SEROLOGIC AND MOLECULAR DETECTION OF TOXOPLASMOSIS AMONG BLOOD DONORS AND HAEMATO-ONCOLOGY PATIENTS IN HOSPITAL UNIVERSITI SAINS MALAYSIA

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by

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LIST OF SYMBOLS AND UNITS

-	Negative
%	Percentage
+	Positive
>	More than
≤	Less than or equal to
≥	More than or equal to
µl	Microlitre
bp	Base pair
IU/ml	International unit per millilitre
min	Minutes
ml	Millilitre
mM	Millimolar
n	Sample size
^{o}C	Degree Celcius
pg	Picogram
sec	Seconds
V	Voltage
x g	Gravitational force
µM	Micromole
nmol	Nanomole
ng	Nanogram
$\chi 2$	Chi square

LIST OF ABBREVIATION

AIDS	Acquired immunodeficiency syndrome
bp	Base pair
CNS	Central nervous system
CSF	Cerebrospinal fluid
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EDTA	Ethylenediaminetetraacetic acid
FAT	Fluorescent antibody test
HIV	Human immunodeficiency virus
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHA	Indirect haemagglutination test
IC	Internal control
LAT	Latex agglutination test
LAMP	Loop-mediated isothermal amplification
OD	Optical density
OR	Odds ratio
PCR	Polymerase chain reaction
SPSS	Statistical Package for the Social Sciences
TE	Toxoplasmic encephalitis
TBE	Tris-Borate-EDTA
UKM	Universiti Kebangsaan Malaysia
USM	Universiti Sains Malaysia
WHO	World Health Organization

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PENGESANAN SEROLOGI DAN MOLEKULAR TOKSOPLASMOSIS DALAM KALANGAN PENDERMA DARAH DAN PESAKIT HEMATO-ONKOLOGI DI HOSPITAL UNIVERSITI SAINS MALAYSIA

ABSTRAK

Toksoplasmosis adalah disebabkan oleh *Toxoplasma gondii* dan penyakit ini telah diketengahkan sebagai satu kesihatan awam yang membimbangkan, kerana satu pertiga penduduk dunia telah dijangkiti *Toxoplasma gondii*. Transmisi daripada penderma darah kepada pesakit terimunokompromi sebagai penerima darah telah menjadi satu kebimbangan. Kajian keratan rentas ini bertujuan untuk menyiasat kelaziman toksoplasmosis dalam kalangan penderma darah dan pesakit hemato-onkologi di Hospital USM. Sejumlah 56 penderma darah dan 56 pesakit hemato-onkologi telah diperiksa dengan kaedah esai imunoserapan terangkai enzim bagi anti-*T. gondii* -imunoglobulin G (IgG) dan antibodi imunoglobulin M (IgM). Sampel yang positif bagi *T. gondii* IgG dan IgM telah seterusnya diuji untuk keavidan IgG. Semua asid deoksiribonukleik (DNA) yang diekstrak daripada sampel darah dianalisis untuk kehadiran gen *Toxoplasma* B1 dan ITS-1 dengan kaedah tindakbalas rantai polimerase. Data sosio-demografi dan ciri-ciri tingkah laku penderma dan pesakit telah dianalisa dengan menggunakan analisa statistik. Daripada 56 penderma darah, 23 (41.07%) penderma adalah IgG+/IgM-, dan 2 (3.57%) penderma adalah IgG+/IgM+. Seorang penderma mempunyai indeks keavidan tinggi yang menunjukkan jangkitan masa lalu lebih daripada 20 minggu, manakala seorang lagi mempunyai indeks keavidan rendah yang menunjukkan jangkitan baru dalam masa 20 minggu. Sementara itu, 28 (50%) pesakit hemato-onkologi adalah seropositif untuk antibodi *T. gondii*, di

mana 27 (48.21%) pesakit adalah IgG+/IgM- dan seorang pesakit (1.79%) adalah IgG+/IgM+ dengan indeks keavidan tinggi. Tiada sampel (penderma dan pesakit) yang telah diuji positif untuk kehadiran gen *Toksoplasma* B1 dan kawasan ITS-1. Analisis Pearson Chi Square dan Fisher Exact Test menunjukkan hanya status pekerjaan dikaitkan dengan kadar seropositif *Toksoplasma* untuk populasi penderma darah. Walau bagaimanapun, untuk pesakit hemato-onkologi tiada faktor sosiodemografi dan ciri-ciri tingkah laku menunjukkan persamaan yang signifikan dengan kadar seropositif toksoplasmosis. Kesimpulannya, penderma darah dalam kajian ini pernah terdedah kepada jangkitan toksoplasmosis tetapi parasit ini mungkin telah dimusnahkan oleh sistem imuniti atau berada di dalam tisu-tisu lain. Oleh itu, darah adalah selamat untuk didermakan. Manakala, pesakit hemato-onkologi juga telah terdedah kepada jangkitan toksoplamosis sebelum ini dan parasit mungkin berada di dalam tisu-tisu lain jika ianya tidak dimusnahkan oleh sistem imuniti pesakit. Pesakit menghadapi risiko besar untuk mengalami pengaktifan semula jangkitan toksoplamosis.

SEROLOGIC AND MOLECULAR DETECTION OF TOXOPLASMOSIS AMONG BLOOD DONORS AND HAEMATO-ONCOLOGY PATIENTS IN HOSPITAL UNIVERSITI SAINS MALAYSIA

ABSTRACT

Toxoplasmosis caused by *Toxoplasma gondii* and it has been highlighted as a public health concern, as one-third of the world population has been infected. Its transmission from blood donors to receiving immunocompromised patients has become a concern. This cross-sectional study aimed to investigate the prevalence of toxoplasmosis among blood donors and haemato-oncology patients in Hospital Universiti Sains Malaysia. A total of 56 blood donors and 56 haemato-oncology patients were screened by an enzyme-linked immunosorbent assay (ELISA) for anti-*T. gondii* immunoglobulin G (IgG) and Immunoglobulin M (IgM) antibodies. Samples that were positive for *T. gondii* IgG and IgM were further tested for IgG avidity using ELISA. All extracted deoxyribonucleic acids (DNAs) from whole blood samples were analyzed for the presence of the *Toxoplasma* B1 gene and the ITS-1 region by PCR. The socio-demographic data and behavioral characteristics of donors and patients were analyzed using statistical analysis. Out of 56 blood donors, 23 (41.07%) donors were IgG+/IgM-, and 2 (3.57%) donors were IgG+/IgM+ with one of the donors having a high avidity index indicating as past infection for more than 20 weeks and the other with a low avidity index indicating as recent infection within 20 weeks. Meanwhile, 28 (50%) of hemato-oncology patients were seropositive for *T. gondii* antibodies, where 27 (48.21%) patients were IgG+/IgM- and one patient (1.79%) was IgG+/IgM+ with high avidity index. None of the samples (donors and patients) tested positive for

the presence of the *Toxoplasma* B1 gene and ITS-1 region. Pearson Chi Square analysis and Fisher Exact Test showed that only employment status was significantly associated with *Toxoplasma* seropositivity rate for blood donors' population. However, for haemato-oncology patients none of sociodemographic factors and behavioral characteristics showed a significant association with *Toxoplasma* seropositivity rate. As for the conclusion, blood donors have been exposed to *T. gondii* infection, but currently, the parasites have been destroyed by the immune system or could reside in other tissues. Thus, blood is considered safe for transfusion. Meanwhile, hemato-oncology patients might have been exposed to *T. gondii* infection, and the parasites may reside in other tissues if patients' immune systems did not destroy it. Therefore, they have a higher risk of reactivation of infection.

CHAPTER 1

INTRODUCTION

1.1 Introduction to *Toxoplasma gondii*

Toxoplasmosis is one of the most common zoonotic diseases that has been highlighted in public health concern due to its high mortality rate or physical and/or psychological sequela in immunocompromised patients and congenitally infected neonates (Elsheikha *et al.*, 2009; Tegegne *et al.*, 2016). It is a parasitic disease caused by *Toxoplasma gondii* (*T. gondii*), a coccidian parasite, and a spore-forming, single-celled obligate intracellular parasite belongs to an apicomplexan class. *T. gondii* is capable of infecting a wide range of warm-blooded animals, including humans. *T. gondii* was first discovered in North Africa by Nicolle and Manceaux in a hamster-like rodent (*gondi).* It was the widespread agent for zoonosis during 1908, which was named a year later (Paniker, 2007; Robert-Gangneux and Darde, 2012).

Its medical importance remained uncertain until it was found and identified in tissues of newborn who were congenitally infected with severe symptoms such as hydrocephalus, retinochoroiditis, and encephalitis in 1939 (Innes, 2010; Robert-Gangneux and Darde, 2012). In the mid-1970s, *T. gondii* were detected in immunocompromised patients, where the concept of reactivation of infection was extensively discovered by immunologists (Robert-Gangneux and Darde, 2012). In the late 1960s, a cat was discovered as *T. gondii* definitive host, which harboured the parasitic sexual cycle and spreading oocyst of *T. gondii* through faeces (Paniker, 2007; Robert-Gangneux and Darde, 2012).

1.1.1 Life Cycle of *T. gondii*

Life cycle of *T. gondii* consists of two parts, the definitive host and intermediate host, and three infectious stages consisting of; sporulated oocyst which contains sporozoites, the rapidly dividing tachyzoites and tissue cyst containing slow-dividing bradyzoites (Dubey, 2016; Gunn and Pitt, 2012). Cats' family are their definitive hosts for its sexual reproduction phase, while human, mouse or other warm-blooded animals are their intermediate hosts for its asexual reproduction phase (Dubey, 1998; Retmanasari *et al.*, 2017; Yohanes *et al.*, 2014). Cats get infected upon ingestion of sporulated oocyst passed in another cats' faeces in the environment or consuming intermediate host harbouring tissue cysts or tachyzoites such as infected birds or mice (*CDC - Toxoplasmosis*, February 28, 2019).

In the definitive host, the sexual reproduction phase starts when the parasites invade the intestinal cells of cats forming male and female gamete (Figure 1.1). Oocysts are formed after fertilization and released by the cells' disruption and shed off in cat faeces as unsporulated forms. After a few days in the environment, an unsporulated oocyst undergoes sporogony process, forming a sporulated oocyst that infects a wide range of intermediate hosts. The sporulated oocyst remains transmissible in the environment for months or even years (Gunn and Pitt, 2012). The contaminated environment may pollute other things such as crops or water, which located nearby, thus causing *T. gondii* to be transmissible to intermediate hosts (Lass *et al.*, 2012; Sahimin *et al.*, 2017). In humans, the parasites undergo an asexual reproductive cycle (Figure 1.1). *T. gondii* infects nucleated cells in humans and does not invade the red blood cells (Gunn and Pitt, 2012). Upon ingestion, oocyst releases sporozoites that penetrate the intestinal epithelium and differentiate into tachyzoites. These rapidly-

dividing tachyzoites transform into tissue cyst containing bradyzoites, and localize in neural or muscle tissue.

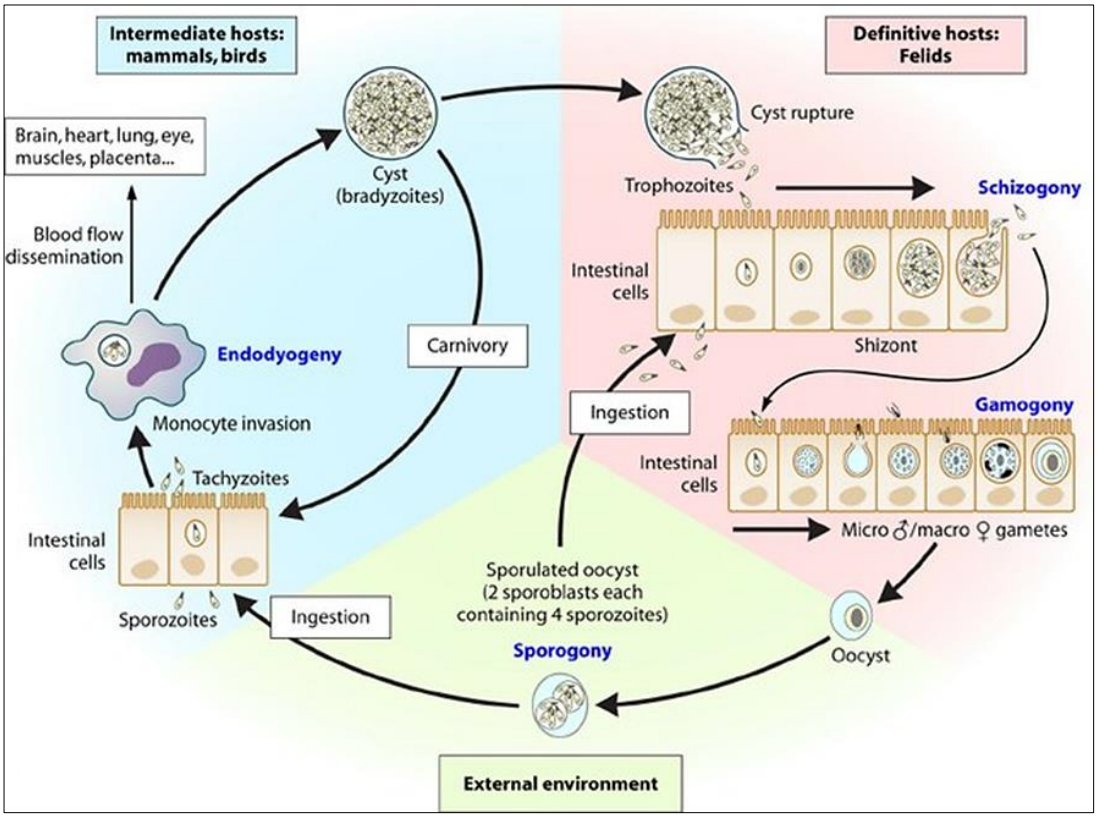

Figure 1.1: The life cycle of *T. gondii* in environment, definitive and intermediate host. Figure adapted from (Robert-Gangneux and Darde, 2012).

1.1.2 Modes of *T. gondii* transmission

Humans get infected with *T. gondii* through horizontal transmission including ingestion of contaminated food or drinks, consumption of raw or undercooked meat, exposure to contaminated soil, close contact with cats or vertical transmission from mother to foetus, organ transplantation and blood transfusion (Figure 1.2) (Abamecha and Awel, 2016; Davami *et al.*, 2015; Dubey, 2004; Foroutan-Rad *et al.*, 2016; Hussain *et al.*, 2017; Jones *et al.*, 2003; Sarkari *et al.*, 2014). Consumption of contaminated foods or drinks that contain oocysts such as unwashed vegetables or fresh fruits from the garden where there are stray cats around could be the source of *T. gondii* transmission (Lass *et al.*, 2012). This parasite remains dormant and resistant to freezing, high temperature, or any physical or chemical treatments in water (Robert-Gangneux and Darde, 2012).

Furthermore, humans can be infected with *T. gondii* by ingesting raw or undercooked meat containing tissue cyst. Toxoplasmosis is considered as a meat-borne disease where tissue cysts of *T. gondii* were found in pork (25%), mutton (10%), and less than 1% in beef and chicken (Ahmad *et al.*, 2014; Brandon-Mong *et al.*, 2015; Champoux *et al.*, 2004; Hussain *et al.*, 2017). Besides, a previous study reported that handling raw meat such as butchers or cooking the meat (tasting undercooked meat) can transmit *T. gondii* tissue cyst (Iddawela *et al.*, 2017). *T. gondii* is also transmitted through their definitive host, a cat species. Infected cats shed a million *T. gondii* oocyst to the environment (Dabritz and Conrad, 2010; Mossalanejad *et al.*, 2017; Paniker, 2007). Kids who play with the cats and contaminated soil expose themselves to *T. gondii* transmission (Ahmad *et al.*, 2014).

In addition, toxoplasmosis was reported to be transmitted through whole blood or white blood cell transfusion, organ transplant, and laboratory accidents even though it is rare (Ahmad *et al.*, 2014; Alvarado-Esquivel *et al.*, 2016; Elsheikha *et al.*, 2009; Sarkari *et al.*, 2014). Previous studies reported that *T. gondii* was transmitted through blood transfusion from seropositive donors to susceptible recipients due to its capability to survive in stored blood (Alvarado-Esquivel *et al.*, 2016; Davami *et al.*, 2015; Elsheikha *et al.*, 2009; Singh and Sehgal, 2010). Tachyzoites of *T. gondii* is one of the infectious stages that can invade all human cell types and survive for several weeks in the stored blood (Elhence *et al.*, 2010; Robert-Gangneux and Darde, 2012). Thus, *T. gondii* tachyzoites in the blood can transmit the parasite to the recipients, especially those requiring multiple transfusions (Sarkari *et al.*, 2014). Infected blood donor in acute phase infection is a significant contributor for this infection, especially in organ transplantation patient who need whole blood or white blood cell transfusion (Alvarado-Esquivel *et al.*, 2016; Derouin and Pelloux, 2008; Sarkari *et al.*, 2014; Yohanes *et al.*, 2014).

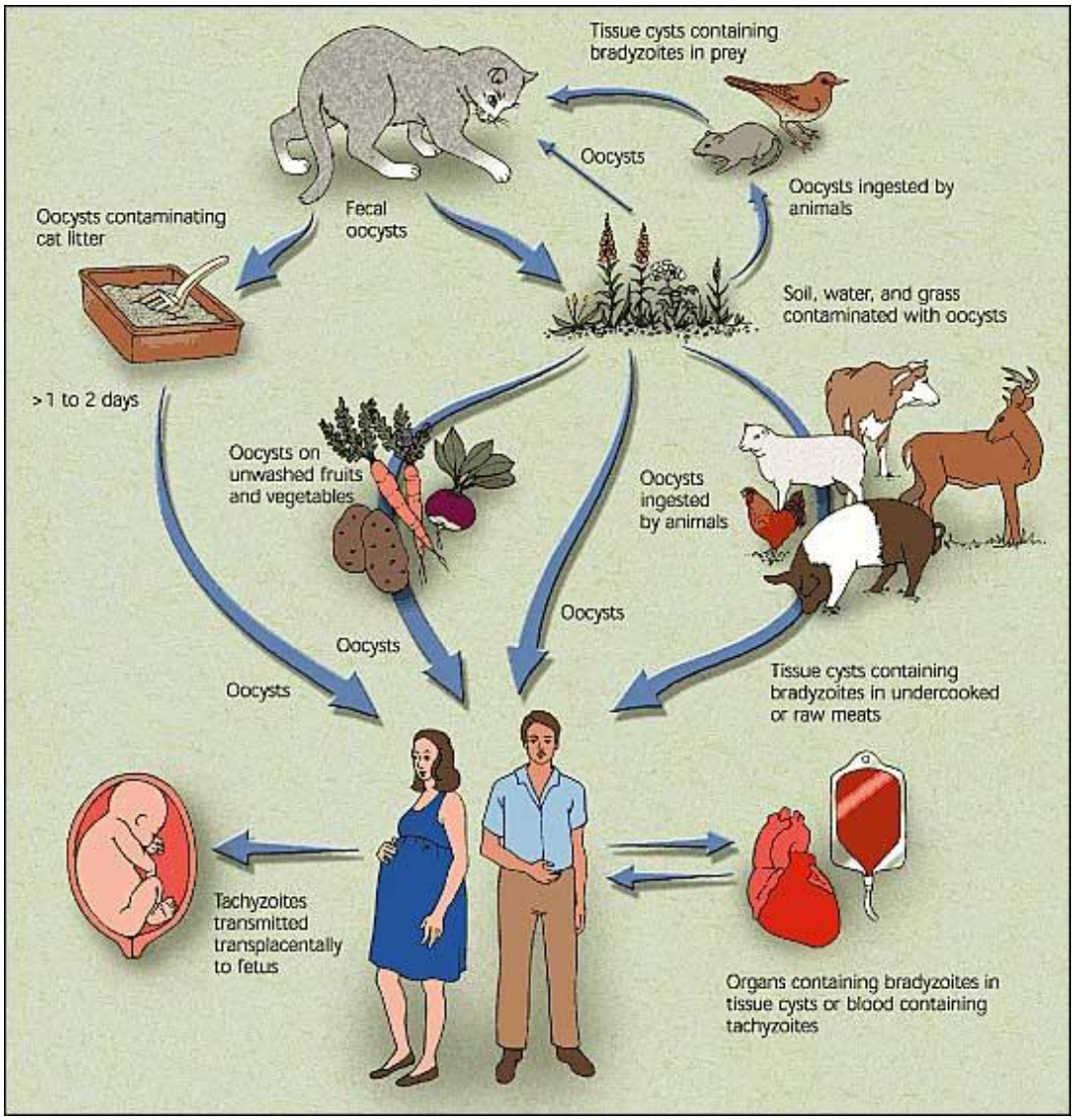

Figure 1.2: Modes of *T. gondii* transmission. Figure adapted from (Jones *et al.*, 2003).

1.1.3 Mechanisms of *T. gondii* in the human body

Tachyzoites of *T. gondii* disseminate quickly (less than 40 s) from the initial site of infection in the intestine to distant tissues through the intestinal mucosa, lymphatic system and bloodstream during early phase of acute infection (Harker *et al.*, 2015; Zenner *et al.*, 1998). *T. gondii* invades those cells and continue to proliferate and eventually disrupt the cells (John and Petri, 2006). In healthy individuals with a normal immune system, most *T. gondii* are destroyed by competent killers of the parasite, macrophages (Ahmad *et al.*, 2014).

A healthy immune response is highly efficient at eliminating tachyzoites. However, some tachyzoites leak from this destruction, causing them to form a tissue cyst containing bradyzoites (Wohlfert *et al.*, 2018). This persistent state, which is cyst-forming bradyzoite, is more resilient and impervious (Betancourt *et al.*, 2019; Blader and Saeij, 2009; Wohlfert *et al.*, 2018). This tissue cyst is commonly found in the central nervous system (CNS), eyes, skeletal or cardiac muscles of the host, causing inflammatory lesions (permanent damage) in immunocompromised patients or the foetus (Dunay *et al.*, 2018). The multiplication process within this tissue cyst in a slow pace produces a cyst that contains thousands of bradyzoites (Ahmad *et al.*, 2014). The tissue cyst remains dormant in healthy individuals, but it can be reactivated in immunocompromised patients (Bavand *et al.*, 2019; Saki *et al.*, 2017).

1.2 Clinical symptoms of toxoplasmosis

One-third of the world population was infected with toxoplasmosis and mostly were asymptomatic (Angal *et al.*, 2016; Sahimin *et al.*, 2017; Sarkari *et al.*, 2014). Clinical symptoms of toxoplasmosis are less commonly reported even though *T. gondii* antibody is widely prevalent in humans throughout the world. Most infections in immunocompetent individuals were asymptomatic, or they present with mild symptoms such as fever, flu-like illness or cervical lymphadenopathy (Angal *et al.*, 2016; Sarkari *et al.*, 2014). The parasites remain dormant for years in the body, but not in immunocompromised individuals and pregnant women, which can cause a life-threatening disease (Davami *et al.*, 2015; Sarkari *et al.*, 2014). Severe complications associated with acute infections or reactivation of past infections were reported in immunocompromised patients such as encephalitis, chorioretinitis, and myocarditis (Arefkhah *et al.*, 2019; Ben-Harari and Connolly, 2019; Ibrahim *et al.*, 2017; Sahimin *et al.*, 2017; Sarkari *et al.*, 2014).

1.2.1 Clinical symptoms of toxoplasmosis in immunocompromised group

In immunocompromised patients, symptomatic toxoplasmosis was reported as acute, sub-acute, or chronic infections. The rapid proliferation of *T. gondii* occurs during infection resulting in various areas of tissue necrosis. The brain is the most common organ affected, which leads to severe complications because of the limited cell capacity to regenerate (Champoux *et al.*, 2004). During acute infection, *T. gondii* may invade the mesenteric lymph nodes and liver parenchyma. The parasite can be found freely in the blood and peritoneal fluids during acute infection for a very short duration. A study on rodents found that *T. gondii* in the bloodstream took less than 40 s to infect the small intestine, 18 hours to transform into tachyzoites, and reaching the brain within six days (Weight and Carding, 2012). Swollen and painful lymph glands

associated with fever, headache, myalgia, anaemia, and lung problems are common symptoms during acute infection. With low immunity, the symptoms and signs may be prolonged, leading to sub-acute infection with severe manifestations. Tachyzoites continue to destroy the host cells and causing extensive lesions in the lung, liver, heart, brain, and eyes. The CNS has a lower immunity response than other tissues, making it prone to extensive damage from the tachyzoites invasion (Bogitsh *et al.*, 2005).

Chronic infection occurs when sufficient immunity is build-up to slow down the proliferation of tachyzoites. These cysts remain in the body without any apparent clinical effect. A cyst wall breaks and releases many bradyzoites, mostly killed by a host immune response, while some form a new cyst. When bradyzoites are killed, an inflammatory reaction occurs where the infected area, such as the brain, is gradually replaced with nodules of glial cells. The presence of many nodules will cause chronic encephalitis (Bogitsh *et al.*, 2005).

An example of a common manifestation of *T. gondii* infection among the immunocompromised group is Toxoplasmic encephalitis (TE). Patients with TE present with fever, headache, lethargy, incoordination due to muscle weakness, loss of memory, dementia, and focal/generalised seizures (Angal *et al.*, 2016; Robert-Gangneux and Darde, 2012; Sarkari *et al.*, 2014). Other than TE, tachyzoites also infect retinal cells causing an extensive infection of a central, macular area, which leads to blindness and ocular toxoplasmosis. In severe cases of ocular toxoplasmosis, there is secondary involvement of the choroid (Furtado *et al.*, 2013).

1.2.2 Clinical symptoms of congenital toxoplasmosis

T. gondii can be transmitted through vertical transmission from mother to foetus, which is called congenital toxoplasmosis. During pregnancy, the innate immunity that protects the mother against *T. gondii* is suppressed, especially in the third trimester, thus making pregnant women more susceptible to infection (Abamecha and Awel, 2016). The effects of *T. gondii* infection among pregnant women depend on the gestational age (Berredjem *et al.*, 2017; Chemoh *et al.*, 2019; Hafez Hassanain *et al.*, 2018). The severity of foetal disease varies between 0–9% during the first trimester, 30% during the second trimester, and 35–59% during the third trimester (Malary *et al.*, 2018; Robert-Gangneux and Darde, 2012). When *T. gondii* infection occurs during the first and second trimesters, it is commonly accompanied by severe manifestations, such as low birth weight, hydrocephaly, intracranial calcifications, and retinochoroiditis, which are recognizable at birth (Sarvi *et al.*, 2019).

In contrast, infection during the third trimester will cause different manifestations such as intracranial calcifications, hearing impairments, developmental delays, and visual disorders later in life (Chemoh *et al.*, 2019). Furthermore, congenital toxoplasmosis can result in abortion, foetal death, and abnormalities, such as blindness and severe cognitive impairment occurring after birth (Sarvi *et al.*, 2019). Serious pathologic complications such as hydrocephaly, microcephaly, chorioretinitis, blindness, mental retardation, epilepsy, jaundice, abortion, and foetal death were reported in previous studies (Awoke et al., 2015; Elhence et al., 2010; Malary et al., 2018; Svobodova and Literak, 1998). Another studies have further confirmed that screening and treatment for toxoplasmosis during pregnancy result in a decline of congenital transmission and clinical manifestation (Olariu *et al.*, 2019). Researchers estimated that prenatal treatment could reduce the risk of severe neurological

complications by 75% (Olariu *et al.*, 2019). Early treatment during pregnancy has been shown to significantly decrease vertical transmission and improve clinical outcomes.

1.3 Epidemiology of toxoplasmosis

Approximately 10 to 70% of the worldwide human population has been exposed to *T. gondii* infection (Sarvi *et al.*, 2019). According to the World Health Organization (WHO), about one million of toxoplasmosis in Europe is caused by contaminated food (Torgerson and Mastroiacovo, 2013). Different seroprevalence rate has been reported which are related to some environmental factors such as climatic conditions, geographical status, disease control and treatment, regional and ethnic customs and human activities (Yan *et al.*, 2016). Globally, low seroprevalence (10 to 30%) of toxoplasmosis has been reported in North America, South East Asia, Northern Europe, and Sahelian countries in Africa. While in countries of central and Southern Europe, a moderate seroprevalence (30 to 50%) of toxoplasmosis was reported, and high seroprevalence was highlighted in Latin America and tropical African countries (Robert-Gangneux and Darde, 2012).

A high prevalence of toxoplasmosis was reported in tropical countries with a humid and warm climate, and conversely, the lower prevalence was found for arid or colder countries (Champoux *et al.*, 2004; Robert-Gangneux and Darde, 2012; Saadatnia and Golkar, 2012). Furthermore, toxoplasmosis is prevalent among kids who live under poor-hygiene conditions, which were probably linked to contaminated soil or waterborne disease (Abamecha and Awel, 2016; Robert-Gangneux and Darde, 2012; Sahimin *et al.*, 2017). This young age group are still not fully aware of good personal hygiene practice or even understand the consequences of exposing

themselves to pathogenic organisms. Besides, in cases where both parents are working and not at home to supervise the children, it leads to a lack of parental guidance and some degree of carelessness, especially in personal hygiene and cleanliness (Sahimin *et al.,* 2017). Thus, they were commonly exposed to infection and reinfection.

1.3.1 Seroepidemiology of toxoplasmosis among blood donors

Blood transfusion services collect blood from blood donors who are at low risk of any infection that is transmissible through transfusion and are unlikely to sacrifice their health through blood donation (WHO, 2013). Asymptomatic donors in acute infection with parasitemia can transmit the parasites to the recipients, especially in prevalent toxoplasmosis area (Robert-Gangneux and Darde, 2012). Blood grouping and compatibility testing, together with HIV, hepatitis B, hepatitis C, and syphilis screening, are among the quality assurance that was done to all donated blood. Since *T. gondii* screening before blood transfusion has not yet been practiced, immunocompromised patients are among susceptible groups that facing greater risk for acute and severe toxoplasmosis through blood transfusion (Foroutan and Majidiani, 2018).

In a global systematic and meta-analysis review, 33% of blood donors worldwide were seropositive for *T. gondii* (Foroutan and Majidiani, 2018; Wang *et al.*, 2018). While in Malaysia, there is only one prevalence study that was conducted among blood donors. This study showed a seroprevalence rate of toxoplasmosis among blood donors was 28.1% (Nissapatorn V *et al.*, 2002). Malaysia has higher seroprevalence among blood donors than Turkey (19.5%) and Iran (19.3%). Other countries reported over 50.0% of toxoplasmosis among their blood donors, such as Northeast Brazil, north India, and Egypt (Alvarado-Esquivel *et al.*, 2016; Foroutan-Rad *et al.*, 2016; Sarkari *et al.*, 2014). Differences in blood donors' behavioural

characteristics that give potential exposure to the infection and different environments could be the reason for the varied seroprevalence rate among blood donors (Alvarado-Esquivel *et al.*, 2016).

1.3.2 Seroepidemiology of toxoplasmosis among immunocompromised patients

T. gondii has been suggested as an important opportunistic pathogen in immunocompromised patients. It causes life-threatening diseases in this group due to the risk of reactivation from the ruptured cyst (Ahmadpour *et al.*, 2014; Wang *et al.*, 2017). A previous study has reported the epidemiological and its clinical aspects, especially among immunocompromised patients. For instance, more than 13 million of HIV patients worldwide were seropositive for *T. gondii* antibodies (Konstantinovic *et al.*, 2019; Wang *et al.*, 2017). The estimated prevalence ranges from 26.0% to 51.2% among cancer, HIV/AIDS, and transplant patients, respectively (Basavaraju, 2016; Wang *et al.*, 2017). The prevalence rate was varied due to the different target groups, the type of assays used, and the year of the study (Basavaraju, 2016). The serological method gives a higher rate of seroprevalence than the molecular method; thus, combining both serological and molecular methods is recommended to accurately diagnose toxoplasmosis (Ghoneim *et al.*, 2010). Furthermore, many types of assays used, such as enzyme-linked assays or haemagglutination assays with different sensitivities, specificities, and cut-off levels to define positive results, affect the prevalence rate (Foroutan-Rad et al., 2016). So far, there is no study reported on the prevalence of toxoplasmosis among haemato-oncology patients.

In Malaysia, the seroprevalence rate of toxoplasmosis among immunocompromised patients was between 41.9 to 51.2% (Daryani *et al.*, 2011). In addition, a study from Hospital Universiti Kebangsaan Malaysia (Hospital UKM) on

oncology patients reported that 41.9 % (54/129) patients were seropositive for toxoplasmosis. From this population, one patient (0.77%) was positive for *T. gondii* IgM antibody, three patients (2.32%) were positive for both IgM and IgG, and fifty patients (38.75%) were positive for *T. gondii* IgG antibody only (Nimir *et al.*, 2010). The prevalence of toxoplasmosis among the immunocompromised group is different between geographical distributions. It was reported in Latin America, Europe, Asia, and Africa with 30.0% to 75.0%, USA with 3.0% to 42.0%, Malaysia with 41.9 % to 51.2%, Thailand with 53.7 %, North India with 21.3%, China with 54.0%, Japan with 5.4% and 50.0% in Ethiopia (Akanmu *et al.*, 2010; Daryani *et al.*, 2011; Uppal *et al.*, 2015; Wang *et al.*, 2017).

1.3.3 Seroepidemiology of toxoplasmosis among other groups

In Malaysia, a few research were conducted to study the prevalence rate of this infection among different target groups such as pregnant women (Andiappan *et al.*, 2014), Orang Asli (Angal *et al.*, 2016), people with close contact to animals (Brandon-Mong *et al.*, 2015), human immunodeficiency virus (HIV) patients (Ngui *et al.*, 2011) and patients who specifically requested for toxoplasmosis screening (Mohamed and Hajissa, 2016). Based on these previous studies, 37.0 % of Orang Asli subgroups living in Peninsular Malaysia were found to be positive with toxoplasmosis (Ngui *et al.* (2011). There is another study focusing on pregnant women in West Malaysia, and 39.73% of them were positive for anti-toxoplasma IgG. In comparison, another 2.74% were positive for both anti-toxoplasma IgG and anti-toxoplasma IgM (Andiappan *et al.*, 2014). A study among people having close contact with animals showed that 19.9 % of 312 subjects were seropositive for T.gondii (Brandon-Mong *et al.* (2015). Another study was conducted in Hospital Universiti Sains Malaysia among patients who suspected to have *T. gondii* infection reported around 0.98%, and 44.2% of the

samples were positive for IgM only and IgG only respectively (Mohamed and Hajissa, 2016).

1.4 Risk factors of toxoplasmosis

Generally, risk factors are divided into two groups; sociodemographic factors and behavioural factors.

1.4.1 Sociodemographic risk factors

Sociodemographic factors are the combination of the population's social and demographic characteristics, such as age, gender, ethnicity, education level, migration background, marital status, economic or income status, and employment status. Studies have shown that age, gender, ethnicity, type of malignancies, residence area, and employment were significantly associated with *Toxoplasma* seropositivity (Brandon-Mong *et al.*, 2015; Chemoh *et al.*, 2019; Retmanasari *et al.*, 2017; Yan *et al.*, 2018).

Older age groups have a low immune system that makes them more susceptible to infection (Alvarado-Esquivel *et al.*, 2016; Sarkari *et al.*, 2014; Zemene *et al.*, 2012). Males are more prone to have *T. gondii* infection as they are actively involved in outdoor activities, sports, or even exposed to contaminated soil (Brandon-Mong *et al.*, 2015; Davami *et al.*, 2015; Minbaeva *et al.*, 2013; Retmanasari *et al.*, 2017). In a multi-racial country like Malaysia, Malay showed a higher prevalence of toxoplasmosis due to their close contact with cats and stray cats around their residential areas (Brandon-Mong *et al.*, 2015; Chemoh *et al.*, 2019). Educational level and employment status are among risk factors that play roles in *T. gondii* infection (Siransy *et al.*, 2016). Patients are more vulnerable to this infection due to a lack of knowledge and awareness about

the risk of transmission. There were also reported cases of *T. gondii* transmission through organ transplant and blood transfusion, although rarely occurs (Alvarado-Esquivel *et al.*, 2016; Sarkari *et al.*, 2014).

1.4.2 Behavioural characteristics factors

It has been discovered that consumption of raw or undercooked meat, eating unwashed vegetables or fruits, close contact with cats, poor hygiene and climatic factors were found to contribute in *T. gondii* transmission (Alvarado-Esquivel *et al.*, 2011; Avelar *et al.*, 2018; Minbaeva *et al.*, 2013; Tammam *et al.*, 2013; Zemene *et al.*, 2012). Undercooked meat might contain *T. gondii* tissue cyst that will be transmitted once ingested. Raw fruits or unwashed vegetables contaminated with oocyst might transmit the parasite to the host.

Since the cat is a definitive host for *T. gondii*, close contact with cats might be a significant risk factor for its transmission (Dabritz and Conrad, 2010). The infection occurs through direct contact with cats' faeces that are shedding oocyst after the first infection, and it needs at least a day for oocyst to sporulate and turn infectious (Avelar *et al.*, 2018). In consequence, the presence of stray cats, or humans that have close contact with cats such as cleaning the cat litter box or having an indoor cat is more prone to *T. gondii* infection. In rural and remote areas, toxoplasmosis is considered a waterborne disease. *T. gondii* can be transmitted through contamination of water tanks with cat faeces or water supply that placed close by a farm where infected cats are around (Sahimin *et al.*, 2017).

1.5 Laboratory detection of toxoplasmosis

There are two standard methods in detecting toxoplasmosis; indirect method (detect antibody) and direct method (detect the parasite). The indirect method is serological testing that measures specific anti-*Toxoplasma* immunoglobulin M (IgM) and immunoglobulin G (IgG) levels. The direct detection method uses tissue samples, cerebrospinal fluid (CSF), or other biopsy materials (Liu *et al.*, 2015).

1.5.1 Serological detection of toxoplasmosis

T. gondii was first discovered in 1948 by the serological method (Sabin-Feldman dye test) before it was studied extensively (Paniker, 2007). Currently, ELISA is one of the most common serological tests used for toxoplasmosis detection (Abdoli *et al.*, 2019; Chemoh *et al.*, 2019). Generally, the serological test for toxoplasmosis is classified into two major groups. Those using disrupted parasites as an antigen source such as ELISA, complement fixation test, latex agglutination test (LAT) and indirect haemagglutination assay (IHA), and another group using complete organisms such as dye test, direct agglutination test (DAT) and a fluorescent antibody test (FAT) (Gillespie and Hawkey, 2002). Serological assay, especially ELISA, offers fast results, lower cost of preparation, high sensitivities and specificities, and it is well-accepted methods by clinicians (Thangarajah *et al.*, 2019).

ELISA is a sensitive and specific analytic immunoassay used for the detection of the particular antibody. It is either quantitative or qualitative analysis of an analyte, usually an antigen, in a sample without the requirement of urbane or costly equipment (Konstantinou, 2017; Shah and Maghsoudlou, 2016). Qualitative determination is mainly detected in the presence or absence of a specific target, while quantitative determination yields the level of that target molecule in the mixture solution

(Konstantinou, 2017). Generally, the antigen is immobilized in a microplate well either directly or indirectly by a specific antibody known as a 'capture antibody.' A 'primary detection antibody' is added, forming an antigen-antibody complex. The primary detection antibody is either directly labelled with an enzyme (direct ELISA) or is itself attached to a secondary antibody known as a ‘secondary detection antibody’ (indirect ELISA). Between each step, the well is washed with a buffer solution. The addition of a substrate produces a colour signal indicating the presence of the antigen in the sample. The measurement of the optical density is proportional to the amount of antibodies in the sample.

ELISA is widely used in clinical laboratories as a routine screening test due to its excellent diagnostic efficiency (100% specificity and 100% sensitivity) (Chemoh *et al.*, 2019; Ghazy *et al.*, 2007; Robert-Gangneux and Darde, 2012; Suresh *et al.*, 2012). For toxoplasmosis, ELISA is used as the routine screening test to detect *Toxoplasma* IgM, IgG, and IgG avidity in infected patients. It was initially described in 1976 (Gillespie and Hawkey, 2002; Rostami *et al.*, 2018; Suresh *et al.*, 2012). Since the IgM and IgG alone cannot be used as an indicator of recent infection, IgG avidity is used to determine a recent infection based on the development of immune response maturity. Technically, IgG avidity evaluates the binding avidity of IgG antibodies against *T. gondii* (Fonseca *et al.*, 2017). Low avidity IgG antibodies indicate early infection (acute), and high avidity IgG antibodies indicate past infection (chronic) (Berredjem *et al.*, 2017; Chemoh *et al.*, 2019; Fonseca *et al.*, 2017).

During the first week of toxoplasmosis infection, IgA and IgM antibodies are produced and reach a plateau within 30 days (Figure 1.3) (Dard *et al.*, 2016; Robert-Gangneux and Darde, 2012). After 1 to 6 months of infection, IgM antibodies decrease

and become negative within less than seven months. Yet, it is still commonly detectable for about a year. Thus, IgM cannot be used as a marker to indicate recent infection unless the titer is very high. IgG antibodies can be detected from 1 to 3 weeks after the first escalation of IgM antibodies. After 2 to 3 months, these IgG antibodies reach plateau and decline but persist for a lifetime at residual titers (Robert-Gangneux and Darde, 2012).

Many other serological tests have been used to detect toxoplasmosis, such as a dye test, which is a complement-mediated neutralizing antigen-antibody reaction (Gillespie and Hawkey, 2002). In addition, indirect fluorescent assay (IFA) is one of the simple serological tests that also measures *Toxoplasma* antibodies. This test requires using a fluorescence microscope (Liu *et al.*, 2015; Saadatnia and Golkar, 2012). Latex agglutination test (LAT) shows agglutination of the latex particle when there is the presence of *T. gondii* antibodies in the serum (Saadatnia and Golkar, 2012). Western blotting is also one of the conventional serological tests. Technically, sera are reacted with *T. gondii* antigen on a membrane transferred from polyacrylamide gel resulting in banding patterns that are matched with known molecular weight (Liu *et al.*, 2015).

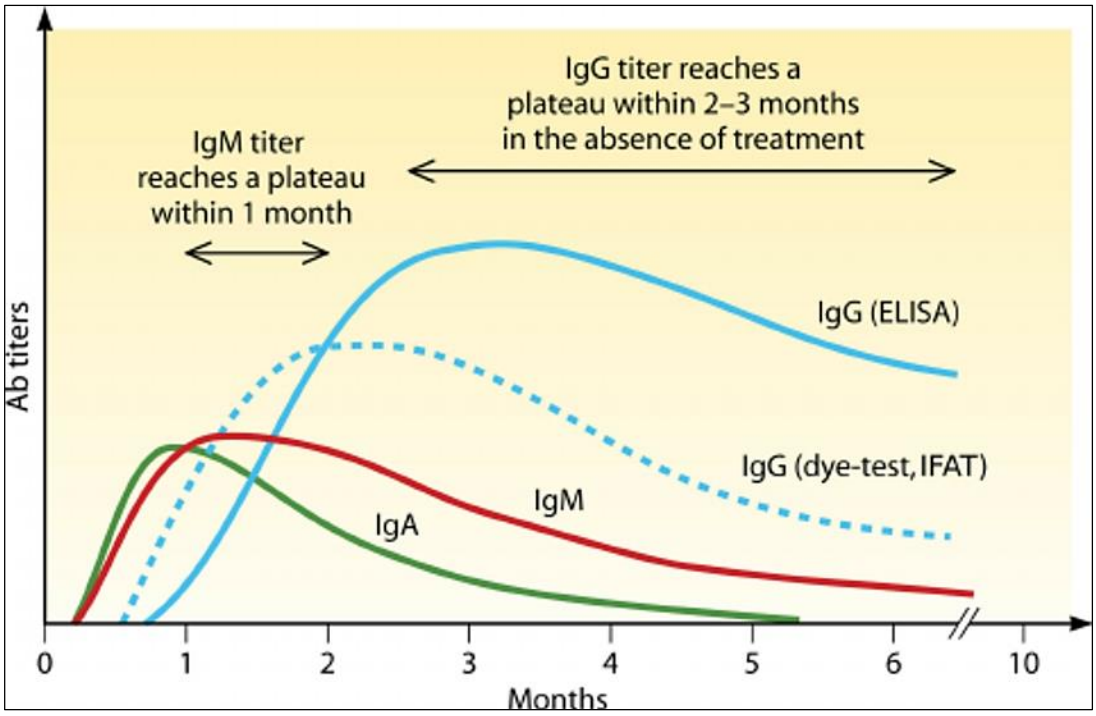

Figure 1.3: Kinetics of the antibody (Ab) response of toxoplasmosis.
The average kinetics of the different isotypes are represented, but they may vary among patients and different serological tests. Figure adapted from (Robert-Gangneux and Darde, 2012).

1.5.2 Molecular detection of toxoplasmosis

Serological tests may fail to detect *T. gondii* infection in immunocompromised patients because of IgG or IgM antibody titer might not increase in these patients (Berredjem *et al.*, 2017; Rahumatullah *et al.*, 2012). To overcome the limitation of serological tests, different molecular techniques have been developed to detect the *T. gondii* DNA directly. The pcr underlies almost all of the modern molecular cloning. It is extensively used for diagnostic purposes to detect the presence of a specific DNA sequence of the organism in a biological fluid (Kadri, 2019). Using PCR, a defined target sequence within a DNA of high complexity and large size can be rapidly and selectively amplified in a quasi-exponential chain reaction that yields millions of copies. The result is easy to set up, inexpensive, and straightforward; the only requirement is some knowledge of the target's nucleotide sequences. In addition to its simplicity, PCR is robust, speedy, flexible, and sensitive (Green and Sambrook, 2020). The PCR is carried out in a reaction mixture which comprises the extracted DNA (template DNA), Taq polymerase, the primers, and the four Deoxyribonucleotide triphosphates (dNTPs) in excess in a buffer solution. The tubes containing the mixture reaction are subjected to repetitive temperature cycles using a thermal cycler machine involves three main steps; denaturation of DNA template, annealing of primers, and lastly, extension of DNA molecules. These steps are repeated for 30–40 cycles (Green and Sambrook, 2020; Gupta, 2019; Kadri, 2019).

Various types of PCR, including conventional PCR, multiplex PCR, and nested PCR, are widely available nowadays. Multiplex PCR is a conventional PCR that used more than one primer to detect multiple targets simultaneously. Performing three PCRs in one mixing saves reagents, which makes it a rational decision for repeating

reactions. Additionally, this method may be adapted for the molecular diagnosis of any infectious disease, providing fast results with a small margin of error (Gonçalves-de-Albuquerque *et al.*, 2014). PCR assay has an advantage in the detection of recent and active infections as a reflection or marker for T.gondii parasitemia (Bakre, 2016). Nested PCR is a modified PCR that intended to reduce nonspecific binding of products that might be due to the amplification of unexpected primer-binding sites (Gupta, 2019). Conventional PCR is a rapid, highly sensitive, and specific in detecting *T. gondii*. When combined with a serological test, it can distinguish chronic, acute, or reactivated toxoplasmosis (Castillo-Morales *et al.*, 2012; Rahumatullah *et al.*, 2012). PCR is useful for identifying or excluding acute toxoplasmosis or estimating the time of seroconversion (Berredjem *et al.*, 2017).

Burg and colleagues discovered PCR in detecting *Toxoplasma* infection in immunocompromised patients, congenital toxoplasmosis, and ocular toxoplasmosis (Montoya, 2002; Rahumatullah *et al.*, 2012). PCR is an *in vitro* enzymatic amplification tool that amplifies specific *T. gondii* DNA from minute amounts of starting materials in a moment. 35-repeat BI gene of *T. gondii* genome was the first to be amplified by PCR and has been widely used for *T. gondii* detection (Robert-Gangneux and Darde, 2012; Rostami *et al.*, 2018). B1 gene is highly specific and well conserved for all *T. gondii* strains, including samples isolated from AIDS patients (Burg *et al.*, 1989; Mesquita *et al.*, 2010; Rahumatullah *et al.*, 2012; Rostami *et al.*, 2018).

Other than B1 gene, several multi-copy targeting genes including 529-bp repeated fragment, ITS-1 region, and P30 are among genes used for *T. gondii* detection in various biological samples (Bavand *et al.*, 2019; Berredjem *et al.*, 2017; Moshfe *et al.*, 2018; Rostami *et al.*, 2018; Steeples *et al.*, 2015; Wells *et al.*, 2015). Furthermore,

a previous study showed that these target genes (B1 gene or the repeated region of *T. gondii*) present good sensitivity for clinical samples (Mesquita *et al.*, 2010). Various clinical specimens have been used in PCR such as serum/plasma, amniotic fluid, placenta, brain tissue, aqueous humour and vitreous fluid for toxoplasmosis (Saki *et al.*, 2017; Shafieenia *et al.*, 2018). In addition, for immunocompromised patients, whole blood, urine, CSF, and other body fluids were used in detecting toxoplasmosis (Liu *et al.*, 2015; Rostami *et al.*, 2018).

Nested PCR is a modified PCR which provides increased sensitivity and specificity of DNA amplification compared to conventional PCR. It is intended to reduce nonspecific binding in their products due to unexpected primer binding site amplification. In nested PCR, it involves two sets of primers where the first PCR product is used as a template for the second PCR (Liu *et al.*, 2015). It is rapid, sensitive, and effective molecular tools in acute toxoplasmosis detection of HIV patients (Alghamdi *et al.*, 2016; Rostami *et al.*, 2018). Recent studies used nested PCR as an additional test to a serological method to detect and confirm the presence of *T. gondii* (Abdoli *et al.*, 2019; Saki *et al.*, 2017; Sarkari *et al.*, 2014; Shafieenia *et al.*, 2018).

Real-time PCR (RT-PCR) is another molecular method that can detect a low concentration of *T. gondii* DNA and amplify a million copies of target DNA in various body fluids (Liu *et al.*, 2015; Steeples *et al.*, 2015; Wells *et al.*, 2015). Loop-mediated isothermal amplification (LAMP) is another type of molecular test which amplified the DNA under isothermal conditions with high efficiency (Liu *et al.*, 2015). However, it is very sensitive to contamination; thus, extra precaution and good quality control are needed (Liu *et al.*, 2015; Rostami *et al.*, 2018). There are recent studies that used LAMP assay for *Toxoplasma* detection using various type of samples such as blood,

urine and also food and water sample (Fallahi *et al.*, 2015; Fallahi *et al.*, 2014; Gallas-Lindemanna *et al.*, 2013; Hu *et al.*, 2012; Lalle *et al.*, 2018).

Nowadays, many ELISA and PCR kits are commercially available for toxoplasmosis, which offers various performances. For example, Platelia™ Toxo IgG, IgM and IgG Avidity (BioRad, USA), Elecsys® Toxo IgG and IgM (Roche, Germany), ELISA kit (ACON Biotech, Hangzhou China) and IgG and IgM µ-capture ELISA (Novalisa, Dietzenbach Germany), ELISA kit (Euroimmune, Germany). Among all these kits, Platelia™ Toxo IgG, IgM, and IgG Avidity (BioRad, USA) have 100% sensitivity and 100% specificity for their packages. There are new rapid PCR kits that are available for *T. gondii* detection. For example, *Toxoplasma gondii* PCR Kit (MyBioSource.com, Canada), *T. gondii* Kit (VetMAX™, US), and *Toxoplasma gondii* kit (PCRmax, UK).

1.6 Prevention and treatment of toxoplasmosis

Since *Toxoplasma* infection can be transmitted through various routes, thus hygienic measures are crucial to avoid this infection. Besides, hygiene is the primary prevention of any parasitic infection. Washing hands regularly after having close contact with cats, gardening, or handling raw meat must be practiced. Furthermore, eating raw or undercooked meat should be avoided. Meat should be cooked appropriately at least 56^{o}C for 15 min or be frozen at -20^{o}C so that *T. gondii* cyst can be eradicated (Ahmad *et al.*, 2014; Robert-Gangneux and Darde, 2012). Individuals are encouraged to wash fruits and vegetables before eating them thoroughly. If the person owns a cat, the litter box should be handled carefully. Health education programs would help deliver useful information regarding possible infections, especially to children, immunocompromised patients, and pregnant mothers. Since *T. gondii* was reported to