RISK OF MOTHER-TO-CHILD TRANSMISSION OF *TOXOPLASMA GONDII* INFECTION AMONG PREGNANT WOMEN IN THE GREATER ACCRA REGION

By

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DECLARATION

I hereby Declare that this submission is my own work towards the MPhil and that, to the best of my knowledge, it contains no material previously published by another person nor material which has been accepted for the award of any other degree of the University, except where due acknowledgement has been made in the text.

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DEDICATION

This thesis is dedicated to God Almighty, my family and my friends

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ABSTRACT

Toxoplasmosis is caused by *Toxoplasma gondii* which can be acquired orally or congenitally. Congenital infection of infants is known to result in several neurological, brain and ophthalmic disorders later in life. This study therefore sought to determine the risk of mother-to-child transmission of *T. gondii* among women at delivery in a hospital facility in the Greater Accra region of Ghana. Ninety- three (93) pregnant women aged 18 to 45 years voluntarily participated with their babies. Maternal Blood, Umbilical cord blood and tissue samples were taken at delivery after the expulsion of each placenta. Finger-prick blood was taken from infants of participating mothers two to six weeks post-natal. ELISA was used to detect anti-*T. gondii* IgG and IgM antibodies in all blood samples while Nested-PCR was used to detect *T. gondii* DNA extracted from placental tissue. Data collected were analysed using SPSS (Version 16). Overall, 37.6% (35/93) maternal blood were positive for anti-*T. gondii* IgG with 39.5% (36/91) umbilical cord blood also positive for anti-*T. gondii* IgG. Fifty-seven percent (23/40) of post-natal infant blood was positive for anti-*T. gondii* IgG. All of the blood samples were negative for IgM. *T. gondii* DNA was detected in 39.8% (35/88) of placental tissues. 38.4% (33/86) of matched maternal and foetal samples were positive for anti-*T.gondii* IgG and/or *T. gondii* DNA. *Toxoplasma gondii* DNA detected in placenta may be largely from cysts and is indicative of infection of the mother in the course of gestation. Placental toxoplasmosis exposes the foetus to the risk of infection which implies that almost 40% of the infants were at risk of congenital infection. Further studies needs to be done to determine the rate of mother-to-child transmission of *T. gondii* in Ghana.

CHAPTER ONE

INTRODUCTION

1.1 Background

Toxoplasmosis is a parasitic disease caused by *Toxoplasma gondii*. The parasite infects most genera of warm-blooded animals, including humans, but the primary host is the felid (cat) family. *Toxoplasma gondii* is a species known to be a potential parasite of man for many years but its true nature as a coccidian was discovered only around 1969-70 (Smyth, 1996). It is an obligate intracellular parasite and was first discovered in 1908 in a desert rodent, the Gondi rat (Roberts and Janovy, 2000).

Toxoplasmosis has been known to be asymptomatic, often associated with short self-limiting illnesses in immunocompetent individuals. This is typical of both the acute and latent forms of the disease. The case is however different in immunocompromised persons such as HIV/AIDS patients (Agyei and Lartey, 2004), persons undergoing chemotherapy, organ transplant recipients, and sometimes in pregnant women in which the disease is usually fatal in their foetuses (Stepick-Biek *et al.*, 2002).

Transmission of the infection involves the acquired (or horizontal) and the congenital (or vertical) types. Acquired toxoplasmosis is either through the ingestion of oocysts shed in infected cats' faeces and sporulated in the soil under suitable conditions or contaminated vegetables and fruits; tachyzoites in unpasteurised milk; cysts in raw or undercooked infected meat (Dubey, 2008). Pork and lamb are known to be the most common sources of contamination although beef and vegetables are potential sources of infection. Insects

such as filth flies and cockroaches also have the capability of carrying oocysts from cat faeces to food (Wallace, 1971).

Congenital toxoplasmosis is a clinical state of the disease in the foetus that results from an acute primary infection acquired by the mother during pregnancy. The materno-foetal transmission rates vary according to gestational age at the time of maternal infection (Stepick-Biek *et al*., 2002) and the severity of congenital toxoplasmosis vary with the trimester during which infection was acquired (CDC, 2002). Maternal infection in the first trimester of gestation results in a transmission rate of 10-15%, which rises up to 68% in the third trimester (Thulliez *et al*., 1992). Although infection of foetuses in the first trimester is rare, infections are of serious consequences when they do occur. This can result in severe consequences such as spontaneous abortion, still-birth, or a child may be born with some degree of abnormalities in the central nervous system including hydrocephalus and mental retardation. Less serious consequences involving the eye are eye lesions. It is however dependent on the age of the foetus when it is infected and the virulence of the *Toxoplasma.*

This notwithstanding, early diagnosis of toxoplasmosis can help treat and manage the disease effectively. Diagnoses in health centers usually involve anti-*Toxoplasma* antibody detection and parasite DNA detection. Treatment regimen following diagnosis involves the use of anti-parasitic agents, of which pyrimethamine and sulfadiazine have been known to be the best over the years. (Remington *et al*., 2006, Mui *et al*., 2008). Prevention of the disease includes avoidance of ingestion of unwashed vegetables and

fruits, and uncooked or improperly cooked meat of infected animals. The practice of good personal hygiene has also been greatly encouraged.

It is estimated that between 30% and 65% of all people worldwide are infected with toxoplasmosis (Tenter *et al.*, 2000). However, there is a large variation between countries, for example in France, the prevalence rate lies at 88%, and in Germany, the Netherlands and Brazil, prevalence rates are around 80%, over 80% and 67% respectively. In Britain about 22% are carriers, and South Korea's rate is 4.3% (Zimmer, 2006).

Studies in Ghana, however, show varying but high sero-prevalence values (51.2% to 92.5%) in humans, particularly pregnant women (Anteson *et al.*, 1978a, 1978b, 1980; Ayi *et al.*, 2005; Ayi *et al.*, 2009) and in animals (Arko-Mensah *et al.*, 2000; Van der Puije *et al.*, 2000). In 2005, Ayi *et al* reported a high sero-prevalence (89%) among eye patients with eye lesions. Toxoplasmic eye lesions in adults have been known to be a consequence of congenital infection at birth.

1.2 Problem Statement

Though toxoplasmosis has potentially significant health risks on the foetuses of women who acquire infection during pregnancy and there is significantly high sero-prevalence among pregnant women in Ghana, not much studies have so far been done in the area of the congenital toxoplasmosis and the risk of infected mothers passing it on to foetuses. For the mother, the toxoplasmosis infection is generally mild, as compared to the infected foetus.

Congenital toxoplasmosis could have grave socioeconomic and psychological consequences on affected families. The human suffering and the cost of care, especially with the issue of hydrocephalus, mental retardation and even blindness could be very enormous. Also, the psychological trauma of having miscarriage(s) or stillbirth(s) cannot be overemphasized.

In countries such as France and Austria, in-depth studies on congenital transmission of toxoplasmosis led to the formulation of policy to routinely screen pregnant women as part of their antenatal care and this has proven to greatly reduce the rate of the congenital form of the disease. In Ghana, despite the risk of Toxoplasmosis to both mother and child, screening of pregnant women for *Toxoplasma* infection is not included in the free-of-charge antenatal care policy, due mainly to ignorance and lack of information on the rate of mother-to-child transmission of congenital toxoplasmosis. Such a lack of screening for risk of congenital toxoplasmosis predisposes countless newborns throughout the country to intra- uterine infection and development of mild to severe consequences, which could have been minimized by an early post-natal intervention. The neglect of toxoplasmosis as a disease of minimum or no public health importance has resulted in the ignorance of the public about the disease and its potential threats on the society.

1.3 Justification

Knowledge about the rare occurrence of congenital toxoplasmosis has been based on studies conducted years ago in developed countries. Not much has been done to determine the real situation of the disease in Ghana. This study will provide data on the risk of congenital transmission and infection status of babies born to *Toxoplasma* positive mothers. Also the knowledge derived from this study will add up to existing information and enhance the prospects of policy formulation to routinely screen pregnant women and women of child bearing age for *Toxoplasma* infection to reduce the effects of the disease which could be pronounced in the later years of the child. Participants' responses to questionnaire will also help determine the *T. gondii* infection-related knowledge and practices among the general populace so as to help create the awareness of the dangers associated with the disease.

1.4 Objectives

1.4.1 Main objective

- To determine the risk of mother-to-child transmission of *Toxoplasma gondii* infection among pregnant women and their newly born babies.

1.4.2 Specific objectives

- To estimate the *T. gondii* sero-prevalence among women at delivery by the detection of anti-*T. gondii* antibodies.

- To estimate *T. gondii* sero-prevalence in babies at birth and 2 – 6 wks post-natal.
- To detect *T. gondii* DNA in placental tissues.
- To assess the risk of mother to child transmission by correlation of infection status of matched maternal and infant tested samples.

CHAPTER TWO

LITERATURE REVIEW

2.1 TOXOPLASMOSIS AND *TOXOPLASMA GONDII*

2.1.1 A Historical Perspective

Toxoplasmosis is a zoonotic parasitic disease caused by the protozoan *Toxoplasma gondii*. The parasite infects most genera of warm-blooded animals, including humans, but the primary host is the felid (cat) family. *Toxoplasma gondii* is a species known to be a potential parasite of man for many years but its true nature as a coccidian was discovered only around 1969-70 (Dubey *et al.*, 1970a; Dubey *et al.*, 1970b; Smyth, 1996). It is an obligate intracellular parasite and was first discovered by Nicolle and Manceaux in 1908 in a desert rodent, the Gundi rat, *Ctenodactylus gundi* (Roberts and Janovy, 2000). Naming of the parasite was based on the morphology (mod. L. Toxo: arc or bow, plasma: life) and the host (Nicolle and Manceaux 1909). In retrospect the correct name for the parasite should have been *T. gundii* but Nicolle and Manceaux in 1908 had incorrectly identified the host as *Ctenodactylus gondi* (Dubey, 2007).

The first case of *T. gondii* infection in humans was identified in an infant girl in New York, USA, in 1938 by three pathologists (Wolf *et al.*, 1939). The girl developed convulsive seizures when she was just three days old and lesions were noted in the maculae of both eyes through an ophthalmoscope. She died at one month old and an autopsy was performed. At post mortem, brain, spinal cord, and right eye were removed for examination. Free and intracellular *T. gondii* were found in lesions of encephalomyelitis and retinitis of the girl. Viable *T. gondii* was isolated in animals

inoculated with tissues from the girl. It was later found out that the child became infected congenitally. Sabin (1942) summarized all that was known of congenital toxoplasmosis in 1942 and proposed typical clinical signs of congenital toxoplasmosis: hydrocephalus or microcephalus, intracerebral calcification, and chorioretinitis. These signs helped in the clinical recognition of congenital toxoplasmosis.

Before then in 1941, Sabin reported toxoplasmosis in a six year old boy with the initials R. H. in Cincinnati, OH. The strain isolated from him took its name after his initials, RH, hence becoming the famous RH strain. These land marks led to other discoveries such as ocular toxoplasmosis (Wilder, 1952; Silveira *et al.*, 1988) and toxoplasmosis in other animals (Hartley and Marshall, 1957; Dubey and Beattie, 1988; Dubey, 2001).

2.1.2 Classification of *T. gondii*

Toxoplasma gondii belongs to the kingdom Protoctista, phylum Apicomplexa which consists of intracellular parasites that have a characteristically polarized cell structure and a complex cytoskeletal and organellar arrangement at their apical end (Dubey *et al.,* 1998). Other members of this phylum include the human pathogens *Plasmodium* (the cause of malaria) and *Cryptosporidium* as well as the animal pathogens *Eimeria* (the cause of chicken coccidiosis) and *Sarcocystis*.

It belongs to the class Conoidasida of which members are obligate intracellular parasites. (They generally complete the sexual stage of their life cycles within a host's intestinal tract) and subclass Coccidiasina. It belongs to order Eucoccidiorida (consists of parasites

of humans, domesticated animals, wild animals, and birds) and the suborder Eimeriorina (includes many species that predominantly parasitize domestic animals, with few human parasites). It also belongs to the family Sarcocystidae (carry out a life cycle that requires more than one obligatory host). *Toxoplasma* is one of the three best known genera; this genus requires transmission between a member of the *felidae* (Frenkel *et al.*, 1970) to carry out its sexual life cycle. Lastly, it belongs to the species *gondii*; the only species in the genus *Toxoplasma*.

2.1.3 Genetic diversity of *T. gondii*

In population genetics, the structure of *T. gondii* is highly clonal, despite a sexual phase in the life cycle (Howe and Sibley, 1995; Sibley and Boothroyd, 1992; Sibley, 2003). There are three major clonal types (I, II, and III) derived from recombination between two highly similar ancestral lineages (Grigg *et al.*, 2001; Su *et al.*, 2003). Type II strains have been identified as the cause of more than 70% of human cases of toxoplasmosis in the United States and France (Ajzenberg, 2002; Darde´, 1992; Howe and Sibley, 1995). Type II strains are relatively avirulent in mice yet they readily establish chronic infections characterized by tissue cysts that are highly infectious by the oral route (Sibley, 2003; Su *et al.*, 2003). Type I strains are more virulent and have a greater capacity to cross tissue barriers in vitro and in vivo (Su *et al.*, 2002, Sibley and Boothroyd, 1992; Barragan and Sibley, 2002).

2.1.4 Morphology of *T. gondii*

Toxoplasma gondii exists in three different forms depending on the stage of development in its lifecycle. These morphological forms include the actively dividing forms known as the tachyzoites, the slowly dividing forms known as the bradyzoites in tissue cysts and oocysts (Dubey *et al.*, 1998). A detailed study of the various forms reveals the basic characteristic differences in their structures.

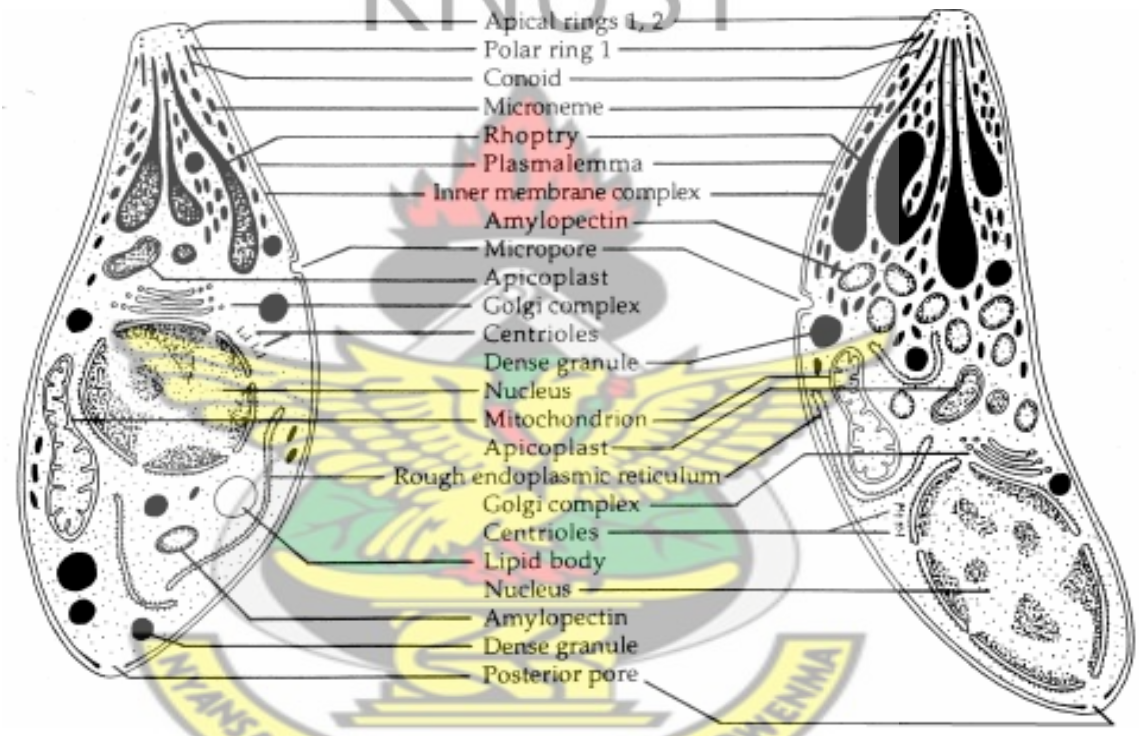

Figure 1: Schematic drawings of a tachyzoite (left) and a bradyzoite (right) of *T. gondii*. The drawings are composites of electron micrographs. (Dubey *et al*, 1998)

2.1.4.1 Tachyzoites

Frenkel (1973) described the stage of the parasite that rapidly multiplied in any cell of the intermediate host and in the non-intestinal epithelial cells of the definitive host as the "tachyzoite" (tachos ; speed in Greek) based on the behavior of these parasites.

The tachyzoite is often crescent shaped, approximately 2 by 6 mm (Fig. 1), with a pointed anterior (conoidal) end and a rounded posterior end. Ultrastructurally, the tachyzoite consists of various organelles and inclusion bodies including a pellicle (outer covering), apical rings, polar rings, conoid, rhoptries, micronemes, micropore, mitochondrion, subpellicular microtubules, endoplasmic reticulum, Golgi complex, ribosomes, rough and smooth endoplasmic reticula, micropore, nucleus, dense granules, amylopectin granules (which may be absent), and a multiple-membrane-bound plastid-like organelle which has also been called a Golgi adjunct or apicoplast. (Dubey *et al.*, 1998)

2.1.4.2Bradyzoites and tissue cysts

The term "bradyzoite" (brady; slow in Greek) was also coined by Frenkel (Frenkel, 1973) to describe the stage of the parasite multiplying slowly within a tissue cyst. The tissue cyst wall is elastic and thin (<0.5 µm thick) and it encloses hundreds of crescent-shaped bradyzoites (Plate 1), each approximately 7 by 1.5 µm in size (Melhorn and Frenkel, 1980). The tissue cyst develops within the host cell cytoplasm. Tissue cysts grow and remain intracellular as the bradyzoites divide by endodyogeny (Ferguson and Hutchison, 1987). Tissue cysts vary in size; young tissue cysts may be as small as 5µm in diameter and contain only two bradyzoites, while older ones may contain hundreds of organisms. (Dubey *et al.*, 1998)

Bradyzoites differ structurally only slightly from tachyzoites (Fig 1). They have a nucleus situated toward the posterior end, whereas the nucleus in tachyzoites is more centrally located. Bradyzoites are more slender than are tachyzoites. Bradyzoites are less

susceptible to destruction by proteolytic enzymes than are tachyzoites (Jacobs *et al.,* 1960), and the prepatent period in cats following feeding of bradyzoites is shorter than that following feeding of tachyzoites (Dubey and Frenkel, 1976). Cysts are usually formed in neural tissues such as the eye and the brain, and the muscular tissues. However, visceral organs including the lungs, kidneys, and liver can also be infected. Intact tissue cysts probably do not cause any harm and can persist for the life of the host without causing a host inflammatory response.

Plate 1: *Toxoplasma* bradyzoites in tissue cyst (Source: Ferguson, 1987)

2.1.4.3 Oocysts

Toxoplasma gondii Oocysts are shed only in the faeces of domestic and wild felids, the definitive hosts. Unsporulated oocysts are almost spherical and are 10 by 12 mm in diameter. When viewed under the light microscope, the oocyst wall consists of two

colorless layers (Plate 2B). There are no polar granules instead sporont almost fills the oocyst. The oocyst does not survive in arid, cool climates and can be destroyed by heating (Wilson and McAuley, 1999).

Sporulation takes place outside the cat within 1 to 5 days of excretion depending upon environmental conditions such as aeration and temperature ($4^{o}C - 37^{o}C$). Sporulated oocysts are subspherical to ellipsoidal and are 11 by 13 um in diameter. Each oocyst contains two ellipsoidal sporocysts without Stieda bodies (Plate 2A). Sporocysts measure 6 by 8 mm. A sporocyst residuum is present; there is no oocyst residuum. Each sporocyst contains four sporozoites. The oocysts become infectious after sporulation and can remain infectious for approximately 1 year under favorable conditions (i.e., in warm, moist soil). (Dubey *et al.*, 1998)

Plate 2: A: *Toxoplasma gondii* sporulated oocyst in an unstained wet mount. **B:** *Toxoplasma gondii* unsporulated oocyst in an unstained wet mount (Source: CDC, 2002)

2.1.5. Life cycle of *T. gondii*

The life cycle consists of two phases, the sexual phase and the asexual phase. The sexual part takes place only in cats, both domestic and wild, which makes cats the parasite's primary (or definitive) host. The second phase, the asexual phase, can take place in other warm-blooded animals, including cats, mice, humans, and birds. The host in which asexual reproduction takes place is called the intermediate host. Rodents are the typical intermediate host.

Cats, the definitive hosts of *T. gondii*, become infected by ingesting sporulated oocysts or (most often) infected animals (containing tachyzoites, bradyzoites or tissue cysts) or infected transplacentally. Proteolytic enzymes in the digestive tract cause the release of the bradyzoites into the stomach and the small intestine. The bradyzoites, after several generations during which the parasites divide by a process known as endodyogeny, are transformed into tachyzoites (Dubey, 1998). Endodyogeny is essentially a budding process in which two daughter cells are formed inside the original or mother cell, which is then consumed by the developing daughter cells (Sheffield and Melton, 1968). After about two to five asexual generations in the cat, a sexual generation of micro – (male) and macro – (female) gametes is also found throughout the small intestine but almost often in the ileum, where they are found 3 – 15 days of infection (Dubey *et al.*, 1998). Fertilization occurs and the zygote develops into a thick walled oocyst. On maturity, the oocysts are discharged through the intestinal lumen in the cat's faeces by the rapture of intestinal epithelial cells. The oocysts become infectious after sporulation and can remain infectious for months even in cold and dry climates (Dubey, 1977).

The asexual life cycle takes place in all intermediate hosts (including humans) as well as in felines. Ingestion of tissue cysts or oocysts is followed by infection of the intestinal epithelial cell by bradyzoites or sporozoites respectively. Through the same process of endodyogeny, these become tachyzoites. Tachyzoites enter host cells, mainly nucleated cells such as immature red blood cells, white blood cells, and macrophages by actively penetrating through the host cell plasmalemma or by phagocytosis (Bonhomme *et al.*, 1992). After entering the host cell, the tachyzoite becomes ovoid and is surrounded by a parasitophorous vacuole (PV), which appears to be derived from both the parasite and the host cell (Dubey, 1998). Inside the vacuole, the tachyzoite replicates itself by endodyogeny until the infected cell fills with parasites and bursts, releasing other tachyzoites which infect other cells and tissues (Dubey *et al.*, 1998). Infected cells distribute tachyzoites throughout the body via the blood or lymph first to the mysentric lymph nodes, followed by the liver and lungs (Fig. 2). In immunocompetent persons, as a response to host acquired immunity, pseudocysts (Fig. 2) are formed enclosing tachyzoites that eventually differentiate into bradyzoites. These cysts are found predominantly in tissues with low immuno-activity, such as the central nervous system and muscle tissues where they may reside for the life of the host, hence the name tissue cysts (Black *et al.*, 2000, Dubey and Frenkel, 1972).

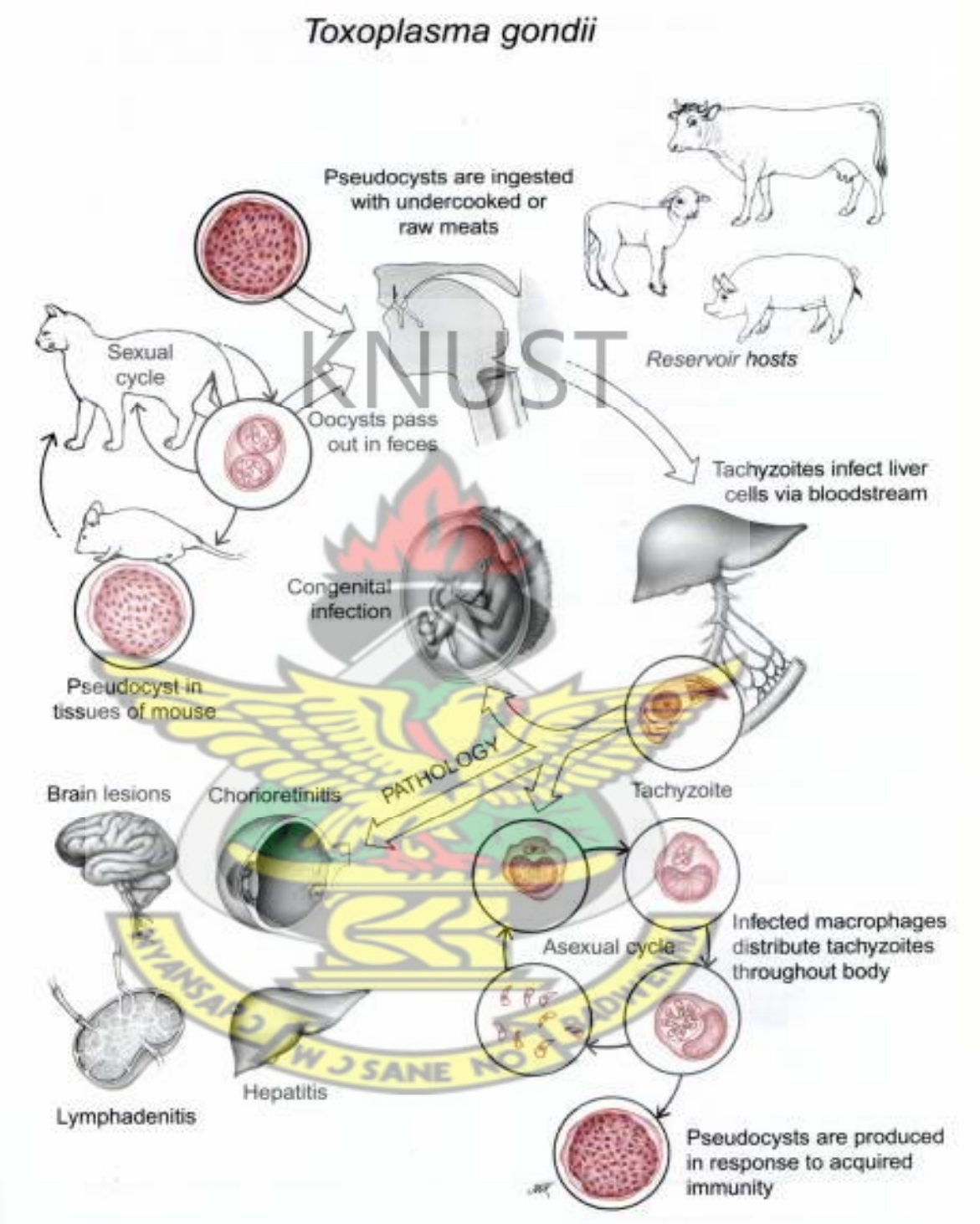

Figure 2: *T. gondii* life cycle and pathogenesis (Source: Racaniello, 2010)

2.1.6. Transmission of *T. gondii*

Transmission of *T. gondii* is by three principal routes: horizontal (tissue cysts), horizontal (oocysts from cats) and vertical (congenital). *Toxoplasma gondii* tissue cysts are horizontally transmitted to humans by the ingestion of infected raw or inadequately cooked meat, such as pork, mutton, beef or that of any other warm-blooded animal. (Dubey, 1994) One can also be infected through uncooked foods that have come in contact with infected meat. Secondly, the parasite is also horizontally transmitted to humans when they inadvertently ingest oocysts that cats have passed in their faeces, either from a litter box or from soil (e.g., garden soil),on unwashed fruits or vegetables, or in unfiltered water. The third route of transmission occurs when an acutely infected pregnant women passes the infection vertically to the fetus in her womb. This can cause serious disease in the fetus (Remington *et al.,* 2001).

Transmission of the parasite is dependent on factors such as climate and rate of exposure to sources of *T. gondii* infection. *Toxoplasma* sero-positivity is reportedly higher in hot and humid areas (Feldman, 1974). As such, some parts of the world are likely to have higher levels of toxoplasmosis prevalence than others. A high prevalence of about 51% was reported for Brazil, a moderate prevalence of approximately 39% for the United States and a low prevalence of 28% for Denmark, while that of Finland was reported at 18.9%, all in separate studies (Zimmer, 2006).

Some countries have reported high prevalence to *T. gondii* infection because of their level of exposure to the sources of infection. France is noted for a very high sero-prevalence of *Toxoplasma* of 70% to 90% and this is attributed to the eating of

undercooked meat (Dubey, 1988). As reported by Krick and Remington, the frequency of *T. gondii* infection increases from 0.5% to 1.0% per year of age (Krick and Remington, 1973). In an investigation by Al-Hamdani *et al*. (1997), the overall prevalence of antibodies gradually increased with age, reaching 23.7% in the age group 35-45years.

2.1.7. Pathogenesis and clinical manifestations

Toxoplasma gondii usually parasitizes both definitive and intermediate hosts without producing clinical signs. Immunocompetent persons develop a powerful cell-mediated immune response to the tachyzoites and control infection, driving the tachyzoites into the tissue cyst or bradyzoite stage (Gazzinelli *et al.*, 1993). In humans, severe disease is usually observed only in congenitally infected children and in immunosuppressed individuals, including patients with acquired immune deficiency syndrome (AIDS) (Dubey and Beattie, 1988).

The tachyzoite stage is responsible for tissue damage; *Toxoplasma gondii* does not produce a toxin (Remington *et al.*, 1995); necrosis is caused by intracellular multiplication of tachyzoites. Clinical signs depend on the number of tachyzoites released, the ability of the host immune system to limit tachyzoite spread, and the organs damaged by the tachyzoites. Because adult immunocompetent animals control tachyzoite spread efficiently, toxoplasmosis is usually asymptomatic. However, in immunocompromised persons, particularly fetuses and persons with AIDS, tachyzoites spread systemically and cause interstitial pneumonia, myocarditis, hepatic necrosis, meningoencephalomyelitis, encephalitis, chorioretinitis, lymphadenopathy, and myositis

(Remington *et al.*, 1995). Lymphadenitis is a common manifestation in humans. Infected nodes are tender and discrete but not painful; the infection subsides in weeks or months. Other clinical signs include fever, malaise, fatigue, muscle pains, sore throat, headache, diarrhoea, cough, dyspnea, icterus, seizures, and death. *T. gondii* is also an important cause of abortions and stillbirths in humans and other intermediate hosts (Daffos *et al.*, 1988).

2.1.8 Pregnancy and congenital toxoplasmosis

According to the CDC, only about 15% of women of childbearing age are immune to toxoplasmosis. Although pregnant women are not immunosuppressed in the classic sense, immunologic changes of pregnancy may induce a state of increased susceptibility to certain intracellular pathogens, including viruses, intracellular bacteria, and parasites. (Daffos *et al.*, 1988,).

Women infected with *T. gondii* before conception, with rare exception (Vogel *et al.*, 1996), do not transmit the infection to their fetuses. Women infected with *T. gondii* during pregnancy can transmit the infection across the placenta to their foetuses. The undeveloped immune system of foetuses leaves them greatly at risk when their mothers become infected for the first time during pregnancy. The risk of congenital transmission is lowest (10–25%) when acute maternal infection occurs during the first trimester and highest(60–90%) when acute maternal infection occurs during the third trimester (Remington *et al.,* 2001, Foulon *et al.*, 1999 Dunn *et al.,* 1999). Nevertheless, the severity of disease is worse if infection is acquired in the first trimester (Remington *et al.,* 2001, Holliman, 1995). The overall risk of congenital infection from acute *T. gondii* infection during pregnancy is 20% to 50%. After infection of a pregnant woman,

tachyzoites spread through the bloodstream to the placenta, causing cell destruction. Infection during the first trimester may lead to spontaneous abortion, still birth, while infection acquired later during pregnancy is usually asymptomatic in the neonate (Daffos *et al.*, 1988). Tachyzoites may also infect the fetus, causing damage in multiple organs (Guerina *et al.*, 1994). Congenitally acquired *T. gondii* often infects the brain and retina and can cause a wide

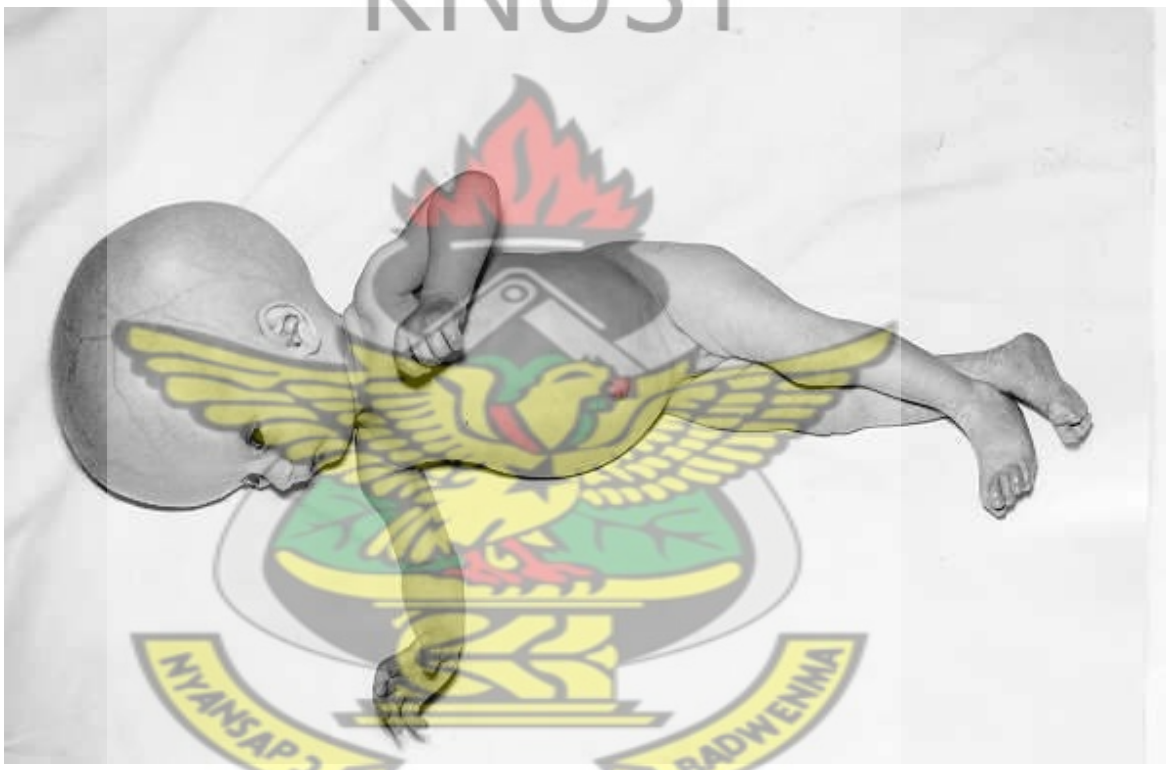

Plate 3. Girl with hydrocephalus due to congenital toxoplasmosis. (Dubey and Beattie, 1988.)

spectrum of clinical disease. Mild disease may consist of slightly diminished vision, whereas severely diseased children may exhibit a classic tetrad of signs: retinochoroiditis, hydrocephalus, convulsions, and intracerebral calcifications

(Remington *et al.*, 1995). Hydrocephalus is the least common but most dramatic lesion of congenital toxoplasmosis (Plate 3). Ocular disease is the most common sequela (Georgie, 1994).

2.1.9 Diagnosis of *T.gondii* infection

Diagnosis of *T. gondii* is very critical in pregnant women who acquire their infection during gestation and in fetuses and new-borns who are congenitally infected (Thulliez *et al.*, 1992; Montoya, 2002; Remington *et al.*, 2001). Diagnosis can be done in the laboratory indirectly by serological methods and directly by polymerase chain reaction (PCR), hybridisation, isolation and histological methods. Indirect methods are used mainly in the diagnosis of immunocompentent persons while direct detection of the parasites is done for definitive diagnosis of immunocompromised persons (Montoya and Liesenfeld, 2004).

2.1.9.1 Serological diagnosis

Different kinds of serological tests have been established for the diagnosis of *T. gondii* infection. Examples include Sabin-Feldman Dye Test, Indirect Flourenscent Antibody Test for IgG and/or IgM detection (IFA), Agglutination Test, Enzyme Immuno-assay (EIA), IgG-Avidity for distinguishing between recent and acute infection and Enzyme Linked Immunosorbent Assay (ELISA). Immunoglobulin G (IgG) and Immunoglobulin M (IgM), and in some cases, Immunoglobulin A (IgA) antibodies are measured for the detection of *T. gondii.* (Araujo and Remington, 1990; Decoster *et al.*, 1988). When an infection occurs for the first time, the first set of antibodies to appear are IgM which persist for a limited time, maybe six months, indicating an acute infection. (Del Bono *et*

al., 1989). IgG titres rise shortly after IgM in a case of acute infection, but declines slowly afterwards and persists for a long time to build immunity against another future parasitemia. Diagnosis of acute toxoplasmosis is based on the demonstration of a significant increase in specific IgG antibody levels and/or the presence of specific IgM antibodies. (Brooks *et al.*, 1987; Remington and Klein, 1990). Serological tests on blood sample of a new born baby or of an umbilical cord can help detect a congenital infection or otherwise, although sometimes results can be ambiguous. IgG are passed from the mother to the baby through the placenta and could be of maternal origin. On the contrary, IgM cannot pass the placenta, so their presence in the baby is suggestive of an infection in the new-born. Nevertheless, not all infected babies produce IgM (Boyer, 1996), that means that the absence of IgM does not exclude congenital toxoplasmosis. In such a case, a follow-up is highly recommended. IgG-titres of maternal origin naturally decline in the first six months of a new-born baby. Persisting (or rising) IgG-titres prove congenital infection of the baby (Boyer, 1996). Therefore serological testing should be repeated until IgG turns negative, exclusion of Congenital Toxoplasmosis cannot be done before that.ss (Trojovsky *et al.*, 1998)

2.1.9.2. Molecular diagnosis

Serological diagnosis can be difficult in prenatal cases or in patients with immunodeficiency. The use of molecular diagnostic techniques is particularly appropriate for such patients, as these techniques do not depend on the immunological status of the host(Switaj *et al.*, 2005). The presence of *T. gondii* in a biological sample can be diagnosed by molecular techniques aimed at detecting its genetic material. The sensitivity and specificity of PCR-based methods depend on an appropriate technique for

isolation of genetic material (DNA) from samples, the characteristics of the DNA sequence chosen for amplification, and the parameters of the amplification reaction itself (Switaj *et al.*, 2005).

For detection of *T. gondii*, the sequence used mostly is the B1 gene, first identified in 1989 by Burg *et al.*, of which there are 35 copies in the genome. It is known to be very specific although its function is not yet known. Homan *et al* (2000) identified a 529-bp sequence, also specific for *T. gondii*, which has over 300 copies in the genome. A real time PCR amplification of the two sequences revealed a ten-fold specificity of the 529-bp sequence than the B1 sequence (Reischl *et al.*, 2003). Several other single-copy sequences, including the SAG1 SAG2, SAG3, SAG4, and GRA4 genes have been used as PCR targets (Pelloux *et al.,* 1998; Howe *et al.*, 1997; Rinder *et al.*, 1995). Recently, a set of markers which can provide a high resolution for the detection and genotyping of *T. gondii* were developed (Su *et al.*, 2006). These markers, capable of distinguishing the three clonal lineages of *T. gondii* include SAG2, SAG3, BTUB and GRA6 among others. In 2007, Dubey *et al* developed a nested PCR-RFLP protocol using these three-way multilocus markers. The nested PCR is to provide higher resolution for the detection and genotyping of *T. gondii*. The reproducibility of the protocol was confirmed by Prestrud *et al* (2008), through their work with brain tissues of Arctic foxes.

2.1.10 Treatment

Over the years, anti-parasitic agents that are able to restrict the growth of actively proliferating parasites which destroy cells and tissues and in so doing, prevent damage to the brain and eye, have been developed (Garin *et al.*, 1985, Araujo *et al.*, 1992, McLeod

et al., 1992, Derouin, 2001, Remington *et al.*, 2006, Meneceur *et al.,* 2008). Pyrimethamine and sulfadiazine have been the main drugs administered for the treatment of toxoplasmosis. Pyrimethamine interferes with the conversion of folic acid to folinic acid through dihydropteroate synthase (DHPS) whereas sulfadiazine interferes with the formation of folic acid from para-amino benzoic acid. Human beings unlike *T. gondii* can utilize exogenous folinic acid for their cells.

The combined therapy of pyrimethamine and sulfadiazine has been seen to be much more active than either pyrimethamine or sulfadiazine alone and has been the "gold standard" to which other antimicrobial agents (alone or in combination) have been compared (Remington *et al*., 2006, Mui *et al*., 2008).

In 1958, Garin and Eyles discovered spiramycin as an antitoxoplasmic agent in mice. Spiramycin has since been been used prophylactically in women during pregnancy to reduce transmission of the parasite from mother to fetus. This is because it is non-toxic and does not cross the placenta.

2.1.11 Prevention and control

Studies of the life cycle of *T. gondii* have led to the acquisition of adequate knowledge on the prevention and control of *T. gondii* infection (Frenkel, 1973). In spite of the multiple transmission routes of *T. gondii* infection, the risk of exposure to toxoplasmosis can be reduced by improved personal and environmental hygiene, meat processing standards and health education (Dunn *et al.*, 1999). Studies conducted have shown temperatures required to kill *T. gondii* oocysts in infected meat by freezing to about -20^{o}C, cooking to about 70^{o}C and by gamma irradiation (Frenkel *et al*., 1970). This information is now being used by regulatory bodies to educate the public about the

safety of meat. Meat should be cooked till the entire pink colour inside disappears and the juice becomes colourless. Tasting of meat while cooking is to be avoided till the meat is completely cooked. Several general sanitation and food safety steps are considered in the prevention of *Toxoplasma* infection. These include, the thorough washing of hands with soap and water, especially, after handling or having contact with a cat and its litter box, raw meat, fresh unwashed fruits and vegetables and soil, before eating. The appropriate treatment of fruits and vegetables before consumption is greatly encouraged. All fruits and vegetables eaten raw should be well washed and peeled of before eating them. The drinking of only treated water has been emphasised.

Routine diagnosis of pregnant women is very important in managing and controlling the infection. In other parts of the world immunisation of babies against *T. gondii* infection has been greatly encouraged (Araujo, 1994).

CHAPTER THREE

MATERIAL AND METHODS

3.1 Study Site

The Obstetrics and Gynaecology Department of the Korle-Bu Teaching Hospital (KBTH) in Accra was the study site. The KBTH is one of the main referral hospitals in Accra which recruits pregnant women from various parts of Accra and its environs. It is considered to be the busiest hospital in the country and its Obstetrics and Gynaecology Department being the busiest in the hospital. The birth delivery rate in this department currently lies between thirty to fifty deliveries per day.

3.2 Study Design

The study takes the form of a cross-sectional screening of pregnant women at the stage of delivery. The study also involved the infants of women at birth and two to six weeks post-delivery. Approval and Ethical clearance were obtained at the Noguchi Memorial Institute for Medical Research (NMIMR) from the Scientific and Technical Committee and the Institutional Review Board, respectively. Permission for the study was also obtained from the appropriate authorities at KBTH and the Ghana Health Service through the study collaborators. Study participants were recruited based on informed consent. Questionnaires were administered to seek personal information, toxoplasmosis-related knowledge, exposure to *Toxoplasma* infection risk factors and available medical history from hospital records. Maternal blood and cord blood samples were taken from placenta and umbilical cord respectively from volunteer women after the expulsion of the placental tissue from the women. Placental tissue samples were also collected from

the women. Blood samples were collected from infants of mothers between two to six weeks after birth. The blood samples were processed appropriately and tested using ELISA and PCR. The results were computerized and analysed appropriately according to the study objectives.

3.3 Study Population

The study population was made up of pregnant women due for delivery and aged between eighteen to forty-five years.

3.4 Study Participants

The study participants were made up of self-volunteered pregnant women within the study population and infants born to these women.

3.5 Sample Size

A hundred women were involved in this study for the cross-sectional screening. This number, based on the previous prevalence of 92.5% reported by Ayi et al in 2009, was generated using the formula:

$N= [Z^2 (P) (1-P)] / (Error)^2$

Where N= Sample size, Z= 1.96, Error= 5%, P= 92.5%

3.6 Informed consent and Questionnaire administration

Written consent was sought after the study has been explained to the understanding of participants and closed-ended questionnaires administered by the interviewer method following informed consent.

3.7 Sample Collection

3.7.1 Maternal Blood

About 2 ml venous blood sample was drawn from the maternal side of each placental tissue after delivery using sterile disposable hypodermic syringe and needle. About half the volume of the blood collected was dispensed into labeled EDTA tubes and the remaining into vacutainers containing serum separators and transported in a cool box containing frozen ice packs to the laboratory of the Parasitology Department of NMIMR for analysis. Serum was obtained by centrifugation of venous blood at 14000 rpm for 20 min and stored at -40ºC until use.

3.7.2 Umbilical Cord Blood

Up to 5 ml cord blood was taken from the vein of the umbilical cord post-delivery (after separation from the infant) by the same processes as described above for analysis.

3.7.3 Infant Blood

Up to 0.5ml of finger-prick blood blot samples were taken from participating babies at two or six weeks old using sterile disposable lancets and blotted on filter papers. The blood blot samples were kept at 4^{o}C until use for serum preparation and antibody detection test by ELISA.

3.7.4 Placental Tissues

A few (tiger nut sized) chunks of placental tissue were taken from the maternal side of each placenta with a pair of sterile surgical scissors and a pair of forceps. Samples of each placenta were put in 15 ml well labelled tubes filled with about 5 ml of saline and placed in a cool box. These were then transported to the Parasitology Department of NMIMR where they were stored frozen at -20^{o}C until processed and examined.

3.8 Analyses of Samples

3.8.1 ELISA

Commercial anti-*Toxoplasma* antibodies detection Enzyme Linked Immunosorbent Assay (ELISA) kits (CTK Biotech, Inc, USA) were used to detect IgG and/or IgM antibodies qualitatively from the serum samples according to manufacturer's instructions. Components of the Kit can be found in Appendix III

3.8.1.1 Anti-Toxoplasma IgG Test

Ninety-six well antigen-coated Microtitre plates provided in the kits were removed and allowed to come to room temperature. ELISA plate maps were designed and samples were loaded into the wells according to the plate maps. All sample-designated wells were filled with 100 ul of sample diluent and 10 ul of each sample respectively. Positive and negative controls-designated wells were filled with 100 ul of positive and negative controls respectively. Plates were rocked for about twenty seconds to mix the samples. Covered plates were incubated at 37^{o}C for 30 min. Plates were then flipped empty, banged and washed with wash buffer (PBS with Tween 20). This was repeated four more times. Wells were then filled with 100 ul of Horse Radish Peroxidase (HRP)-anti human IgG conjugate. Covered plates were then incubated for 20 min at 37^{o}C. 50 ul microliters of TMB substrate A and 50 ul of TMB substrate B were added to each well. Covered plates were incubated at 37^{o}C for 10 min in darkness. The reaction was stopped by adding 50 ul of stop buffer to each well. The plates were then read for absorbance at a wavelength of 450 nm against the blank well within 15 minutes after adding the stop solution using the Spectrophotometer, Multiskan assent (Thermo systems)

3.8.1.2 Anti-Toxoplasma IgM Test

The Procedure was the same for the anti- *Toxoplasma* IgG test, except that serum samples were loaded into the wells undiluted as per manufacturer's instructuions.

3.8.1.3 Cut-off Value

The cut-off value was set using the formula: **0.15 + N**. where N is the mean optical density (OD) of the negative controls, as instructed by the manufacturer

3.8.1.4 Calculation of specimen OD ratio

The OD ratio of each specimen was calculated by dividing the OD value by the cut-off value as provided by the manufacturer and shown below:

$$\textit{Specimen OD ratio} = \frac{\textit{Specimen OD}}{\textit{Cut-off Value}}$$

3.8.1.5 Assay Validation and Intepretation of results.

The mean OD of the Toxo IgG/IgM positive controls had to be ≥ 1.00/0.80 respectively. The mean OD of the Toxo IgG/IgM negative controls also had to be ≤ 0.10. Once these requirements were met the results were considered valid and interpreted as follows: Positive samples were those whose specimen OD ratios were greater than or equal to 1.0 while samples with specimen OD ratio less than 1.0 were considered negative.

3.8.2 Extraction of DNA

Genomic DNA was extracted from tissue samples using DNeasy® blood and tissue kit (QIAgen, USA). The protocol used was as follows:

Twenty-five milligrams of cut placental tissue from each participant's sample was put into a 1.5 ml microcentrifuge tube. Two hundred microliters of buffer ATL (tissue lysis buffer) was added to the tissues in the tube. Tissues were mechanically ground with

QIAgen protenase K was aliquoted into a 1.5 ml microcentrifuge tube. Two hundred microlitres of buffer AL (cell lysis buffer) was added to the mixture and mixed by pulse vortexing for 15 s.The mixture was incubated at 56ºC for 10 min and snap centrifuged to include condensed drops from inside the lid. Two hundred microlitres of absolute ethanol was added to precipitate the DNA molecules in the solution and mixed again by pulse-vortexing for 15 s and centrifuged. The mixture was carefully applied to the Dneasy® blood and tissue kit spin column (in a 2 ml collection tube) without wetting the rim. The cap was closed and centrifuged at 8000 rpm for 1 min. The spin column , with trapped DNA molecules, was placed in a clean 2ml collection tube and the tube containing the filtrate was discarded. Five hundred microlitres of Buffer AW1(first wash Buffer) was added to the spin column without wetting the rim and centrifuged at 14000 rpm for 1 min and placed in a clean 2 ml collection tube. The collection tube containing the filtrate was discarded. Five hundred microlitres of buffer AW2 (second wash buffer) was added to the spin column and centrifuged at 14000 rpm for 3 min. The spin column was placed in a clean 1.5 ml microcentrifuge tube. Fifty microlitres of buffer AE (DNA elution buffer) was added and incubated at room temperature for 5 minutes and centrifuged at 8000 rpm for 1 min. The purified DNA was stored at -20 ºC and used within a three weeks for PCR amplification.

3.8.3 Nested Polymerase Chain Reaction

The extracted DNA was applied in a Nested PCR (nPCR) using the appropriate primer sets in accordance with Prestrud *et al* (2008) with modification as follows. Reagents used and their concentrations are found in Table 3.1:

Table 3.1 Nested PCR reagents showing their various concentrations

Reagents	Nest 1	Nest 2
PCR Buffer	1x	1x
$MgCl_2$	2.5mM	2.5mM
DNTPs (dATP, dTCP, dCCP, dGTP)	2.0mM each	2.0mM each
Forward primer	0.1uM	0.3uM
Reverse primer	0.1uM	0.3uM
Taq polymerase	0.5units	0.5units
Template	5ul of DNA	1ul of nest 1 amplicon

The Nest 1 reaction condition was maintained at 95^{o}C for 4 min, followed by 25 cycles of 94^{o}C for 30 s, 55^{o}C for 1 min, and 72^{o}C for 1.5 min. The Nest 2 reaction condition was maintained at 95 ^{o}C for 4 min, followed by 35 cycles of 94 ^{o}C for 30 s, 60 ^{o}C for 1 min, and 72 ^{o}C for 1.5 min.

3.8.4 Agarose gel electophoresis

The PCR Products were then ran on a 2% agarose gel. Briefly, 100 ml of 1X Tris Acetate EDTA (TAE) buffer was measured into a conical flask. Two grams (ie 2% of 100 ml) of agarose powder was then weighed and added to the buffer. The suspension was heated in a microwave to get a uniform solution. The solution was allowed to cool and transferred into a gel cast system with combs having appropriate number of protrutions inserted into it to form wells. The gel was left for about 20 min to solidify. The cast gel was then transferred to a gel tank filled with buffer. Ten microliters of each nPCR product was loaded into the wells and then allowed to ran for 30 min at a voltage of 100 v. After electrophosesis, the gel was stained with Etidium Bromide for 10 mins

and viewed under UV to identify any bands corresponding to *T. gondii* (225bp for SAG3 gene and 344bp for GRA6 gene).

3.9 Data Analyses

Descriptive analyses for prevalence of *T. gondii* infection of were done using SPSS (version 16). The Phi coefficient (ø) was used to test the degree of association in the occurrence of IgG positives in matched maternal and foetal samples.

CHAPTER FOUR

RESULTS

4.1 General Characteristics of Study Participants

A total of hundred (100) women aged 18 to 45 years and their newly-born babies were involved in the study. The mean age of the women was 29.85 years, with 48.7% (37/76) and 23.7% (18/76) of the women having attained elementary and secondary educational levels respectively. 68.4% (52/76) are also doing some sort of vocational jobs. About 77% of the women were multi-gravid. The birth weight of babies born to the women ranged from 1.4 kg to 4.8 kg with an average weight of 3.2 kg. 55.2% (48/87) of the babies were males with 44.8% (39/87) being females. A total number of 312 samples were good for processing: 93 maternal (Placental) blood samples, 91 cord blood samples, 88 placental tissues and 40 infant blood samples.

4.2 Seroprevalence of Anti – *T. gondii* IgG in maternal blood

ELISA conducted on maternal blood samples to detect anti-*T. gondii* IgG and IgM showed 37.6% (35/93) to be positive for IgG antibodies(Table 4.1) while none of them were positive for IgM antibodies (Plate 1). Age distribution of the participating mothers are shown in table 4.2. The highest prevalence within the various age groups was seen in the 41-45 year olds with 100%.

Table 4.1 Overall Prevalence of Anti – *T. gondii* IgG in maternal blood

	No. tested	%
Positive	35	**37.6**
Negative	58	**62.4**
Total	93	**100**

Table 4.2 Prevalence of anti – *T. gondii* IgG in maternal blood by age groups

Age Group (yrs)	No. tested	No. positive (%)	Overall Prevalence (%)
18-24	16	6 (37.5)*	6.5
25-30	28	13 (46.4)*	13.9
31-35	24	10 (41.7)*	10.7
36-40	13	4 (30.7)*	4.3
41-45	2	2 (100)*	2.2
Missing ages	10	0 (0)*	0
Total	93	35 (37.6)*	**37.6**

* Prevalence within age group in parenthesis

4.3 Seroprevalence of Anti-*T. gondii* IgG in cord and Infant blood samples

Out of the 91 cord blood samples examined, 39.5% (36) were positive for IgG (Table 4.3) while all were negative for IgM (Plate 2). 57.5% (23/40) of blood samples

collected from infants 2-6 weeks post-natal were positive for IgG ELISA while none were positive for IgM

Table 4.3 Seroprevalence of anti -*T. gondii* IgG in cord and infant blood samples (IgG)

Type of Sample	No. tested	No. Positive	Overall Prevalence (%)
Cord Blood	91	36	39.5
Infant Blood (Finger Prick)	40	23	57.5

4.4 Prevalence of *T. gondii* DNA in placental tissue samples by PCR

Nested PCR done on total DNA from 88 Placental tissues to detect *T. gondii* showed 39.8% (35/88) of the tissues to be positive for either SAG3 or GRA6 primer regions (Table 4.4).

Table 4.4 Prevalence of *T. gondii* DNA in Placental tissues

Target genes	No. Positive	Percentage positive (%)
Only SAG 3	7	8.0
Only GRA 6	23	26.1
SAG 3 and GRA 6	5	5.7
total	35	39.8

Table 4.5 Summary of tests done on maternal and foetal samples

Sample origin	Sample tested	Test Performed	No. Tested	No. Positive (%)*
Maternal	Placental blood	IgG ELISA	93	35 (37.6)
	placental tissue	Nested PCR on extracted DNA	88	35 (39.8)
Foetal	Cord blood	IgG ELISA	91	36 (39.5)
	Infant Blood (Finger-Prick)	IgG ELISA	40	23 (57.5)
Total			312	

*- Percentage positive (prevalence) in parenthesis

4.5 Risk of Mother-to-Child transmission of *T. gondii*

To assess the risk of mother-to-child transmission, matched maternal (Placental blood and tissues) and foetal (cord and infant finger-prick blood) samples were used. Table 4.5 shows the categorization of the various biological samples obtained into maternal and foetal samples. Results showed 38.4% (33/86) of the matched samples to be positive for *T. gondii* in both mother and the infant (Table 4.6). 4.6% (4/86) of the matched samples came out to be positive for *T. gondii* in the mother and negative in the infant. The reverse was the case in 9.3% (8/86) of the matched samples.

Table 4.6 *T. gondii* prevalence in matched maternal and foetal samples

Test results	Number	Percentage (%)
*M(+) F(+)	33	38.4
M(+) F(-)	4	4.6
M(-) F(+)	8	9.3
M(-) F (-)	41	47.7
Total	86	100

(ø=0.722, P < 0.05) * - Maternal and Foetal samples' results

4.6 Exposure to Risk factors and infection status of pregnant women

Some of the risk factors assessed included the exposure of the women to cats either in their homes or vicinities, handling and eating of meats and vegetables either in their raw state or cooked. Results showed 30.6% (22/72) of the positive mothers (either for IgG ELISA or PCR) as having been exposed to cats whereas the other 12.5% (9/72) of the positive mothers not exposed to cats. 47.2% (34/72) of negative mothers have also been exposed to cats (Table 4.7). Only 6.5% (2/31) positive mothers questioned said they had sand boxes for their cats. 96.7% (30/31) of the positive mothers admitted to have handled raw meat in the recent. Out of the 93.1% (67/72) of women who admitted eating meat, only 41.8% (28/67) were positive for anti-*Toxoplasma* IgG. When asked whether the loved vegetables, all the women answered in the affirmative. 43.1% showed anti- *T. gondii* IgG seropositivity while 56.9% did not.

Table 4.7 Exposure to cats and infection status of both Mother and Baby

Infection Status of Mother and Baby	Exposure to cats (%)*	No Exposure to cats (%)*	Total (%)*
M (+) B (+)	19 (33.9)	9 (56.2)	28 (38.9)
M (+) B (-)	3 (5.4)	0 (0)	3 (4.2)
M (-) B (+)	6 (10.7)	1 (6.2)	7 (9.7)
M (-) B (-)	28 (50.0)	6 (37.5)	34 (47.2)
Total	56 (100)	16 (100)	72 (100)

*- Percentage within groups

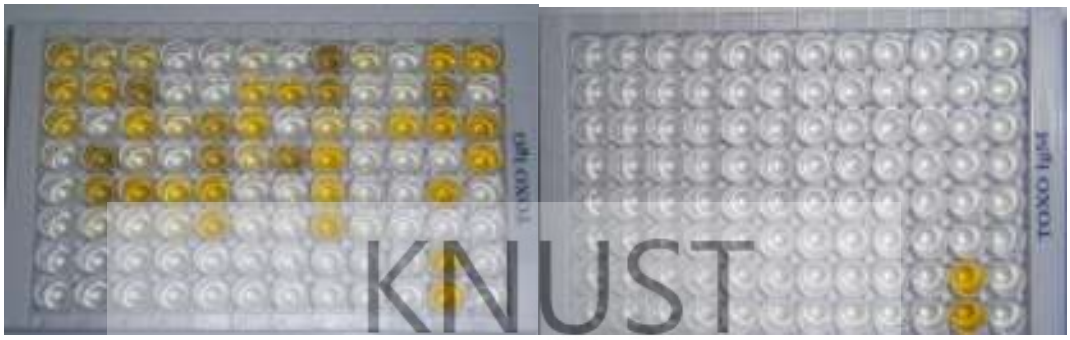

Plate 4: ELISA Plates showing the results of some maternal samples taken through the *T. gondii* IgG and IgM tests.

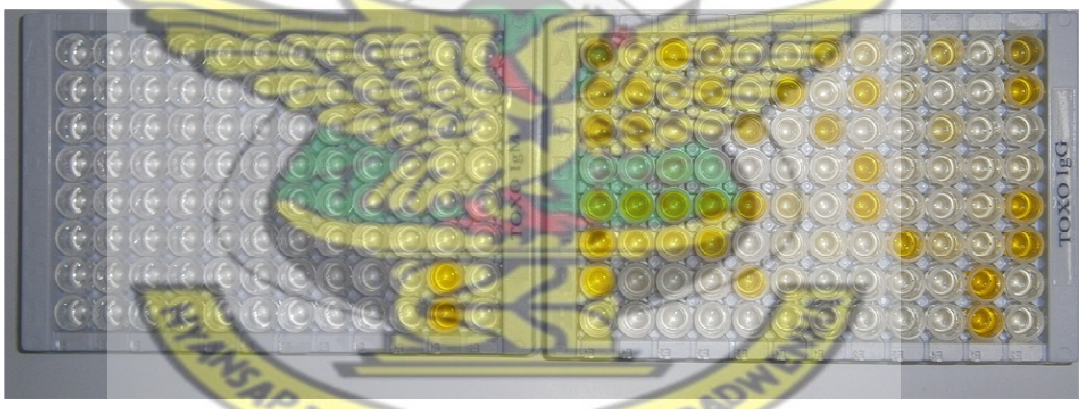

Plate 5: ELISA Plates showing the results of some Foetal samples taken through the *T. gondii* IgM and IgG tests.

CHAPTER FIVE

DISCUSSION

Toxoplasma gondii infection acquired by pregnant women during gestation and its transmission to the fetus continue to be the cause of tragic yet preventable disease in the offspring (Remington *et al*, 2006). The aim of this study is to assess the risk of mother-to-child transmission of *T. gondii*. An anti-*T. gondii* IgG sero-prevalence value of 37.6% (35/93) was found among the pregnant women, which included the only 2 women aged between 41 and 45 years. This sero-prevalence value is lower compared to the 73.6% IgG prevalence reported by Ayi *et al*, 2009, which included pregnant women in their first, second and third trimesters as opposed to only pregnant women due for delivery (third trimester) in this study. Seroprevalence of anti-*T. gondii* IgG among the cord blood samples was also recorded at 39.5% (36/91). Although results did not confirm the presence of anti-*T. gondii* IgM, the presence of anti-*T. gondii* IgG alone suggests a chronic infection in the pregnant women. The prevalence of *T. gondii* infection in placental tissues taken from the participating women after birth was found to be 39.8% (35/88). This is quite interesting because this is the first time placental tissues have been screened with PCR in Ghana.

The absence of IgM suggests that *T. gondii DNA* detected in placental tissues could be from bradyzoites contained in tissue cysts, which is an indication of latent infection in the pregnant women. The presence of anti-*T. gondii* IgG alone and *T. gondii DNA* indicate that the women might have had the infection during the pregnancy. In 2008,

Montoya and Remington advised that if serological test results suggest a recently acquired infection, an effort should be made to determine whether the infection was likely acquired during gestation or shortly before conception. If so, the foetus is at risk. Congenital infection of the foetus occurs if the mother acquires acute infection during pregnancy. An acute infection may result from a primary infection or re-activation of latent (chronic) infection in any case of immuno-depression (Luft and Remington, 1992; Isrealski and Remington, 1993). In a case where the women got infected before the pregnancy, the presence of tissue cysts still poses a high risk of congenital transmission by increasing the chances of re-activation, that is, the release of bradyzoites (which will later be transformed into tachyzoites) into the bloodstream, should there be a suppression of one's immune system.

Almost all anti-*T. gondii* IgG-positive maternal samples, 94.6% (35 out of 37) had corresponding anti-*T. gondii* IgG-positive foetal samples. Anti-*T. gondii* IgG in foetal samples showed a strong association (ϕ=0.722, $P < 0.05$) with the occurence of anti-*T. gondii* IgG in matched maternal blood samples, suggesting a high risk of congenital transmission of the infection to the infants. These anti-*T. gondii* IgG in umbilical cord and two weeks post-natal infant blood samples could be from maternal origin but that does not rule out the possibility of infection in the newly born baby. Follow-up on these babies for about a year could help one really conclude on infectivity, as was done and published by Trojovsky *et al* (1998). They followed up on babies with anti-*T. gondii* IgG at birth for about a year. Babies that turned sero-negative eventually were declared negative while those that remained sero-positive were declared positive and treated.

Until adequate follow-up screening is done, exclusion of congenital transmission cannot be done (Trojovsky *et al.*, 1998).

The 9.3% (8 out of 86 maternal-foetal matched samples) IgG-negative maternal samples with corresponding IgG-positive cord blood samples supports the finding that cord blood IgG level at term tends to exceed the respective maternal concentration, suggesting active placental transport from mother to fetus (Palmeira *et al.*, 2012). This goes to further indicate the risk of congenital transmission, though, it could also be that the anti-*T. gondii* IgG found in the 2-6 wk old babies could be produced as a result of external sources of infection.

None of the risk factors assessed showed any correlatation with the infection status of the participating mothers. The difference between *T. gondii* positive mothers and *T. gondii* negative mothers was not statistically significant ($P > 0.05$) for any of the risk factors assessed. The situation was not different with the most prevalent risk factor for *T. gondii* infection, possession or association with cats. Though, majority (19/28) of positive mothers indicated some sort of exposure to cats, the difference was not statistically different ($P > 0.05$) from negative mothers. McAllister (2005) indicated that a high risk to *Toxoplasma* infection is imposed on human communities that come into contact with cats. In contrast, most previous studies have established that cat ownership was less predictive for *Toxoplasma* Infection. It is important to note that recent epidemiological studies have not shown cat ownership to be a high risk factor for *T. gondii* infection (Jeffrey *et al.,* 2003). The risk of infection was not related in owning a cat but rather being exposed to the faeces of a cat shedding oocysts (Dubey, 1994).

Meat and vegetable consumption has also been widely reported as a risk factor for *T. gondii* Infection (Dubey and Beattie, 1988). In current study meat consumption recorded an IgG seropositivity of 41.8%. This prevalence was lower compared to those who eat meat but were negative for anti-*T.gondii* IgG. No association was found between meat consumption and *Toxoplasma* infection, although, previous studies in Ghana and elsewhere had reported infection in most farm animals especially pigs, sheep and goats. (Arko-Mensah *et al.*, 2000; Van der Puije *et al.*, 2000). In Ghana, it is a common practice to freeze meat and cook thoroughly before consumption. This practice could have significantly reduced the risk of meat-eating-related *Toxoplasma* infection and could have contributed to the lack of association of *Toxoplasma* infection. The case of vegetable consumption also showed no association to *Toxoplasma* infection. Since the study was conducted in an urban setting, it could be that people usually treat their vegetables through the process of washing and the application of salt solution or vinegar in some cases. Others to love to steam their vegetables before eating. These are a few reasons that could account for the lack of association to *T. gondii* infection.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

This study has showed at least a third of the participating pregnant women to be chronically infected with toxoplasmosis. It also established that more than 90% of women with anti-*T. gondii* IgG have corresponding babies also with anti-*T. gondii* IgG. Although IgG antibodies found in new-borns could be from maternal origin, it still does not rule out the possibility of congenital transmission of the Infection. It also established that more than 90% of women with anti-*T. gondii* IgG also harbour *T. gondii* DNA which may be from cysts.

There is a weak association between risk factors (exposure to cats, eating meat and vegetables etc.) and infection status of participating women. These results suggest an indication of high risk of congenital transmission of *T. gondii* in Ghana and also provide baseline data for future work to really ascertain the rate of mother-to-child transmission in Ghana.

6.2 Recommendation

To really ascertain the rate of congenital transmission of *Toxoplasma gondii* infection in new borns, it is important to do follow-up screening of the babies for at least one year. It is also important to expand the sample size and be extended to other hospitals and health

centers in the Greater Accra region. It will be also useful to compare the rate of Congenital transmission of the infection in urban and rural areas.

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APPENDICES

Appendix Ia : Raw Data of all samples Tested

	Sample ID	Maternal (IgG)	Cord (IgG)	Placenta (PCR)	2 weeks (IgG)
1	F1 001	-	Negative	-	-
2	F1 002	-	Positive	-	-
3	F1 003	Negative	Negative	Negative	Negative
4	F1 004	Negative	Negative	Negative	-
5	F1 005	Positive	Positive	Positive	-
6	F1 006	Negative	Negative	Negative	-
7	F1 007	Negative	Negative	Negative	-
8	F1 008	Negative	Negative	Negative	-
9	F1 009	Negative	Negative	Negative	-
10	F1 010	Negative	Negative	Negative	-
11	F1 011	Negative	Negative	Negative	Negative
12	F1 012	Negative	Negative	Negative	Positive
13	F1 013	Positive	Positive	Positive	Positive
14	F1 014	Negative	Negative	Negative	-
15	F1 015	Negative	Negative	Negative	-
16	F1 016	Positive	Positive	Positive	-
17	F1 017	Positive	Negative	Negative	-
18	F1 018	Positive	Positive	Positive	Positive
19	F1 019	Negative	Negative	Positive	Positive
20	F1 020	Negative	Negative	Negative	-
21	F1 021	Positive	-	positive	-
22	F1 022	Positive	Positive	Positive	-
23	F1 023	Positive	Positive	Negative	-
24	F1 024	Negative	Negative	Negative	-
25	F1 025	Negative	Negative	Negative	Negative
26	F1 026	Negative	Negative	Negative	-
27	F1 027	Negative	Negative	Negative	Negative
28	F1 028	Negative	Negative	Negative	Negative
29	F1 029	Negative	Negative	Negative	-
30	F1 030	negative	Positive	Negative	Negative
31	F1 031	Negative	Negative	Negative	Negative
32	F1 032	Positive	Positive	Positive	Positive
33	F1 033	Negative	Negative	Negative	Positive
34	F1 034	Negative	Negative	Negative	-
35	F1 035	Negative	Negative	Negative	-

36	F1 036	Negative	Negative	Negative	Negative
37	F1 037	Positive	Positive	Negative	Positive
38	F1 038	Negative	Negative	Positive	-
39	F1 039	Negative	Negative	Negative	Negative
40	F1 040	Positive	Positive	Positive	-
41	F1 041	Positive	Positive	Positive	Positive
42	F1 042	Negative	Negative	Positive	Negative
43	F1 043	Positive	negative	Negative	Positive
44	F1 044	Positive	Positive	Positive	-
45	F1 045	Negative	Negative	Negative	Negative
46	F2 001	Negative	Negative	Negative	-
47	F2 002	Negative	Negative	Negative	-
48	F2 003	Positive	Positive	Positive	-
49	F2 004	Negative	Negative	Negative	-
50	F2 005	Negative	Negative	Negative	Positive
51	F2 006	Positive	Positive	Positive	-
52	F2 007	Positive	Positive	Positive	-
53	F2 008	Positive	Positive	Positive	Positive
54	F2 009	Negative	Negative	Negative	Negative
55	F2 010	-	-	-	-
56	F2 011	Positive	Positive	Positive	-
57	F2 012	Positive	Positive	Positive	-
58	F2 013	Negative	Positive	Positive	Negative
59	F2 014	Negative	Negative	Negative	-
60	F2 015	Negative	-	Negative	-
61	F2 016	Positive	Positive	Positive	Positive
62	F2 017	Negative	Negative	Negative	-
63	F2 018	Positive	Positive	Positive	Positive
64	F2 019	Positive	Positive	Positive	-
65	F2 020	Negative	Negative	Negative	Negative
66	F2 021	Negative	Negative	Negative	-
67	F2 022	Negative	Negative	Negative	Negative
68	F2 023	Negative	Negative	Negative	Positive
69	F2 024	Negative	Negative	Negative	Negative
70	F2 025	Negative	Negative	Negative	-
71	F2 026	Positive	Positive	Positive	Positive
72	F2 027	Negative	Negative	Negative	-
73	F2 028	-	-	-	-
74	F2 029	Positive	Positive	Positive	-
75	F2 030	Negative	Negative	Negative	Positive
76	F2 031	Negative	Negative	Negative	-
77	F2 032	Positive	Positive	Positive	-

78	F2 033	Positive	Positive	Positive	Positive
79	F2 034	Positive	Positive	Positive	-
80	F2 035	Positive	Positive	Positive	-
81	F2 036	-	Positive	-	-
82	F2 037	Negative	Negative	Negative	-
83	F2 038	Positive	Positive	Positive	-
84	F2 039	Positive	-	Positive	Negative
85	F2 040	Positive	Positive	Positive	-
86	F2 041	Positive	Positive	Positive	Positive
87	F2 042	Positive	Positive	Positive	Positive
88	F2 043	Negative	Negative	Negative	-
89	F2 044	Negative	Negative	Negative	Positive
90	F2 045	Negative	Negative	Negative	Positive
91	F2 046	Positive	Positive	Positive	Positive
92	F2 047	-	Positive	-	Positive
93	F2 048	Negative	Negative	negative	-
		87 total	88 total	87 total	40 total
		35 positve	36 positve	35 positive	23 positive

- No sample collected

Appendix I b : Raw Data on Matched samples

	Sample ID	Maternal (IgG)	Cord (IgG)	Placenta (PCR)	2 weeks (IgG)
1	F1 003	Negative	Negative	Negative	Negative
2	F1 004	Negative	Negative	Negative	-
3	F1 005	Positive	Positive	Positive	-
4	F1 006	Negative	Negative	Negative	-
5	F1 007	Negative	Negative	Negative	-
6	F1 008	Negative	Negative	Negative	-
7	F1 009	Negative	Negative	Negative	-
8	F1 010	Negative	Negative	Negative	-
9	F1 011	Negative	Negative	Negative	Negative
10	F1 012	Negative	Negative	Negative	Positive
11	F1 013	Positive	Positive	Positive	Positive
12	F1 014	Negative	Negative	Negative	-
13	F1 015	Negative	Negative	Negative	-
14	F1 016	Positive	Positive	Positive	-
15	F1 017	Positive	Negative	Negative	-
16	F1 018	Positive	Positive	Positive	Positive
17	F1 019	Negative	Negative	Positive	Positive
18	F1 020	Negative	Negative	Negative	-
19	F1 022	Positive	Positive	Positive	-
20	F1 023	Positive	Positive	Negative	-
21	F1 024	Negative	Negative	Negative	-
22	F1 025	Negative	Negative	Negative	Negative
23	F1 026	Negative	Negative	Negative	-
24	F1 027	Negative	Negative	Negative	Negative
25	F1 028	Negative	Negative	Negative	Negative
26	F1 029	Negative	Negative	Negative	-
27	F1 030	negative	Positive	Negative	Negative
28	F1 031	Negative	Negative	Negative	Negative
29	F1 032	Positive	Positive	Positive	Positive
30	F1 033	Negative	Negative	Negative	Positive
31	F1 034	Negative	Negative	Negative	-
32	F1 035	Negative	Negative	Negative	-
33	F1 036	Negative	Negative	Negative	Negative
34	F1 037	Positive	Positive	Negative	Positive
35	F1 038	Negative	Negative	Positive	-
36	F1 039	Negative	Negative	Negative	Negative
37	F1 040	Positive	Positive	Positive	-

38	F1 041	Positive	Positive	Positive	Positive
39	F1 042	Negative	Negative	Positive	Negative
40	F1 043	Positive	negative	Negative	Positive
41	F1 044	Positive	Positive	Positive	-
42	F1 045	Negative	Negative	Negative	Negative
43	F2 001	Negative	Negative	Negative	-
44	F2 002	Negative	Negative	Negative	-
45	F2 003	Positive	Positive	Positive	-
46	F2 004	Negative	Negative	Negative	-
47	F2 005	Negative	Negative	Negative	Positive
48	F2 006	Positive	Positive	Positive	-
49	F2 007	Positive	Positive	Positive	-
50	F2 008	Positive	Positive	Positive	Positive
51	F2 009	Negative	Negative	Negative	Negative
52	F2 011	Positive	Positive	Positive	-
53	F2 012	Positive	Positive	Positive	-
54	F2 013	Negative	Positive	Positive	Negative
55	F2 014	Negative	Negative	Negative	-
56	F2 016	Positive	Positive	Positive	Positive
57	F2 017	Negative	Negative	Negative	-
58	F2 018	Positive	Positive	Positive	Positive
59	F2 019	Positive	Positive	Positive	-
60	F2 020	Negative	Negative	Negative	Negative
61	F2 021	Negative	Negative	Negative	-
62	F2 022	Negative	Negative	Negative	Negative
63	F2 023	Negative	Negative	Negative	Positive
64	F2 024	Negative	Negative	Negative	Negative
65	F2 025	Negative	Negative	Negative	-
66	F2 026	Positive	Positive	Positive	Positive
67	F2 027	Negative	Negative	Negative	-
68	F2 029	Positive	Positive	Positive	-
69	F2 030	Negative	Negative	Negative	Positive
70	F2 031	Negative	Negative	Negative	-
71	F2 032	Positive	Positive	Positive	-
72	F2 033	Positive	Positive	Positive	Positive
73	F2 034	Positive	Positive	Positive	-
74	F2 035	Positive	Positive	Positive	-
75	F2 037	Negative	Negative	Negative	-
76	F2 038	Positive	Positive	Positive	-
77	F2 040	Positive	Positive	Positive	-
78	F2 041	Positive	Positive	Positive	Positive
79	F2 042	Positive	Positive	Positive	Positive

80	F2 043	Negative	Negative	Negative	-
81	F2 044	Negative	Negative	Negative	Positive
82	F2 045	Negative	Negative	Negative	Positive
83	F2 046	Positive	Positive	Positive	Positive
84	F2 048	Negative	Negative	negative	-
		33 positive	33 positive	33 positive	22 positive

- No sample collected

Appendix II: Nested PCR Primer sets

Marker	Primers (external forward, external reverse, internal forward, internal reverse
SAG3	P43S1: CAACTCTCACCATTCCACCC; P43AS1: P43S2: TCTTGTCGGGTGTTCACTCA; P43AS2: CACAAGGAGACCGAGAAGGA
GRA6	GRA6-F1x: ATTTGTGTTTCCGAGCAGGT; GRA6-R1: GCACCTTCGCTTGTGGTT GRA6-F1: TTTCCGAGCAGGTGACCT; GRA6-R1x: CGCCGAAGAGTTGACATAG

Appendix III: List of Reagents contained in the RecombiLISA Toxo Test Kit (CTK Biotech, Inc., USA)

IgG Test Kit	**IgM Test Kit**
96 well Toxo antigen coated microtitre plates	96 well Toxo antigen coated microtitre plates
11 mL Sample diluent	6 mL HRP-anti human IgM conjugates
11 mL HRP-anti human IgG conjugates	0.5 mL Toxo IgM positive control
0.5 mL Toxo IgG positive control	0.5 mL Toxo IgM negative control
0.5 mL Toxo IgG negative control	20 mL Concentrated wash buffer (30X)
20 mL Concentrated wash buffer (30X)	6 mL TMB substrate A
6 mL TMB substrate A	6 mL TMB substrate B
6 mL TMB substrate B	6 mL Stop solution
6 mL Stop solution	2 sets ELISA working sheet
2 sets ELISA working sheet	1 set Product insert (instruction of use)
1 set Product insert (instruction of use)	

Appendix IV: Questionnaire for pregnant women

RISK OF MOTHER-TO-CHILD TRANSMISSION OF *TOXOPLASMA GONDII* INFECTION AMONG PREGNANT WOMEN IN THE GREATER ACCRA REGION

QUESTIONNAIRE FOR PREGNANT WOMEN

Kindly provide the needed information in the questionnaire below. You may tick the appropriate box or boxes (☑) as indicated. Thank you.

PART A: PERSONAL INFORMATION

ID No:.:…… Date:……/……..…/…....… (dd/mm/year)

Name:…… Age (years):…..…

Area of residence: …… Duration at residence: ……

Contact address: …….. Telephone No: ……..

Educational background

No formal education ☐

Elementary ☐

Secondary/Vocational ☐

Tertiary (Polytechnic, university, etc) ☐

Other (specify): ……..

Where do you work?

Office (bank, school, etc) ☐

Hospital (labour ward, theatre, accident/emergency centre, blood bank, cardio centre)	
Hospital (records, OPD, nurse/mid-wife/theatre, wards)	
Slaughter House	
Garden/farm	
Market (sell vegetables, raw meat)	
Vocational Centre (carpentry, fitting mechanic)	
Other (specify)……	

PART B: TOXOPLASMOSIS-RELATED INFORMATION

Have you ever heard of "*Toxoplasma or Toxoplasmosis"*? Yes No

Have you ever been tested for *Toxoplasma* infection? Yes No

If your answer to Question (Qu.) 2. is "No", please skip Qu. 3, 4and 5. Go to Qu. 6

If "Yes", please answer Qu. 3, 4 and 5 before continuing with Qu. 6

When was the test conducted?

3 to 6 months ago

Up to a year ago

More than a year ago

Other (specify)……..

What was the result? Positive Negative No idea

If positive, did you receive any treatment? Yes No

Would you like to be tested for *Toxoplasma* infection during this pregnancy?

Yes No

How many pregnancies have you had before the current pregnancy?

None (this is my first)

One

Two

Three

More than three

Have you ever had a stillbirth, spontaneous abortion(s) [miscarriage(s)]?

Once

Twice

Three times or more

Never

How old is your current pregnancy?

1 to 3 months

4 to 6 months

7 to 9 months

Do you own a cat or have cats in your house or your neighbourhood?

Yes No

If "Yes" to Qu. 10, please answer Qu. 11. If "No" skip Qu. 11

Do you have a sand box for your cat?Yes No

Have you ever handled raw meat from pig, sheep, goat or cow?

Yes No

Do you eat meat? Yes No, I am a vegetarian

If your answer to Qu. 13 is "No'' please skip and go to Qu. 16

In which form do you often eat your meat?

Cooked but tough

Cooked till soft

Cooked tough or soft

Which meat do you eat? (Please c*hoose as many as applicable.*)

Pork

Goat meat/mutton

Beef

Chicken

No idea

Others (specify)……

Do you eat khebabs? Yes No

If yes, what type do you enjoy most?

Pork

Goat meat/mutton

Beef

Chicken

No idea

Others (specify)……

Are you a lover of vegetables? Yes No

In what state do you prefer your vegetables before eating them?

Fresh and raw

Steamed

Cooked

Where will you attend your post-natal clinic after delivery?

In this hospital (Ada Health Centre)

Faith Kope (Dangme East District Hospital)

In this Hospital (Korle-Bu Hospital)

Korle-Bu Polyclinic

Other (specify)……

Will you allow a blood sample to be taken from your baby at 2 or 6 weeks and between seven and twelve months after birth? Yes No

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Thank you very much

Appendix V: CONSENT FORM

Title: Risk Of Mother-To-Child Transmission Of *Toxoplasma Gondii* Infection Among Pregnant Women In Two Health Facilities In The Greater Accra Region

Principal Investigator: Kofi Dadzie Kwofie (MPhil. Student).

Address: 1. Department of Clinical microbiology, School of Medical Sciences, KNUST, Kumasi

2. Department of Parasitology, NMIMR, CHS, University of Ghana. Legon, Accra.

General Information about Research

Dear Madam,

You/your child (in the future) are/is kindly invited to take part in this study voluntarily and you are at liberty to opt out without any consequence.

Purpose of study: To find out how many pregnant women who become infected with *Toxoplasma gondii* during pregnancy will give the infection to their babies by the time they are born.

Study Background: Toxoplasmosis is a serious disease that affects all types of animals including humans. It is caused by *Toxoplasma gondii, a small germ* that can live in the blood, brain and the flesh of infected animals, including humans. If a pregnant woman gets the infection for the first time during pregnancy, through eating undercooked meat, unwashed vegetables or accidentally, soil which has cat faeces, she can also give it to the unborn child in the womb. This can cause the baby to die in the womb, or born alive with an abnormally big head, or eye problems as s/he grows, and many more abnormalities. The disease can be treated with appropriate drugs but many Ghanaians seem to know very little about toxoplasmosis.

How it will be done: You will answer questions from a questionnaire for yourself. Samples of your blood and placental tissues (about half a teaspoon full – 2.5 ml) will be taken from the placenta when you come to deliver your baby and from the cord attached to the placenta after it is separated from your baby. Some blood samples (less than half a teaspoon – 0.5 ml) will also be taken from the heel or arm vein of your child at 2 or 6 weeks after birth to check if s/he has the infection. Blood will be taken by an expert using a new sterilized hypodermic needle and syringe. Samples will be tested for *Toxoplasma* infection and the test results will be communicated to you through your doctor.

Possible Risks and Discomforts

There is no risk involved but your child will experience temporary pain when blood is being drawn. You are at liberty to opt out whenever you wish to.

Possible Benefits

Results of the study will inform your doctor to give you/r child the necessary medication and/or advice.

Confidentiality

Your personal information including your name and all other details provided in the questionnaire will be kept confidential for reference purposes by investigators and not disclosed to anyone. All samples will be given identification codes.

Voluntary Participation and Right to Leave the Research

Participation in this research is fully voluntary therefore you have the right to leave the research whenever you wish to.

Contacts for Additional Information

If you have any questions about the study, you may contact any of the following people:

Mr. Kofi Dadzie Kwofie (Noguchi Memorial Institute of Medical Research;

Tel 024 4115532)

Dr. Irene Ayi (Noguchi Memorial Institute of Medical Research; Tel 024 3670493)

Prof. Obed (Obstetrics/Gynaecology Department, Korle-Bu Teaching Hospital; Tel 020-8190973)

Your rights as a Participant

This research has been reviewed and approved by the Institutional Review Board of Noguchi Memorial Institute for Medical Research (NMIMR-IRB). If you have any questions about your rights as a research participant you can contact the IRB Office between the hours of 8am-5pm through the landline 0302916438 or email addresses: nirb@noguchi.mimcom.org or HBaidoo@noguchi.mimcom.org . You may also contact the chairman, Rev. Dr. Ayete-Nyampong through mobile number 0208152360 when necessary

Appendix VI: VOLUNTEER AGREEMENT

The above document describing the benefits, risks and procedures for the research title (Risk Of Mother-To-Child Transmission Of *Toxoplasma Gondii* Infection Among Pregnant Women In The Greater Accra Region) has been read and explained to me. I have been given an opportunity to have any questions about the research answered to my satisfaction. I agree to participate as a volunteer.

_____ _____

Date Name and signature or mark of volunteers

If volunteers cannot read the form themselves, a witness must sign here:

I was present while the benefits, risks and procedures were read to the volunteer. All questions were answered and the volunteer has agreed to take part in the research.

_____ _____

Date Name and signature of witness

I certify that the nature and purpose, the potential benefits, and possible risks associated with participating in this research have been explained to the above individual.

_____ _____

Date Name /Signature of Person Who Obtained Consent