USE OF PAIRED SERUM SAMPLE FOR THE DIAGNOSIS OF *Toxoplasma* INFECTION IN SELECTED POPULATION BY USING ELECSYS TOXO IgG/IgM ASSAY IN HUSM

by

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ABRREVIATIONS

µl	Microliter
AIDS	Acquired Immunodeficiency Syndrome
AUC	Area under the curve
CMV	Cytomegalovirus
COI	Cut off index
CT scan	Computed Tomography scan
CTL	Cytotoxic lymphocytes
DAT	Direct Agglutination Test
DM	Diabetes Mellitus
DNA	Deoxyribonucleic Acid
E. coli	*Escherichia coli*
ECLIA	Electro- chemiluminescence Immunoassay
ELISA	Enzyme – Linked Immunosorbent Assay
HIV	Human Immunodeficiency Virus
HLA-G	Human Leucocyte Antigen - G
HSV	Human simplex virus
HUSM	Hospital University Sains Malaysia

ICAM	Intercellular Adhesion Molecule
IFA	Indirect Fluorescent Assay
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHAT	Indirect Haemagglutination test
IL-10	Interleukin 10
INF_{γ}	Interferon Gamma
IU/ml	International units to ml
IUD	Intrauterine death
IUGR	Intrauterine Growth Retardation
LAT	Latex Agglutination Test
mm^3	Cubic milimetre
NK	Natural Killer
NNJ	Neonatal Jaundice
NPV	Negative Predictive Value
PCR	Polymerase Chain Reaction
PPV	Positive Predictive Value

RBC	Red Blood Cell
ROC	Receiver Operative Characteristic
$(Ru(bpy)^{2/3+})$	Ruthenium complex
SAG1	Surface Antigen 1
SFDT	Sabin – Feldman Dye Test
SGA	Small for Gestational Age
SLE	Systemic Lupus Erythematous
T. gondi	*Toxoplasma gondii*
TB	Tuberculosis
TE	Toxoplasmosis Enchephalitis
TGF- β1	Transforming Growth Factor Beta - 1
Th -1	T Helper cell- 1
Th -2	T Helper cell -2
TPA	Tripropylamine

ABSTRACT

USE OF PAIRED SERUM SAMPLE FOR THE DIAGNOSIS OF *Toxoplasma* INFECTION IN SELECTED POPULATION BY USING ELECSYS TOXO IgG/IgM ASSAY IN HUSM

Introduction

Toxoplasmosis is one of the most common worldwide parasitic infection due to *Toxoplasma gondii*, an obligate intracellular parasite. The mode of transmission is through consumption of food or water or undercooked meat contaminated with the parasite. Maternal infection with vertical transmission depends on age of gestation. Severity of infection is during early gestation, may lead to intense complications such as intrauterine death (IUD) or later with increased risk of congenital infection. In ocular toxoplasmosis, reactivation of infection are common among immunocompromised and immunocompetent patients. Serology is still the mainstay for the diagnosis of *Toxoplasma* infection. Therefore, this method was applied with paired serum sample in selected population using Elecsys Toxo IgG/IgM assay in HUSM. The paired serum sample was to classify the infection into early, acute, reactivation, recent, latent, possible congenital infection and passive immunity from mother. This study aimed to describe the clinical manifestation, determine the seroprevalence classification of infection among the selected populations, validity of the test and percentage of patient on treatment after first and paired serum sample.

Methodology

This is a prospective cohort study held in Microbiology Laboratory, Hospital Universiti Sains Malaysia. Paired serum sample with interval of 2 weeks for clinically suspected cases among selected populations were collected from 1st January 2016 till 31st December 2016. Elecsys and cobas e 601 analyser was used to perform the Elecsys Toxo IgG/IgM ECLIA assay. The classification of infection was generated based on the study flow chart. Patients clinical data were obtained from clinical notes or folders.

Results

A total of 482 patients with paired serum sample were included in the study. The highest seroprevalence was in latent infection, 54%. New born or infants were majority having passive immunity from mother and 4.3% were classified into possible congenital infection. Ocular toxoplasmosis were mainly classified into reactivation and latent infection. Acute infection was successfully detected especially among new born, pregnant women and immune compromised patients. Paired serum sample were compared to clinically confirmed cases as gold standard have given high sensitivity (100%), low specificity (77.1%),high negative predictive value (NPV) (100%), and low positive predictive value (PPV) (53.5%). The ROC curve analyses of paired serum sample showed (AUC) was 0.932 (95% confidence interval between 0.802 and 1.000, p – value 0.001). Majority of patients classified into early, acute and reactivation of infection were treated after the first serum sample.

Conclusion

Paired serum sample using Elecsys Toxo IgG/IgM assay is a potential diagnostic test for *Toxoplasma* infection due to high sensitivity and specificity. Furthermore is a good tool to classify the infection among selection populations. The classification of infection is mainly to provide a better understanding regarding the infection and guide the clinician to start treatment promptly.

ABSTRAK

PENGGUNAAN SAMPEL SERUM BERPASANGAN UNTUK DIAGNOSIS JANGKITAN *Toxoplasma* DI KALANGAN POPULASI TERPILIH DENGAN MENGGUNAKAN UJIAN ELECSYS TOXO IgG / IgM DI HUSM

Pengenalan

Toxoplasmosis adalah salah satu daripada jangkitan parasit yang paling mudah ditemui. Penyakit ini disebabkan oleh *Toxoplasma gondii*, sejenis parasit intraselular obligat. Cara penyebarannya adalah melalui pengambilan makanan atau minuman serta daging yang kurang masak tercemar dengan parasit tersebut. Di samping itu, jangkitan daripada ibu kepada anak dalam kandungan boleh terjadi dan bergantung pada usia kehamilan**.** Jangkitan di awal kehamilan boleh membawa kepada komplikasi yang parah seperti kematian janin dalam kandungan, Dalam kes toxoplasmosis okular, kebiasaannya terjadi reaktivasi jangkitan di kalangan pesakit kurang daya tahan tubuh termasuk yang sihat juga. Serologi masih menjadi ujian utama bagi tujuan diagnosis jangkitan toxoplasmosis. Oleh itu, serologi digunakan dengan sampel serum berpasangan dalam populasi terpilih dengan menggunakan ujian Elecsys Toxo IgG / IgM di HUSM. Sampel serum yang berpasangan adalah untuk mengklasifikasikan jangkitan ke awal, akut, reaktivasi, jangkitan terkini, jangkitan latensi, kemungkinan jangkitan kongenital dan imuniti pasif dari ibu. Kajian ini bertujuan untuk menggambarkan manifestasi klinikal, menentukan kelaziman klasifikasi jangkitan di kalangan populasi terpilih, kesahihan ujian sampel serum berpasangan dan peratusan pesakit yang menerima rawatan selepas sampel serum yang pertama dan berpasangan.

Metodologi

Kajian kohort prospektif telah dijalankan di Makmal Mikrobiologi, Hospital Universiti Sains Malaysia. Sampel serum yang berpasangan dengan selang 2 minggu untuk kes yang disyaki secara klinikal di kalangan populasi terpilih telah dikumpulkan dari 1 Januari 2016 hingga 31 Disember 2016. Mesin analisis Elecsys dan cobas e 601 digunakan untuk melakukan ujian Elecsys Toxo IgG / IgM ECLIA. Klasifikasi jangkitan dibuat berdasarkan carta alir kajian. Data klinikal pesakit diperoleh dari nota klinikal.

Keputusan

Sejumlah 482 pesakit dengan sampel serum berpasangan dimasukkan dalam kajian ini. Klasifikasi jangkitan yang tertinggi adalah jangkitan latensi, 54%. Bayi baru lahir majoriti mempunyai imuniti pasif dari ibu dan hanya 4.3% diklasifikasikan ke dalam jangkitan kongenital. Kebanyakan kes toxoplasmosis okular diklasifikasikan ke dalam reaktivasi dan jangkitan latensi terutamanya di kalangan pesakit kurang daya tahan tubuh dan juga yang sihat. Jangkitan akut telah berjaya dikesan di kalangan bayi yang baru lahir, ibu mengandung dan pesakit kurang daya tahan tubuh. Sampel serum yang berpasangan berbanding dengan kes yang disahkan secara klinikal berdasarkan kepiawaian telah memberikan sensitiviti yang tinggi (100%), spesifisiti yang rendah (77.1%), nilai ramalan negatif (NPV) yang tinggi (100%) dan nilai ramalan positif (PPV) yang rendah (53.5%). Analisis lengkung ROC sampel serum berpasangan menunjukkan (AUC) adalah 0.932 (selang keyakinan 95% antara 0.802 dan 1.000, p - nilai 0.001). Majoriti pesakit yang diklasifikasikan kepada jangkitan awal, akut dan reaktivasi jangkitan telah merima rawatan selepas sampel serum yang pertama.

Kesimpulan

Sampel serum berpasangan menggunakan Elecsys Toxo IgG /IgM adalah ujian diagnostik yang berpontensi untuk jangkitan *Toxoplasma* kerana menunjukkan sensitiviti dan spesifisiti yang tinggi serta mampu mengklasifikasikan jangkitan di kalangan populasi terpilih. Tujuan wujudnya klasifikasi jangkitan *Toxoplasma* adalah untuk memberi pemahaman yang lebih terperinci mengenai jangkitan itu sendiri dan membimbing pakar klinikal untuk memulakan rawatan dengan segera.

CHAPTER ONE

INTRODUCTION

1.1. Background of the study

Toxoplasmosis is a common worldwide infection caused by the parasite *Toxoplasma gondii*. Approximately 25 to 30% of the world's human population is infected by *Toxoplasma gondii* (*T. gondii*) (Montoya and Liesenfeld, 2004). This obligate intracellular parasite can invade various types of human cells as well as all warm-blooded animals, including mammals and birds. The majority of horizontal transmissions to humans is caused either by the ingestion of tissue cysts in infected meat or by the ingestion of soil, water, or food contaminated with sporulated oocysts derived from the environment or, less frequently, directly from cat feces. The seroprevalence of toxoplasmosis was estimated to vary from <2% up to 70% in the Southeast Asian population (Nissapatorn, 2007). Higher prevalence observed in tropical countries with humid and warm climate as similar to Malaysia.

The seroprevalence of toxoplasmosis in Malaysia among general healthy population is 20% to 30% (Nissapatorn *et al.*, 2002; Shamilah *et al.*, 2001). The disease has been recognised to be a major cause of morbidity in congenital infection as a result of primary acquired maternal infection during gestation and also among immunocompromised patients. The seroprevalence of toxoplasmosis in pregnant women is significantly increasing with estimation of 27.9% to 49.0%. The frequency of seroprevalence according to trimester of pregnancy is increasing, 1st trimester is 33.3% , 2nd trimester 45.2% and 3rd trimester 49.0% (Khairul Anuar *et al.*, 1991; Nissapatorn *et al.*, 2003b).

The frequency of foetal infection increases with gestational age but risk of serious foetal anomalies and prenatal death occurs in early maternal infection. There was one study conducted among 405 congenitally defective Malaysian infant age 0 – 4 month old, showed 2% positivity for toxoplasmosis. In a different study, a total of 8.2% intrauterine toxoplasmic infection per 1000 live births detected with one third presented with liver, eye and brain damage (Tan and Mak, 1985). Among immunocompromised patients, reactivation of toxoplasmosis encephalitis (TE) is the most common opportunistic infection. Anti-*Toxoplasma* IgG antibody by ELISA technique shows prevalence 41.2% (301) (Nissapatorn *et al.*, 2003a). Congenital ocular toxoplasmosis with cicartical stage 63.3% as the most common presentation and chronic congenital infection with acute recurrences 36.7% occurs below 40 years old, (Lim and Tan, 1983). A total of 134 patients with 72% were seropositive for *Toxoplasma* infection, with most apparent symptoms of chorioretinitis and vitritis both having 100% correlation with seropositivity (Zurainee *et al.*, 2017). In a study, patients with ocular diseases showed that 12.5 to 31.1% (IgG) and 3.1 to 19.3% (IgM) of toxoplasmosis prevalence were found in Malaysia and Thailand (Nissapatorn, 2007).

The strategies for diagnosis of toxoplasmosis infection depends on the immune status of the patient and clinical setting especially among pregnant women, new born and immunocompromised patients. Nowadays, serological tests stands as primary approach and most clinical laboratories uses ELISA for the routine screening of *T. gondii* - specific IgG and IgM, whereas other techniques are mostly reserved for reference laboratories (Robert-Gangneux and Dardé, 2012). Presence of *T.gondii* specific- IgM antibody indicates acute or recent infection where else a positive IgG indicates a past or latent infection and provide immunity to reinfection (Remington *et al.*, 2001; Wong and Remington, 1994). By understanding the kinetic of antibody response and the need

of precise diagnosis for *Toxoplasma* infection in in our setting, the requirement for paired serum at 2 weeks interval was made available during this study. It is to establish the seroconversion of (IgM and/ or IgG) and significant rise or 4 fold rise of Ig G antibody titer. Confirmation is still by molecular testing but due to lack of facilities, laborious and expensive, serology remains as a screening test and by this study a better accuracy for age of infection is made.

Elecsys and cobas e 601 analyser was used to perform the Elecsys Toxo IgG / IgM electro chemiluminescence immunoassay (ECLIA) technique. This assay was used to detect the in vitro quantitative IgG and IgM antibodies respectively to *T. gondii* in human serum. During the acute phase of infection, the dominant surface protein on tachyzoites is SAG 1 (surface antigen 1), formerly called p30 are highly immunogenic properties of SAG 1 induces IgM as well as IgG specific antibodies.

1.2. Rationale of the study

Congenital infection is the most important part of the disease burden due to *Toxoplasma* infection in human (Robert-Gangneux and Dardé, 2012). The severity of infection depends on age of gestation and the risk of vertical transmission to foetus. In new born, presences of IgG antibody explains the passive immunity from mother whom has previous exposure to *Toxoplasma* infection (Lago *et al.*, 2014). Among immunocompromised patients with reactivation of infection, IgM antibodies are rarely found (Roth *et al.*, 1994).

However, in IgM positive cases, it is important to establish whether the infection is acute or recent because IgM antibody may persist over a year (Meek *et al.*, 2001; Montoya and Liesenfeld, 2004). Apart from IgM antibodies, another way to appreciate the age of infection is to analyse the IgG titer (Robert-Gangneux and Dardé, 2012). Thus, the presences of IgG and /or IgM *Toxoplasma* antibodies in a single serum

sample may suggest but not clarify or define whether the infection was early, acute, recent, reactivation or latent infection and passive immunity from maternal (Remington *et al.*, 2001).

Serological test as a screening tool for *Toxoplasma* infection has been routinely done in our department. Since last year a new analyser e cobas 601 was introduced using Elecsys Toxo IgG / IgM electrochemiluminescence immunoassay (ECLIA). This analyser provides a cut off value for reactive, indeterminate or nonreactive and also titer for semiquantitative calculation. Therefore, the product information recommended to take second sample at 2 weeks interval to establish the seroconversion of (IgM and/ or IgG) and significant rise or 4 fold rise of IgG antibody titer. Consequently, this recommendation was circulated to the wards in the form of 'Surat Pekeliling' to request for second sample at 2 weeks interval (Appendix A). The importance of paired serum sample, compared to single serum sample is to classify the infection into early, acute, reactivation, recent, latent, passive immunity from mother and possible congenital infection.

The benefits from these classification of *Toxoplasma* infection by routine serological screening is to provide an early detection of early, acute, recent, reactivation of infection especially in congenital toxoplasmosis and pregnant women. This classification is very crucial among pregnant women in initiation of treatment and intervention in management. Whereas for new born and infants, the presence of IgG antibody in their blood confirms as a passive immunity from mother and long term repeated follow up for blood taking can be avoided. Among immunocompromised patients, cerebral toxoplasmosis is a major concern as a reactivation of latent infection. Significant high titer of IgG antibody without presences of IgM, supported with radiological imaging assures the infection and guides the clinician to start treatment

without delay. In summary, this classification will assist physician in the treatment of toxoplasmosis in those patients.

1.3. Literature review

1.3.1. *Toxoplasma gondii*

Toxoplasma gondii is an obligate intracellular protozoan responsible for a common parasitic infections throughout the world. It was classified in the coccidian subclass, phylum *Apicomplexa*, class *Sporozoasida*, order *Eucoccidiorida*, and family Sarcocystidae (Frenkel, 1990). Only in the late 1960s the discovery of cat as a definitive host and spreading the oocysts through feces was acknowledged. The importance of toxoplasmosis in human, was discovered through a congenital infection and the whole spectrum of disease was revised by (Weiss and Dubey, 2009). Recently a breakthrough in the evolution of *T. gondii* has brought us to the understanding of particular virulences associated with some genotypes (Mercier *et al.*, 2011).

1.3.2. Three parasitic stages

The three parasitic stages are rapidly dividing tachyzoites, slowly dividing bradyzoites in tissue cysts and sporozoites which are protected in an oocyst in the environmental condition. Generally these three infective stages are crescent – shaped cells, with a pointed apical end involves in cell invasion and a rounded posterior end. They are limited by pellicle as a membrane and cytoskeleton involved in the structural integrity and motility. They are built in with nucleus, mitochondrion, Golgi complex, ribosomes and an endoplasmic reticulum. Tachyzoites are the rapid proliferative and dissemination form, which can invade all vertebral cell types by penetrating the plasma membrane or by phagocytosis and multiple rapidly causing cell rupture and continuous infection to neighbouring cells. Bradyzoites are the slow proliferative stage and form tissue cyst as a

result from the conversion of tachyzoites. These cysts contain hundreds to thousands of densely packed bradyzoites that have a latent metabolism and remain intracellular for life span. Their high affinity towards central nervous system, muscular tissues including heart and skeletal muscle, eyes and placenta explains the clinical manifestation of these sites as tropism for reactivation of infection. Sporozoites are located in mature oocysts which has a multilayer wall for protecting the parasite from mechanical and chemical damage in the environment. As a result these oocysts can survive for more than a year in a moist condition (Mai *et al.*, 2009)(Figure 1.1).

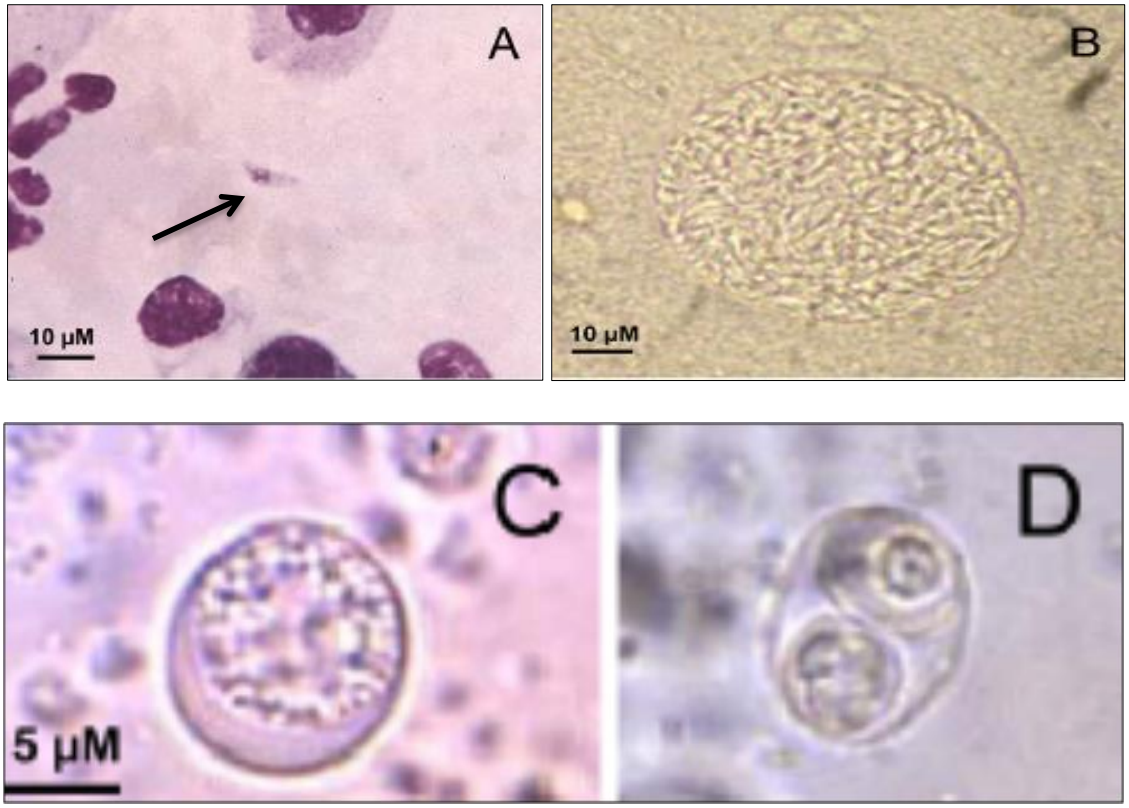

Figure 1.1: Three parasitic stages of *Toxoplasma gondii*. Microscopic examination of Tachyzoite (A), Bradyzoite in tissue (B), an unsporulated (C) and sporulated oocysts (D) (Adopted from Robert-Gangneux, F. and Dardé, M.L., 2012).

1.3.3. Life cycle of *T. gondii*

The life cycle alternates between definitive (sexual reproduction) and intermediate (asexual replication) hosts. Sexual reproduction occurs only in guts of felids (domestic and wild cats). After the ingestion of cysts present in an intermediate host or oocyst from the environment, the cell wall is destroyed by gastric enzyme and the parasite invades the intestinal mucosa of the definitive host. In the enterocytes the gametocytes are formed and fertilize into oocyst and excreted as unsporulated oocysts in cats feces. The shedding of oocysts begins 3 to 7 days after ingestion of tissue cysts and continues up to 20 days. Infected cat can shed more than 100 millions oocysts in their feces (Jones and Dubey, 2010).

Meanwhile within the intermediate hosts, there is only asexual reproduction occurs. After ingestion of oocyst from contaminated environment, sporozoites are released. Similarly, ingestion of uncooked or raw meat contains tissue cysts releases bradyzoites and these two forms of parasite penetrate the intestinal epithelium where they differentiate into tachyzoites which replicate rapidly and disseminate through the body. Once they reach the respective tropism sites, conversion to bradyzoites occurs as early as 7 to 10 days post infection and remain throughout life (Figure 1.2).

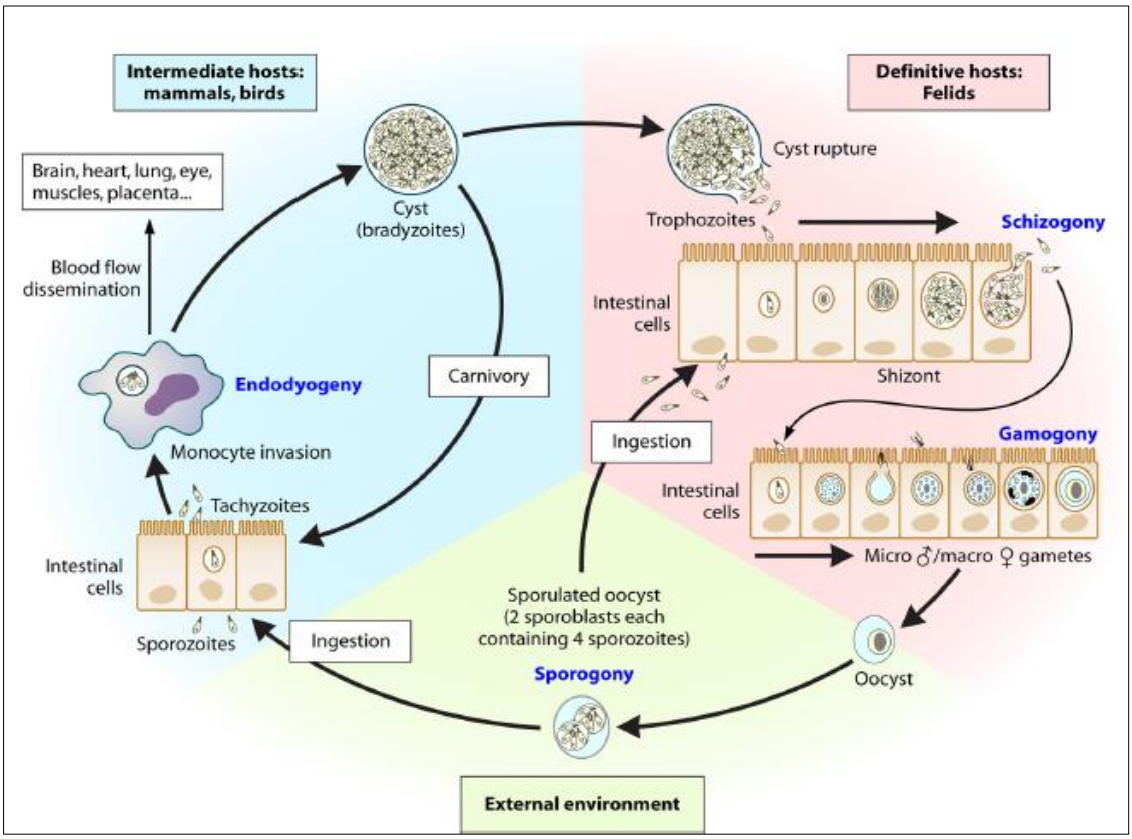

Figure 1.2: Life cycle of *Toxoplasma gondii*. Shown are the biology, infection, and replication of the three infective stages of the parasites in their respective hosts. (Adopted from Robert-Gangneux, F. and Dardé, M.L., 2012).

1.3.4. Transmission

The horizontal transmissions to humans are cause by ingestion of tissue cysts in infected meat or by ingestion of soil, water or food contaminated with sporulated cysts from environment. Another way is by solid organ transplantation , where by a cyst-containing organ from a donor to a non - immunised recipient in particular heart transplant patient (Fernàndez-Sabé *et al.*, 2011). Where else, vertical transmission occurs through tachyzoites colonized at placental tissue to foetus by primary infection or reactivation of latent infection.

1.3.5. Clinical features of *Toxoplasma* infection

In general, *T. gondii* infections are asymptomatic and self - limiting among healthy immunocompetent individuals, in others cases, patients may experience fever or cervical lymphadenopathy, sometimes associated with myalgia, asthenia, or other nonspecific clinical signs. However among pregnant women, new born, infants and immunocompromised patients the infection may cause severe clinical manifestation.

1.3.5.1. Manifestation during pregnancy and congenital toxoplasmosis

Classically, congenital infection occurs only if a pregnant women develops primary acquired infection during pregnancy or 8 weeks before conceiving. Placenta plays a major role as a barrier at the beginning of the gestation leading to less parasites transmission but later it becomes more permeable allowing transmission of 30% in second trimester and 60 to 70% third trimester (Dunn *et al.*, 1999). Congenital infection from a reactivation of chronic infection in an immunocompetent pregnant is a rare event. This condition is postulated due to decreased cellular response during pregnancy that interferes the parasitic control leading to increased risk of vertical transmission (Garweg *et al.*, 2005). No doubt this phenomenon might attribute to

reinfection with exposure to different genotypes or reactivation of chronic infection (Elbez-Rubinstein *et al.*, 2009).

Foetal infection during early gestation are at high risk of developing foetal anomaly such as miscarriage, stillbirth, intrauterine growth retardation and birth defect. Major squeal include mental retardation, seizures (80%), microcephalus and hydrocephalus (28%), deafness, and psychomotor deficiency (Remington *et al.*, 2001). Eye lesion is more severe in early pregnancy where microopthalmia, cataract, increase intraocular pressure, strabismus, optic neuritis, retinal necrosis, uveitis and retinochoroiditis can be observed (Delair *et al.*, 2011; Roberts *et al.*, 2001). Clinical manifestation during second trimester includes epilepsy, anaemia, thrombocytopenia induced petechial, hepatic disorder, severe sepsis, pneumonitis or retinochoroiditis (Remington *et al.*, 2001). By contrast, late maternal infection mostly in third trimester results in subclinical toxoplasmosis in new born, sometimes goes unnoticed , but later in life may develop chorioretinitis (Montoya and Liesenfeld, 2004).

1.3.5.2. Role of placenta in *T. gondii* transmission and pathophysiology

Placenta is a key tissue in the mother-to-foetus relationship, apart from trophic role it also provides the tolerant immune microenvironment necessary for gestation (Entrican, 2002). During primary infection, parasites cross intestinal barrier, invade monocytes in contact with lamina propria, disseminates throughout the body including placenta. Infection of placenta tissue leads to placentitis and subsequently infect the trophoblast lining which interface with foetus compartment, proceeds to congenital infection. This important process has two main consequences, firstly placental infection may adversely affect this tenuous equilibrium between maternal and foetal compartments; and secondly placenta is directly involved in parasite transmission to the foetus.

By immune response, interferon $_{\gamma}$ (IFN- $_{\gamma}$) produced by natural killer (NK) cells or CD8+ cytotoxic lymphocytes (CTL) directly controls both invasion of monocytes and trophoblasts by *T. gondii* and replication of the parasite in infected cells. Massive IFN-$_{\gamma}$ release has immunopathological effects, of which apoptosis of decidual cells and spiral artery dilation (Senegas *et al.*, 2009). Some of these are essential immunomodulatory mechanisms that compensate for the Th-1 inflammatory cytokines induced by *Toxoplasma*, and could avoid foetal loss, particularly when infection occurs in early pregnancy. Human trophoblast cells produce interleukin 10 (IL-10) and transforming growth factor β1 (TGF-β1), which promote a Th-2 immune response to ensure maternal–foetal tolerance but induce a significant increase in both *T. gondii* intracellular replication and invasion (Barbosa *et al.*, 2008). IFN$_{\gamma}$ secretion induces intercellular adhesion molecule (ICAM)-1 up regulation on the trophoblast surface, enhancing adhesion of infected monocytes to the trophoblast cell surface. Infected trophoblast cells then lose the ability for apoptosis, which results in parasite persistence in placental tissues (Pfaff and Candolfi, 2008), and this can be a reservoir for immediate or delayed congenital infection. The strong expression of human leukocyte antigen-G (HLA-G) on trophoblast cells can inhibit lysis by maternal NK cells and can mediate suppression of the allocytotoxic T cell response against the foetus. HLA-G expression also drives mononuclear phagocytes into suppressive pathways (Hunt *et al.*, 2005) (Figure 1.3).

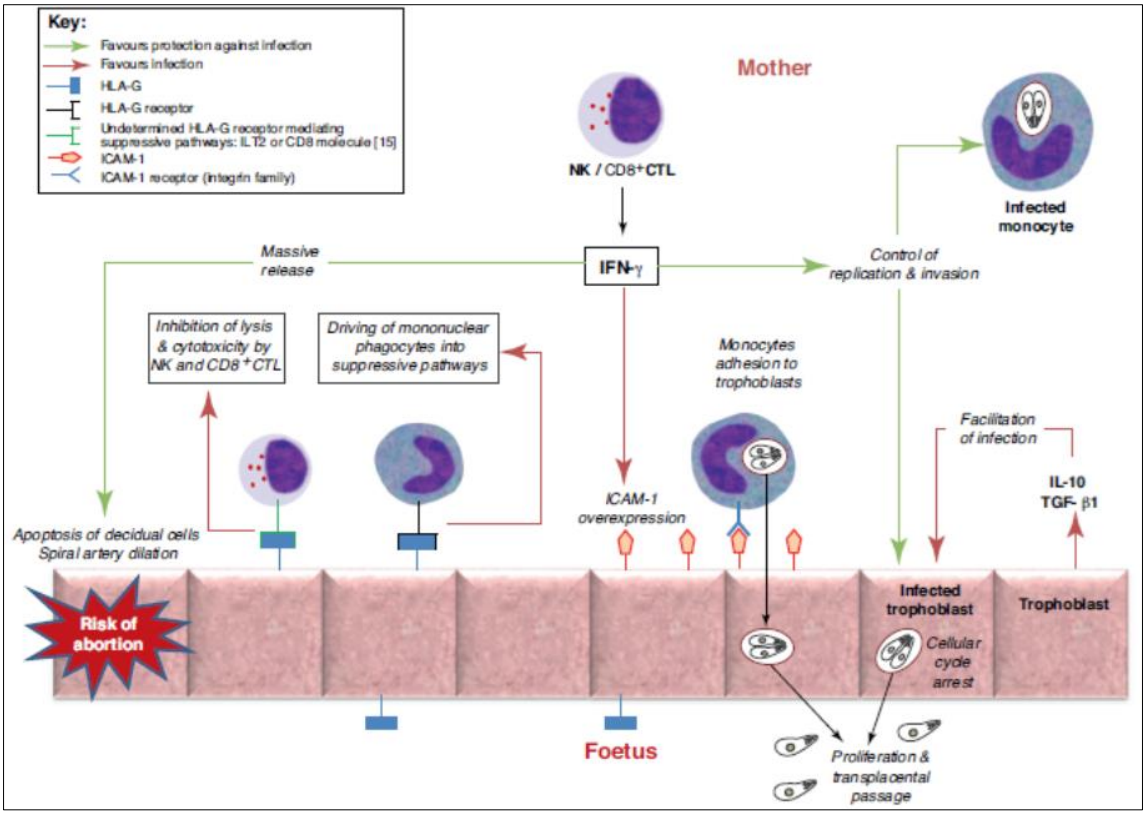

Figure 1.3:Tentative scheme of the pathophysiological hypotheses controlling trophoblast–cell infection and transplacental transfer of *Toxoplasma gondii*.(Adopted from Robert-Gangneux et al., 2011)

1.3.5.3. Toxoplasmosis in immunocompromised patients

Typically occurs as a reactivation of a chronic disease leading to life threatening condition. The immunocompromised patients consists of HIV – infected patient, tuberculosis patient, on prolonged immune suppressive therapies such as Systemic Lupus Erythematous (SLE), haematological malignancy, non-communicable disease such as diabetes mellitus and even old aged individuals. The major factor promoting to the reactivation of infection is profoundly impaired cellular immunity. In HIV – infected patients, the incidence is closely related to CD4 T cell counts, especially when count falls under100 cell/ul. Toxoplasmosis encephalitis (TE) most predominant manifestation with various symptoms, ranging from headache, lethargy, incoordination, or ataxia to hemiparesis, loss of memory, dementia, seizure usually associated with fever (Luft and Remington, 1992).

1.3.5.4. Ocular toxoplasmosis

T. gondii is the most common pathogen of retinochroiditis leading to reccurent posterior uveitis worldwide (Butler *et al.,* 2013). Common complaints are eye redness, blurring of vision and ocular pain. Chorioretinal lesion may develope from congenital or postnatal acquired infection and occur during the acute or latent stage of infection. Generally it is difficult to determine the infection was congenital or acquired with recurrences of chorioretinitis. Patients whom present with acute toxoplasmosis between the fourth and sixth decade of life, often have unilateral eye involvement, usually spare the macula with no scars. Whereas those acquired infection postnatally, often subclinical, may result in partial or complete loss of vision (Delair *et al.,* 2008). By contrast in congenital infection, the chorioretinitis is bilateral and they might face severe complications such as optic nerve atrophy, glaucoma, cataract and retinal detachment. Among immunocompromised patients, ocular toxoplasmosis occur with atypical and severe necrotizing form of retinochoroiditis (Antoniazzi *et al.,* 2008).

1.3.6. Diagnostic methods

There are several diagnostic approaches including detection of parasitic agent and *Toxoplasma* antibodies. Parasitic agents can be detected through histological identification, isolation from tissue culture and molecular technique by polymerase chain reaction (PCR). Whereas the serodiagnostic test are mainly to detect different classes of antibodies or antigen.

1.3.6.1. Serology diagnosis

Understanding the kinetic of antibody response is the basis of serological test. Interpretation of the results depends on patient's immune background and disease setting followed by clinical signs. Many serology tests are to measure different types of antibody, including IgG, IgM, IgA, and IgE, which show unique increase or decrease

during the course of infection. The established serological methods available include Sabin – Feldman Dye test (SFDT), Indirect fluorescent Assay (IFA) agglutination tests, Enzyme-linked immunosorbent assays (ELISA) and Avidity of *Toxoplasma* IgG. In avidity of *Toxoplasma* IgG, a high avidity ratio exclude a recent infection preceding 4 months, test is performed during first trimester of pregnancy. Whereby, if index is low or intermediate, the interpretation is ambivalent. This cannot exclude an infection acquired in the preceding 4 months, or prove that it is recent, unless the index is extremely low. Also should be kept in mind that treatment delays IgG avidity maturation (Meroni *et al.*, 2009).

1.3.6.2. Kinetic of antibody responses

T. gondii-specific IgM antibodies rises from day 5 to weeks following acute infection (Paquet *et al.*, 2013) peaks at 2 months and disappear within 6-9 months, more rapidly than IgG. A positive IgM result indicates an early, acute or a recent *Toxoplasma* infection. In recent infection there will be presence of persistent IgM (Meek *et al.*, 2001; Montoya and Liesenfeld, 2004). IgM antibodies can persist over years without any clinical significance and thus hamper the interpretation of test results especially in case of pregnancy screening (Remington *et al.*, 2001; Wong and Remington, 1994) *T. gondii*-specific IgG antibody are detectable within 1 or 2 weeks after infection, peaks within 12 weeks to 6 months and persist for decades (Paquet *et al.*, 2013). (Figure 1.4). A positive result indicates a previous exposure (latent infection) and provide immunity to reinfection unless from a different genotype and virulence genes. It may also indicate recent or reactivation of latent infection. In case of recent or reactivation of infection usually a significant rise or 4 fold titer in IgG antibody level can be expected in serial samples obtained in 2 – 3 weeks apart (Montoya and Liesenfeld, 2004). Passive immunity is detection of IgG antibody in newborn or infant, is from mother and may

persist up to 6 – 12 months. The IgG antibody titer reduces to 50% every month till 6 month or 12 months of age. A negative IgG antibody at 1 year of age essentially rules out congenital toxoplasmosis (Lago *et al.*, 2014). In recent years, IgE and IgA antibody has proved a better age of infection especially in new born because they are specific with no cross immunity from mother. However, they are produced during first week of infection and disappears early.

Reinfection during pregnancy are associated with exposure to a large number of parasites, more virulent strain or a different genotype (Lebas *et al.*, 2004). Where by reactivation occurs from a latent infection. Both these conditions have been reported in an immunocompetent mother which later caused congenital infection to her new born. The serological profile of reinfection is by detecting (IgM and /or IgA antibodies, and high IgG level) compared to reactivation (no IgM and /or IgA antibodies, and high IgG level) (Andrade *et al.*, 2010).

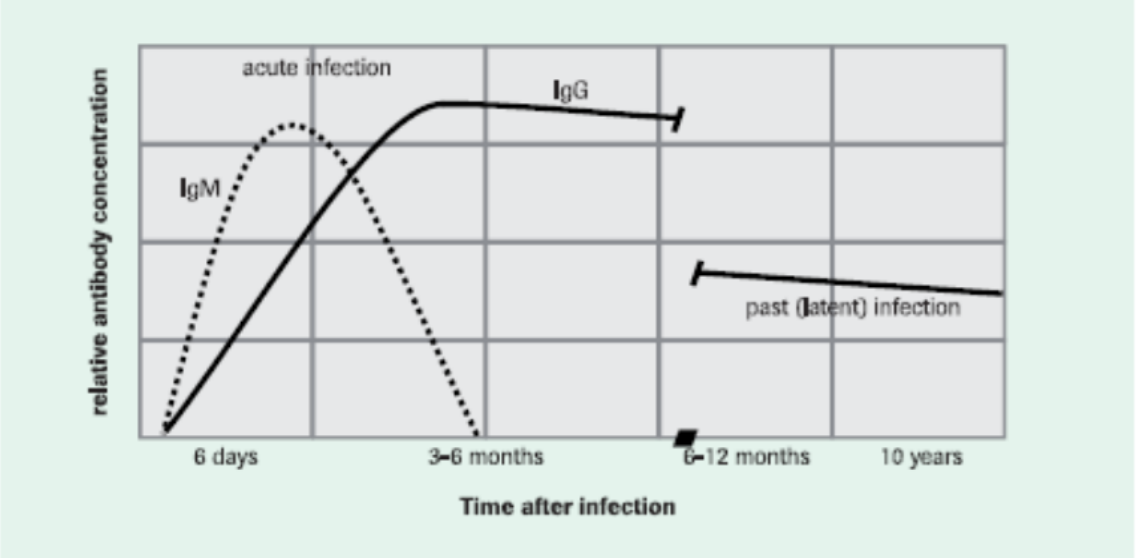

Figure 1.4:*Toxoplasma* infection serological profile (Adopted from Elecsys Toxo IgG, IgM Immunoassay, Elecsys and e cobas analyser, Roche, 2010)(Product Information, 2010)

1.3.6.3. Sabin – Feldman dye test (SFDT)

Sabin – Feldman dye test (SFDT) is the first assay developed and still considered as the gold standard with high sensitivity and specificity. This test is applied based on incubation of live tachyzoites on the slide with patients serum and complement. If the serum contains specific antibodies against *T. gondii*, the coated tachyzoite will be lysed by the complement system and fail to stain with dye methylene blue. Finally the stained and unstained tachyzoites are counted and used as end-point titer. The limitation for this test is application of live tachyzoites which is considered as biohazard and fails to determine the stage of infection (Udonsom *et al.*, 2010).

1.3.6.4. Indirect fluorescent assay (IFA)

This method is applied based on killed tachyzoites fixed on a glass slide to react with the antibody in the serum. The detection of this reaction is by fluorescence – labelled anti human IgG and IgM antibodies and reviewed under a fluorescence microscope. This test is simple, safe and inexpensive but it is operator dependent. There are also possibilities of false positive results in cases of patients with rheumatoid factors or antinuclear antibodies (Rorman *et al.*, 2006).

1.3.6.5. Agglutination tests

The available tests are direct agglutination test (DAT), indirect hemagglutination test (IHAT) and latex agglutination test (LAT). Development of these tests are to identify antibodies against *T. gondii*. DAT starting with coating of microtiter plates containing formalinized *Toxoplasma* tachyzoites will agglutinate if antibodies are present in the diluted patients serum. In IHAT, sensitised red blood cells (RBCs) are used that shows agglutination if serum are positive with anti – *T. gondii* antibodies (Liu *et al.*, 2015). In LAT, tachyzoites are fixed to latex beads and visible flocculation reveals positive serum containing specific IgG antibody. The agglutination test are generally simple and

inexpensive but there a tendencies for false positive results to occur especially in immunocompromised individual.

1.3.6.6. Enzyme – linked immunosorbent assay (ELISA)

The principle of this assay is a microtiter plate is coated with antigens, and diluted sera are applied. The anti – *Toxoplasma* antibodies will bind to the antigen and detected by secondary antibody. Unbound reagents are washed and substrate is added colour reaction occurs which correlates with the quantity of the antibody. The determination of results depends on optic density of the serum. This test is highly sensitive, measures quantitative and semiquantitative antibody, detects IgG, IgM, IgA and IgE antibodies and applied as a large scale of samples in short duration (Sudan *et al.*, 2013). In current settings, the latest generation of ELISA, electrochemiluminescence immunoassay "ECLIA" is applied in most laboratories as a screening tool for *Toxoplasma* infection. The initial principle is similar to ELISA but the end result is measured with a cell that uses electrodes and magnet to gather the bound immunocomplexes. Finally application of a defined voltage induces the electrochemiluminescent reaction and the resulting light emission is measured directly by the photomultiplier.

A study conducted by the new Roche Elecsys Toxo IgG and IgM immunoassay was compared with Sabin–Feldman dye test and immunosorbent agglutination assay-IgM as a reference test. Single serum samples were analysed from 927 pregnant women, including 100 negative, 706 chronic, and 121 acute infections. The combination of both Elecsys IgG and Ig M assays demonstrated high sensitivity and specificity of 97.1% and 100.0%, respectively, and a positive and negative predictive value of 100.0% and 81.3%, respectively (Prusa *et al.*, 2010).

1.3.6.7. Parasite isolation

Isolation of *T. gondii* from blood, body fluids and tissue always indicate acute infection. Isolation is by inoculation of the parasites from patients sample in the tissue cultures or laboratory animals. In mice, the test is performed by injecting the clinical samples either intraperitoneally or subcutaneously. The mice will be subjected for serological test after 3 to 6 weeks to detect the presence of anti *T.gondii* antibodies. Tissues from positive antibodies will be tested with PCR or microscopic examination to look for cysts for final confirmation (Hill and Dubey, 2002).

1.3.6.8. Histologic diagnosis

Demonstration of tachyzoites in tissue or smears of body fluids establish the diagnosis of acute infection. Conventional staining is difficult to demonstrate tachyzoites, therefore the immunoperoxidase technique with antisera is applied successfully to detect the parasite in toxoplasmosis encephalitis cases of HIV patients (Montoya, 2002).

1.3.6.9. Molecular diagnosis

PCR amplification for detection of *T. gondii* DNA in body fluids and tissues are used to diagnose congenital, ocular, cerebral and disseminated toxoplasmosis. The most popular used gene is the 35 – fold repetitive gene B, one of the first gene used in the PCR detection of *T.gondii* (Sarkari *et al.*, 2014). PCR has evolved in the diagnosis of intrauterine *Toxoplasma* infection by early detection in placenta tissue or amniotic fluids and helped to guide for prompt treatment and avoid unnecessary invasive procedures (Montoya, 2002). Amniotic fluids from amniocentesis is the cornerstone for diagnosis of congenital toxoplasmosis. If maternal primary infection is suspected and abnormal ultrasound findings were diagnosed (intracranial calcification, microcephaly, hydrocephalus, ascites, hepatosplenomegaly or severe intrauterine growth retardation), PCR should be offered to identify *Toxoplasma* infection in the

amniotic fluid. This procedure is not performed at less than 18 weeks gestation because of high rate of false positive results, nevertheless should be done not less than 4 weeks after time of suspected maternal infection (Paquet *et al.*, 2013). Performance of amniocentesis after 18 weeks has sensitivity of 91%, specificity 96 % (Romand *et al.*, 2001).

1.3.7. Treatment

Treatment in immunocompetent pregnant women with previous infection with *T. gondii* should not be necessary. Women who are immunosuppressed or HIV-positive should be offered screening because of the risk of reactivation and toxoplasmosis encephalitis. A non-pregnant woman, diagnosed with an acute toxoplasmosis should be counselled to wait 6 months before attempting to become pregnant. Each case should be considered separately in consultation with an expert. The existing indication for treatment in pregnant women is to achieve two goals. Firstly, if maternal infection occurred but foetus is not infected, aimed at preventing vertical transmission, before foetal infection. Spiramycin, a macrolide, does not readily cross the placenta, therefore is not reliable for treatment of foetal infection. As a foetal prophylaxis, is used to prevent spread of organisms across the placenta (Goldstein *et al.*, 2008). Secondly if foetal infection has been confirmed or is highly suspected by amniotic fluid PCR or foetal ultrasound, pyrimethamine and sulfadiazine is indicated. Pyrimethamine is a folic acid antagonist acts synergistically with sulfonamides. This drug not be used in the first trimester, potentially teratogenic and a reversible dose related depression of the bone marrow and therefore must be combined with folinic acid. The combination of pyrimethamine and sulfadiazine results in a significant decrease in disease severity. Neonates with congenital toxoplasmosis, even asymptomatic at birth should be treated early to reduce long term squeal (Paquet *et al.*, 2013) (Figure 1.5).

In patients with AIDS whom developed reactivation of cerebral toxoplasmosis, CD4 cell count of < 100 cells/mm^3 has a strong association as a prognostic marker in progression of the disease, especially with other opportunistic infection. Toxoplasmosis encephalitis is a preventable disease when adequate chemoprophylaxis of trimethoprim-sulfamethoxazole or dapsone-pyrimethamine plus folinic acid is administered (Passos *et al.*, 2000).

The diagnosis for retinochoroiditis depends on an accurate opthalmological examination which shows typical of white focal lesion associated with a vitreous inflammatory reaction and *Toxoplasma* seropositivity. This indicates for treatment and further confirmed with therapeutic response (Robert-Gangneux and Dardé, 2012). Intravitreous clindamycin with dexamethasone seems to be as effective as systemic treatments, where as other preferred oral antibiotic would be trimethoprim-sulfamethoxazole, azithromycin or clindamycin which prevents recurrence of the disease (Harrell and Carvounis, 2014).

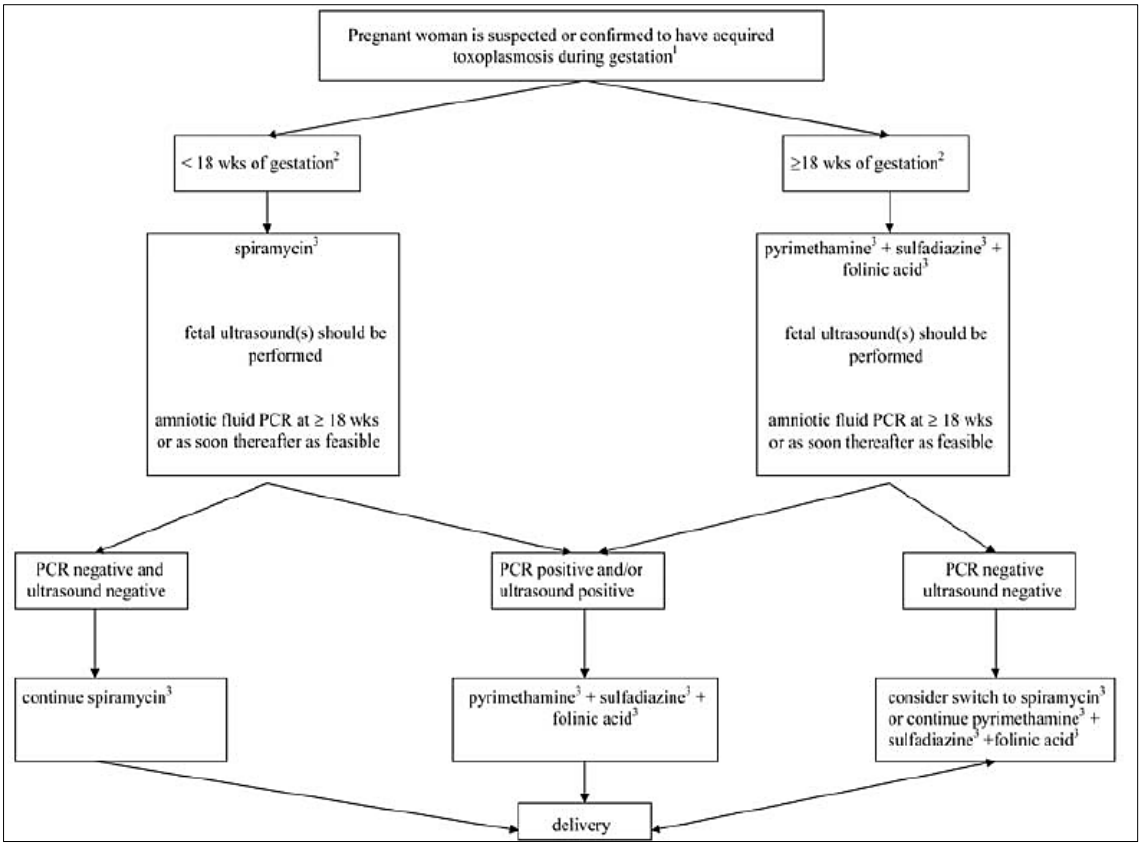

Figure 1.5: Treatment approach for pregnant women suspected or confirmed to have toxoplasmosis acquired during gestation. (Adopted from Goldstein, E. J., Montoya, J. G. & Remington,J. S. 2008).

1.3.8. Prevention and control measures

Various efforts have been taken to reduce the burden of *Toxoplasma* infection among all population. Good hygiene measures such as hand washing after contact with raw meat, after gardening or other external activity in contact with soil and close contact with cats. Thorough washing of fruits and vegetables is strongly advised and cat litter box need to be changed every 2 days. Primary care is the first line to explain the preventive measures to pregnant women or those plan to be pregnant. Besides counselling, written information is helpful to reduce congenital toxoplasmosis. Based on a survey regarding knowledge and practice on *Toxoplasma* infection in pregnant women from Malaysia, Philippines, and Thailand, only 11% of these pregnant women had read, heard, or seen information regarding toxoplasmosis and 3.5% of them were aware of being tested for

the infection (Andiappan *et al.*, 2014). Screening for pregnant women and neonate is implemented in many European countries. Hence the decisions on implementation of screening in a country are considered based on prevalence data, disease burden, technical resources and diagnostic costs.

1.4. Conceptual framework

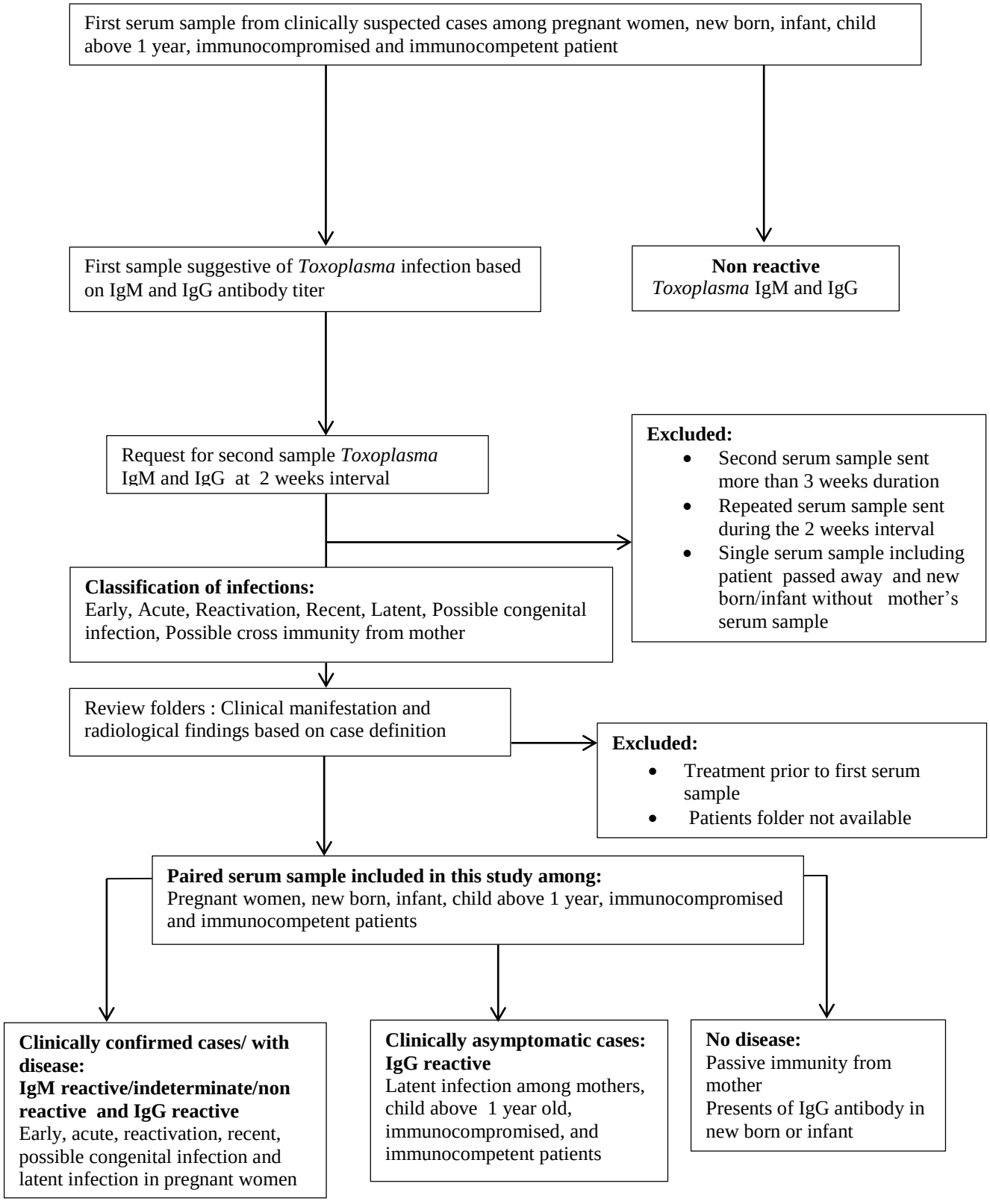

Figure 1.6: Conceptual framework for case selection process

1.5. OBJECTIVES

1.5.1. General objectives

To describe the clinical manifestation and determine the seroprevalance classification of infection among the selected populations, validity of the test and percentage of patient on treatment after first and paired serum sample.

1.5.2. Specific objectives

1. To describe the clinical manifestation of *Toxoplasma* infection among pregnant women, new born, infant, child above 1 year old, immunocompromised and immuno - competent patients.
2. To determine seroprevalence of early, acute, reactivation, recent, latent, passive immunity from mother and possible congenital infection with paired serum sample using Elecsys IgM / IgG assay among pregnant women , new born, infant, child above 1 year old, immunocompromised and immunocompetent patients.
3. To determine the validity of paired serum sample to diagnose *Toxoplasma* infection by calculating the sensitivity, specificity, PPV, NPV and ROC curve analyses in clinically confirmed cases (by gold standard).
4. To describe the percentage of patient on treatment after the first and paired serum sample.

1.5.3. Research hypothesis

The classification of *Toxoplasma* infection into early , acute, reactivation, recent , latent, passive immunity from mother and possible congenital infection can be established by using paired serum sample of *Toxoplasma* IgM and IgG antibody titer in pregnant women , new born, infant, child above 1 year old, immunocompromised and immunocompetent patients.