desorption ioization	MALDI
17	Microgram	µg
18	Microlitre	µl
19	Mililtre	ml
20	Milimolar	mM
21	Minute	min
22	Nanogram	ng

23	One-dimensional electrophoresis	1-DE
24	Optical density	O.D.
25	Peroxidase	HRP
26	Rehydration buffer	RS
27	Sample buffer	SB
28	Second	sec
29	Serum sample	SS
30	Sodium doedecyl sulphate polyacrylamide gel electrophoresis	SDS-PAGE
31	Surface antigen 1	SAG1
32	Time of flight	TOF
33	Two-dimensional gel electrophoresis	2-DE
34	Volt	V
35	Volume	v
36	Watt	W
37	Weight per volume	w/v
38	α_1-antitrypsin	AAT
39	α_1-β glycoprotein	ABG
40	α_2-HS glycoprotein	AHS

PENGESANAN DAN PENGENALPASTIAN ANTIGEN-EDARAN *Toxoplasma gondii* DAN PROTEIN KHUSUS PERUMAH DALAM TOXOPLASMOSIS MELALUI PENDEKATAN PROTEOMIK SERUM

ABSTRAK

Toxoplasma gondii merupakan parasit intrasel yang tersebar meluas di dunia. Jangkitan pada wanita hamil boleh mengakibatkan keguguran dan kecacatan janin dan boleh mengancam nyawa dalam perumah yang rentan daya tahan imun. Pelbagai kajian telah dilakukan untuk membangunkan asai pengesanan antigen *T. gondii*. Walaubagaimana-pun, asai-asai ini tidak boleh didapati secara komersil, satu sebab kemungkinan kurangnya data yang baik berkaitan antigen-edaran *Toxoplasma* dalam darah pada individu terjangkit aktif. Kaedah proteomik sangat membantu dalam penemuan petanda jangkitan penyakit untuk tujuan diagnosa, dan pendekatan ini telah digunakan dalam kajian untuk mengenalpasti petanda jangkitan dalam serum yang berpotensi dalam toksoplasmosis. Pada awalnya, ELISA, menggunakan anti-*Toxoplasma* SAG1 (p30) monoklonal sebagai antibodi penangkap untuk mengesan antigen-edaran *T. gondii* di dalam sampel serum telah dibangunkan. Seterusnya, kajian profil protein bagi serum daripada jangkitan *T. gondii* telah dijalankan menggunakan elektroforesis 2-dimensi (2-DE) diikuti dengan kajian identifikasi dan pencirian protein-edaran *T. gondii* dan protein khusus perumah di dalam sampel serum pesakit toksoplasmosis aktif menggunakan blot Western dan analisis spektrometri jisim. Menggunakan serum yang dicampurkan dengan protein larut *Toxoplasma* dan protein rekombinan SAG1, takat pengesanan ELISA

masing-masing ialah 31.25 ng/ml and 62.50 ng/ml. Keputusan positif diperolehi sebanyak 28% (21/75) dan 11% (23/206) masing-masing bagi sampel serum toxoplasmosis aktif dan kronik. Analisis blot Western pada kumpulan sampel serum positif-antigen menunjukkan jalur antigenik dengan berat molekul 25 dan 75 kDa daripada sera pesakit dengan jangkitan aktif dan lima jalur antigenik dalam lingkungan saiz daripada 26 hingga 33 kDa daripada serum pesakit dengan jangkitan kronik. Selanjutnya untuk menganalisa dan mencirikan antigen-edaran *T. gondii*, elektroforesis 2-dimensi digunakan untuk membandingkan profil protein serum bagi subjek terjangkit (n=31) dan normal (n=10). Daripada 21 sera jangkitan aktif, 29 kDa, 33 kDa dan 38 kDa, masing-masing kelihatan dalam 19 (90.5%), 14 (66.7%) dan 11 (52.4%) gel 2-DE yang diwarnakan. Protein antigenik dikesan melalui pemblotan imuno menggunakan kumpulan sera dan IgM-HRP anti-manusia monoklonal. Tompok-tompok protein terpilih dicirikan menggunakan spektrometri jisim. Perbezaan yang ketara diperhatikan apabila sampel serum daripada pesakit terjangkit *T. gondii* dan kawalan normal dibandingkan, yang terdahulu menunjukkan peningkatan ekspresi yang signifikan bagi protein perumah α_2-HS glycoprotein (AHS) and α_1-B glycoprotein (ABG). Tiada perbezaan yang signifikan diperhatikan di dalam gel-gel pewarnaan perak bagi protein khusus perumah daripada jangkitan aktif dan kronik, walaubagaimanapun yang terdahulu menunjukkan tindakbalas imun di dalam blot Western. Tambahan pula, tiga kelompok protein *T. gondii* dikesan di dalam sampel serum daripada jangkitan aktif, dinamakan (a) protein hipotetikal chrXII: 3984434-3 TGME49, (b) protein dual specificity phosphatase TGME49, catalytic domain TGME 49 dan (c) NADPH-cytochrome p450 reductase TGME 49. Kajian ini telah mengenalpasti lima petanda

jangkitan yang berpotensi dalam diagnosa toksoplasmosis, tiga adalah protein khusus-parasit dan dua adalah protein khusus-perumah.

DETECTION AND IDENTIFICATION OF CIRCULATING *Toxoplasma gondii* ANTIGENS AND HOST SPECIFIC PROTEINS IN TOXOPLASMOSIS *VIA* SERUM PROTEOMIC APPROACH

ABSTRACT

Toxoplasma gondii is an intracellular parasite of world-wide distribution. Infection in pregnant women may result in abortion and foetal abnormalities, and may be life-threatening in immunocompromised hosts. Many studies have been performed on developing an antigen detection assay for *T. gondii*. However, such assay is unavailable commercially, one reason may be due to lack of good data regarding *Toxoplasma* circulating antigens in the blood of actively infected individuals. Proteomics has greatly aided in discovery of disease infection markers for diagnostic purposes, thus this approach was used in this study to identify potential serum infection markers in toxoplasmosis. Initially, an ELISA employing monoclonal anti-*Toxoplasma* SAG1 (p30) as the capture antibody to detect *T. gondii* circulating antigens in serum samples was developed. Subsequently a study on protein profiles of serum from *Toxoplasma gondii* infection using two dimensional electrophoresis (2-DE) was performed, followed by identification and characterization studies of the circulating *T. gondii* proteins and host specific proteins in the serum samples of active toxoplasmosis patients by Western blot and mass-spectrometry analysis. Using serum spiked with *Toxoplasma* soluble and with SAG1 recombinant proteins, the ELISA detection limits were found to be 31.25 ng/ml and 62.50 ng/ml

respectively. Positive results were obtained in 28% (21/75) and 11% (23/206) of serum samples from active and chronic toxoplasmosis respectively. Western blot analysis on pooled antigen-positive serum samples showed antigenic bands of molecular weights 25 and 75 kDa from sera of patients with active infection and five antigenic bands ranging in size from 26 to 33 kDa from sera of patients with chronic infection. To further analyse and characterize the circulating *T. gondii* antigens, a two dimensional electrophoresis was used to compare serum protein profiles of infected (n=31) and normal (n=10) subjects. Antigenic proteins were identified by immunoblotting using pooled sera and monoclonal anti-human IgM-HRP. Out of the 21 sera from active infection, 29 kDa, 33 kDa and 38 kDa were seen in 19 (90.5%), 14 (66.7%) and 11 (52.4%) respectively of the stained 2-DE gels. Selected protein spots were characterized using mass spectrometry. Prominent differences were observed when serum samples from *T. gondii* infected patients and normal controls were compared, the former showed significant up-regulation of host proteins α_2-HS glycoprotein (AHS) and α_1-B glycoprotein (ABG). No significant difference was observed in the silver-stained gels of host-specific proteins from active and chronic infections, however only the former showed immunoreactivity in Western blots. In addition, three clusters of *T. gondii* proteins were detected in serum samples from active infection, namely (a) hypothetical protein chrXII: 3984434-3 TGME49, (b) dual specificity protein phosphatase TGME49, catalytic domain TGME 49 and (c) NADPH-cytochrome p450 reductase TGME 49. The study has identified five potential infection markers for diagnosis of toxoplasmosis, three are parasite-specific proteins and two are host-specific proteins.

CHAPTER ONE

INTRODUCTION

1.1 General Introduction on Toxoplasmosis

Toxoplasmosis is a worldwide disease caused by *Toxoplasma gondii*, an intracellular protozoan parasite in the order Coccidia (phylum Apicomplexa). It was documented that over half a billion of the human population have serum antibodies to *T. gondii* (Dubey, 1997b). In recent years, the importance of toxoplasmosis has been increasingly recognized. The course of infection by *T. gondii* is generally benign, as the vast majority of the infected individual remains asymptomatic or presents only with mild symptoms. However, the infection can cause significant morbidity and mortality in the developing foetus and in immunocompromised individuals.

Infection during pregnancy in serologically negative women can lead to parasite transmission to the foetus *via* placenta, and thus can give rise to congenital toxoplasmosis (CT) of the newborn to a severe extent (Remington *et al.*, 1995). Immunocompromised persons, such as those with acquired immune deficiency syndrome (AIDS), some organ transplant recipients and cancer patients receiving immunosuppressive therapy may suffer recrudescent active toxoplasmosis (Bretagne, 1995; Maschke *et al.*, 1999). Therefore, by understanding the nature of *T. gondii* and overall aspect of toxoplasmosis may lead to a better prevention, diagnosis and treatment of the disease.

1.2 History of the Discovery of *Toxoplasma gondii*

T. gondii was first described in 1908 by Nicolle and Manceaux (Nicolle and Manceaux, 1909) in a rodent *Ctenodactylus gundi*, and by Splendore in a rabbit. Based on the morphology, the name *Toxoplasma* was derived from a Greek word "toxo" meaning arc or bow, referring to the curved shape of the trophozoite (Figure 1.1) and "plasma" referring to life (Nicolle & Manceaux, 1909). The pathogenicity of the protozoan, *T. gondii* to human infants was pointed out by Wolf *et al.*, (1939) who isolated the organism from children who had died from congenital encephalomyelitis. The discovery of Sabin-Feldman dye test as the first serological assay for *Toxoplasma* antibody in 1948 (Sabin and Feldman, 1948), opened wide the scope and extent of the study on this infection.

1.3 Epidemiology of Human Toxoplasmosis

T. gondii infection is widespread in human and prevalence varies substantially according to countries. It is estimated that more than 60 million people in the United States are infected with *T. gondii* (CDC, 2007) and that approximately one-third of the world's population has been exposed to the parasite (Tenter *et al.*, 2000). In the United Kingdom, it was reported that 16-40% of people are infected whereas in continental Europe, the infection estimates reached up to 50-80% (Dubey and Beattie, 1988; Jones *et al.*, 2001b; Tenter *et al.*, 2000). The Centre for Disease Control (CDC) in the USA estimates that anywhere from 400 to 4,000 cases of congenital toxoplasmosis occur each year (CDC, 2007). The incidence of prenatal infection with *T. gondii* is estimated to be anywhere from 1-120 in every 10,000 births. In the early stages of pregnancy, about

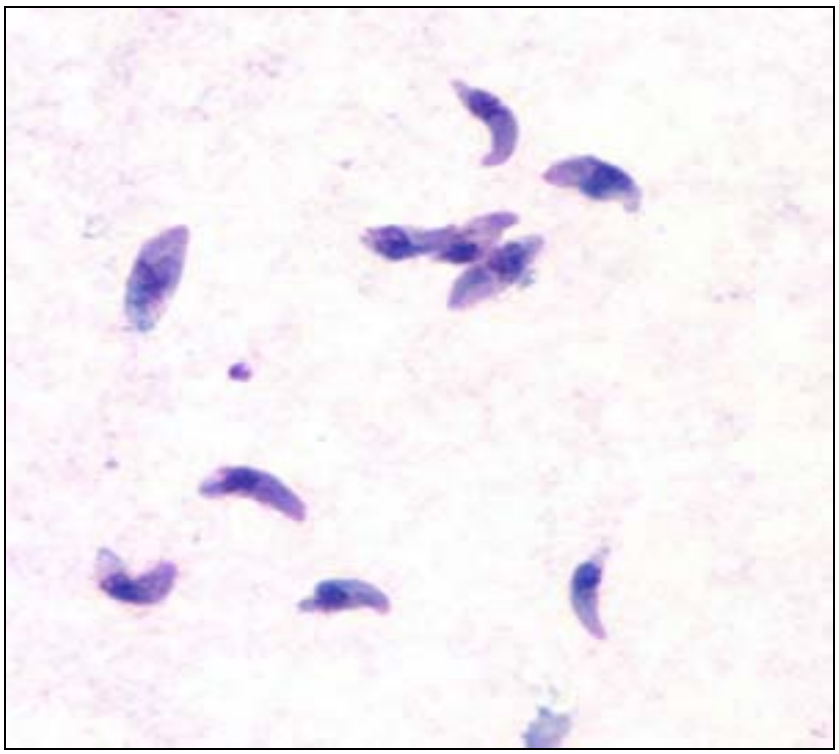


Figure 1.1. *Toxoplasma gondii* tachyzoites, stained with Giemsa, from a smear of peritoneal fluid obtained from a laboratory-inoculated mouse. Reference:www.dpd.cdc.gov/DPDx/HTML/ImageLibrary/SZ/ Toxoplasmosis/

10% of the fetal infection with *T. gondii* results in abortion or neonatal death. Another 10-23% of infected infants show signs at birth like encephalomyelitis, retinochoroiditis, convulsions, hydrocephalus, splenomegaly, hepatomegaly, fever, anaemia, jaundice or lymphadenopathy. About 12 to 16% of infants born die from the disease, and survivors suffer from progressive neural disorders such as mental retardation or seizures (Tenter *et al*., 2000). In immunocompromised individuals, such as those with HIV/AIDS, organ transplant and chemotherapy patients, toxoplasmosis is an opportunistic infection that poses a significant risk. Latent infection can be reactivated to cause encephalitis, which can be fatal (Singh *et al.*, 2005). Severe encephalitis occurs in up to 40% of AIDS patients and it is estimated that 10-30% of AIDS patients worldwide are infected with *T. gondii* (Tenter *et al*., 2000).

In Malaysia, anti-*Toxoplasma* antibodies have been shown to be present in the local population (Osman *et al.*, 1992), as well as among the pregnant women and women of child-bearing age. The seroprevalence of toxoplasmosis in pregnant women was found to be 49%, in which 39%, 4% and 6% were positive for anti-*Toxoplasma* IgG, IgM and both anti-*Toxoplasma* IgG and IgM antibodies, respectively (Azmi *et al*., 2003). It was also reported that there was a significant difference ($p<0.05$) in rates of *Toxoplasma* seroprevalence among races; the highest rate was in the Malays (55.7%), followed by the Indian (55.3%) and the Chinese (19.4%) (Azmi *et al*. 2003). The relatively higher rate of *Toxoplasma* infection in Malays may be attributed to their close association with cats as compared to the other races. In spite of the fact that pigs have the highest infection rate among domestic animals, the Chinese population who consume pork more than any other meat had the lowest infection rate (Tan and Mak, 1985). This could be

due to the fact that the Chinese in Malaysia usually cook their meat thoroughly before eating, thus avoiding infection.

A finding reported by Osman *et al*. (1992) revealed that in congenital toxoplasmosis (IgM positive), only 12 (0.35%) of 3420 infants were clinically suspected of acquiring congenital infection. The main clinical features of congenital abnormalities in Malaysia are hepatomegaly, neonatal jaundice, hydrocephalus, microcephalus, ocular lesion and general illness (Osman *et al*., 1992; Tan and Mak., 1985). A study conducted in 2003 by (Nissapatorn *et al.*, 2003) reported that the seroprevalence of toxoplasmosis among 406 AIDS patients in Kuala Lumpur Hospital was 208 (51.2%). This seroprevalence was much higher than in other similar studies e.g. 15-37% in France (Leport and Remington, 1992), 21% in Malaysia (Nissapatorn *et al.*, 2002), 22.4% in Thailand (Nissapatorn *et al.*, 2001) and 10-40% in USA (Luft and Remington, 1988). The majority of the patients were males (82.6%), Malays (47.5%), single (52.4%), unemployed (47.6%) and heterosexuals who engaged commercial sex workers (46.6%) and were at risk to HIV infection.

The socioeconomic impact of toxoplasmosis in terms of human suffering and long term care of children with mental retardation and blindness are enormous (Roberts *et al.*, 1994). Testing of all pregnant women for *T. gondii* infection is compulsory in France and Austria, and the cost benefits of such mass screening are being debated in many countries (Remington *et al.*, 2001). For immunocompromised patients due to HIV/AIDS, the risk of toxoplasmosis however has decreased after introduction of

primary prophylaxis against *T. gondii* and effective antiretroviral therapy (ART) (Jones *et al.*, 1999).

1.4 Life Cycle of *Toxoplasma gondii*

T. gondii is a coccidian parasite and capable of infecting a wide range of hosts and many different host cells (Dubey and Beattie, 1988; Dubey *et al.*, 1998a)**.** Generally, coccidia have a complex life cycle. In order to understand the transmission of *T. gondii* and how it infects the body, it is important to understand its life cycle. The life cycle of *T. gondii* is facultatively heteroxenous (Figure 1.2). All warm-blooded animals including most livestocks and humans serve as its intermediate hosts. Definitive hosts are members of the family Felidae, for example domestic or stray cats, including wild Felidae such as lions and tigers (Dubey and Beattie, 1988; Dubey *et al.*, 1998b; Frenkel, 2000; Jackson and Hutchison, 1989; Levine, 1961). One study revealed that only felids were found to shed *T. gondii* oocysts (Frenkel *et al.*, 1970; Miller *et al.*, 1972). Results of epidemiologic studies indicated that most cats in the wild become infected soon after they are weaned by eating infective animal tissues. During controlled studies, most uninfected cats that are fed tissues containing bradyzoites will shed oocysts, whereas less than half of the cats that are fed oocysts will shed oocysts (Dubey and Frenkel, 1972). Moreover, the number of oocysts shed by a cat after ingestion of oocysts is far fewer than after ingestion of bradyzoites in infective tissues. The ingestion of infected rodents and birds by cats can lead to excretion of large numbers of environmentally resistant oocysts. Transplacental infection can develop in cats, and kittens infected in utero can shed *T. gondii* oocysts after birth (Dubey *et al.*, 1993; Sato *et al.*, 1993). Although cats can shed *T. gondii* oocysts after reinfection (or even without reinfection),

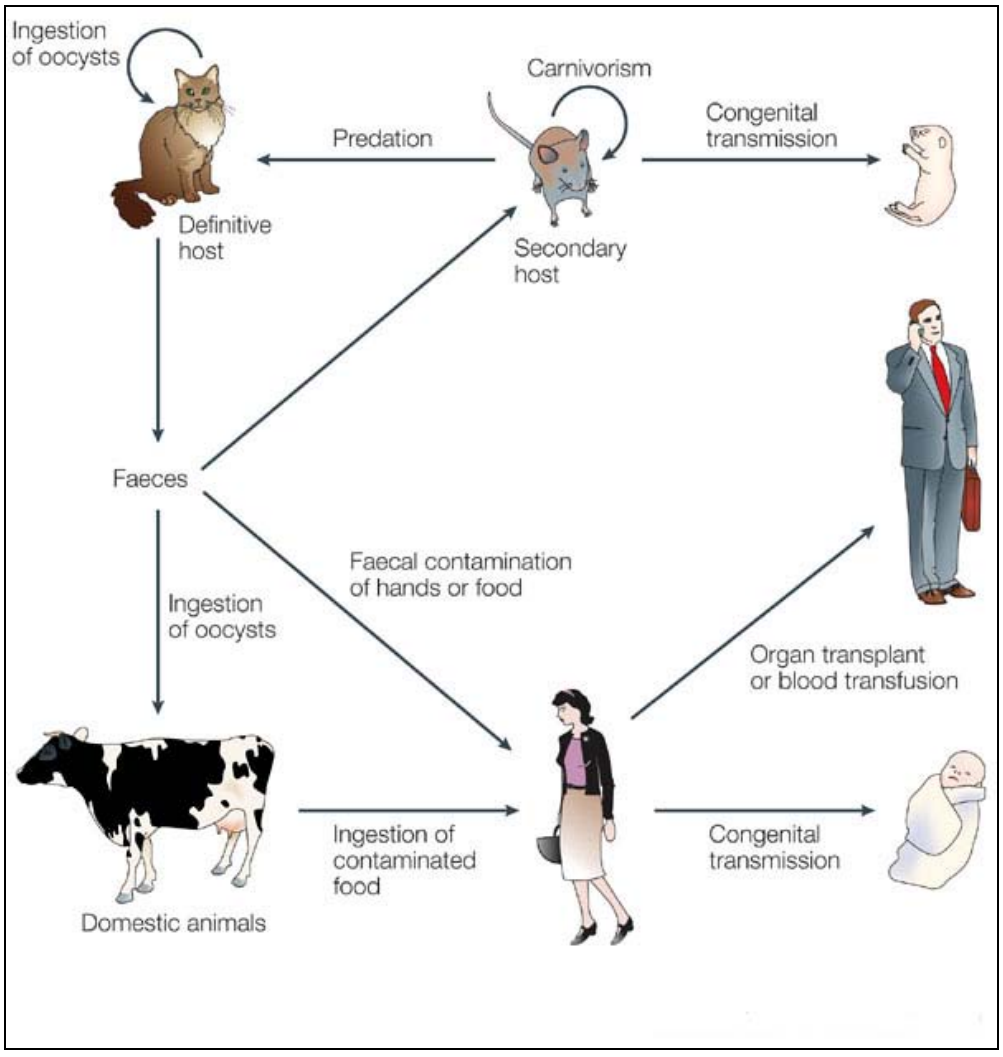


Figure 1.2. Life cycle of *T. gondii*

Reference: (Aliberti, 2005)

shedding of oocysts in in cats in the wild is unknown (Dubey and Frenkel, 1974).

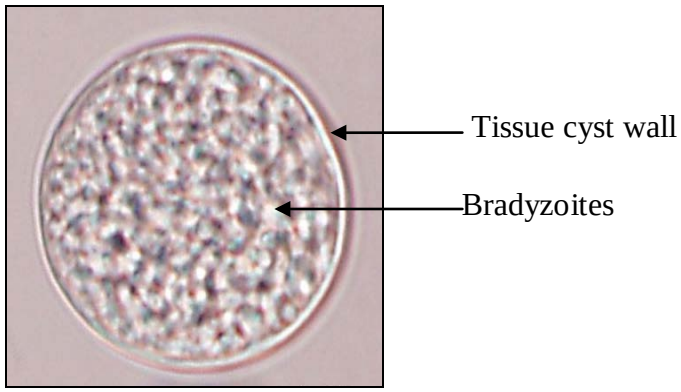
In intermediate hosts, *T. gondii* undergoes two phases of asexual development. In the first phase, tachyzoites multiply rapidly by repeated endodyogeny in many different types of host cells. Tachyzoites of the last generation initiate the second phase of development which results in the formation of tissue cysts. Within the tissue cysts, bradyzoites multiply slowly by endodyogeny (Dubey, 1986; Dubey, 1993; Dubey and Beattie, 1988; Frenkel, 2000; Levine, 1961). These cysts are most often located within the eye, central nervous system and skeletal or cardiac muscles. To a lesser extent, it may occur in the liver, kidney and lungs (Dubey, 1993; Dubey, 1998b; Dubey *et al.*, 1998; Levine, 1961). In some intermediate host species, bradyzoites may persist for life in the host. However, it is still unknown how the persistence mechanism occurs. Many investigators believed that tissue cysts break down periodically, with bradyzoites transforming to tachyzoites that reinvade host cells and again transform to bradyzoites within new tissue cysts (Dubey, 1998a; Dubey, 1998b; Dubey *et al.*, 1998; Evans, 1992; Frenkel, 2000; Levine, 1961; Remington and Desmonts, 1990; Weiss *et al.*, 1988). If ingested by definitive hosts, the bradyzoites initiate another asexual phase of proliferation which consists of initial multiplication by endodyogeny followed by repeated endopolygeny in epithelial cells of the small intestine. The terminal stages of this asexual multiplication initiate the sexual phase of the life cycle.

Sporogony and oocyst formation take place in the epithelium of the small intestine of felines. Unsporulated oocysts are released into the intestinal lumen and passed into the environment with the faeces. Sporogony occurs outside the host and leads to the

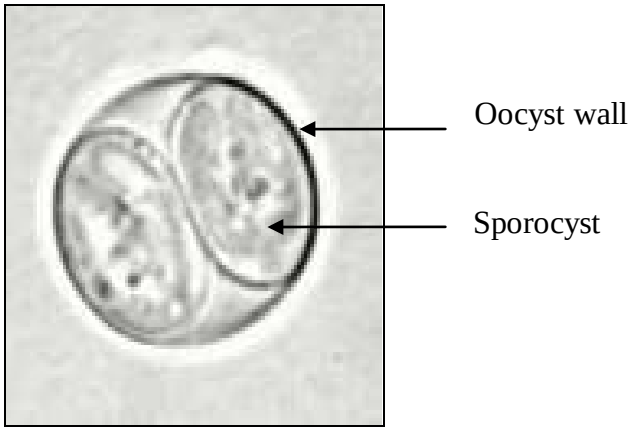
development of infectious oocysts which contain two sporocysts, each containing four sporozoites (Dubey, 1993; Dubey and Beattie, 1988; Dubey *et al.*, 1998; Evans, 1992; Jackson and Hutchison, 1989; Levine, 1961). Oocysts can spread in the environment and contaminate water, soil, fruits, vegetables and herbivores are infected following consumption of contaminated plants material. Oocysts have been found to be very stable, especially in warm and humid environments, and resistant to many disinfecting agents (Dumetre and Darde, 2003), but survive poorly in arid, cold climates (Jones *et al.*, 2001a).

1.5 Transmission of Human Toxoplasmosis

Transmission of *T. gondii* in human beings can occur by several routes. The routes of infection rely on the infectious stages of the parasite. There are three infectious stages of *T. gondii* for all hosts: tachyzoites, bradyzoites (in tissue cysts) and sporozoites (in oocysts). Figure 1.3 shows the images of bradyzoites and oocyst of *T. gondii*. All three stages are infectious for both intermediate and definitive hosts. In most cases, infection with *T. gondii* occur inadvertently, thus the specific route of transmission cannot usually be established. A person may acquire a *T. gondii* infection mainly *via* one of the following routes; horizontally by oral ingestion of infectious oocysts from the environment, horizontally by oral ingestion of tissue cysts contained in raw or undercooked meat or vertically by transplacental transmission of tachyzoites (Dubey, 1991; Dubey, 1993; Dubey and Beattie, 1988; Dubey *et al.*, 1998; Evans, 1992; Jackson and Hutchison, 1989; Remington and Desmonts, 1990).



a) The tissue cyst of *T. gondii* contains bradyzoites surrounded by the cyst wall. (Reference: www.parasite.org.au)



b) *T. gondii* sporulated oocyst in an unstained wet mount (Reference: http://www.dpd.cdc.gov)

Figure 1.3. Images of tissue cyst and oocyst of *T. gondii*

1.5.1 Faecal-Oral Route

Seroprevalence reports of human toxoplasmosis indicated a correlation with eating and hygiene habits of a population. This finding lends support to the contention that the faecal oral route is the major source of infection (Cook *et al.*, 2000; Desmonts *et al.*, 1965; Remington *et al.*, 2001). A study in Bombay, India found that the prevalence of *T. gondii* in strict vegetarians to be similar to that in non-vegetarian (Rawal, 1959). However, a later study by Roghmann *et al.* (1999) has proved that avoidance of meat in daily diet resulted in significant reduction in risk of infection to toxoplasmosis. The widespread infection of toxoplasmosis in vegetarian is explained by this route of transmission.

Epidemics of human toxoplasmosis attributed to exposure to infected cats indicate an important role of oocyst excretion by cats in the propagation of infection in nature and man (Teutsch *et al.*, 1979). Infected cat sheds large numbers of oocysts which are able to survive for months in moist soil (Frenkel *et al.*, 1970) and becomes a source of infection to a variety of animals and man. Chronically infected cats have been shown to reshed *T. gondii* oocysts in the absence of reinfection (Dubey, 1976) increasing the chances of environmental contamination of oocysts. Several outbreaks of toxoplasmosis in human beings also have been linked to drinking of unfiltered water (Bahia-Oliveira *et al.*, 2003; Bowie *et al.*, 1997). Toxoplasmosis may be equally important in many other developing countries, where the lack of adequate sanitary conditions expose populations to a variety of diseases. Contamination of household items with oocysts from soil is likely to occur in floods or runoffs that are frequent after rainfall. Since oocysts can survive for long

periods in water (Dubey, 1988), the lower socioeconomic population may frequently be exposed to *T. gondii* oocysts from drinking water (e.g. river and well).

1.5.2 Carnivorism

Transmission of *T. gondii* through carnivorism was first suggested by Weinman and Chandler, (1954). The idea was supported by demonstrating the resistance to proteolytic enzymes of *T. gondii* derived from cysts (Jacobs *et al.*, 1960a). They found that the cyst wall was immediately dissolved by such enzymes but the released bradyzoites survived long enough to infect the host. Ingestion of viable cysts present in meat may result in infection, particularly among people who habitually eat raw or undercooked meat. *T. gondii* is killed when the internal temperature of the meat reaches 66^{o}C (Dubey, 1988). *T. gondii* however may survive in improperly grilled or barbecued meat. Durfee (1976) reported that in South Kalimantan (Indonesia), goat meat acts as a source of infection to man due to eating undercooked goat 'sate'.

1.5.3 Congenital Transmission

Congenital transmission of toxoplasmosis can occur transplacentally during primary infection of a pregnant woman. The mechanism of this vertical transmission is not yet understood. A probable scenario is that temporary parasitaemia in a primary infected pregnant women may result in invasion of the placenta by tachyzoites which then multiply within cells and placenta. Eventually, some of these may cross the placenta and enter the fetal circulation or fetal tissues (Ebbesen, 2000; Remington and Desmonts, 1990). For a primary *Toxoplasma* infection, the mother has no antibodies against *T. gondii,* thus the organisms (tachyzoites) are able to cross the placental barrier into the

foetus. However, if the mother was infected previously, the organisms are destroyed before it can cross the placenta. The foetus of a previously infected mother is therefore protected.

Early maternal infection (first and second trimester) may result in severe congenital toxoplasmosis and can result in death of the foetus in utero and spontaneous abortion. By contrast, late maternal infection (third trimester) usually results in normal appearing newborns (Montoya and Liesenfeld, 2004). Infection initially goes unnoticed, but if it is not treated, babies can develop chorioretinitis or physical growth can be delayed in the second or third decade of life (Remington *et al.*, 2001; Wilson *et al.*, 1980). Vertical transmission of *T. gondii* in the setting of chronic infection is only recorded in immunocompromised women- i.e, those with AIDS or receiving immunosuppressive drugs. However, the rate of vertical transmission in this setting seems to be fairly low (Minkoff *et al.*, 1997).

1.5.4 Organ Transplantation and Blood Transfusion

In recent years, it has been found that organ transplantation of heart, kidney, liver and bone marrow from a seropositive donor to a seronegative recipient may be complicated by *T. gondii* infections. In these cases either tachyzoites or tissue cysts may be involved (Dubey and Beattie, 1988; Ho Yen, 1992). Reactivation of latent infection in the recipient is the most usual mechanism for toxoplasmosis to arise in bone marrow, haematopoietic stem cell and liver transplant patients and in people with AIDS.

Although rare, *T. gondii* can also be transmitted *via* blood or leukocytes from immunocompetent and immunocompromised donors (Raisanen, 1978; Siegel *et al.*, 1971). However, parasitaemia usually occurs for only a short period of time after primary infection. Therefore it has been suggested that there is only a low risk of acquiring an infection with *T. gondii via* ordinary blood transfusion (Dubey and Beattie, 1988).

1.5.5 Other Routes of Transmission

Toxoplasma is also known to be able to enter the body by several other ways, for instance through conjunctiva, respiratory and cutaneous routes (Beverly, 1973). Accidental laboratory infections have been reported and arisen from various laboratories by contact with contaminated needles and glassware or infected animals (Kayhoe *et al.*, 1957; Remington and Gentry, 1970). There was also a report of toxoplasmosis in a breast-fed infant whose mother acquired primary infection with *T. gondii* (Bonametti *et al.*, 1997).

1.6 Clinical Manifestations, Pathogenesis, Treatment and Prevention of Toxoplasmosis

Toxoplasmosis is a systemic disease. Clinically, infection with *T. gondii* can go unnoticed or could cause signs and symptoms depending on the immune status of the patient and the clinical settings - eg, immunocompetent, congenital toxoplasmosis, immunocompromised or ocular disease.

1.6.1 Immunocompetent Adults and Children

Primary infection of *T. gondii* in children and adults (including pregnant women) is asymptomatic in most patients since particularly large numbers of the population with *T. gondii* antibody do not recall an illness with relevant symptoms (Frenkel, 1973). It causes a self-limited and non-specific illness and rarely needs treatment. General symptoms of toxoplasmosis in immunocompetent individuals are fever, lymphadeno-pathy, headache, sore throat, cough, myalgia, and dizziness. However, when patients with lymphadenopathy were studied, non-specific gastrointestinal symptoms such as nausea, vomiting, and abdominal pain were prominent (Tenhunen, 1964). Very infrequently, myocarditis, polymyositis, pneumonitis, hepatitis or encephalitis can arise in healthy individuals. (Montoya and Liesenfeld, 2004).

1.6.2 Congenital Toxoplasmosis

Congenital *T. gondii* infection in a human was initially described by Wolf *et al.*, (1939). Infection acquired during pregnancy may cause severe damage to the foetus, which can lead to a wide array of manifestations, ranging from mild chorioretinitis which can present many years after birth, to miscarriage, mental retardation, microcephaly, hydrocephalus and seizures. In congenital toxoplasmosis cases, data on clinical manifestations in both maternal and foetal infection are important to aid diagnosis of the disease.

1.6.3 Maternal Infection

Congenital infection may occur following maternal infection during pregnancy. Every instance of primary maternal toxoplasmosis occurring during pregnancy exposes the

foetus to the risk of infection *via* the transplacental route, whereas the foetus is protected when maternal infection occurs prior to pregnancy. The severity of the disease may depend upon the stage of pregnancy at the time of infection. The likelihood of clinical symptoms in the newborn is reduced when infection occurs later in the pregnancy. Infection acquired during the first trimester by women not treated with anti *T. gondii* drugs results in congenital infection in 10 to 25% of cases. For infection occurring during the second and third trimesters, the incidence of foetal infection ranges between 30-54% and 60-65%, respectively (Lynfield and Guerina, 1997). The consequences are more severe when fetal infection occurs in early stages in pregnancy, when it can cause miscarriage (Remington and Desmonts, 1990), severe disease, intra-uterine growth retardation or premature birth. The highest frequency of severe abnormalities at birth is seen in children whose mothers acquired a primary infection between 10^{th} and 24^{th} week of gestation (Remington and Desmonts, 1990).

Primary infection acquired during pregnancy is asymptomatic in about 60% of cases (Daffos *et al.*, 1988). Like in immunocompetent adult, infected pregnant women are asymptomatic or have only mild and non-specific symptoms such as fatigue, malaise, low grade fever, myalgia and lymphadenopathy. Although signs of active infections are commonly unrecognized and not noticed by patients, however, when the serology of pregnant woman suggests a recent infection, it is important to search for symptoms by retrospective questioning, since the time of their onset may reliably indicate the time of infection with respect to the date of conception (Thulliez, 2001). Since the clinical features of maternal infection are not diagnostic, identification of primary infections

occurring in a given population of pregnant women requires systematic serological screening.

1.6.4 Foetal Infection

The risk of fetal infection is multifactorial, depending on the time of maternal infection, immunological competence of the mother during parasitaemia, parasite load and strain virulence (Tenter *et al*., 2000). Infection of the placenta is a prerequisite for congenital transmission as the placenta acts as a source of parasites which are transmitted to the foetus almost immediately after maternal infection but possibly with a delay of several weeks or longer (Remington *et al.,* 1995). A wide spectrum of clinical disease occurs in congenitally infected children (Jones *et al.*, 2001a; Remington *et al.*, 2001). Mild disease may consist of slightly diminished vision only whereas severely diseased children may have the full tetrad of signs including retinochoroiditis, hydrocephalus, convulsions and intracerebral calcification. Of these, hydrocephalus is the least common but most dramatic lesion of congenital toxoplasmosis (Dubey, 2004).

Foetuses with congenital toxoplasmosis usually look normal in prenatal ultrasound. If present, ultrasonographic findings suggestive of congenital disease include intracranial calcifications, ventricular dilatation, hepatic enlargement, ascites and increased placental thickness (Gay-Andrieu *et al*., 2003). Neonatal clinical manifestations of congenital toxoplasmosis vary widely and include hydrocephalus, microcephaly, intracranial calcifications, chorioretinitis, strabismus, blindness to thrombocytopenia and anaemia (McAuley *et al.*, 1994; Swisher *et al.*, 1994). The classic triad of chorioretinitis, hydrocephalus and cerebral calcification is rather rare. None of the signs described in

newborns with congenital disease is pathognomonic for toxoplasmosis and can be mimicked by congenital infection with other pathogens, including cytomegalovirus, herpes simplex virus, rubella and syphilis (Montoya and Liesenfeld, 2004).

1.6.5 Immunocompromised Patients

By contrast with the favourable course of toxoplasmosis in almost all immunocompetent individuals, toxoplasmosis can be life-threatening in those who are immunocompromised (Liesenfeld *et al.*, 1999). In patients infected with AIDS, the overwhelming majority (>95%) of toxoplasmosis cases occur as a consequence of reactivation of a dormant (latent) infection (Luft and Remington, 1992). Encephalitis is the clinically important manifestation of toxoplasmosis in AIDS patients and in fact, the most common cause of death among toxoplasmosis patients with AIDS (Dubey, 2004).

The clinical manifestations of toxoplasmic encephalitis depend upon the location, number and size of the lesions. Toxoplasmic encephalitis may present with focal or generalized signs and symptoms of central nervous system dysfunction (Levy *et al.*, 1985; Luft and Remington, 1992; McArthur, 1987; Navia *et al.*, 1986). Clinical presentation of toxoplasmic encephalitis varies from a subactive gradual process evolving over weeks to an active confusional state, with or without focal neurological deficit, evolving over days. Clinical manifestations include mental status changes, seizures, focal motor deficits, cranial nerve disturbances, sensory abnormalities, cerebellar signs, movement disorders and neuropsychiatric findings. Meningeal signs are rare. Constitutional symptoms and signs such as fever and malaise can vary. The most

typical focal neurological findings are hemiparesis and speech abnormalities (Luft *et al.*, 1993).

1.6.6 Ocular Toxoplasmosis

Toxoplasmic chorioretinitis can be seen in the setting of congenital or postnatally acquired disease as a result of active infection or reactivation (Holland, 1999; Montoya and Remington, 1996). Focal retinochoroiditis is the most frequent outcome of infection of the eye by *T. gondii*. Retinochoroiditis in individuals with active acquired toxoplasmosis can arise sporadically or in the context of an outbreak of active disease (Burnett *et al.*, 1998). The natural history of the disease is for an active inflammatory lesion of retina to slowly resolve, leaving a focus of retinochoroidal scarring (Koch *et al.*, 1943). Recurrent lesions are usually recorded at the borders of chorioretinal scars, which are typically found in clusters. Patients suffering from active toxoplasmic retinochoroiditis characteristically present with symptoms of unilateral, mild ocular pain, blurred vision and new onset of floating spots which may be associated with discomfort (Dutton, 2001). Further clinical signs which may develop include segmental periarteritis (O'Connor, 1970), retinal and vitreous haemorrhage (Rieger, 1952), choroidal or retinal neovascularization (Fine *et al.*, 1981; Gaynon *et al.*, 1984; Malbrel, 1981), branch artery occlusion (Braunstein and Gass, 1980; Willerson *et al.*, 1977) and retinochoroidal anastomoses (Kennedy and Wise, 1971; Owens *et al.*, 1979).

Retinochoroiditis in adults has been traditionally deemed a late manifestation and reactivation of congenital disease; however, it has been reported with increasing frequency in association with active infection (Montoya and Remington, 1996). It is

difficult to establish whether the original infection was congenital or acquired in patients who have recurrences of chorioretinitis.

1.6.7 Toxoplasmosis and Brain Diseases

Schizophrenia and related psychotic illness are a class of pervasive neuropsychiatric disorders of uncertain origin (Buka *et al.*, 2001). Early exposure to several infectious agents has been associated with the later development of schizophrenia (Mortensen *et al.*, 2007). Epidemiological studies have also found that infections known to cause congenital central nervous system abnormalities in humans, including rubella, herpes simplex, polio and varicella zoster virus, might be related to the risk of schizophrenia (Amminger *et al.*, 2007; Flegr *et al.*, 2003). Schizophrenia is also associated with maldevelopment of the central nervous system and ecological data has led to the suggestion that *T. gondii* might be involved in its aetiology (Brown *et al.*, 2005).

One study has found that patients with psychosis had significantly elevated levels of IgG antibodies to *T. gondii* compared with control (Tamer and Dundar, 2008). The finding is in accordance with a previous research which has shown an increase in the antibody response to *Toxoplasma* proteins in schizophrenia (Yolken *et al.*, 2001). This suggests that *T. gondii* could play a role in the aetiology of schizophrenia. Interestingly, anti- *T. gondii* IgM antibody, a key indicator of active acquired infection, is not elevated in the sera of patients with first-onset schizophrenia (Torrey *et al.*, 2006; Wang *et al.*, 2006) implying that the patients are not in the active stage of a newly acquired infection. Therefore, a reactivation of chronic infection with the parasite (proliferation of tachyzoites caused by cyst rupture) in the brain might be involved in the onset of the

disease. In support of this possibility, it is noteworthy that individuals with congenital *T. gondii* infection often develop ocular toxoplasmosis later in life (Remington *et al.*, 2001) and the disease is considered to be due to reactivation of infection. These results are consistent with studies indicating that *T. gondii* infection of the brain can result in behavioral alterations in experimentally infected animals (Holliman, 1997). A very interesting study by Webster *et al.*, (2006) has demonstrated that several commonly used medications of antipsychotics such as Fluphenazine HCl, Chlorpromazine and Clozapine, for treating schizophrenia have the ability to inhibit the replication of *T. gondii*.

The onset of toxoplasmic chorioretinitis is most frequent during the ages of 20–30 years (Remington *et al*., 2001) correlating well with the age of onset of schizophrenia (Hafner *et al.*, 1993). Therefore, congenital infection with *T. gondii* may be involved in the aetiology of schizophrenia. Another brain disease, epilepsy has also been associated with *T. gondii*. In Turkey, Yazar *et al.*, (2003) reported that in a study of cryptogenic epilepsy cases and healthy individuals the level of *T. gondii* IgG showed a statistically significantly high value in epileptic patients. However, a contradicted finding by Akyol *et al.*, (2007) reported that there wasn't any relationship between epilepsy and positive *T. gondii* serology in epileptic patients. All of the above reports had revealed recent discoveries of association between toxoplasmosis and brain diseases which warrant further study for confirmation.

1.7 Laboratory Diagnosis of Toxoplasmosis

The diagnosis of toxoplasmosis may be established by various methods including serological tests, polymerase chain reaction (PCR) and histological demonstration of the organism or isolation of the organism. For instance, the *Toxoplasma* Serology Laboratory of the Palo Alto Medical Foundation Research Institute, United States (TSL-PAMFRI) offers a panel of tests, the *Toxoplasma* Serological Profile (TSP) which comprised of the Sabin-Feldman Dye Test (SFDT), double sandwich IgM ELISA, IgA ELISA, IgE ELISA and AC/HC test (http://www.pamf.org/serology). The discriminatory power of the TSP has been clinically helpful to differentiate between recent and chronic infections to any single serological test.

1.7.1 Sabin Feldman Dye Test (SFDT)

SFDT is the first test developed for the laboratory diagnosis of *T. gondii* infection (Sabin and Feldman, 1948) and it is still considered as the "gold standard". The principle of this assay is based on binding of specific *T. gondii* antibodies to the surface of viable trophozoites and fixes complement with resultant damage to the parasite cell wall. When this reaction occurs the organism is not capable of retaining a vital stain, alkaline methylene blue. Performance of dye test requires a source of live, *Toxoplasma* and detects the presence of anti- *T. gondii* specific antibodies. This is the reason why this assay is usually performed in reference centres. SFDT in infected pregnant woman is performed by taking consecutive serum samples at least 3 weeks apart to determine the change in antibody titer for the evaluation of the infection. A "significant" change is considered to be at least a four-fold difference in titre. The absolute antibody titer is also important whereby values over 250 IU/ml are considered "high" and suggestive of

recent infection (Rorman *et al.*, 2006). SFDT is able to determine early arising IgG antibody as its reactivity appears within 1 to 2 weeks of infection. IgG antibody usually appear within 1 to 2 weeks of the infection, peaks within 1 to 2 months, decline gradually over many months or years and usually low titres commonly persist for life (Holliman, 1990).

The tested sera is serially diluted and incubated with live tachyzoites (carrying *Toxoplasma* antigens) in the presence of separated human plasma from "sero-negative" donors (providing complement components). If the patient's serum contains antibodies to *Toxoplasma*, formation of the antigen-antibody-complement complexes subsequently lysed the tachyzoites in the presence of the dye methylene blue. End point titer is determined by counting the number of dead (unstained) and live (stained) parasites. End point titer can be converted to international units (IU): additional standardization is achieved by preparation of a standardised control serum (consisting of a pool of sera), tested by numerous reference centres, and adjusted so that the SFDT value of this control serum is set at 1000IU/ml (Reiter-Owona *et al.*, 1999).

1.7.2 Indirect Fluorescent Assay (IFA)

The IFA is widely used to demonstrate *T. gondii* –specific antibodies. Serially diluted serum samples are incubated with live, inactivated *Toxoplasma* fixed to a glass slide. Binding of specific *T. gondii* antibodies present in the serum to the inactivated parasite occurs and the complex is then detected using fluorescein isothiocyanate-labeled anti-human immunoglobulin (or anti-IgG or anti-IgM). IFA is safer to perform and more economical than SFDT. It appears to measure the same antibodies as the dye test, and its

titres tend to parallel dye test titres (Araujo *et al.*, 1980; Dubey and Beattie, 1988). However, the interpretation of this assay involves subjective assessment of the degree of fluorescent present and is time consuming.

False positive reactions were reported to associate with polar staining in sera containing antinuclear antibodies and rheumatoid factor (Wilson *et al.*, 2003). Moreover, some false positive reactions due to polar staining have been found to involve the expression of Fc receptors by *Toxoplasma.* The Fc receptor of higher affinity was found to accumulate at the polar cap and pre-treatment of the trophozoites with Fc abolished non-specific binding of the immunoglobulin (Budzko *et al.*, 1989). False negative reaction of IFA for IgM may also occur due to competitive inhibition or blockage by high levels of *T. gondii* specific IgG antibodies (Remington *et al.*, 1985), which may lead to up to 40% of false negative results (Van Loon *et al.*, 1983). The IgM fluorescent antibody test has been shown to be less sensitive than enzyme linked immunosorbent assays (ELISA) or agglutination system (Desmonts *et al.*, 1981).

1.7.3 Complement Fixation Test (CFT)

The original description of this technique, applied to the measurement of *Toxoplasma* specific antibodies, utilised *T. gondii* infected hens eggs as an antigen source. Subsequently, the sensitivity of the assay was improved with parasites obtained from mouse peritoneal inoculation (Fleck and Kwantes, 1980). Antibodies reactive in the complement fixation test rise later than those detected in the dye test as the former assay measures both membrane and cytoplasm directed immunoglobulin (Welch *et al.*, 1980).