DEVELOPMENT OF CONVENTIONAL, REAL-TIME AND THERMOSTABILISED MULTIPLEX PCR FOR THE DETECTION OF *Toxoplasma gondii*

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DEVELOPMENT OF CONVENTIONAL, REAL-TIME AND THERMOSTABILISED MULTIPLEX PCR FOR THE DETECTION OF *Toxoplasma gondii*

by

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DEDICATIONS

This thesis is dedicated to my mother, Patumutu Ibrahim, my late father Rahumatullah Othman and my lovely sisters, Rafizah and Rumaisyah who has been a great source of inspiration and motivation.

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TABLE OF CONTENTS

CHAPTER TWO

MATERIALS AND METHODS

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSION

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

	Description	Abbreviations
1	Ampicilin	Amp
2	Amniotic fluid	AF
3	Annealing temperature	T_a
4	Approximately	~
5	Base pair(s)	bp
6	Basic Local Alignment Search Tool	BLAST
7	Cerebral spinal fluid	CSF
8	Coefficient of correlation	R^2
11	Degree Celcius	^oC
12	Deoxyribonucleic acid	DNA
13	Deoxynucleotide triphosphate	dNTP
14	Dulbecco's Modified Eagle Media	DMEM
15	Ethylenediamine tetraacetic acid	EDTA
16	Fanto gram	fg
17	Fetal bovine serum	FBS
18	gram	g
19	Heat-inactivated fetal bovine serum	HIFBS
20	Hour(s)	hr
21	Internal control	IC
22	kilobase	kb
23	Limit of detection	LoD
24	Liter	L
25	Luria Bertani	LB
26	Maximum	max
27	Melting temperature	T_m
28	Microgram(s)	µg
29	Microlitre(s)	µl

30	Miligram(s)	Mg
31	Mililitre(s)	ml
32	Milimolar(s)	mM
33	Minor goove binding	MGB
34	Minute(s)	min
35	Nanogram(s)	ng
36	Nanometre(s)	nm
37	Nanomolar	nM
38	No amplification	NA
39	Non-template control	NTC
40	Percentage	%
41	Phosphate-buffered saline	PBS
42	Pico gram	pg
43	Pico mole	pmole
44	Polymerase chain reaction	PCR
45	Revolutions per minute	Rpm
46	Room temperature	RT
47	Second(s)	sec.
48	Unit	U
49	Ultra violet	UV
50	Volume	vol
51	Volt(s)	V

PEMBANGUNAN KAEDAH MULTIPLEKS KONVENSIONAL PCR, REAL-TIME PCR DAN THERMOSTABIL PCR UNTUK MENGESAN *Toxoplasma gondii*

ABSTRAK

Toxoplasma gondii merupakan patogen yang penting dalam bidang veterinar dan perubatan. Parasit ini tersebar dengan meluasnya di seluruh dunia dan mempunyai pelbagai perumah yang merangkumi semua haiwan yang berdarah panas sebagai perumah perantara dan *felid* (kucing) sebagai perumah tetap. Jangkitan parasit ini biasanya tidak menunjukkan sebarang simptom di kalangan individu yang sihat tetapi boleh menyebabkan morbiditi dan kematian terhadap individu yang rentan daya tahan imun dan bayi yang dijangkiti semasa di dalam kandungan ibu. Kini kaedah biologi molekul semakin meluas digunakan sebagai asai pengesanan diagnostik untuk toksoplasmosis, terutamanya bagi spesimen cecair amnion, cecair sum-sum tulang, tisu plasenta, darah dan cecair mata. Walaubagaimanapun, asai PCR yang sedia ada untuk mengesan DNA *Toxoplasma* menunjukkan prestasi yang berlainan di antara makmal. Selain itu, penggunaan asai PCR untuk mendiagnosis *Toxoplasma* adalah masih jarang di Malaysia. Oleh itu, tujuan kajian ini adalah untuk membangunkan asai PCR yang pantas, spesifik dan sensitif untuk mengesan DNA *T. gondii* dan seterusnya mendiagnosis penyakit toksoplasmosis. Tiga asai PCR iaitu konvensional multipleks PCR, "real-time" PCR dan termostabil PCR untuk tujuan pengesanan DNA *T. gondii* telah dibangunkan. Semua "primers" dan "probes" yang digunakan dalam kajian ini baru direka dan kemudiannya dioptimisasikan. Kesemua asai PCR yang dibangunkan,

menunjukkan 100% spesifisiti di mana tiada sebarang timdakbalas silang dengan organisma yang lain di perhatikan. Bagi asai konvensional multipleks PCR, tahap pengesanan minimum untuk DNA dan parasit, sama ada spesimen yang tulen atau *spiked* adalah 10 pg dan 10^4 parasit. Seterusnya, tahap pengesanan minimum bagi termostabil PCR adalah 100 pg dan 10^4 parasit. Tahap sensitiviti bagi "real-time" PCR yang di nilai melalui kaedah "standard curve" yang di lakukan menggunakan cecair badan yang ditambah (*spiked*) dengan parasit *T. gondii* mencatatkan tahap pengesanan sehingga 1 parasit dengan nilai R^2 daripada 0.975 hingga 0.999. Tahap sensitiviti bagi asai yang dibangunkan di uji lagi dengan menggunakan pelbagai organ (otak, hati, limpa, hati, and ginjal) daripada haiwan yang terinfeksi. Bagi asai konvensional multipleks PCR, DNA parasit hanya dapat di kesan pada hati dan limpa mencit yang di jangkiti oleh strain RH dan tiada parasit yang dikesan pada organ mencit yang di jangkiti oleh strain ME49. Seterusnya, asai "real-time" PCR dapat mengesan kehadiran parasit dalam semua organ mencit yang dijangkiti tetapi pada tahap infeksi yang berbeza. Mencit yang dijangkiti dengan strain RH menunjukkan bilangan kehadiran parasit yang lebih tinggi bagi semua organ kecuali otak, yang mana ianya lebih tinggi dalam mencit yang dijangkiti oleh strain ME49. Kesimpulannya, kajian ini telah berjaya membangunkan tiga asai PCR bagi pengesanan DNA *T. gondii*. Asai PCR yang baru ini menawarkan ujian diagnostik molekul yang pantas, spesifik dan sensitif bagi pengesanan penyakit toksoplasmosis.

DEVELOPMENT OF CONVENTIONAL, REAL-TIME AND THERMOSTABILISED MULTIPLEX PCR FOR THE DETECTION OF *Toxoplasma gondii*

ABSTRACT

Toxoplasma gondii is an important pathogen in veterinary and human medicines. It is widely distributed throughout the world and has a broad range of hosts that includes warm-blooded animals as intermediate hosts and felid (cat) as the definitive host. Infection with this parasite is usually asymptomatic in healthy individuals but can cause morbidity and mortality in immunocompromised patients and in congenitally infected infants. Molecular biology is increasingly being used as a diagnostic method for diagnosis of toxoplasmosis, especially for specimens collected from amniotic fluid, cerebrospinal fluid, placenta tissue, blood and eye fluid. However, the existing PCR-based methods for the detection of *Toxoplasma* DNA suffer from variations in performance among the laboratories. Indeed, the use of PCR-based assay for diagnosis of *Toxoplasma* is still uncommon in Malaysia. The aim of this study is to develop rapid, specific and sensitive PCR-based assays to detect *T. gondii* DNA for diagnosis of toxoplasmosis. Three PCR-based assays namely conventional multiplex PCR, real-time multiplex PCR and thermostabilised PCR for the detection of *T. gondii* DNA were developed. All the primers and probes used were newly designed and optimized. All three PCR assays showed 100% specificity whereby no cross-reactivity with other organisms was observed. For conventional multiplex PCR, the detection limit for *T. gondii* DNA and whole parasite, regardless whether the *T. gondii* DNA and whole

parasite were used in pure form or spiked into specimens, were 10 pg and 10^4 parasites, respectively. Similarly, for thermostabilised PCR, the detection limit for *T. gondii* DNA and whole parasite were 100 pg and 10^4 parasites, respectively. However, for the real-time multiplex PCR assay which was evaluated by standard curve constructed using human body fluids spiked with *T. gondii* whole parasite, the detection level for all types of samples was as low as 1 parasite with R^2 value in the range of 0.975 to 0.999. The sensitivity of the developed assays was further evaluated using organs (brain, liver, spleen, heart, and kidney) of experimentally infected mice. For conventional multiplex PCR assay, the parasite DNA was only detected in liver and spleen of RH strain-infected mice and no amplification was observed in the organs of the ME49 strain-infected mice. On the other hand, the real-time multiplex PCR assay detected the presence of parasite DNA for both strains in all the organs but at different infection levels. The organs of the RH strain infected mice showed higher parasite load except for brain whereby ME49 strain infected mice showed more parasite load. In conclusion, the present study had successfully developed three PCR-based assays for the detection of *T. gondii* DNA. These newly designed PCR-based assays offer rapid, specific and sensitive molecular diagnostic tests for the diagnosis of toxoplasmosis.

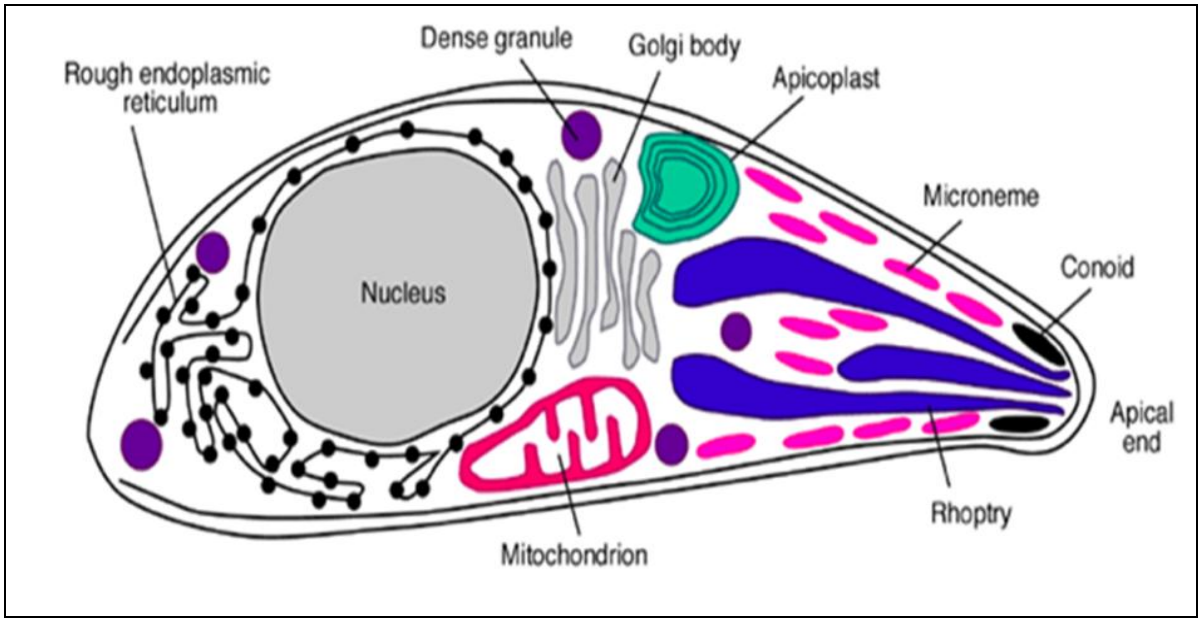
CHAPTER ONE

INTRODUCTION

1.1 Overview of toxoplasmosis

Toxoplasmosis is a disease caused by the protozoan parasite *Toxoplasma gondii* (*T. gondii*). This intracellular parasite, in the order Coccidia (Figure 1.1), is an important pathogen in both veterinary and human medicine. *T. gondii* is widely distributed throughout the world, and it has a broad range of hosts that includes all warm-blooded animals (mammal and birds) as intermediate hosts and felid (cat) as the definitive host. It has been estimated that as many as one-third of the human population harbors this zoonosis. A study from Malaysia reported a seroprevalence in Malays of 55.7% (Petersen, 2007). In addition, the recent calculations of the disease burden of toxoplasmosis rank this foodborne disease at the same level as *salmonellosis* or *campylobacteriosis*, according to Kijlstra and Jongert (2008).

In general *T. gondii* can be divided into three distinct clonal lineages, type I, II and III (Dubey *et al.,* 2007). The differences among these three genotypes at the genome sequences level is less than 1% but different types have different virulence phenotypes in mice (Dubey *et al.,* 2007). The course of *T. gondii* infection 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 fetus and immunocompromised individuals. Therefore, it is very crucial to understand the nature of *T. gondii* which includes the overall aspect of



Scientific Classification	
Kindom	*Protista*
Phylum	*Apicomplexa*
Class	*Conoidasida*
Subclass	*Coccidiasina*
Order	*Eucoccidiorida*
Family	*Sarcosystidae*
Genus	*Toxoplasma*
Species	*T. gondii*
Binomial name	*Toxoplasma gondii*

Figure 1.1: Ultrastructure and scientific classification of a *Toxoplasma gondii*. The above figure is adapted and modified from Ajioka *et al.*, (2001).

toxoplasmosis in order to provide a better prevention, diagnosis and treatment of this infection.

1.2 Epidemiology of *Toxoplasma* infection

Toxoplasmosis is a zoonosis that is widespread in human and its prevalence varies substantially according to countries. The prevalence of the infection rises with age and does not vary greatly between genders. There are considerable geographical differences in prevalence rate of *Toxoplasma* infection. Montoya and Liesenfeld (2004) recorded that different incidence in different climates, with lower incidence in cold regions, hot and arid areas, or at high elevations. For instance, the rates of positive seroprevalence were 18-75% in Western Europe countries, 16-93% in Central and Eastern Europe countries, 34-93% in Central American countries and 29.7-72% in South American countries. Low seroprevalence, 10.9-29.4% was reported in cold climate areas such as Scandinavian countries. Southeast Asia countries include Indonesia, Malaysia and Thailand was reported to have 2.3-58% seropositivity rates (Figure 1.2). The differences in the rate of incidence with the population group within the same environment may be explained by differences in exposure to sources of infection (Saadatnia, 2010).

In the National Health and Examination Survey (NHANES) in USA from 1999-2000, 4234 sera samples (12 to 49 years old) were collected. This survey found that 84.2% of childbearing age women in the United State to be seronegative, and thereby were at risk of acquiring *T. gondii* infection during gestation. On the other hand, *T. gondii* antibody

Continents	Year	Seropositivity (%)
Western Europe		
Austria	1998	43
Belgium	1997	50
France	2001	Up to 75
Germany	2004	26-54
Italy	2001	18-60
The Netherlands	2004	40.5
Spain	2004	28.6
Switzerland	1995	46
UK	1992	23-33
Scandavania		
Denmark	1999	27.8
Finland	1995	20.3
Norway	1998	10.9
Sweden	2001	14.0-29.4
Central and Eastern Europe		
Croatia	2000	38.1
Poland	2001	46.4-58.5
Slovenia	2002	34
Yugoslavia	1998	57-93
The Americas		
USA	2004	16-40
Central America		
Costa Rica	1996	76
Cuba	1993	60
Mexico	2001	35
Panama	1988	90 (at 60 years of age)
South America		
Argentina	2001	72
Brazil	2001	59
West Indies	1991	29.7
Southeast Asia		
Indonesia	2000	58*
Malaysia	2004	44.8
Thailand	1992, 1997, 2000, 2001	2.3-21.9
***Male: Female ratio= 63:52**		

Figure 1.2: *Toxoplasma* seropositivity rates in Europe, the Americas and Southeast Asia (Petersen, 2007).

prevalence was higher among non-Hispanic black persons than among non-Hispanic white persons, and increase with age (Jones *et al.,* 2003). Lopez *et al.,* (2000) conducted an interesting study in the year 2000 and estimated that the rate of congenital toxoplasmosis in the United States between 1-10 per 10,000 live births and 400 to 4000 infants would be born each year with congenital toxoplasmosis.

In Malaysia the rates of toxoplasmosis seroprevalence have been reported in several studies. Based on the survey conducted by Nissapatorn *et al.,* (2004) from January 2001 to December 2002, the seroprevalence rate of toxoplasmosis among 505 HIV/AIDS patients that were admitted to Hospital Kuala Lumpur, was 44.8% (n=226). This seroprevalence rate was much higher compared with other similar studies, e.g. 15-37% in France (Leport & Remington, 1992), 21% in Malaysia (Nissapatorn *et al.*, 2002), 22.4% in Thailand (Nissapatorn *et al.,* 2001) and 10-40% in USA (Luft & Remington, 1988). The majority of the patients were male (75.7%), Chinese (53%), married (51.3%), unemployed (51.3%), heterosexual who engaged commercial sex workers (59.3%) and who are at risk of HIV infection (Nissapatorn *et al.,* 2002). On the other hand, Azmi *et al.,* (2003) found that the seroprevalence of toxoplasmosis in pregnant women in Malaysia was 49%, in which 39%, 4% and 6% were positive for anti-*Toxoplasma* IgG, IgM and both IgG and IgM antibodies, respectively. In addition there was significant difference (p <0.05) in rates of *Toxoplasma* seroprevalence among three major races in Malaysia. The highest rate was recorded by Malays at 55.7% and closely followed by Indians with 55.3% and the prevalence in Chinese was 19.4% (Azmi *et al.,*

2003). The highest rates in Malays can be explained by their very close association with cats as compared to the other two races.

1.3 Discovery of *Toxoplasma gondii*

The discovery of *T. gondii* was in 1908, by a scientist, Charles Nicolle, who was working in North Africa and was searching for the reservoir of Leishmania in a native rodent, *Ctenodactylus gundi* at the Pasteur Institute in Tunis (Innes, 2008). At about the same time, Alfonso Splendore working in Sao Paulo also discovered a similar parasite in rabbits. There were several other reports appeared following the discovery of the parasite and species of *Toxoplasma* were named with reference to the host species in which they were initially detected (Innes, 2008).

The name *Toxoplasma* was derived from a Greek word "toxo" meaning arc or bow, since it resembled the crescent shaped morphology of the tachyzoite and bradyzoite stages of the organism while "plasma" referred to form or life (Nicolle and Manceaux, 1909). The pathogenicity of the protozoan *T. gondii* to human was pointed out by three pathologists, Wolf, Cowen and Paige from New York, USA, who first isolated the organism from an infant girl who had died of congenital encephalomyelitis (Dubey, 2009). Wolf and Cowen then outlined the classic triad of symptoms associated with human congenital toxoplasmosis namely hydrocephalus, retinochoroiditis and encephalitis (Innes, 2008).

1.4 Strain type

Toxoplasma has its own ability to manipulate the immune system and establish a chronic infection in host. The parasite can be divided into at least three distict clonal lineages which are type I, type II and type III. Type I can be further characterized as RH and GT-1 strains. Type II strain, in the other hand, as ME 49 strain and its derivatives PDS, PLK and PTg. Type III characterized as CEP (CTg) strain and VEG strain. These strains were either completely or partially sequenced (ME49, RH; Toxo DB http://www.toxodb.org) and were part of *T. gondii* expressed sequence tag projects (ME49, RH, VEG), and/or have been used as the parents in genetic crosses and subsequently genotyped with more than 130 markers (GT-1, CEP, ME49; *Toxoplasma* Genome Map http://www.toxomap.wustl.edu) (Saeij *et al.*, 2005). Genetic exchanges between different strains can only occur in the rare event of a feline becoming infected simultaneously with more than one strain. According to Saeij *et al.*, (2005), millions of stable and highly infectious oocysts that are secreted in the cat feces as a result of meiosis contain different sporozoite genotypes and huge number of genetically distinct organisms can be formed from a single infected cat. Animals ingesting these oocysts can subsequently function to select the most successful of these genotypes.

1.5 Virulence and pathogenesis

T. gondii virulence is normally defined based on LD_{50} (lethal dose) in mice. The *T. gondii* parasite burden is a major contributor of *Toxoplasma* pathogenesis in mice related to an over stimulation of the immune system leading to high levels of T helper cell type 1 (Th1) cytokines, increased apoptosis and organ damage (Weiss and Kim,

2007). Most of the authors focused their research on type I RH strain, followed by type II ME49 strain and type III VEG strain. The popularity of type I RH strain among the researches may be due to certain factors such as growth rate and virulence in mice. One tachyzoite of a type I strain is sufficient to generate high parasite loads and high levels of Th 1 cytokines but increased inoculation needed to obtain high parasite loads of Type II strain in which will result in equally high levels of cytokines and pathology. Several studies have noted that type I strain grow faster than type II or type III. In order to determine the growth factor and capability of a parasite to infect an organism, factors to be considered are time taken by the parasite to lyse the host cells, number of parasites in a culture and the doubling time. Routinely observed, the type I RH strain completely lyses a flask of cultured cells much faster than the type II or type III and others. The higher growth rate of type I parasites in cell culture have been suggested to be due to a higher reinvasion rather than to a shorter doubling time. Besides, the extracellular type I parasites remain infectious for a longer time compared with type II and type III which enable the parasites to disseminate more efficiently to new cells after infected cells are lysed. Type I strains are also found in higher number in the peritoneum than type II strain parasites. This ability to cross epithelial barriers rapidly and reach the blood stream within hours post-infection might be an important predeterminant of parasite dissemination *in vivo* in susceptible host species. Type I also has a lower rate of inter-conversion from tachyzoite to bradyzoite than type II strains (Weiss and Kim, 2007).

Although these *in vitro* studies demonstrated different intrinsic properties of the different strain, the host response is essential for expression of virulence. It is crucial to

understand that strain virulence is not the same across host species. As an example type I strains, which are highly virulent in mice, are not pathogenic in rats. Besides, congenital toxoplasmosis cases are predominantly associated with type I and type II strains (Djurkovic´-Djakovic´ *et al.,* 2006). This shows that type II strains, which are non-virulent in mice, can sometimes be highly pathogenic to the human fetus.

1.6 Life cycle of *Toxoplasma gondii*

T. gondii is a coccidian parasite that can infect a wide range of hosts and many different host cells (Dubey and Beattie, 1988; Dubey *et al.,* 1998). Coccidia in general have a complex life cycle. Life cycle of *T. gondii* clearly illustrates the transmission mode and how it infects the body (Figure 1.3). The life cycle of *T. gondii* is facultatively heteroxenous (Frenkel and Dubey, 2000) means it has more than one obligatory host in its life cycle. Examples of members of Felidae are domestic or stray cats, including wild cat such as ocelots, margays, jaguarandi, bobcats, Pallas cats and

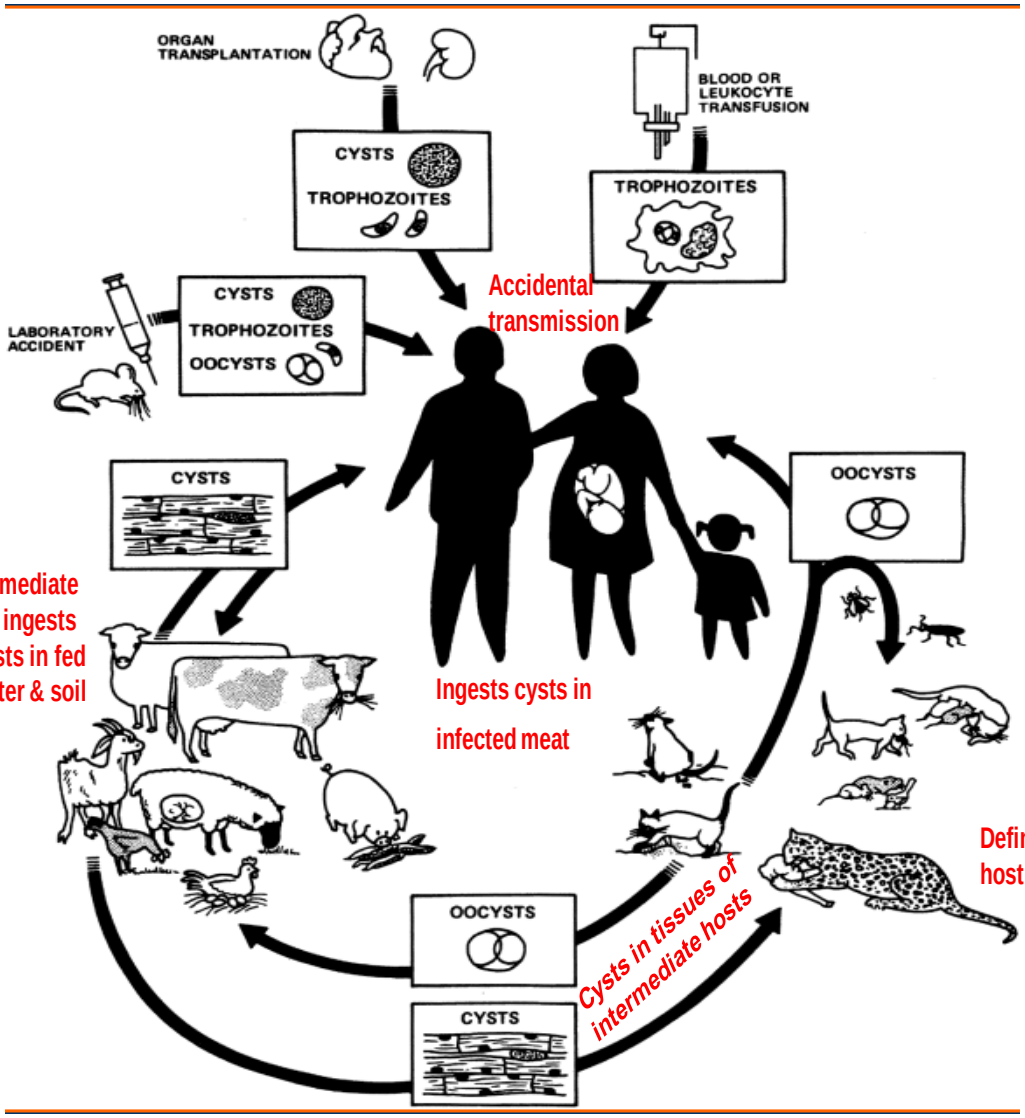


Figure 1.3: Life cycle and transmission routes of *T. gondii.*

(Adapted and modified from http://www.recenttrendsofparasitology.com)

Bengal tigers. Based on the study conducted by Dr. J.K. Frenkel, of the many species of animals experimentally infected with *T. gondii* only, felids shed oocysts (Dubey, 2009). The study described several seroepidemiological studies that had been conducted to confirm the role of cat in the natural transmission of *T. gondii*.

The results of epidemiologic studies indicated that *T. gondii* is transmitted most efficiently by carnivorism in the cat and by faecal-oral (oocysts) route in other hosts. During controlled studies, most uninfected cats that were fed tissues containing bradyzoites shed oocysts, but only less than half of the cats that were fed oocysts will shed oocysts (Dubey and Frenkel, 1972). In addition, the number of oocysts shed by a cat after ingestion of bradyzoites in infected tissues was more compared to cats that ingested oocysts. Thus the ingestion of infected rodents and birds by cats can lead to excretion of large numbers of environmentally resistant oocysts. Besides, transplacental infection can develop in cats, and kittens infected *in utero* can shed oocysts after birth (Dubey *et al.,* 1993; Sato *et al.,* 1993). However the frequency of repetitive shedding of oocysts in cats in the wild is unknown even though cats can shed oocysts after reinfection (or even without reinfection).

Sexual reproduction occurs only in the feline intestinal tract. Oocyst shed by cat occur after ingesting of any of three infectious stages of *T. gondii* i.e. tachyzoites, bradyzoites and sporozoites (Figure 1.4 a, b, 1.5 a, b, 1.6 a, b respectively). The frequency of oocysts shedding varies according to the stage of *T. gondii* ingested.

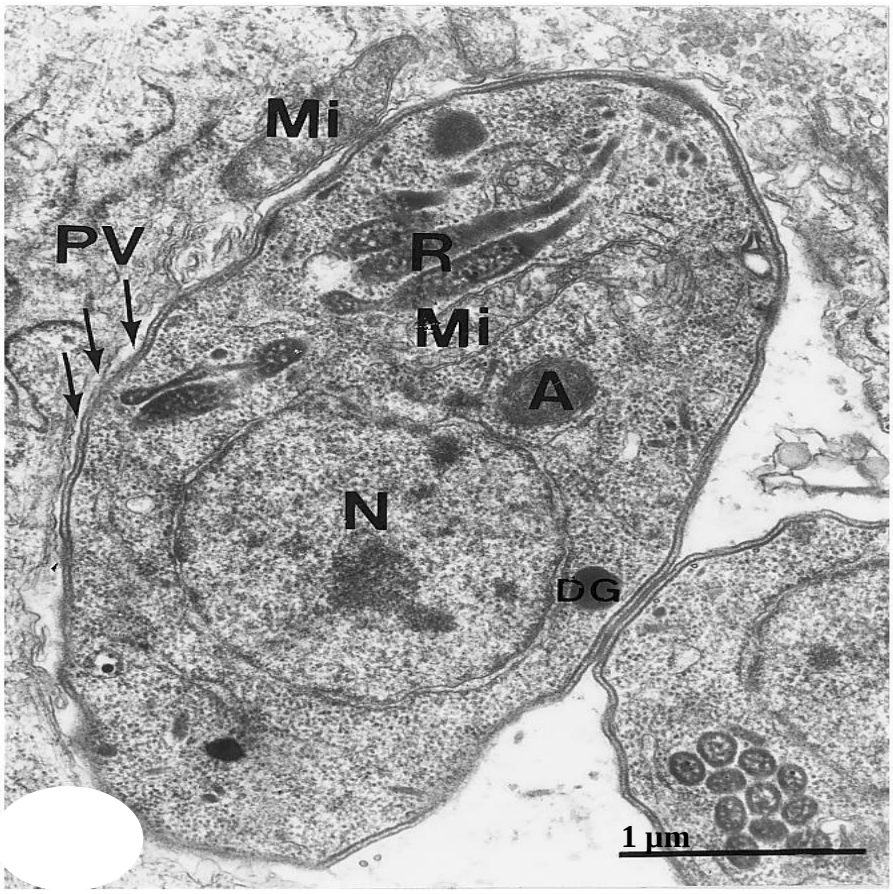


Figure 1.4 a: Transmission electron micrographs of an *in vitro* tachyzoite within a parasitophorus vacuole.

PV: Parasitophorus vacuole; **Mi**: Mitochondrion; **R**: Rhoptry; **N**: Nucleus; **A**: Apicoplast; **DG**: Dense granules.

(Adapted and modified from Yahiaoui *et al.,* 1999)

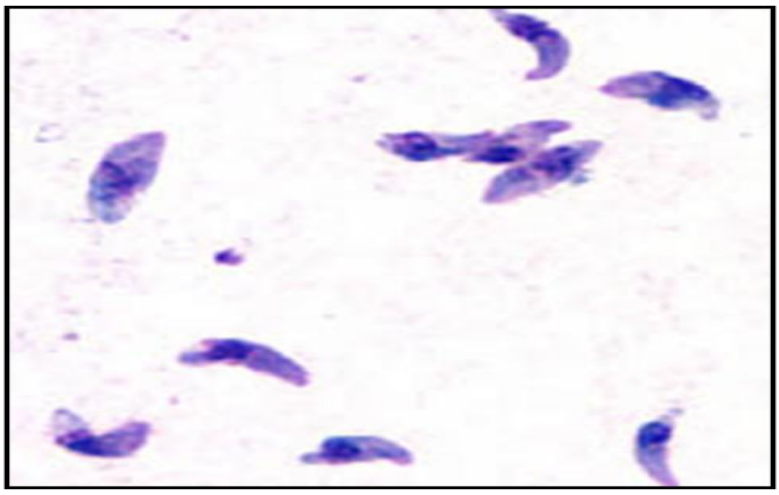


Figure 1.4 b: *Toxoplasam gondii* tachyzoites, stained with Giemsa, from a smear of peritoneal fluid obtained from a laboratory-inoculated mouse.

(Adapted from www.dpd.cdc.gov/DPDx/html/Toxoplasmosis.com)

Tachyzoites (trophozoites) of *T. gondii* are approximately 4-8 µm long by 2-3 µm wide, with a tapered anterior end, a blunt posterior end a large nucleus. They may be found in various sites throughout the body of the host.

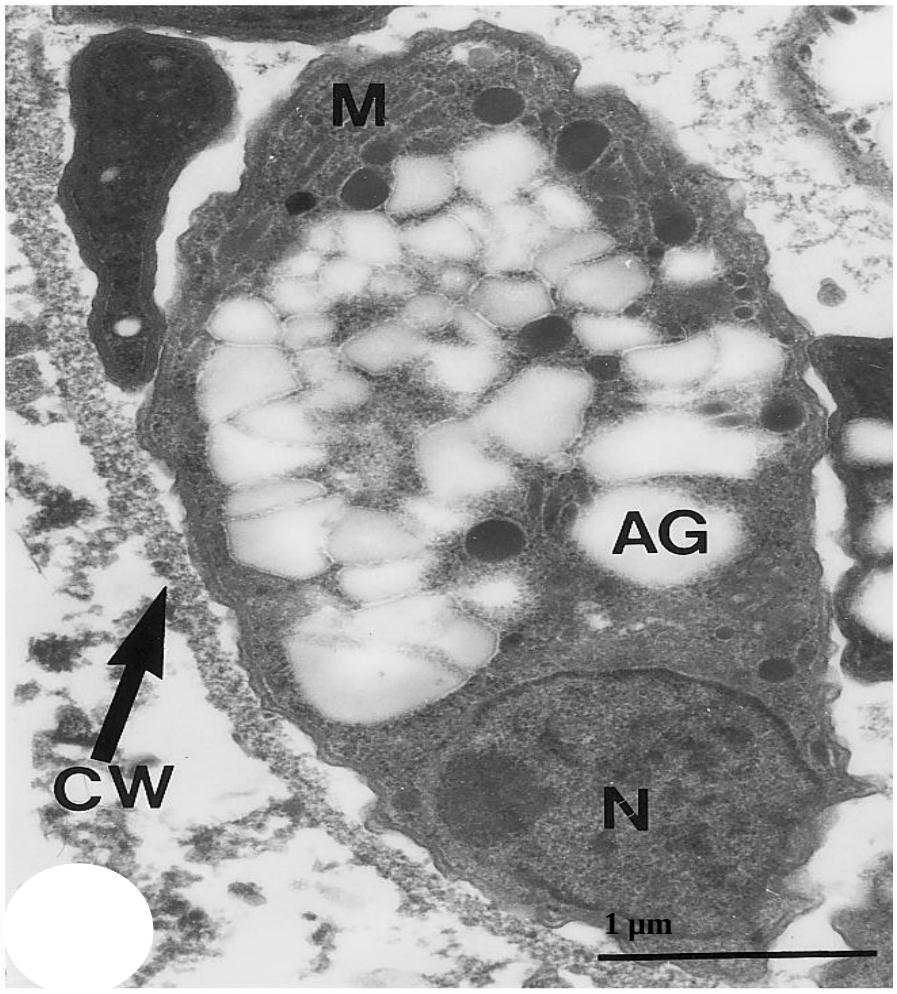


Figure 1.5 a: Transmission electron micrographs of an *in vitro* bradyzoite.

M: Micronemes; **CW**: Cyst wall; **AG**: Amylopectin granules; **N**: Nucleus.

(Adapted and modified from Yahiaoui *et al.,* 1999)

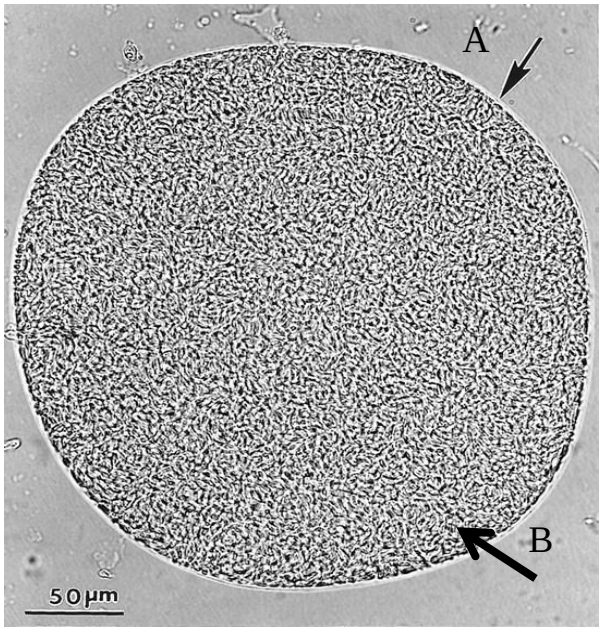


Figure 1.5 b: Tissue cyst obtained from a smear of brain homogenate from a rat 14 months after infection with the VEG strain of *T. gondii*.

A: Barely visible cyst wall; **B**: Bradyzoites.

(Adapted and modified from Yahiaoui *et al.*, 1999)

Cysts of *T. gondii* usually range in size from 5-50 µm in diameter. Cysts are usually spherical in the brain but more elongated in cardiac and skeletal muscles. They may be found in various sites throughout the body of the host, but are most common in the brain and skeletal and cardiac muscles.

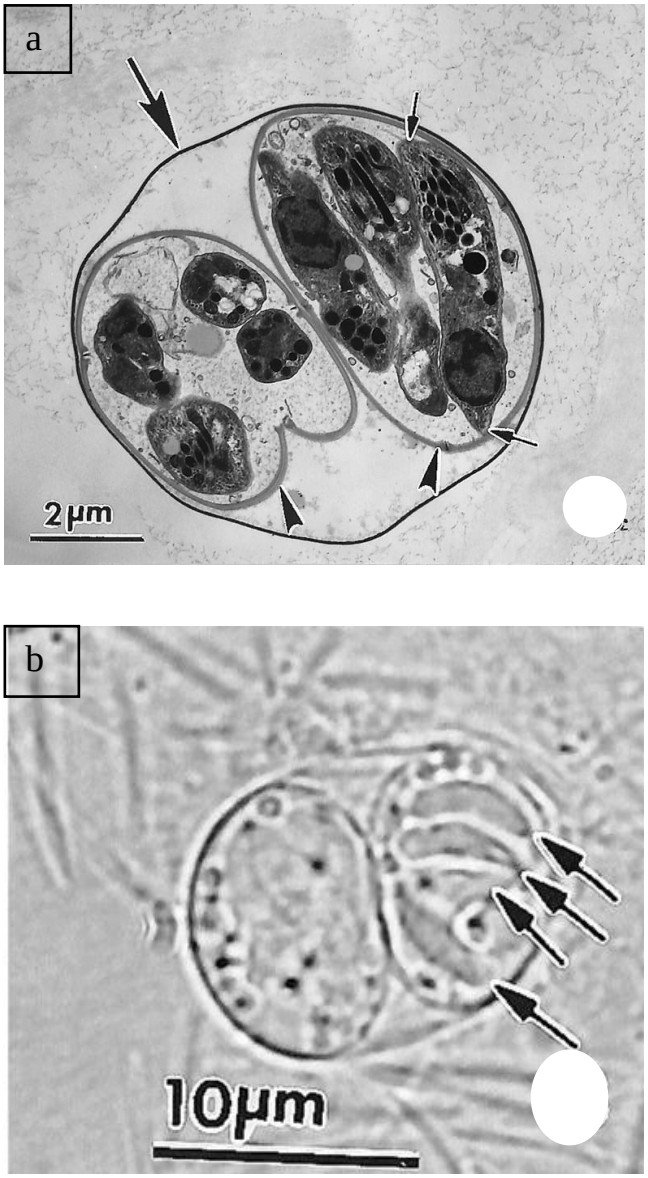


Figure 1.6: *T. gondii* oocyst. (a)Transmission electron micrograph of a sporulated oocyst. Note the thin oocyst wall (large arrow), two sporocysts (arrowheads), and sporozoites, one of which is cut longitudinally (small arrows). (b) Sporulated oocyst with two sporocysts. Four sporozoites (arrows) are visible in one of the sporocysts.

(Adapted from Dubey *et al.*, 1998)

The pre-patent periods after ingesting tissue cysts are 3-10 days, 18 days or longer after ingesting oocysts, irrespective of the dose. The pre-patent period after ingesting tachyzoites may vary. After ingestion of tissue cysts, the parasites invade the cat enterocytes, undergo several rounds of division and differentiation into microgametocytes and macrogametocytes. The gametocytes fuse to form zygote or oocysts that are shed into environment with the cat feces. The oocysts undergoes meiosis, producing an octet of highly infectious sporozoites that are resistant to environmental damage and may persist for years in warm and humid environment. These oocysts spread and contaminate water, soil, fruits, vegetables and animals (especially herbivores) following consumption of infected plant material or any of those mentioned above.

Asexual reproduction occurs in various tissues of intermediate hosts of numerous warm-blooded animals. It begins after the ingestion of oocysts in cat feces, contaminated water, or soil. The sporozoites or oocyst differentiate into rapidly dividing tachyzoites (also called as trophozoite). The tachyzoites will initiate the formation of tissue cysts. Then bradyzoites multiply slowly within the tissue cyst by a process called endodyogeny (Dubey, 1986; Dubey, 1993; Dubey and Beatie, 1988; Frenkel, 2000; Levine, 1961). These cysts are most often located within the eye, central nervous system, skeletal or cardiac muscles. To a lesser extent it may be present in the liver, kidney and lungs (Dubey, 1993; Dubey, 1998b; Dubey *et al.*, 1998; Levine, 1961). Bradyzoites may persist for life in some intermediate hosts, mainly within the muscles and brain. The cysts are very difficult to eradicate entirely because they rest inside the host cells.

However, it is still unknown how the persistence mechanism occurs. Many investigators believed that tissue cysts breakdown periodically, with brayzoites transforming to tachyzoites that reinvade host cells and again transform to bradyzoites within new tissue cysts (Dubey, 1998; Dubey *et al.*, 1998b; Evans, 1992; Frenkel, 2000; Remington and Desmonts, 1990; Weiss *et al.*, 1998). Ingestion of bradyzoites by definitive hosts will initiate another asexual phase of proliferation which consist of initial multiplication by endodyogeny followed by repeated polygeny in epithelial cells of the small intestine. The terminal stages of this asexual multiplication initiate the sexual phase of the life cycle.

1.6.1 Transmission route of *Toxoplasma gondii* in human

Transmission of *T. gondii* in human occur *via* three principal routes, the first route is by eating raw or inadequately cooked infected meat, especially pork, mutton, and wild game, or uncooked foods that have come in contact with infected meat. Second, humans can inadvertently ingest oocysts that cats have passed in their feces, either from a litter box or from soil (e.g. soil from gardening, on unwashed fruits or vegetables, or unfiltered water). Third, women can transmit the infection tranplacentally to their unborn fetus. In adults, the incubation period for *T. gondii* infection ranges from to 10 to 23 days after the ingestion of undercooked meat and from 5 to 20 days after the ingestion of oocysts from cat feces.

1.6.1.1 Faecal-oral route

Epidemic 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 environmentally stable and becomes a source of infection to a variety of animals and man. Based on a 10 year study, the risk of infection is increased when the weather is both warm and moist, or moderate and less moist (Afonso *et al.,* 2006). This suggests that moisture increases oocysts survival during periods of heat. For maximum temperatures of 35^{o}C, oocysts persist longer in moist than in dry areas. Prevalence depends on variables that influence the survival of oocysts including maximum temperatures, rain and location of the animals.

Several outbreaks of toxoplasmosis in human beings have been linked to drinking of unfiltered water (Bahia-Oliveira *et al.,* 2003; Bowie *et al.,* 1997). Toxoplasmosis also may occur in developing countries that lack adequate sanitary conditions which expose populations to a variety of diseases. For example, an epidemic of *T. gondii* infection in Atlanta, Georgia, was attributed to dust from the floor of a horse stable that had been contaminated with cat feces containing oocysts. It was hypothesized that the dust was inhaled, then eventually swallowed, although hand to- mouth transmission or dust contamination of food or drinks in the stable (Petersen *et al.,* 2000).

1.6.1.2 Carnivorism

Weinman and Chandler (1954) was the first to suggest the transmission of *T. gondii* through carnivorism. Later, the idea was supported by demonstrating the *T. gondii* derived from cysts to proteolytic enzymes (Jacobs *et al.*, 1960). They found that the proteolytic enzymes digested the cyst wall immediately while releasing the bradyzoites that were able to survive long enough to infect the host. Therefore, ingestion of viable cysts present in meat may result in infection, particularly among people who habitually eat raw or undercooked meat. Serological studies have found widespread of *T. gondii* infection in meat-producing animals, particularly pigs, sheep and goats (Aspinall *et al.*, 2002). Older animals, which are found to have a higher prevalence of the organism, are routinely used in the production of sausages, salami, and cured meats. *T. gondii* is killed when the internal temperature of meat reaches 66^{o}C (Dubey, 1998) but it may survive in improperly grilled or barbecued meat. A study in South Kalimantan (Indonesia), reported that goat meat acts as a source of infection to man due to eating undercooked goat 'sate' (Durfee, 1976).

1.6.1.3 Congenital transmission

A study published in 2000 reported that 30% to 63% of *T. gondii* infections among pregnant women, at various centers in Europe, could be attributed to ingestion of undercooked or inadequately cured meat, while 6% to 17% could be attributed to contact with soil (Petersen *et al.*, 2000). Generally, congenital transmission of toxoplasmosis can occur transplacentally during primary infection of a pregnant woman. However, the mechanism of this vertical transmission is not yet well described. The probable scenario

for primary infected pregnant women may result in invasion of the tachyzoites into placenta and multiplication within cells and placenta because the mother has no antibodies against *T. gondii* during the first time infection. Then, some of these tachyzoites may cross the placenta and enter the fetal circulation or fetal tissue (Ebbesen, 2000; Remington and Desmonts, 1990). After the first infection, the antibody is produced and the organisms may be destroyed before it can cross the placenta, therefore the fetus is protected.

Infection during early maternity (first and second trimester) may result in severe congenital toxoplasmosis which can lead to the death of the fetus *in utero* and spontaneous abortion. Conversely if infection occurs during late maternal (trimester), the newborns may not be infected and are born normal (Montoya and Liesenfeld, 2004). Infection may initially appear asymptomatic. Babies can develop chorioretinitis or delayed growth in the second or third decade of life if no proper treatment is applied (Remington *et al.,* 2001; Wilson *et al.,* 1980). This type of infection is also recorded in immunocompromised women with AIDS or receiving immunosuppressive drugs but the rate of vertical transmission in this setting seems to be fairly low (Minkoff *et al.*, 1997).

1.6.1.4 Organ transplantation and blood transfusion

Another route of *T. gondii* transmission to human is through organ transplantation. It has been found that organ transplantation of heart, kidney, liver and bone marrow from a seropositive donor to a seronegative recipient is complicated by tachyzoites or tissue cysts of *T. gondii* infections (Emelia, 2009; Duey and Beattie, 1998; Ho Yen, 1992). Reactivation of latent infection in the recipient is the common reason behind the incidence of toxoplasmosis in bone marrow, haematopoietic stem cell and liver transplant patients and in AIDS patients (Emelia, 2009). *T. gondii* can also be transmitted via blood or leucocytes from immunocompetent and immunocompromised donors (Raisanen, 1978; Siegel *et al.,* 1971). However it has been suggested that the risk of transmission via blood transfusion is very low (Dubey and Beattie, 1988) because the parasitemia only occurs for a short period of time after the primary infection.

1.6.1.5 Other routes of transmission

Other possible ways that *T. gondii* parasite can make entry are through conjunctiva, respiratory and cutaneous routes (Beverly, 1973). Laboratory accidents also have been reported by contact with *T. gondii* contaminated needles and glassware or during handling of infected animals (Kayhoe *et al.,* 1957; Remington and Gentry, 1970). Bonametti *et al.,* (1997) reported a case of toxoplasmosis in a breast-fed infant whose mother was having acquired primary infection with *T. gondii*.

1.7 Clinical manifestations and pathogenesis of human toxoplasmosis

Clinical manifestations and pathogenesis of toxoplasmosis in humans depends on the immune status of the patient or the clinical setting such as immunocompetence, congenital toxoplasmosis, or ocular disease.

1.7.1 Immunocompetent adults and children

Most persons infected after birth are asymptomatic established with no symptoms or an illness which is so mild that it is looked upon as harmless and a very temporary problem ascribed to a virus (Dubey and Jones, 2008). Humans are believed to remain infected for life and the organism reactivated only when immunosuppression occurs. It causes a self-limited and non-specific illness which rarely needs treatment. General symptoms of toxoplasmosis in immunocompetent individuals are fever, lymphadenopathy, headache, sore throat, cough, myalgia, and dizziness. According to Tenhunen (1964), non-specific gastrointestinal symptoms such as nausea, vomiting and abdominal pain are prominent in patients with lymphadenopathy. Very infrequently, myocarditis, polymyolitis, pneumonitis, hepatitis or encephalitis can arise in healthy individuals (Montoya and Liesenfeld, 2004).

1.7.2 Maternal infection and congenital toxoplasmosis

The severity of the maternal infection depends upon the stage of pregnancy at the time of infection. The chances of congenital infection are 10 to 25% in untreated women who acquired the infection in the first trimester (Emelia, 2009). If the infection occurs during

the second and third trimesters, the incidence of fetal infection ranges between 30 to 54% and 60 to 65% respectively (Lynfield and Guerina, 1997). If the infection occurs in early stages of pregnancy, the consequences are more severe including miscarriage (Remington and Desmonts, 1990), severe disease, intra-uterine growth retardation or premature birth. The highest frequency of severe abnormalities at birth is observed in children whose mothers acquired a primary infection between 10^{th} and 24^{th} week of gestation (Remington and Desmonts, 1990). A wide range of clinical manifestations are seen such as chorioretinitis which can be present for many years after birth, mental retardation, microcephaly, hydrocephalus and seizures.

Factors related to the fetal infection are the time of maternal infection, immunological competence of the mother during parasitemia, parasite load and strain virulence (Tenter *et al.,* 2000). Placenta as the medium transmits the parasites to the foetus almost immediately after maternal infection but possibly with a delay of several weeks or longer (Remington *et al.,* 1995). Mildly infected children may consist of slightly diminished vision whereas severely infected children may have the full tetrad of signs including retinochorioditis, convulsions and intracerebral calcification. Among these, hydrocephalus is the least common but most dramatic lesion of toxoplasmosis (Dubey, 2004).

Generally, fetuses with congenital toxoplasmosis will look normal in prenatal ultrasound. However examples of ultrasonographic findings suggestive of congenital

infection are intracranial calcifications, ventricular dilation, hepatic enlargement, ascites and increased placental thickness (Gay-Andrieu *et al.,* 2003). None of the signs described in newborns with congenital disease is pathogonomic for toxoplasmosis and can be mimicked by congenital infection with other pathogens such as cytomegalovirus, herpes simplex virus, rubella and syphilis (Montoya and Liesenfeld, 2004).

1.7.3 Immunocompromised patients

Toxoplasmosis can be life-threatening in almost all immunocompromised individuals. A person becomes severely immunosuppressed when the CD4+ T-lymphocyte count drops below 50 cells per microliter (Dubey and Jones, 2008). In AIDS patients, the overwhelming majority (>95%) of toxoplasmosis cases occur as a consequence reactivation of latent infection (Emelia, 2009). Toxoplasmic encephalitis (TE) is the most common clinical manifestations in AIDS patient and in fact the most common cause of death among toxoplasmosis patients with AIDS (Dubey, 2004). The clinical presentation often includes focal encephalitis with headache, confusion, motor weakness and fever and if it is not treated can progress to seizures, stupor and coma. The most common focal neurological findings are speech abnormalities and hemiparesis (Luft *et al.,* 1993). The primary lesion is cerebral necrosis, particularly of the thalamus and pneumonia, other disseminated systemic disease or retinochoroiditis. Clinically severe symptoms also can be observed in immunosuppressed patients with malignancies and after transfusions or transplant with immunosuppressive therapy (Dubey and Jones, 2008). However after the introduction of primary prophylaxis against *T. gondii* and