CYTOKINE RESPONSES TO *TOXOPLASMA GONDII* IN IMMUNE-COMPETENT AND IMMUNE-COMPROMISED INDIVIDUALS AT KORLE-BU TEACHING HOSPITAL, GHANA

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 work is dedicated to my wonderful family. To my parents; Mr. and Mrs. Napoleon and Charity Kartey. My brothers and cousins; Eli, Patrick, Jonathan, Fidelia and Seyram. Without you this thesis would not have been possible.

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ABSTRACT

Toxoplasmosis, a disease known to impart a significant burden to public health, is caused by the parasite *Toxoplasma gondii*. An understanding of the relationship between the cellular immune response and pathology of the disease is needed to control this disease. The study thus sought to characterize the cellular immune responses to *T. gondii in* immune-competent and immunecompromised persons in Ghana. Venous blood was collected from 54 blood donors (HIV seronegative) and 38 HIV seropositive (ART naïve) participants and transported to the Noguchi Memorial Institute for Medical Research. Laboratory analysis was performed for the presence or absence and further genotyping of *T. gondii* using PCR. ELISA was done to obtain levels of cytokines in supernatants of stimulated PBMCs. Socio-demographic data and information on risk factors were obtained from the participants through questionnaires and hospital folders. The results did not show any statistically significant associations between *Toxoplasma gondii* positivity and exposure to any known risk factors such as cat ownership ($p<0.05$). The overall prevalence of *T. gondii* DNA was 26.3% and 14.8% in the HIV seropositive and blood donor participants; respectively. Levels of cytokines IL-10, TNF-α, IFN-γ, TGF-β were found to be significantly higher ($p<0.05$) in HIV seropositive participants than blood donor participants, however, differences in the cytokine levels of *T. gondii* positive, HIV-seropositive individuals compared against *T. gondii* positive, blood donor individuals, showed no statistical significance indicating that other pathogens may play contributing roles to the cytokine levels in the HIV seropositive participants. TGF-β levels for *T. gondii* positive persons differed significantly ($p<0.05$) suggesting that TGF-β may influence the immune status of *T. gondii*-infected persons. The patterns of the cytokine profiles in *T. gondii*positive and *P. falciparum*-positive individuals were found to be comparable indicating a similar mechanism of action of these parasites.

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LIST OF ABBREVIATIONS

ABA	Abscisic acid
AIDS	Acquired Immune Deficiency Syndrome
BSA	Bovine Serum Albumin
DAT	Dye Agglutination Test
DNA	Deoxyribonucleic Acid
ELISA	Enzyme Linked Immunosorbent Assay
GRA	Granule Antigen
HIV	Human Immune-deficiency Virus
IFA	Indirect Fluorescent Antibody
IHA	Indirect hemagglutination test
IL	Interleukin
INF-γ	Interferon gamma
IRB	Institution Review Board
ISAGA	Immunosorbent Agglutinatin test
KBTH	Korle-Bu Teaching Hospital
KNUST	Kwame Nkrumah University of Science and Technology
MAT	Modified Agglutination test
mRNA	Messenger Ribonucleic Acid
NBS	National Blood Service
NMIMR	Noguchi Memorial Institute for Medical Research

NO	Nitric Oxide
OPD	Out Patients Department
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate buffered Saline
PCR	Polymerase Chain Reaction
RDT	Rapid Diagnostics Kit
Rh D	Rhesus Factor
RFLP	Restriction Fragment Length Polymorphism
SAG	Surface Antigens
STC	Science Technical Committee
TBS	Tris Borate EDTA buffer
TGF-β	Transforming Growth Factor-beta
Th	T helper
TMB	3,3',5,5'-tetramethylbenzidine
TNF-α	Tumor Necrosis Factor alpha
WHO	World Health Organization

CHAPTER ONE

INTRODUCTION

1.1 Background

Parasitic protozoans are unicellular organisms that cause a variety of devastating and lifethreatening diseases in humans as well as animals (Doerig *et al.,* 2000). *Toxoplasma* is one of the most common protozoan parasites (Flegr *et al.*, 2014) and causes the disease Toxoplasmosis.

Toxoplasma gondii is an issue of public health concern because of its significant impact on society (Hill *et al.*, 2005; Torgerson and Mastroiacovo, 2013). There have been reported outbreaks of toxoplasmosis in 1995 (Bowie *et al.,* 1997) and between 2001 and 2002 (de Moura *et al.*, 2006) in a number of countries. It is estimated that between 25 to 30% of the world's populace is infected with the parasite; however, the prevalence varies widely among countries with the highest prevalence of 50-80 % being found in Latin America and in sub-Saharan Africa (Krueger *et al.*, 2014; Robert-Gangneux and Dardé, 2012). In Ghana, a sero-prevalence of 6687% of anti-*Toxoplasma gondii* antibodies was detected in ocular toxoplasmosis patients (Anteson *et al*., 1980; Abu, *et al.*, 2014) whereas an overall anti-*Toxoplasma gondii* antibody IgM, IgG and IgA sero-prevalence of 92.5% was recorded in pregnant women (Ayi *et al*., 2005; 2009). In HIV sero-positive patients and in sero-negative individuals from the Korle-bu Teaching Hospital in Ghana, a prevalence of 54.7% and 3.4%, respectively, was detected using Polymerase Chain Reaction (PCR) (Ayi *et al.*, 2016).

Toxoplasma gondii infects a wide range of warm blooded animals but employs domestic and wild cats in the felidae family as its definitive host (Frenkel *et al.*, 1970; Dubey, 2006; Krueger *et al.*, 2014). Two characteristically distinct features of *Toxoplasma* life cycle enable the parasite to be

ubiquitous; its ability to infect and survive in a vast number of intermediate hosts and its ability to produce several environmentally resistant oocysts through its definitive host (Dubey, 2009). Dormant bradyzoite cysts found in the tissues of animals can be passed on to the consumer, if the meat is not properly cooked, leading to infection (Hill *et al.*, 2005). Others may also be infected when they accidentally consume sporulated oocysts found in food, soil and water contaminated with cat faeces (Khan *et al.*, 2005b; Robert-Gangneux & Dardé, 2012). Tachyzoites, (actively dividing trophozoites), could also be transmitted via blood transfusions or from mother to child congenitally (Dubey *et al.*, 2012; Torgerson & Mastroiacovo, 2013). In the infected host, parasites usually encyst in various organs and tissues, but are most commonly found in the brain (Webster *et al.*, 2006; Krueger *et al.*, 2014).

Approximately 90% of individuals infected with the parasite are asymptomatic, however the disease can be distressing in congenitally infected children and immune-compromised individuals (Arko-Mensah *et al.*, 2000; Weiss and Dubey, 2009; Flegr *et al*., 2014). In HIV/AIDS sero-positive patients, persons with haematological malignancies and patients who undergo transplantations, toxoplasmic encephalitis may be the most frequent clinical presentation indicative of parasite infection (Ajzenberg *et al.*, 2009).

Toxoplasma gondii, comprises 15 distinct clonal population genetic structures referred to as types. Types I, II and III are predominantly human strains with the Type I lineage strains being highly virulent (Grigg and Ganatra, 2001; Ivović *et al.*, 2007; Xiao and Yolken, 2015). Mixed infections with different clonal lineages of *T. gondii* have been shown in mice (Khan *et al*., 2005a). Such infections result in very different clinical outcomes (Khan *et al.*, 2005a) with heightened virulence of Type I strains partly due to overstimulation of pro-inflammatory Th1 immune response that leads to pathology (Hill *et al.*, 2012). Studies suggest that Type I strains are more likely to cause

severe disease in immune-compromised patients and hence an effective immune system is essential for controlling the parasite (Su *et al.*, 2006). In an immunecompetent host, the periodic rupture of the bradyzoite cysts is thought to be the origin of sustained immunity against toxoplasmosis (Filisetti and Candolfi, 2004).

The hosts' regulation of the disease is complex and depends on factors including the genetic background of the host, immune status and parasite factors such as virulence (Filisetti and Candolfi, 2004; Robert-Gangneux and Dardé, 2012). Following the lysis of an infected cell, the antibodies generated by the host act on the extracellular tachyzoite stage by opsonizing the parasites for phagocytosis, and also blocking the invasion of other cells by the tachyzoites to limit the proliferation of the parasite. The antibodies can also initiate the complement system (Filisetti and Candolfi, 2004; Dupont *et al*., 2013). Antibodies have also been shown to stimulate the development of intra-cerebral cysts (Mozaffarian *et al.*, 2014). The formation of these cysts act as an evasive mechanism for the parasite and promotes its survival as the parasite tends to hide itself in parts of the host with very little immune activity such as the brain and muscles (Black and Boothroyd, 2000).

In the immune response to *Toxoplasma,* antibodies play a minor role but are essential for the diagnosis of the disease in humans. Cellular immunity is therefore the major immune response to the infection (Filisetti and Candolfi, 2004). The T-lymphocytes, natural killer cells, and macrophages are the main immune cells involved in cellular immune response mediated by cytokines with key actors being $CD8^+$ TL cells (Filisetti and Candolfi, 2004; Dupont *et al*., 2013).

Interferon gamma (IFN-γ) was the first cytokine associated with resistance to *T. gondii* and has been found to increase the phagocytic and cytotoxic activity of macrophages and $CD8^+$

Tlymphocytes respectively (Denkers *et al.*, 1998). This cytokine which is produced by $CD8^{+}$T lymphocytes (Dupont *et al.*, 2013) is also known to trigger the transformation of tachyzoites to bradyzoites. A high level of IFN-γ production has also been implicated with virulent Type-1 strains (Filisetti & Candolfi, 2004). Interleukin-12 (IL-12) has been found to play a foremost role againt *T. gondii* during acute and chronic stages of the infection (Tait & Hunter, 2009). Tumor necrosis factor-α (TNF-α) induced by interleukin-6 (IL-6) acts in synergy with interleukin-1 (IL1) to fight against the parasites by activating human microglia which are known to inhibit proliferation of tachyzoites within the cell (Suzuki *et al*., 2012). Other cytokines implicated to have a protective activity against *T. gondii* are IL-2, IL-15, IL-17 and IL-18. Cytokines which have been implicated to have an anti-inflammatory regulatory function during toxoplasmosis are

IL-4, IL-10 and transforming growth factor β (TGF- β) (Filisetti *et al.,* 2004; Kelly *et al.*, 2004). These Th2 type immune responses are associated with protective immunity and also aid in down regulating the severe immunopathology mediated by Th1 cytokines (Suzuki *et al.*, 2012)

1.2 Problem Statement

Studies have shown that toxoplasmosis elicits a Th1 response primarily from the cell-mediated branch of the human immune response system. Th1 responses are generally characterized by the production of interferon gamma (IFN-γ), lymphotoxin, and tumor necrosis factor alpha (TNF-α) which in turn activate the parasiticidal activities of macrophages leading to opsonization of the microbe

Toxoplasma gondii is ubiquitous in nature and as such all groups of persons are at risk of infection. In Ghana studies report high prevalence of anti-*Toxoplasma* sero-positivity among a cross-section

of the Ghanaian population and also reports evidence of congenital disease caused by *Toxoplasma gondii* infections ranging from 52%-90% (Anteson *et al*., 1978, Ayi *et al*., 2005, 2009, 2016; Blay *et al*., 2015). Ayeh-Kumi *et al*., (2010) also reported that the seroprevalence of anti-*Toxoplasma* gondii antibodies IgG among the healthy Ghanaian population was 32.7%.

Armed with these high prevalence rates of sero-positive anti-*Toxoplasma* antibodies it is important to determine the immune responses that the human body elicits during *Toxoplasma gondii* infection. Possible immune responses that are generated with co-infection with other apicomplexan haemoparasites such as *Plasmodium* also need to be investigated. This will help understand whether co-infections produce synergistic or antagonistic effects.

1.3 Justification of study

Knowledge of cytokine responses elicited during toxoplasmosis is still very limited. Furthermore, information from the co-infection of *Toxoplasma gondii* with other parasites such as *Plasmodium spp* and soil transmitted helminths, which could either have a synergistic or antagonistic effect on the nature of immune response mounted in infected individuals (Abruzzi *et al.*, 2011; Lescano *et al.,* 2012) is scarce. The study therefore, seeks to provide information on the specific cytokines elicited during *T. gondii* infection in the different categories of study participants in Ghana. Data generated from the study will also contribute to understanding the nature of immune response during infection with *T. gondii* only as well as during co-infection with other parasites. In addition, the knowledge obtained will contribute to the formulation of better control and management of *T. gondii* infections in humans.

1.4 Research questions.

- Are there differences in the cytokine levels in response to infection with *T. gondii* in the immune- competent compared to immune-compromised individuals?
- Are there differences in the cytokine levels of single or mixed *T. gondii* and *Plasmodium spp* infections?

1.5 Objective

1.5.1 Main objective

The main objective of this study is to characterize the cytokine responses to *Toxoplasma gondii* in immune-competent and immune-compromised persons.

1.5.2 Specific objectives

The specific objectives are:

- To detect and genotype *T. gondii* infections in the study participants using PCR with specific genetic markers.
- To screen participants for other parasitic infections such as *Plasmodium* infections that may elicit a Th1 response, using a rapid diagnostic kit and molecular techniques (PCR).
- To measure levels of specific cytokines in the supernatants of stimulated PBMCs of participants *in vitro* by cytokine ELISA.
- To assess exposure to risks of infection by obtaining medical history and administration of questionnaires.
- To compare the immune responses data obtained from *T. gondii* infected study participants with *T. gondii* uninfected study participants as well as for those co-infected with

other parasites.

CHAPTER TWO

LITERATURE REVIEW

2.1 The History of Toxoplasmosis

Toxoplasma gondii is an obligate intracellular parasite that causes the disease, toxoplasmosis, and infects many warm blooded mammals including humans (Khan *et al*., 2005; Flegr *et al*., 2014). It is a parasite of medical and veterinary importance and has been studied in both molecular and cellular biology (Dubey, 2008).

This protozoan parasite, first described by Nicolle and Manceaux in 1908, was isolated from the tissues of a small hamster-like North African rodent (*Ctenodactylus gundi*) whiles working on *Leishmania* (Dubey, 2008). It was also independently isolated from an infected rabbit (*Oryctolagus cuniculus*) by Splendore in the same year. Nicolle and Manceaux in the year 1908, discovered that it was an organism which had not been taxonomically classified and proposed *Toxoplasma gondii* as its nomenclature based on its morphology and the host it was initially isolated from (Bortero-Kleiven, 2006; Dubey, 2009; Wyrosdick and Schaefer, 2015). *T. gondii* was found to be a parasite of medical importance after it was isolated and conclusively identified from the tissues of a congenitally infected infant in New York City, USA in 1939 (Wolf *et al*, 1939 as cited by Dubey and Jones, 2008)

Toxoplasmosis is endemic throughout the world and is known to infect a large proportion of adults (Bowie *et al*., 1997). There have been several reports over the years of outbreaks of the disease; in 1966 through to 2012, there were reports of outbreaks in Brazil, Canada, Korea, China, Turkey, among other countries (Bowie *et al*., 1997; de Moura *et al*., 2006; Doganci *et al*.,

2006; Quan *et al.,* 2008; Ekman *et al*., 2012).

Statistics show that there were over 6 million individuals infected with the parasite around the world, with 14% being seropositive by 40 years of age and 750 deaths related to food borne diseases annually (Furtado *et al*., 2011).

2.2 Toxoplasmosis in Ghana

Toxoplasmosis in Ghana was first published in 1978 by Anteson *et al* who reported a prevalence of 76% in pregnant women and their neonates. This finding increased the awareness of the public health importance of toxoplasmosis in Ghana. Over the years' other research findings have been published showing prevalence of the disease in both humans and animals. Work done in sheep, goats and pigs from three ecological zones in Ghana showed an overall sero-prevalence of 30.5% in sheep and goats and 39% in pigs (Arko-Mensah *et al.,* 2000; Van Der Puije *et al.,* 2000). Abu *et al*, in 2014 reported a sero-prevalence of 85% in certain indigenes in the Central R egion and also confirmed a significant positive correlation between sero-positivity and different modes of transmissions. Ayi *et al.,* (2005, 2009 and 2016) reported varying prevalence of *T. gondii* infections ranging from as low as 3.4% to as high as 92% in a wide array of study participants including pregnant women, eye lesion patients, blood donors and HIV seropositive individuals. The studies conducted by Ayi *et al.,* (2005, 2009, 2016) used different detection techniques such as PCR for the detection of parasite DNA from extracted blood blots and ELISA for the detection of *Toxoplasma gondii* antigen and *Toxoplasma* antibodies in serum and urine samples.

2.3 Classification of *Toxoplasma gondii*

Toxoplasma gondii can be classified under the kingdom Protista, which mostly consists of unicellular organisms. They are eukaryotic protozoans that have mitochondria for cellular respiration (Black & Boothroyd, 2000) and can also form cysts in adverse conditions. The parasite amongst others like *Plasmodium* and Coccidia belongs to the phylum Apicomplexa (Tan *et al.*, 2011) which consists of organisms known to be single-celled animal-like, mostly obligate endoparasites. Those that are heterotrophs, have a polarized cell structure and a single nucleus and also have organelles such as rhoptries, micronemes, and sub-pellicular microtubules in their apical complex (Robert-gangneux & Dardé, 2012). Further classifications are the class Sporozoasida, characterized by presence of spores. They belong to the order Eucoccidiorida, which are characterized by their complicated life cycles and their fecal-oral mode of transmission. They are found in the family Sarcocystidae, genus *Toxoplasma* and there exists only one species of the parasite which is *T. gondii* (Hill *et al*., 2005).

2.4 Life Cycle of *Toxoplasma gondii*

This parasite is known to be capable of infecting and replicating within a wide diversity of nucleated mammalian or avian cells (Robert-gangneux & Dardé, 2012). It has a complex life cycle which involves a sexual and asexual phase alternating between a feline (domestic and wild cats) and non-feline (others mammals or avians) infections (Black & Boothroyd, 2000). There are mainly three infective stages of the parasite: the tachyzoite which facilitate parasite proliferation during acute phase of the infection by rapidly dividing, the bradyzoite, sustain chronic infection and the sporozoites found in the oocyt which are spread into the environment

(Wang *et al.*, 2015). All these stages are linked to the life cycle of the parasite (Fig 2.1) and are responsible for the sexual and asexual phases of the parasite (Black and Boothroyd, 2000; Flegr *et al*., 2014).

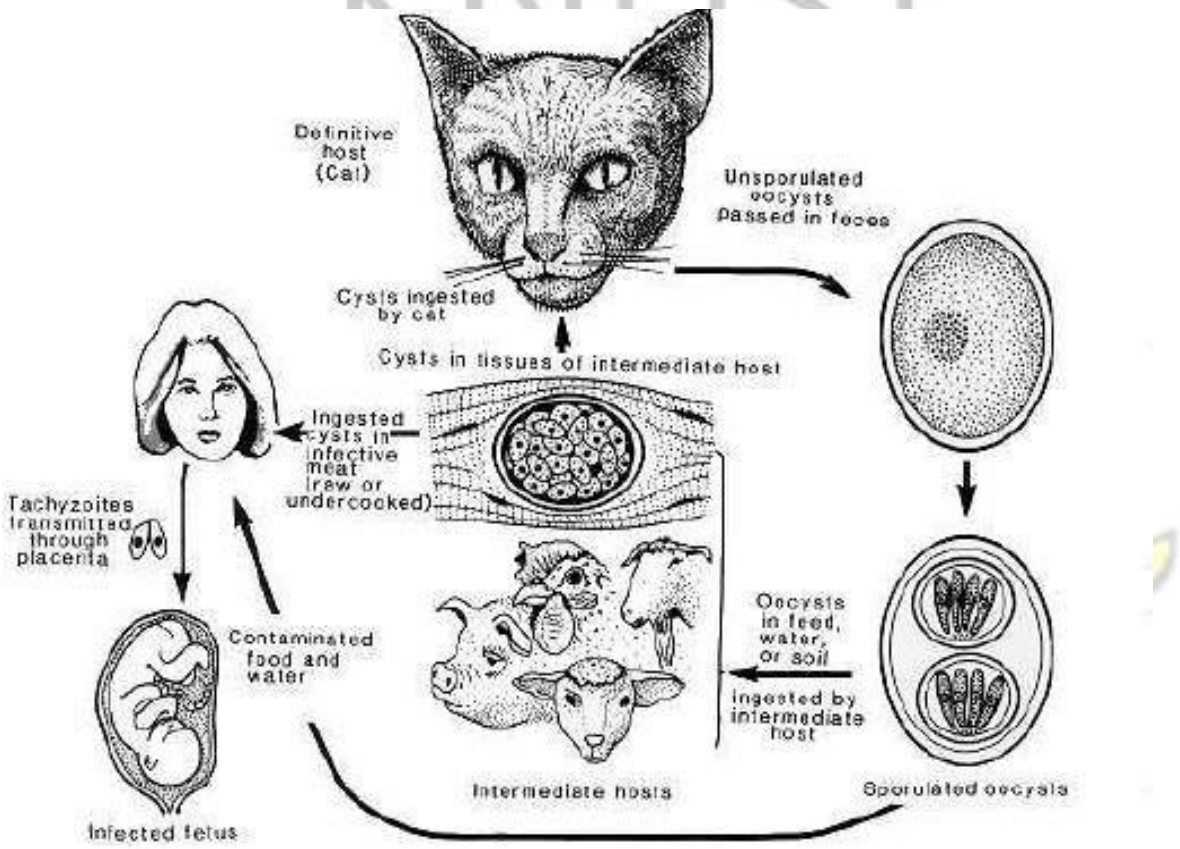

Figure 2.1: *Toxoplasma gondii* parasite life cycle (From Dubey *et al*., 1998)

2.4.1 Sexual Cycle

This stage which occurs only in the definitive host, that is members of the Felidae family (Denkers and Gazzinelli, 1998) is initiated by the ingestion of bradyzoite cysts found in the flesh of an infected animal (Mozaffarian *et al.*, 2014). After the cysts are ingested, they travel through the gut of the host to the cat's enterocytes where they divide rapidly and differentiate into macrogametocytes and micro-gametocytes. These then fuse forming the zygote (oocyst) which is

shed into the environment by the cat during faecal excretion. The oocysts undergo meiosis which results in the production of extremely infectious sporozoites which are resistant and may persist for years in unfavourable environmental circumstances (Dubey *et al.*, 1998; Jones and Dubey, 2010) . When these sporozoites are ingested by an intermediate host, either from the soil or on leaves of vegetables, it completes the sexual cycle (Khan *et al.*, 2005).

2.4.2 Asexual Cycle

This cycle begins when an intermediate host such as humans, dogs or goats ingests the sporozoites in cat faeces either directly from the soil or from contaminated plant parts. The intermediate host can also be infected when the bradyzoite cysts are ingested or from transplants as well as congenitally *via* tachyzoite infection (Jones and Dubey, 2010). These bradyzoites or sporozoites then penetrate the host's intestinal epithelial cells and multiply by differentiating into rapidly dividing tachyzoites within twenty-four hours of infection (Hill *et al*., 2005). The chronic phase is characterized by the differentiation of the tachyzoites into the slowly dividing bradyzoites which persist within the host tissue (Xia *et al*., 2008). The alternation between tachyzoites and bradyzoites in conjunction with the host's immune system results in the symptoms of the chronic phase (Weiss and Dubey, 2009). The dormant bradyzoites usually found in the tissues of the host, are encased in a cyst (small thick-walled sac) and are very difficult to eradicate, and are adapted to long term survival as a result of their latent metabolism, thus persisting throughout the lifespan of the host. The death of the host may result in the disruption of the cyst wall and cause the release of the bradyzoites (Robert-Gangneux and Dardé, 2012). The transmission of the cyst into the gut of the dominant host completes the asexual cycle.

2.5 Morphology of Infective stages

There are three different forms in which *T. gondii* exist which are dependent on the parasite's stage in its host. The morphological forms are the tachyzoites and bradyzoites (in the tissue cyst) usually found in the intermediate host. The final stage which is the sporozoite (in the oocysts) is found only in the definitive host (Dubey, 2009). The three stages are similar in their ultrastructure with very slight differences in their possession of certain organelles and inclusion bodies (Dubey *et al.*, 1998)

2.5.1 The Oocyst

The oocysts which can develop aerobically are shed from the faeces of only the definitive host which is the domestic or wild felids (Frenkel *et al.*, 1970). They shed the oocysts after ingestion of any of infectious stages which include the tachyzoites, bradyzoites and sporozoites. The oocysts are however less infective to the felids than other host (Dubey, 2006). The time and regularity of shedding is dependent on the stage of parasite ingested. It has been shown that oocysts are shed within 3 to 10 days after ingesting bradyzoites, 13 days after ingesting tachyzoites and 18 days after ingesting oocysts (Dubey *et al*., 1998),

The oocysts before sporulation are found, under the light microscope, to consist of two colourless layers and are10 x 12mm in diameter. They are also subspherical and the sporozoites are contained within the oocyst with an absence of polar granules (Dubey *et al.*, 1998). When the oocysts are shed, sporulation then takes place within 1 to 5 days depending on the environmental conditions, with favourable temperatures between 4^{o}C and 37^{o}C. Sporulated oocysts are 11 to 13 µm in

diameter and are subspherical to ellipsoidal in shape. There are two ellipsoidal sporocyst containing four sporozoites each within the oocysts (Fig 2.2). The sporocysts measure 6 by 8 µm and have a residuum present (Frenkel *et al*., 1970; Robert-Gangneux and Dardé, 2012; Van Kessell, 2012) z

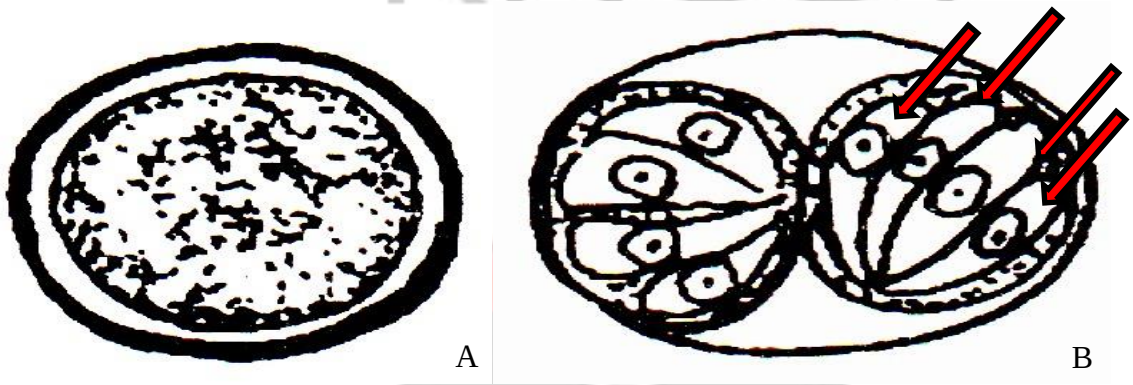

Figure 2.2: (A) Unsporulated oocyst (with a double layered oocyst wall enclosing its central and undivided mass) obtained from cat faeces. (B). Sporulated oocyst with a thin oocyst wall, two sporocysts. (Each sporocyst has four sporozoites (arrows) that are not in complete focus (Source: Hill et al., 2005)

2.5.2 The Tachyzoites

The tachyzoite stage is the rapidly dividing stage of the parasite. It is capable of dividing in any nucleated cell of an intermediate host and in only the non-intestinal epithelial cells of the definitive host. The word "tachyzoite was first termed so from the Greek word '*tachos'* meaning 'speed' (Saade *et al.*, 2013) based on the behavior of the parasite (Dubey *et al.*, 1998). Within 6 to 8 hours, roughly 64 to 128 cells would have replicated within a cell (in vitro) and after exiting, infect new cells (Black and Boothroyd, 2000).

Tachyzoites are found to be roughly 5um long and 2um wide (Black and Boothroyd, 2000) and crescent shaped having a pointed anterior end and a rounded posterior end (Fig 2.3 A and B). It

has organelles and inclusion bodies such as; micropore, pellicle (which is the outer covering) micronemes, rhoptries, mitochondrion, golgi-complex, dense granules and endoplasmic reticulum. It's nucleus is located in the middle area of the cell and has clumps of chromatin and a central nucleolus (Fig 2.3 C) (Dubey *et al.*, 1998).

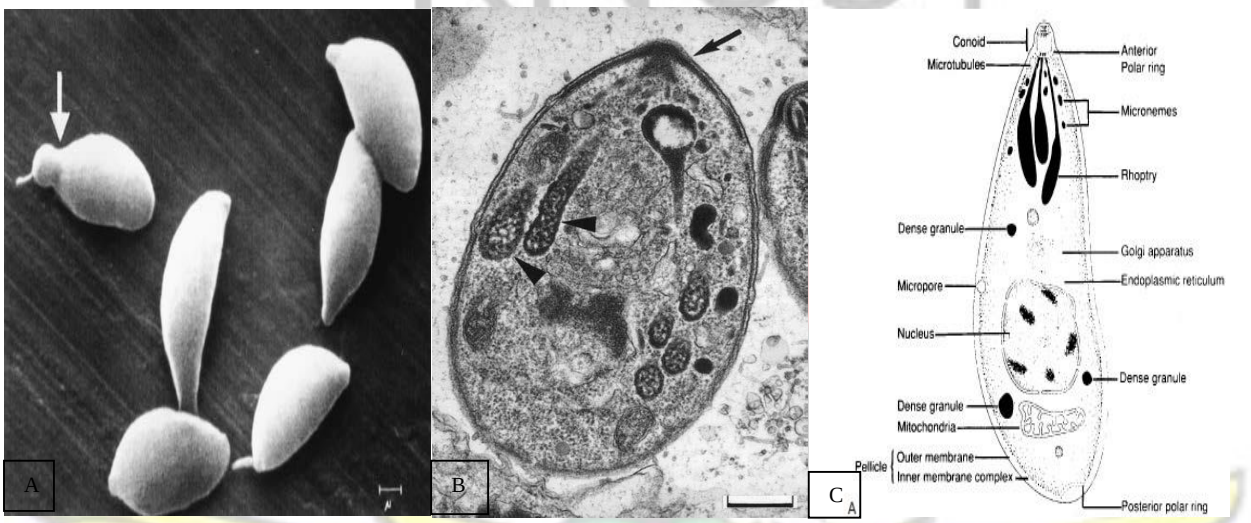

Figure 2.3: Morphology of *Toxoplasma gondii* tachyzoite. (A) Scanning electron micrograph of extracellular tachyzoites. (B) Transmission Electron Micrograph of a *Toxoplasma gondii* tachyzoite. (C) Schematic diagram showing the various structures within the organism (Sources:J P Dubey et al., 1998; Medina *et al,* 2001)

2.5.3 The Bradyzoites (Tissue Cysts)

The tissue cysts (bradyzoites) of *Toxoplasma gondii* usually appear between 7 to 10 days postinfection. To avoid the immune system, they are found predominantly in neural and muscular tissues like the muscle tissue, ocular tissues and brain as well as visceral organs such as the lungs and liver, (Black and Boothroyd, 2000) sometimes even in the gonads. The tissue cysts vary in sizes ranging from 10-20 µm with each cyst containing as many as 3000 bradyzoites approximately 7 by 1.5 µm in size (Fig 2.4). The cyst wall is 0.5 µm thick and is elastic.

Morphologically, bradyzoites are only slightly different from tachyzoites. They are slenderer than tachyzoites with their nucleus located more towards the posterior end. Bradyzoites contain amylopectin granules that stain red with periodic acid-schiff reagent. They are also less vulnerable to being destroyed by proteolytic enzymes than tachyzoites (Dubey *et al*., 1998).

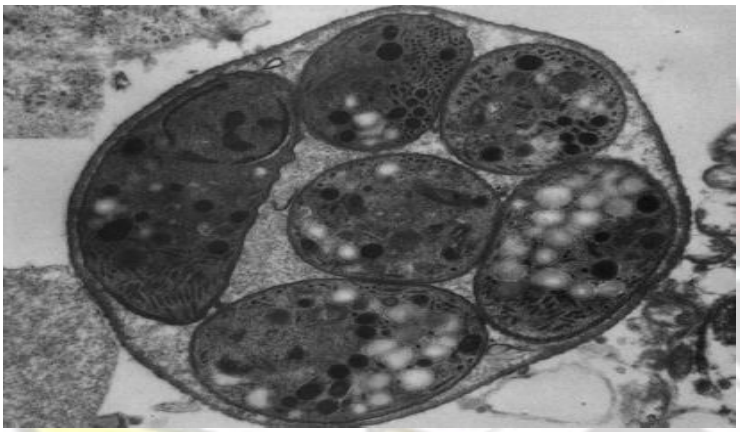

Figure 2.4: Higher power transmission electron micrograph of a cyst showing portions of six bradyzoites with their internal organelles within the cyst (Source: Moorth *et al*,. 1993).

2.6 Population structure of *T. gondii*

The population structure of *T. gondii* were initially known to be highly clonal with low genetic variety and were classified into three main lineages or clonal genotypes known as types I, II and III (Howe *et al.*, 1997; Ajzenberg *et al.*, 2009). These were believed to have originated from the natural genetic crosses of similar parental types, coupled with population curves and biogeography (Sibley *et al.*, 2009). However, in recent years, with new genotypic methods, 15 haplogroups under 6 major clades were identified, including the 3 main clonal lineages as well as other atypical genotypes and new clonal lineages. Studies have supported that these genotypes are different from

the main 3 in transmissibility and pathogenicity(Ivović *et al.*, 2007; Xiao and Yolken, 2015). The dominance though, of the 3 main lineages found in humans over other genotypes is reported to have been as a result of heightened fitness and ability to survive (Dardé, 2004; Robert-Gangneux and Dardé, 2012). Studies have shown that type II and III lineages are more common throughout the continents as compared to type I (Wang *et al.*, 2015) with type II being associated with more than 70% of human toxoplasmosis in some parts of Europe, Africa and North America (Fuentes *et al.*, 2001). Ayi *et al* (2016) also established the prevalence of type II within the Ghanaian population.

Even though the genetic divergence of the 3 clonal lineages is approximately 1 to 2% at the DNA sequence level, the three lineages have very different virulence phenotypes exhibited in mice (Quan *et al.*, 2008). The pathogenicity difference has been shown to be determined by polymorphism as well as expression differences in proteins such as the dense granule and rhoptry. Type I strains are highly virulent, have a faster rate of growth *in vitro* and have been shown to reach greater parasite loads in laboratory mice. They also have enhanced migratory capacity with greater ability to cross the tissues barrier and have been postulated to be responsible for the infections in immune-compromised individuals (Khan *et al.*, 2005a; Xiao and Yolken, 2015).The Type II and III strains are however considered to be relatively avirulent in mice; but are capable of establishing chronic infections characterized by tissue cysts in the host (Sibley, 2003)

2.7 Mode of Transmission

There are diverse ways by which the parasite is transmitted to its hosts, however there are only two foremost routes which can be described as either vertical or horizontal (Bruna-romero and

Oliveira, 1970; Andiappan *et al*., 2014; Blader *et al.*, 2015). The horizontal routes are from consumption of tissue cysts or oocysts from felids. The vertical route is primarily congenital, through blood transfusion or organ transplants (Astrid and Anja, 2011).

The tissue cysts or bradyzoites are transmitted to the hosts through the ingestion of infected raw or undercooked meat from a wide variety of warm blooded animals or avians. The oocysts are transmitted to the host through contact with contaminated soils, for instance, garden soils or cat litter. The infection from oocysts can also be from unwashed or improperly washed vegetables or fruits and also through unfiltered contaminated water (Bowie *et al.,* 1997). The vertical route of transmission occurs when an infected woman passes the infection vertically to the fetus through the placenta resulting in serious disease in the fetus or even miscarriages. The parasite can also be transmitted when contaminated blood is transfused to an individual or also when an organ contaminated with bradyzoite cysts are passed on from one individual to another (Mccabe *et al*., 1983; Dubey *et al*., 2012; Cabral *et al.*, 2013; Flegr *et al*., 2014).

Transmission of the parasite is known to be dependent on factors such as climate and rate of exposure to sources of *T. gondii* infection. *Toxoplasma* sero-positivity is also reportedly higher in hot and humid areas, resulting in higher sero-prevalence levels in some parts of the world as compared to others. Comparatively, prevalence of the disease was found to be as low as 18.9% in Finland and as high as 51% in Brazil (Weiss *et al.* 2011; Flegr *et al.,* 2014).

2.8 Lytic Life Cycle to *Toxoplasma gondii*

T. gondii is among the most unusual pathogens known for its ability to invade a wide variety of hosts cells (Hill *et al*., 2005; Carruthers and Suzuki, 2007). *In vitro* experiments have shown that

they are able to invade many mammalian cell types as well as insect and fish cells (Blader, *et al*., 2015) suggestive of the possibility of the presence of a highly preserved host cell receptor or a diversity of receptors all recognized by the parasite. *T. gondii* is also highly adapted to invade and develop within the host cell. The main stages involved in the lytic cycle of the parasite are: invasion, replication and egress (Fig 2.5) as described by Black and Boothroyd (2000) and Blader *et al*., (2015). The lytic cycle may take between 24 to 48 hours and result in the production of between 16 and 128 progeny originating from a single parasite (Sibley, 2003). There are two distinct biological states involved in the lytic cycle of the parasite which are the intracellular and extracellular parasitic states. The extracellular parasites are extremely motile, non-proliferative organisms which excrete their conoid. The intracellular parasites however are non-motile, proliferative organisms which do not excrete their conoid or secrete micronemes. The switch from one state to another during the lytic cycle is motivated by changes in gene expression, mRNA availability for translation and translocation of the glycolytic enzymes from the cytoplasm to the cortex (Blader *et al.*, 2015)

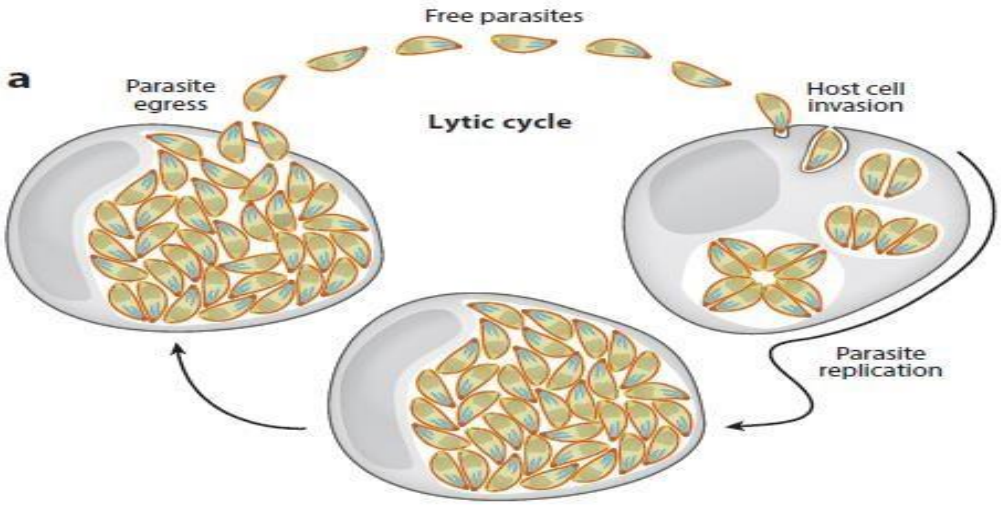

Figure 2.5: The lytic cycle of *Toxoplasma gondii* showing invasion, replication, and egress (Blader *et al*., 2015).

2.8.1 Invasion

Toxoplasma gondii has the ability to actively invade host cells by formation of a vacuole through penetration of the host cell plasma membrane (Sibley, 2003). During invasion, the host cell is primarily passive with no detectable change in the membrane ruffling, phosphorylation of host cell proteins or the actin cytoskeleton unlike other parasites which require the host cells' participation (Sibley, 2003). The main organelles involved in invasion are the rhoptries and micronemes. The micronemes which are thin vesicular organelles are adjacent to the rhoptries positioned at the apical end of the parasite. On contact with the host cell, the parasite re-orients itself such that the apical complex directly opposes the host cell membrane to allow the secretion of contents from the rhoptries into a newly formed parasitophorous vacuole (Blader *et al.*, 2015). Surface antigens SAG1 and SAG2 have been described as affecting the invasion prior to apical re-orientation. Other proteins found to be associated with the parasite membrane are a cluster of proteins found in the dense granules. These proteins with the prefix GRA either extend from the parasite surface or are inserted into the parasitorous vacuole (Carruthers, 1999).

2.8.2 Replication

Upon entering the cell, parasites replicate in the absence of an immune response, by a unique method of cell division known as endodyogeny (Dubey, 2008). The process is initiated by the establishment of two pellicular membranes in the middle of the cell defining the daughter cells within the cytoplasm of the parent cell. The parent cell gradually becomes more spherical as the single mitochondrion and nucleus divide between the daughter cells. The cells begin to mature

until the cytoplasm and the other organelles are divided amongst them. As this occurs, the parent cell loses its inner membrane complex. The budding of the daughter cells is motivated by the cortical cytoskeleton against which the divided organelles are anchored. The entire cytoplasm eventually divides forming two new daughter cells (Black and Boothroyd, 2000; Blader *et al.*, 2015).

2.8.3 Egress

Egress for *Toxoplasma gondii* is a process that results in the disruption of the host cell allowing the discharge of very motile parasites (Blader *et al.*, 2015). Parasite egress from the host cell is mediated by several factors separated into those occurring in the presence of the host immune response and those occurring in the absence of the host immune response (Blader *et al.*, 2015). Cytotoxic $CD8^+$ T cell responses against the host cell results in a damaged cell which causes a drop in the cytoplasmic K^+ triggering the egress of the parasite. In the absence of an immune response however, the parasites proliferate within the cell and reach maturity. During replication, the intracellular abscisic acid (ABA) concentration increases above threshold resulting in the egress of the parasite (Blader *et al.*, 2015).

Another factor which has been recognized to play an significant role in the egress of *Toxoplasma* parasites from the host cell is the intracellular Ca^{2+} concentration. During the process, the host plasma membrane is pushed out from the surface eventually rupturing the cell membrane resulting in cell lysis and subsequent escape of the parasite (Black and Boothroyd, 2000; Sibley, 2003; Robert-Gangneux and Dardé, 2012).

2.9 Immunity to *T. gondii* infection

During toxoplasmosis infection, the immune system plays a principal role in the control of the parasites, without which the infection can develop into a severe disease resulting in mortality (Denkers and Gazzinelli, 1998). However, the immune systems' response to the parasites infection is dependent on the kind of host, particularly due to genetic differences as well as current health status. Another challenge is the inherent ability of *Toxoplasma gondii* to infect a wide range of cells or tissues, each having its own exact immune response especially for the placenta and the central nervous system. Finally, research has identified that the strain of parasite, having variable virulence also contributes to the varying immune responses elicited (Filisetti and Candolfi, 2004). The innate immune system is able to recognize *T. gondii* by sensing certain molecules that are unique to protozoan parasites (Yarovinsky, 2014). The immune response in most immune-competent hosts causes the tachyzoites to differentiate into bradyzoite cysts that can go into hiding and persist throughout the lifetime of the host. This is however not the case in chronically infected hosts as the immune system is lacking (Tait and Hunter, 2009). There are two arms of the immune system which are responsible for fighting diseases and these consist of the humoral and cell mediated immune response

2.9.1 Humoral Response

This is also referred to as the antibody-mediated immune system. This system is usually mediated by macromolecules secreted by antibodies or complement proteins into extracellular fluids. It has been established that *T. gondii* promotes the production of antibodies that aid in killing the parasite (Holec-Ga, 2013). These immunoglobulins are essential for diagnosis however play a minor role

in eliminating the parasite. They have been found to protect against the parasite by blocking invasion, opsonizing the parasite for phagocytosis as well as activating the complement pathway (Filisetti and Candolfi, 2004; Luptakova *et al.,* 2012; Dupont *et al.,* 2013). Studies have proposed that the antibodies hinder invasion by blocking the activity of secretory-excretory substances that enhance host cell penetration. They work on the extracellular parasites however, are not able to protect against pathogenic *T. gondii* infection (Parker *et al.*, 1991). The presence and serological levels of a particular immunoglobulin are usually dependent on the immune status as well as the time of diagnosis of the individual (Holliman *et al.,* 1994). Immunoglobulin (Ig) M is the first antibody to be produced, appearing after one week of infection. Ig A and IgE appear next and are used for diagnosing the acute phase of the disease. IgG appears later and persists throughout the lifetime of the host. It is also able to pass from the blood to tissue spaces, crossing from the placenta of the mother to the fetus. It is hence the only antibody that is able to protect the fetus (Cheesebrough, 2006; Luptakova *et al.*, 2012)

2.9.2 Cell Mediated Response

One unique characteristic of *T. gondii* is its ability to elicit a persistent and strong cell mediated immune response that contributes to protecting the host against the rapidly dividing tachyzoite stage and its subsequent effects (Denkers and Gazzinelli, 1998). During the response, the macrophages, T-lymphocytes, natural killer cells and cytokines are all involved. Several studies have indicated that T cells are key contributors in conferring resistance to *Toxoplasma gondii* and ultimately determine the survival of the host as well as the parasite (Denkers and Gazzinelli, 1998; Parker *et al.*, 1991). Studies indicate that the production of cytokines and chemokine causes

inflammatory cells to be activated and accumulate leading to parasite depletion (Kasper *et al.*, 2004).

The innate response to toxoplasmosis is facilitated by macrophages and dendritic cells (DC) which are activated to produce IL-12 and TNF-α. These also activate natural killers (NK) cells to secrete INF- γ inducing T-cells to kill the parasites (Parker *et al.*, 1991). Studies with mice have shown that INF- γ is central to *T. gondii* resistance, without which the mice are unable to survive acute infection. They also show a high correlation between the depletion of this cytokine and reactivation of cysts resulting in death. One mechanism proposed by researchers show that INF- γ induces the enhanced production of Nitric Oxide (NO) as well as the degradation of host cell tryptophan which have a toxoplasmocidal effect (Denkers, 1999; Carruthers and Suzuki, 2007; Yarovinsky, 2014).

The acquired immunity against *Toxoplasma gondii* is characterized by $CD4^+$ and $CD8^+$ T cell activity with the cytokine IFN-γ still being significant in resistance to the parasite during both acute and chronic stages of the infection. The cytokine initiates the differentiation of $CD4^+$ T cells specific to the parasite antigens which recruit other Th1 related cytokines. $CD8^+$ T cells function by developing cytotoxic activity against infected host cells as well as acting as an extra source of INF-γ (Bruna-Romero and Oliveira, 2012). Other cytokines secreted during the immune response include TNF-α, IL-(2, 7, 12, and 15) which act as up-regulators or proinflammatory cytokines. Cytokines which down-regulate (anti-inflammatory) the immuneresponse include TGF-β, IL-(4, 6 and 10) (Liu *et al.*, 2012). TNF-α acts in synergy with INF-γ to kill tachyzoites by macrophages through enhanced production of free oxygen radicals and NO

(Denkers and Gazzinelli, 1998). IL-12 acts by maintaining the INF-γ production and mediate resistance to chronic infection (Suzuki *et al.*, 2012). IL-10 is produced by T-lymphocytes

expressing the Th2 phenotype. They antagonize or down-regulate the production of Th1 type cells and the cytokines produced by these cells and inhibit the synthesis of INF-γ by NK cells.

They also suppress $CD4^+$ T cell proliferation (Filisetti and Candolfi, 2004; Kasper *et al.*, 2004; Tait and Hunter, 2009).

2.9.3 Immune Responses in Immuno-Competent Hosts

Toxoplasmosis is a disease caused by a protozoan parasite, however unlike other protozoan parasites such as *Plasmodium spp, T. gondii* under normal circumstances is unable to elicit significant immunopathologic changes in immune-competent hosts, with symptoms such as fatigue, lymphadenopathy and fever usually being the only indicators of disease state (Denkers, 1999). Majority of infected individuals, roughly 80% of cases are asymptomatic (RobertGangneux and Dardé, 2012) The parasite during infection spreads throughout the host without specificity but as acquired immunity and the host develops protective immunity; the parasite perseveres in the bradyzoite form inside the cysts hiding within the central nervous system or skeletal muscles (Denkers, 1999; Filisetti and Candolfi, 2004).

2.9.4 Immune Responses in Immuno-Compromised Hosts

T. gondii has been identified as one of the important opportunistic pathogens in pregnant women, fetuses, newborns, and patients with various acquired immuno-deficiencies (Petersen and Dubey 2001). Patients at most risk of the disease however include transplant patients and HIV seropositive individuals with their $CD4^+$ T cell counts below 200 cells/ µl (Wreghitt *et al.*, 1986; Derouin and Pelloux, 2008; Miller *et al.*, 2009; Baliu *et al.*, 2014).

There have been several studies on the immunobiology of Toxoplasmosis considering its clinical importance especially in the immune-compromised group. Studies have proven that immune compromised patients developing clinical disease have defects in their T cell function and with depreciating cellular immunity; severe toxoplasmosis is always existent indicating that lymphocytes are important in the control of the infection. The disease is always life threatening in this case however, the immune background of the host also playing a critical role. Most of the infections are from cyst reactivation rather than from newly acquired infections (Tait and Hunter, 2009; Robert-Gangneux and Dardé, 2012)

2.10 Diagnosis of Toxoplasmosis

Toxoplasmosis can be diagnosed using the clinical signs (Johnson and Dubey, 2000); however symptoms are not pathognomonic hence laboratory analysis is essential in diagnosing the disease, particularly in immuno-compromised individuals (Arko-Mensah, 1999; Furtado *et al.*, 2011). Over the years, several methods have been developed by which toxoplasmosis is diagnosed. These methods can be categorized into the direct and indirect techniques (Ayi *et al.*, 2009). The direct methods include culture of the parasite, ophthalmic tests and also detection of *T. gondii* DNA from the fluids of samples using techniques such as PCR. Demonstration of the organism through inoculation in mice can also be done. The indirect methods developed are serological methods for detection of anti- *T. gondii* antibodies. Such tests are the serological test for immunoglobulin M (IgM) detection, Enzyme Linked Immuno-Sorbent Assays (ELISA), indirect Fluorescent antibody (IFA), Dye-Agglutination Test (DAT) and Sabin-Feldman dye test (Silva *et al.*, 1997; Ayi *et al.*, 2005, 2009; Dai *et al.*, 2012).

The Sabin-Feldman dye test is one of the earliest serological tests developed for detection of *T. gondii*. This test which has been used as the gold standard for several years with no evidence of false positives in humans was based on lysing the parasite with serum antibodies in the presence of a complement and measures primarily IgG antibodies (Mccabe *et al.*, 1983; Van Kessell, 2012). Other serological tests for antibody detection are enzyme linked immunosorbent assay (ELISA), immunosorbent agglutination test (ISAGA), indirect fluorescence assay (IFA), indirect hemagglutination test (IHA), latex agglutination test (LAT), modified antibody agglutination test (MAT) or direct agglutination test (DAT) (Wyrosdick and Schaefer, 2015).

For some individuals however, for instance HIV seropositive patients, detection using serological tests could be unreliable due to the possibility of very low titers of specific antibodies being produced. Molecular techniques, such as PCR, which detects genetic material of *T. gondii* in a biological sample is less invasive yet more specific and sensitive, are being used (Alfonso *et al.*, 2009). PCR is highly efficient and able to amplify DNA from small amounts of starting material in a short time (Liu *et al.*, 2015) after that it is visualized using agarose or polyacrylamide gel following staining (Switaj *et al.*, 2005).

Other direct methods such as culture of parasites or parasite detection histology could be cumbersome as they may require histology expertise, biopsy for tissue collection and/ or live tachyzoites or laboratory animals (Wyrosdick and Schaefer, 2015).

2.11 Treatment of Toxoplasmosis

In spite of documentation showing toxoplasmosis-related morbidity and mortality, strong evidence indicates that active infection with the parasite can be treated and the consequence decreased (Mcleod *et al.,* 2009). The clinical severity of the disease is however a very strong determinant for the type and duration of treatment (Mccabe *et al.,* 1983).

2.11.1 Treatment of Toxoplasmosis in Immune-Competent Hosts

Pyrimethamine, which can be used as a single therapy drug in the treatment of Toxoplasmosis in immune competent hosts, has been shown to inhibit the conversion of folic acid to folinic acid through dihydropteroate synthase depriving the parasite of its source of folinic acid (GuexCrosier, 2009). Unlike *T. gondii*, humans are able to use external folinic acid for their cells. The drug is however considered less efficient as compared to when combined with other drugs (Derouin and Pelloux, 2008; Song *et al.,* 2013). The most effective regimen for the treatment of toxoplasmosis in the immune competent host is thus the combination of pyrimethamine and sulfadoxine or with sulfadiazine or trisulfapyrimidines: sulfamerazine, sulfamethazine and sulfapyrazine (Mccabe *et al.*, 1983; Guex-Crosier, 2009).

2.11.2 Treatment of Toxoplasmosis in Immune-compromised Host

Drugs that have been shown to be effective in the treatment of toxoplasmosis amongst immunesuppressed individuals are spiramycin (for acutely infected pregnant women with uninfected foetuses), spiramycin (for maternal infections), pyrimethamine and folinic acid (congenital toxoplasmosis in neonates) a combination of pyrimethamine and clindamycin/

clarithromycin/azithromycin (in the case of hypersensitivity to sulfonamides) (Mcleod *et al.*, 2009). Trimethoprim-sulfamethoxazole has also proven to be an effective drug in the treatment of toxoplasma seropositive HIV-infected pregnant women with $CD4^+$ cells less than 200 cells/ mm^3 (Montoya and Remington, 2008; Mcleod *et al.*, 2009; Van Kessell, 2012).

2.12 Prevention and Control of Toxoplasmosis

The prevention and control of the incidence of toxoplasmosis employs measures that have been classified into two main categories; primary and secondary prevention (Montoya and Remington, 2008; Robert-Gangneux and Dardé, 2012).

2.12.1 Primary Prevention

Maintaining good hygiene is one of the principal ways by which infection is avoided. Individuals who handle raw meat should ensure that their hands are protected before handling (Mccabe *et al.*, 1983). Meat should be either cooked above 67^{o}C or cooled to -13 to -20^{o}C to destroy tissue cysts. Fruits and vegetables, especially those grown in contact with the soil should be washed very well before consumption. Protective clothing particularly garden shoes and gloves should be worn anytime working in the garden soil and hands washed thoroughly after gardening. Individuals who keep pet cats should ensure that a litter box is provided for the animals and the litter box changed or cleaned every two days. Appropriate protective wear such as masks and gloves should be worn before undertaking the task (Mccabe *et al.*, 1983; Hill *et al.*, 2005; Robert-Gangneux and Dardé, 2012).

Due to the challenge of the unavailability of drugs that can entirely cure or prevent against *T. gondii* infections in humans, another preventive measure, still being studied is the use of vaccines. Effective vaccines can contribute immensely to the control and spread of the disease. Researchers have identified that studies into pathogenesis of the disease in susceptible patients may be key in development of new vaccines and in immuno-therapy. Studies which have been conducted are mostly in animal models and even though some have been successful in protecting animal models such as mice from the disease, no human vaccine has been developed as yet (Tait and Hunter, 2009; Tan *et al.*, 2011; Liu *et al.*, 2012) . Various methods are hence being employed in vaccinology, some of which include use of attenuated whole parasites, soluble parasite antigens, recombinant purified proteins or recombinant live vectors. Currently some of the tools are used in combination (Bruna-Romero and Oliveira, 2012)

2.12.2 Secondary Prevention

Secondary prevention focuses on management of patients to prevent re-infection or re-activation of the parasite after a past infection (Robert-Gangneux and Dardé, 2012) These programs rely on chemoprophylaxis and regular serological screening to verify immune status against the parasite, especially in HIV/AIDS patients. Screening and intensive health education activities must be conducted for pregnant women to prevent reactivation and vertical transmission of the parasite from mother to the foetus (Pawlowski *et al.,* 2001; Governor, 2012).

CHAPTER 3

MATERIAL AND METHODS

3.1 Study Site

The study was carried out in two units of the Korle-Bu Teaching hospital (KBTH) in Accra, Ghana. These were the blood bank (haematology unit), under the operation of the National Blood Service (NBS) and the Fevers Unit of the Physician Specialist department under the Medical Sub Budget Management Centre (Sub-BMC).

KBTH, established in the year 1923, is currently the third largest hospital in Africa and the leading national referral center in Ghana. The hospital was upgraded to a teaching hospital status in the year 1962 when the University of Ghana Medical School was established, for the training of medical doctors. The hospital has 17 clinical and diagnostic departments with a bed capacity of 2000 and an average daily attendance of 1,500 patients and roughly 250 patient admissions (http://:www.kbth.gov.gh).

The fevers unit of KBTH treats patients with diseases such as Cryptococcus meningitis, cerebral toxoplasmosis and HIV/AIDS. The facility is well equipped for counseling and running various laboratory tests, such as viral load count and $CD4^+$ T-cell count, needed for the management of various cases admitted in the unit. On its clinic days, which are Mondays, Wednesdays and Fridays for adults, also Tuesdays and Thursdays for children, the fevers unit has an average patient visitation rate of 300/day and HIV/AIDS patients' visitation rate of 200/day (retrieved from the fever's unit data room on 28th September, 2015).

The National Blood Service is one of the newest agencies under the Ministry of Health, established in the year 1992, for the provision of blood and blood products as well as research and training. It

has the blood bank of the KBTH under its wings and is responsible for the individuals that come in to donate blood for relatives that may require transfusion for various reasons including surgical treatments and child deliveries. The blood bank of the KBTH has an average blood donation rate of 30/day. All blood is thoroughly screened for its ABO and Rh D status as well as for diseases of public health importance that can be transfused such as, HIV (types 1 and 2), Hepatitis B and C and Syphilis (http://:nbsghana.org). Toxoplasmosis is however not screened for.

3.2 Study Design

The study took the form of a hospital based cross-sectional screening conducted over a two-year period in collaboration with the appropriate authorities of the selected study sites. The samples were collected from October 2015 to April 2016. Scientific approval and ethical clearance were sought from the Scientific and Technical Committee (STC) and the Institutional Review Board (IRB) of the Noguchi Memorial Institute for Medical Research (NMIMR) respectively. Permission was obtained from the medical officers in charge of the National Blood Service and the Fevers unit. Study participants who met the inclusion and exclusion criteria were recruited upon willingness and informed consent. The data collected from the participants included socio demographic details, occupational information and medical history which highlighted tests conducted. Blood samples were also obtained from participants. Portions of the blood were tested for plasmodium antigens in situ also blotted on labeled filter paper whilst the larger volume was dispensed into anti-coagulated sterile tubes and transported in a cool box to NMIMR laboratory for PBMC isolation and other appropriate tests.

3.3 Study Population and Participants

The study population comprised of both male and female out-patients department attendees, at the blood bank of the Haematology section, representing the immune-competent group and the Fevers unit representing the immune-compromised group.

3.3.1 Inclusion Criteria

Immune competent Group

- Blood donors at the KBTH between 18 and 60 years
- Individuals who had been screened and approved to donate blood.

Immune Compromised group

- HIV seropositive individuals between 18 and 60 years
- Individuals who were anti-retroviral treatment naïve
- Individuals whose $CD4^+$ cell count were less than 200 cells/mm^3
- Individuals who had consented to partake in the study

3.3.2 Exclusion Criteria

Immune competent Group

- Individuals who did not pass the screening tests and were disqualified to donate blood
- Individuals who were aged below 18 and above 60 years
- Individuals who were HIV seropositive after blood donation and screening

Immune Compromised group

- HIV seropositive individuals
- Individuals who had been put on anti-retroviral treatment
- Individuals whose $CD4^+$ cell count were above 200 cells/mm^3

3.4 Sample Size Determination

Prevalence of *T. gondii* infection in Ghana among HIV seropositive individuals and healthy blood donors in Ghana, was estimated at 54.7% and 3.4% respectively (Ayi *et al.,* 2016).

Therefore, assuming a minimum *Toxoplasma* infection rate of 10% and a maximum of 90% (based on prevalence estimates in Ghanaians, Anteson *et al*, Ayi *et al*, 2005; 2009, Abu *et al*, 2014, 2016) at a confidence level of 95% and a margin of error of 5%, 138 persons are needed for the study.

The sample size (SS) was estimated using the formula

SS (n) = $[Z^2P \times P (1-P)] / d^2$ (Naing *et al.,* 2006) where

SS (n) = sample size,

Z (1.96) = Z statistic for a 95% level of confidence,

P = expected prevalence or proportion and *d* =

precision (0.05)

3.5 Ethical Consideration

Scientific approval and ethical clearance were obtained from the NMIMR Scientific and Technical Committee and Institutional Review Board respectively (NMIMR-IRB 074/14-15, see appendix I)

and clearance also from the Research and Development Department of the NBS (NBSGRD/150423/01, see appendix II). Permission for the study was also obtained from the appropriate authorities at the Fevers Unit (KBTH). Written informed consent was obtained from study participants preceding collection of blood samples.

3.6 Medical History and Questionnaire Administration

Questionnaires (see appendix IV) were administered to each of the participants aided by trained research assistants after the consent forms (see appendix III) had been signed. Data collected included the age, gender, residence and basic knowledge on Toxoplasma infection risks factors. The medical history information was obtained from the folders of the HIV Seropositive participants. Data collected included date of first visit, opportunistic diseases diagnosed and laboratory tests conducted.

3.7 Blood Sample Collection

Five ml of venous blood was drawn from the cubital veins of HIV seropositive participants into 6 ml sodium heparin tubes with the help of a phlebotomist/laboratory technologist at the Fevers unit. For blood donor participants, 5 ml of blood collected using the extension tubes was transferred into the sodium heparin tubes. This was done by the medical personnel during the process of blood donation.

Approximately 60 µl of blood from each participant were spotted on filter papers appropriately labeled with the participant's unique code. Five microlitres of the blood was also used for

Plasmodium infection detection using a One Step Malaria HRP-II (*P. f*) and pLDH (Pan) Antigen Rapid Test kit (Standard Diagnostics, Korea). The samples were then stored in a cool box containing ice packs and transported to NMIMR Parasitology laboratory, within four hours of the collection of the first whole blood sample, for isolation of PBMC.

3.8 Peripheral Blood Mononuclear Cell Isolation from Whole Blood

Peripheral blood mononuclear cells (PMBCs) were isolated using a modified Ficoll-Hypaque density gradient centrifugation method as described by Gazzinelli *et al*., (2016). Blood sample was diluted in a 15 ml falcon tube using an equal volume of RPMI-1640 (1:1 dilution) containing 1% each of L-Glutamine and penicillin –streptomycin solutions at room temperature (22-28ºC). The mixture was then gently over-laid with Lymphrophep, 1/3 its volume in a 50 ml falcon tube and centrifuged at 2000 revolutions per minute (rpm) for 10 min, without break (Being careful not to disturb the layers before and after centrifugation). A separation of the blood into three layers of plasma, lymphocyte and packed red cells was observed. The plasma was gently aliquoted into 2 ml micro-centrifuge tubes and stored at -20ºC with the dilution factor indicated. The lymphocyte layer was then collected into a 15 ml falcon tube containing 10 ml wash medium made up of RPMI-1640 with 5% heat inactivated Fetal Bovine Serum (FBS) and 1% each of L-Glutamine and penicillin –streptomycin solutions at room temperature. This was centrifuged at 2000 rpm for 10 min and the supernatant discarded. Fifteen millilitres of the wash medium was then added to the cells after they had been loosened, the mixture was centrifuged at 2000 rpm for 10min and the supernatant discarded. The loosened cells were then re-suspended in 2 ml of the wash medium. Ten microlitres of the sample was then added to 90 µl of trypan blue

solution and cell viability and concentration estimated using neubauer counting chamber (haemocytometer) with a light microscope. During PBMC estimation, the rest of the cell suspension was kept at 4^{o}C until use.

3.9 PBMC Cryo-Preservation

After cell estimation, an appropriate volume of RPMI-1640 with 5% Fetal Bovine Serum (FBS) and 1% each of L-Glutamine and penicillin-streptomycin and 10% dimethyl sulfoxide (at room temperature) was added to the cells to achieve a concentration of $1x10^{6}$ cells/ml. One milliliter of this suspension was stored in 1.5 ml cryotubes which was transferred into pre-cooled strata coolers and then placed in a -80^{o}C freezer overnight. The cryo-preserved PBMC was then transferred from the freezer within 24 hrs into liquid nitrogen at -196^{o}C for long term storage.

3.10 Recovery of Cryo-Preservation PBMC

The cryo-preserved cells were removed from the liquid nitrogen and immediately thawed by shaking the vial in a 37^{o}C water bath until most of the ice was melted. The cell suspension was then gently transferred to a 15 ml falcon tube containing 5 ml of RPMI-1640 with 5% heat inactivated Fetal Bovine Serum (FBS) and 1% each of L-Glutamine and penicillin-streptomycin solutions at room temperature. This mixture was then centrifuged at 2000 rpm for 10 min after which supernatant was discarded and pelleted cells loosened. The cells were similarly washed again and supernatant discarded. Pelleted cells were loosened, re-suspended in 2 ml wash medium and incubated at 37^{o}C with 5% CO_2 in air with caps loosened for at least 1 hr before plating. Cell concentration and viability was estimated in a 90% trypan blue solution (1:10 dilution) using a haemo-cytometer and light microscope.

3.11 PBMC Stimulation with *T. gondii* Antigen

Cells were cultured in the presence of 5 µg/ml of *T. gondii* (Mybiosource Inc USA), active antigen and 3.2 µg/ml Concavalin A, as positive control in a 24 well culture plate at a concentration of 1 x 10^6 cells/well, in a final volume of 1.0 ml. The parasite antigen concentration (5 µg/ml) and length of incubation were predetermined based on titration and kinetic experiments. The supernatant was harvested at 48 hours to measure the cytokines; IFN- ɤ, TNF-α, TGF-β and IL-10.

3.12 Cytokine Enzyme Linked Immunosorbent Assays

The ability of the stimulated cells to produce cytokines *in vitro* was quantified using commercially available ELISA kits. The following cytokines were measured: IFN- γ, TNF-α, TGF-β and IL-10 (R and D Systems, Minneapolis, USA).

The capture antibody was diluted to the working concentration (2 µg/µl for IL-10 and TGF-β; 4 µg/µl for TNF-α and IFN- γ) in 1X sterile PBS and used immediately to coat a 96 well microplate, for each of the different cytokines at 100 µl per well (22 – 28^{o}C). The plates were sealed and incubated overnight at room temperature. The wells were aspirated, washed three times with wash buffer (0.2 µm filtered 0.05% Tween® in 1X PBS, PH 7.2-7.4) and blotted against clean paper towels. The wells of respective plates were then blocked with 300 µl of blocking buffer, 1% BSA in PBS for TNF-α and IL-10; 1% BSA in TBS for IFN- γ and 1% Tween® 20 in PBS for TGF-β. The plates were incubated for 1 hr at room temperature, washed three times and blotted against clean paper towels.

Standards were prepared to working concentrations of 220 ng/ml for TGF-β, 75 ng/ml for IL-10, 67.5 ng/ml for TNF-α and 65 ng/ml for IFN- γ. After the addition of standards and test supernatants from participants, guided by the plate map, the plates were incubated for 2 hrs at room temperature and washed and blotted. Hundred microlitres of the detection antibody which was diluted in reagent diluent (0.2µm filtered 1% BSA in PBS, pH 7.2-7.4) and added to each well on the respective plates, covered and incubated for 2 hrs at room temperature. The plates were then washed, blotted and 100 µl of 1X Streptavidin conjugated to horseradish-peroxidase solution added. The substrate solution (Tetramethylbenzidine) was added in the dark at 100 µl/ well and incubated covered with aluminum foil for 20 min at room temperature after which 50 µl of stop solution was added to each well. The optical density was determined immediately after addition of the stop solution using a microplate reader (1A110 WAKO, Japan) set to 450nm wavelength.

3.13 Interpretation of ELISA Results

The results obtained from the microplate reader were interpreted according to the instructions of the manufacturer (R and D Systems, Minneapolis, USA). This was done by constructing a standard curve with the mean absorbance (optical density) and concentration of the standards plotted on the y-axis and x-axis respectively. The curve of best fit was drawn and used to obtain the respective cytokine concentration of the test samples (supernatants from participants' antigen stimulated PBMCs).

3.14 Extraction and Purification of Genomic DNA from Blotted Filter Paper.

Deoxyribonucleic Acid (DNA) was extracted and purified from the blood blots using a DNeasy® Blood and Tissue Kit (Qiagen QIAamp, Hilden, Germany) with a modified protocol.

Three pieces of the 5 mm sized dried blood blots from each participant were cut into labeled sterile 1.5 ml micro-centrifuge tubes and 180 µl of Buffer ATL added. This was then incubated in a pre-heated heat block at 85^{o}C for 10 min and centrifuged for 30 secs to remove droplets from the lid. Twenty microlitres of proteinase K stock solution was added, mixed by vortexing briefly and incubated at 56^{o}C for one hour and centrifuged for 30 secs to remove droplets from lid. Two hundred microlitres of lysis Buffer AL was then added to the mix and thoroughly vortexed immediately for about 15 sec. This was then incubated at 70^{o}C for 10 min, after which 200 µl of absolute ethanol (96-100%) was added and thoroughly vortexed briefly to mix. This was then centrifuged for 30 secs to remove droplets from the lid. The supernatant was then transferred into a QIAamp Spin Column (in a 2 ml collection tube). This was then centrifuged at 8000 rpm for 1 min and the flow through along with the collection tube was discarded. The QIAamp spin column was then placed in a new 2 ml collection tube and 500 µl of wash buffer 1 (AW1) was added. This was centrifuged at 8000 rpm for 1 min and the flow through and collection tube discarded. The QIAamp spin column was again placed into a new 2 ml collection tube and 500 µl of wash buffer 2 (AW2) was added. This was centrifuged at 14,000 rpm for 3min. The flow through and the collection tube were then discarded and the spin column placed into well labeled sterile 1.5 ml micro-centrifuge tubes and 200 µl of elution buffer (AE) added and incubated at room temperature for 1 min. The DNA was eluted by centrifuging at 8000 rpm for 1 min. The eluted DNA was then placed into cardboard cryo-boxes and stored at -20^{o}C until analyzed for *T. gondii* and *Plasmodium spp* by PCR methods.

3.15 Detection of *Toxoplasma gondii* DNA by Polymerase Chain Reaction

Toxoplasma gondii detection from the extracted DNA was done using a nested PCR method as described by Ayi *et al,* (2016). This method was used because it is appropriate for population genetics and epidemiological studies and has a high resolution in typing of clonal lineages (Ayi *et al.*, 2016).

3.15.1 Nest One (Outer Reaction) Procedure

The nest 1 PCR was carried out in a 25 µl solution containing 1X Green GoTaq® reaction buffer comprising PCR buffer, $MgCl_2$ and dNTPs (Promega, USA), 0.1 µM each of the external primers of SAG3 (forward; CAACTCTCACCATTCCACCC, reverse; GCGCGTTGTTAGACAAGACA) and GRA6 (forward; ATTTGTGTTTCCGAGCAGGT, reverse; GCACCTTCGCTTGTGGTT) and 5 µl of the genomic DNA extracted. The nest 1 reaction thermocyclic conditions were 95ºC for 4 minutes, followed by 25 cycles of 94ºC for 30 sec, 55ºC for 1 min, and 72ºC for 2 min.

3.15.2 Nest Two (Outer Reaction) Procedure

The nest 2 PCR was carried out in a 25 µl solution containing 1X Green GoTaq® reaction buffer, 0.3 µM each of the external primers of SAG3 (forward; TCTTGTCGGGTGTTCACTCA, reverse; CACAAGGAGACCGAGAAGGA) and GRA6 (forward;TTTCCGAGCAGGTGACCT, reverse; CGCCGAAGAGTTGACATAG) with 2 µl of nest 1 amplicon. The reaction conditions were 95ºC

for 4 min, followed by 27 cycles of 94ºC for 30 sec, 58ºC for 1 min and 72ºC for 2 min. Seven microlitres of the nPCR products from nest 2 were ran on 2% Agarose gel stained with 0.3ug/ml ethidium bromide solution and visualized under ultraviolet light to identify bands corresponding to *T. gondii* (SAG 3; 225 bp and GRA6: 344 bp)

3.16 Restriction Fragment Length Polymorphism

For clonal type detection, the specific and distinctive patterns obtained from RFLP within each locus were used. The amplified fragments of the nested PCR samples that tested positive for the markers used were digested with appropriate restriction enzymes for SAG 3. The 20 µl digest reaction mix was made up of 3 µl of nPCR products, 1X NEB buffer, 0.1 mg/ml BSA and 1 unit of the restriction enzyme (Ncil enzyme, New England BioLab, Beverly, MA, USA). The reaction was incubated at 37ºC for one hour. The fragments were analyzed on a 3% Agarose gel electrophoresis stained with 0.3 µg/ml ethidium bromide and visualized under the UV transilluminator. The patterns of digestion of the samples were compared to the digest patterns of two reference strains RH (as an isolate of type I lineage) and PTG (as an isolate of type II lineage)(Cabral *et al.*, 2013). The RH strain was characterized as genotype I when the 3'-5' ends of the SAG3 amplified fragment digested with the Ncil restriction enzyme (5'-3': CCSGG; 3'-5': GGSCC) yielded 3 bands of sizes in the regions <50bp, <80bp, and approximately 100bp respectively. The PTG strain was characterized as genotype II when amplified fragments of the SAG3 gene remained undigested with the Ncil restriction enzyme.

3.17 Detection of *Plasmodium species* DNA by PCR

Plasmodium falciparum DNA were detected using a modified nested PCR method as described by Snounou *et al*, (1993).

3.17.1 Nest One (Inner Reaction) Procedure

The reaction for the nest 1 was carried out in a 20µl solution mix containing 1X Green GoTaq® reaction buffer comprising PCR buffer, $MgCl_2$ and dNTPs (Promega, USA) and 0.25 µM each of the external forward and reverse generic primers of Plasmodium (PLU5: CCTGTTGTTGCCTTAAACTTC and PLU6: TTAAAATTGTTGCAGTTAAAACG).with 1 µl of extracted DNA sample were also added to the mix. The reaction conditions for nest 1 were 94ºC for 4 min, followed by 35 cycles of 94ºC for 30 sec, 58ºC for 1 min and 72ºC for 5 min.

3.17.2 Nest Two (Outer Reaction) Procedure

The nest 2 PCR was carried out in a 20 µl solution containing 1X Green GoTaq® reaction buffer, 0.25 µM each of the forward and reverse internal primers of rFAL1 (forward primer: TTAAACTGGTTTGGGAAAATATATT) and rFAL2 (reverse primer: ACACAATGAACTCAATCATGACTACCCGTC) with 1 µl of nest 1 amplicon. The reaction conditions were 95ºC for 4 min, followed by 27 cycles of 94ºC for 30 sec, 58ºC for 1 min and 72ºC for 2 min. Five microliters of the nPCR products from nest were ran on 2% Agarose gel stained with 0.3ug/ml ethidium bromide solution and viewed under ultraviolet light to identify any bands corresponding to *Plasmodium falciparum* known band size (205 bp)

3.18 Gel Electrophoresis and UV Visualization

For electrophoresis, 5 ul of PCR products were run on a 2% agarose (Sigma-Aldrich) gel, stained with ethidium bromide, using a 1 X Tris Acetate EDTA (0.8 mM Tris, 0.8 mM glacial acetic acid, 10 mM EDTA) as running buffer at 100V (Power Pac 3000, Biorad) for 45 min. The gel was visualized under a UV transilluminator (Clear View, Cleaver Scientific Ltd). For each run, 100 bp ladder was loaded alongside. The gel picture was taken using Gel Logic Imaging System (Kodak) and band sizes independently scored with the ladder as reference point.

3.19 Data Analysis

Data was entered into Microsoft Excel® 2010 spreadsheet and analyzed using the statistical software packages SPSS version 20 (SPSS Inc.) and GraphPad Prism 5 (GraphPad Software, Inc). For demographic data, the geometric mean and standard deviations of the ages were determined using Microsoft Excel® 2010. The prevalence of *Toxoplasma* and *Plasmodium spps* were also determined using SPSS. The chi-square test was used to determine association between age and infection status as well as gender and parasite infection status. A Student's t-test was calculated to determine statistical significance with a set confidence interval (CI) of 95% and 1 degree of freedom (df) and *p* values less than 0.05 were considered statistically significant.

Cytokine optical density (OD) was converted to concentration using a 4-parameter logistic curve fit using excel. As cytokine concentrations were not normally distributed, nonparametric tests were used. Differences between infection groups in cytokine concentrations and non-infection groups were tested by Mann-Whitney U test. Spearman's p was calculated to estimate the correlation between cytokine responses and infection status and graphs were drawn using

GraphPad.

CHAPTER FOUR

RESULTS

4.1 General Characteristics of Study Participants

A total of 92 participants from different parts of the country were involved in this study, 54 were recruited from the blood bank of the National Blood Service and 38 from the Fevers Unit, all of the KBTH. The participants consisted of 54 (58.7%) males and 38 (41.3%) females aged 20 to 60 years (Mean: 36.5 ± 9.8). The general characteristics of study participants are presented in Table 1.

The age distribution of the participants is shown in Figure 4.1. Individuals within the age group of 30 to 39 years formed the majority whilst the 50 to 60 year old group had the least number among the blood donor participants. The HIV seropositive participants had majority among the 30 to 39 as well as 50 to 60 year olds with the 20 to 29 year olds being the least in number.

Table 2 shows the responses to questionnaire on the exposure of participants to *T. gondii* infection. The results did not show any statistically significant associations between *Toxoplasma* positivity and known the risk factors ($p > 0.05$). Out of the 92 individuals sampled, only 2 (2.2%) had any knowledge about Toxoplasmosis, both being HIV seropositive individuals.

Table 1: General Characteristics of Study Participants

Characteristic		HIV Seropositive	Blood Donors
Age (years)	Mean	41.1±10.1	33.2±8.2
	Range	23-60	20-52
Gender	Male (%)	10 (26.3)	44 (81.4)
	Female (%)	28 (73.7)	10 (18.6)
Educational Level	No formal Education (%)	4 (10.5)	1 (1.9)
	Elementary (%)	10 (28.3)	8 (14.8)
	Secondary/Tertiary/Vocational (%)	24 (63.2)	45 (83.3)
Occupation	Office (bank, school, etc) (%)	5 (13.2)	16 (29.6)
	Hospital (OPD, labour ward, blood bank) (%)	1 (2.6)	1 (1.9)
	Market (sell vegetables and meat) (%)	10 (26.3)	7 (13.0)
	Vocational centre (%)	12 (31.6)	20 (37.0)
	Other (self-employed, unemployed) (%)	10 (26.3)	10 (18.5)

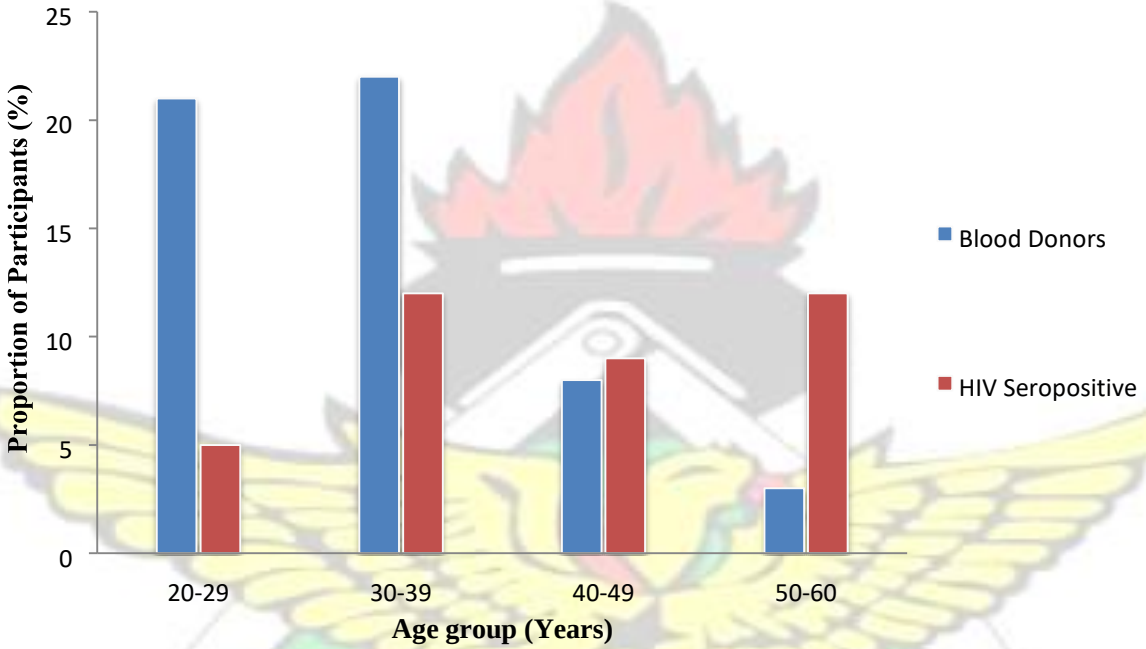

Figure 4.1: Age distribution among study participants

Table 2: Exposure to risk of *T. gondii* infection (responses to questionnaire)

Study Participants	Number examined (N)	*Toxoplasma gondii* positivity (n/N; %)	χ^2 Value	P value (CI:95%)
Cat ownership				
Yes	40	7 (17.5)	0.234	0.629
No	52	12 (23.1)		
Sandbox for cat(s)				
Yes	54	7 (12.9)	0.661	0.843
No	38	11 (28.9)		
Eating Meat				
Yes	4	1 (25.0)	0.078	0.779
No	88	17 (19.3)		
Eating Vegetables				
Yes	8	2(25.0)	0.164	0.685
No	84	16(19.1)		
Handling Raw Pork				
Yes	7	0(0)	1.870	0.171
No	84	18(21.4)		
Handling Raw Chevon				
Yes	25	2(8)	3.015	0.083
No	66	16(24.2)		
Handling Raw <u>Mutton</u>				
Yes	18	1(5.6)	2.861	0.091
No	73	18(21.4)		
Handling Raw Beef				
Yes	16	2 (12.3)	0.648	0.421
No	75	16(21.3)		

4.2: Prevalence of *Toxoplasma gondii* in Study Participants by PCR

An overall DNA positivity of 19.6% (18/92) as detected by SAG 3 only was recorded among the participants. There was no DNA positivity by GRA 6. The percentage prevalence of *T. gondii* DNA in the blood-donor and HIV seropositive participants was found to be 14.8% (8/54) and 26.3% (10/38) respectively (Fig 4.2). All the *T. gondii* DNA positive samples were of clonal type II.

Frequency of *T. gondii* infections categorized by sex and age are represented in Figures 4.3 and 4.4 respectively. Majority of infections were within the 30-39 and 40-49 age brackets for Blood donor and HIV seropositive participant respectively. Males had more *T. gondii* DNA positives in the blood donor group whereas females had more *T. gondii* DNA positives in the HIV seropositive group. There was no statistically significant difference ($p > 0.05$) for any of the analysis.

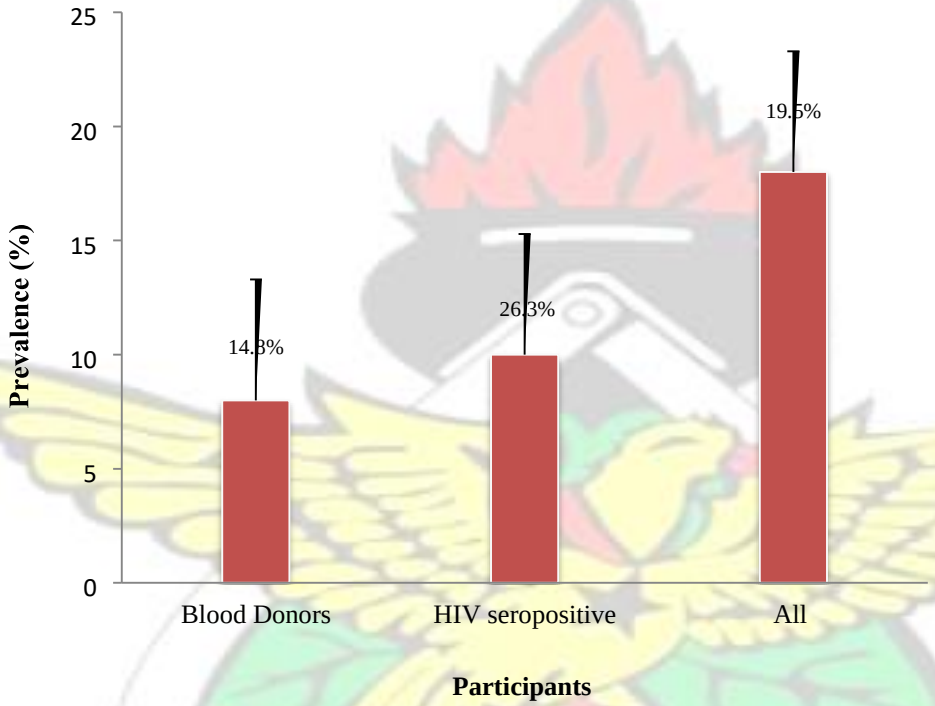

Figure 4.2: Prevalence of *Toxoplasma gondii* DNA in study participants

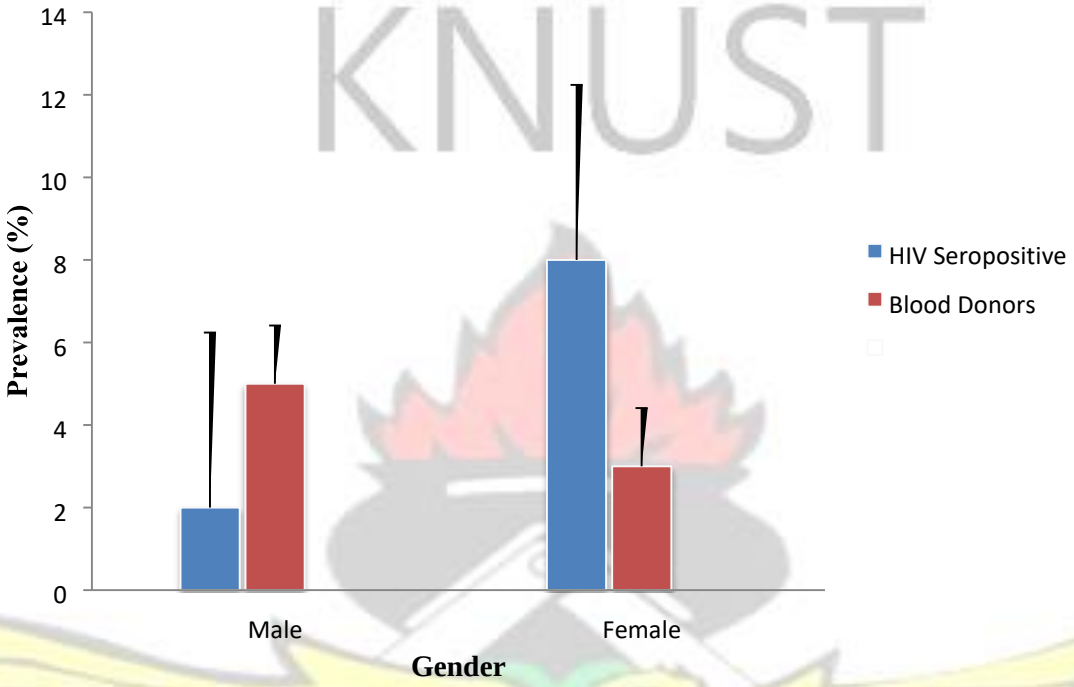

Figure 4.3: Prevalence of *Toxoplasma gondii* DNA by gender among study participants

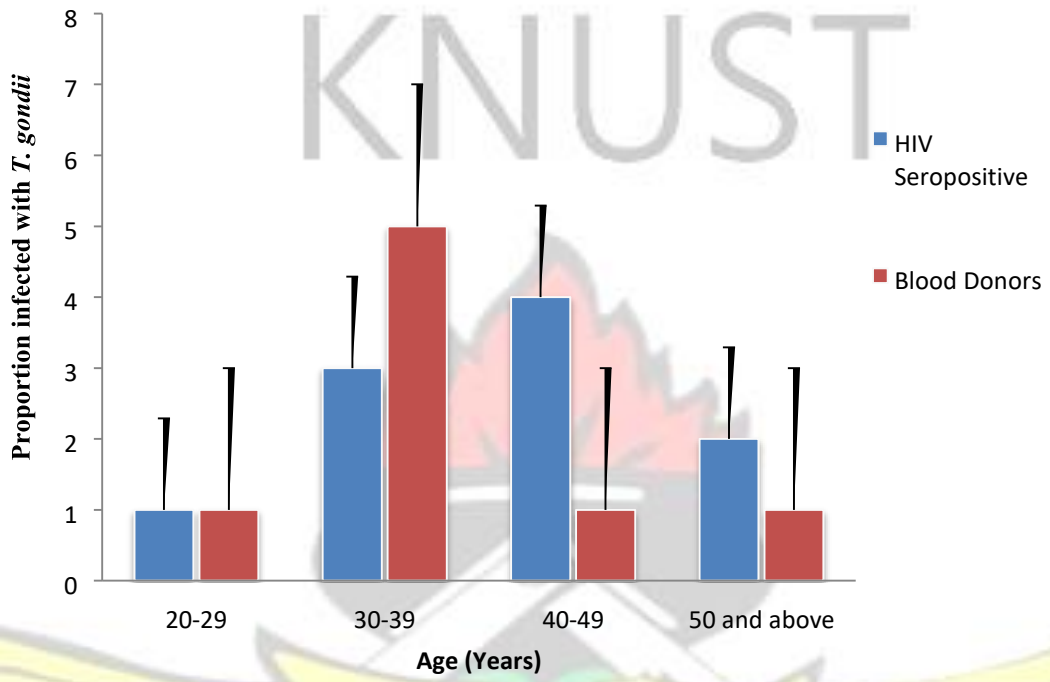

Figure 4.4: *Toxoplasma gondii* DNA positivity by age group among study participants

4.3: Prevalence of *Plasmodium falciparum* in Study Participants using RDT and PCR

Malaria RDT showed 5.4% (5/92; 3 blood donors and 2 HIV seropositive) participants had *P. falciparum* infections while nested PCR detected *P. falciparum* DNA in 18.5% (17/92) of participants. The percentage prevalence of *Plasmodium falciparum* DNA in the blood donor and HIV seropositive participants was 14.8% (8/54) and 23.6% (9/38) respectively (Figure 4.5). All who tested positive by RDT were also positive for PCR. The frequency of *P. falciparum* infection differed between age groups in the blood donor group (Figure 4.6). There was no statistically significant difference in any of the analysis.

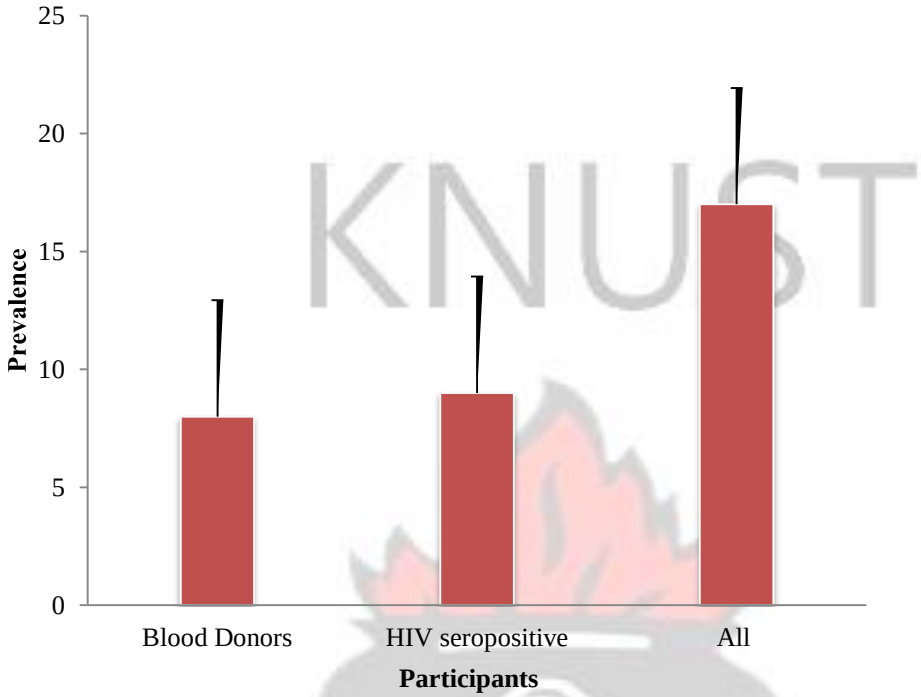

Figure 4.5: Prevalence of *Plasmodium falciparum* DNA infection detected by PCR

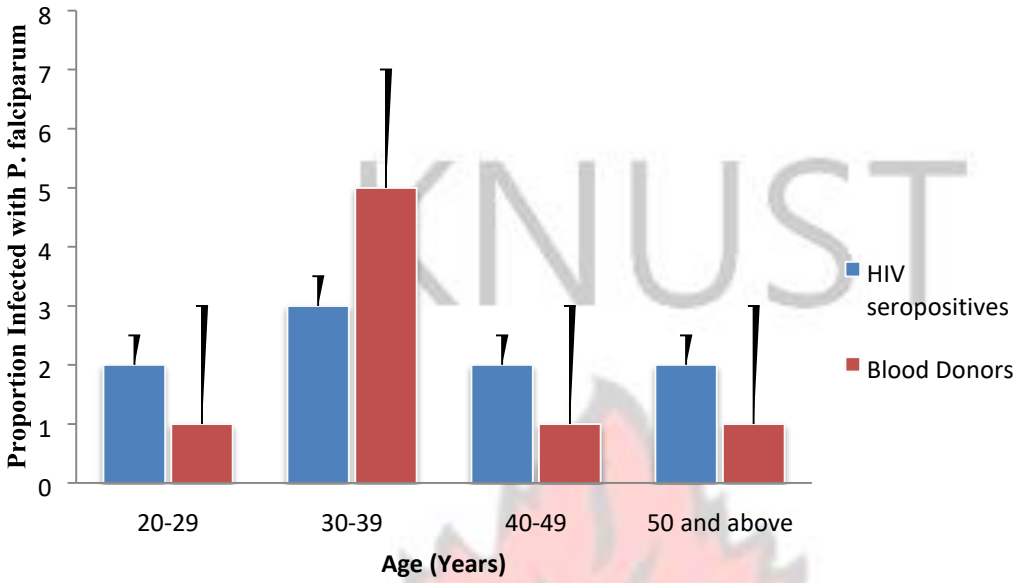

Figure 4.6: *Plasmodium falciparum* DNA positivity by age groups among Study Participants

4.4 Prevalence of *Toxoplasma gondii* and *Plasmodium falciparum* co-infections in Study Participants

Two (3.7%) out of 92 participants were positive for both *P. falciparum* and *T. gondii* infections by PCR. Both were HIV seropositive participants.

4.5 Cytokine Levels in Study Participants.

Levels of cytokines IL-10, TNF-α, IFN-γ, TGF-β in response to *T. gondii* antigen were measured from stimulated PBMCs of 34 out of 38 HIV seropositive and 28 out of 54 Blood donor participants. PBMCs from HIV seropositive participants were found to produce high levels of all four cytokines as compared to the PBMCs from the Blood donor participants (Table 3).

Figure 4.7 shows the levels of cytokines measured in *T. gondii*-DNA positive HIV-seropositive and blood donor participants. In both HIV-seropositive and Blood Donor participants, TGF-β recorded the highest levels in pg/ml whilst IL-10 recorded the least. There was also a significant difference between the levels of TGF-β in HIV seropositive participants as compared to Blood donor participants. There was no statistically significant difference, however, recorded for the other cytokines

Table 3: Cytokines levels in Response to *T. gondii* antigen across Study Participants

	Mean Cytokine Level (Range) Cytokine		
	HIV Seropositive	**Blood Donors**	***p* value**
TGF-β *	493.1 (399.4-586.9)	118.0 (97.8-138.2)	<0.001
TNF-α *	560.6 (181.0-940.3)	31.5 (12.2-50.8)	<0.001
IFN- γ *	81.5 (46.8-116.3)	16.6 (12.6-20.5)	<0.001
IL10 *	52.3 (26.8-77.9)	4.8 (3.7-5.9)	<0.001

There was a significant difference ($P < 0.05$) in the levels of cytokines of HIV seropositive participant compared with Blood donor participants

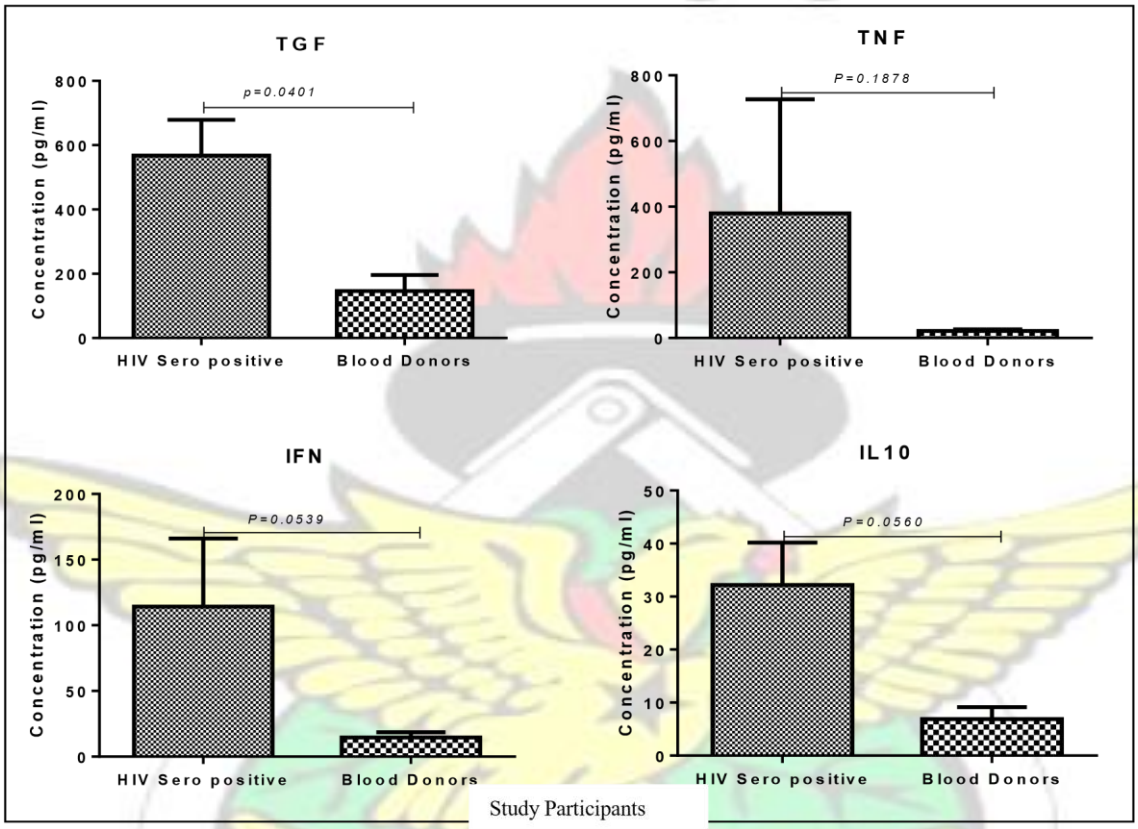

Figure 4.7: Comparison of cytokine levels in Study Participants. Only the data for TGF-β was significant *(P < 0.05)* though generally HIV seropositive participants had higher cytokine levels.

CHAPTER FIVE

5.0 DISCUSSION

Toxoplasmosis, one of the neglected tropical disease, is increasingly becoming a disease of public health concern (Flegr *et al.,* 2014) with a greater disease burden among immune compromised individuals (Baliu *et al.,* 2014; Ayi *et al.,* 2016). Several studies have been conducted on this disease, however, most immune studies have been done in murine models which may not be reflective of the situation within humans. Studies have however indicated that cellular immune response is central to the control of the parasite (Filisetti and Candolfi, 2004). This study therefore aimed at characterizing cytokine responses to *Toxoplasma gondii* in immune competent and immune compromised individuals in Ghana.

The prevalence of *Toxoplasma gondii* in the HIV seropositive participants and Blood donors in this study was 26.3% and 14.8% respectively. The prevalence among HIV seropositive individuals was lower but that among blood donors is significantly higher in this study than those reported (54% in HIV seropositive and 3.4% in Blood donor participants) by Ayi *et al.*, (2016). $CD4^+$ T cell counts of 200 cells/mm^3 in blood or less have been implicated to increase the risk of reactivation of the *T. gondii* parasite in HIV seropositive participants and hence could account for the higher prevalence recorded among the HIV seropositive participants (Weiss and Dubey, 2009) as these had $CD4^+$ T cell counts less than 200 cells/mm^3 of blood. This is comparative with studies that found an increasing risk of the disease among individuals with lower $CD4^+$ T cell counts (Sullivan and Jeffers, 2013). The increasing prevalence among the Blood Donor participants is however an issue of public health concern as one of the main routes of transmission of *Toxoplasma gondii* is via blood transfusions (Dubey *et al.*, 2008; RobertGangneux and Darde, 2012) and *Toxoplasma*

tachyzoites can survive within body fluids for up to a day; however they can persist in whole blood for approximately 50 days at 4^{o}C (Karimi *et al.,* 2016). Majority of those requiring blood transfusions are individuals who are immune suppressed and hence could be at risk of parasite reactivation and consequent disease-associated effects. The observation of SAG 3 showing more positives as compared to GRA 6 is consistent with literature in Ghana (Ayi *et al*, 2016). This is also however an issue for public health concern as SAG 3 is known to be a surface antigen and arbitrates the attachment between the parasite and the host cell (Jacquet *et al*, 2001). This could therefore be indicative of active infections hence implying that approximately 15% of donated blood could transmit the parasite to the recipient if the blood is transfused. A comparison however between the prevalence of *T. gondii* between the two study groups revealed no significant difference.

Overall, females had a higher prevalence of *T. gondii* infection than males though it was not statistically significant. The prevalence patterns differed across gender for the two study groups mainly due to the skewed nature of the samples obtained from each site. A higher prevalence of *T. gondii* infection was seen in males for the Blood donor participants whereas HIV seropositive participants recorded a higher prevalence among females. This observation of more males seen in Blood donor groups and vice versa for HIV seropositive groups have been reported in other studies (Bani and Giussani, 2010; Madrona *et al.,* 2014) Such studies have shown that men are more likely to donate blood because they usually make such decisions on their own, whereas women are more influenced by others and are more susceptibility to vasovagal reactions. The studies also indicated that there are more restrictions placed on women preventing them from donating blood, such as the need for higher haemoglobin concentrations (Bani *et al.,* 2010; Madrona *et al.,* 2014). In HIV

infected persons, a report generated by Country AIDS response progress (2014) indicates that an estimated 2,983 new female infections will be recorded as opposed to the 2,391 cases of men by the end 2016. This suggests that females have higher odds of being infected with HIV as compared to males.

The prevalence of *T. gondii* infection was higher in the 30 to 39 age bracket for all the study participants possibly due to the fact that most participants were within this age bracket.

This study showed no significant association between *T. gondii* positivity and known risk factors such as presence of domestic cats, handling of raw meat and proximity to cat sand boxes. This is in contrast with the results of studies reported by Abu *et al.,* (2014) and other studies conducted in different countries such as Tanzania (Mwambe *et al*., 2013).

Detection of *P. falciparum* using PCR showed a prevalence of 18.5% of infection whereas the use of the Malaria RDT kit showed a prevalence of 5.4%. The lower prevalence obtained by RDT tests is possibly due the fact that the RDT kit is targeted at detecting antigens which are known to persist in the blood for up to two weeks (Humar *et al.,* 1997) whereas PCR is known to detect parasite DNA which could even persist for a very long period of time after parasite has been opsonized. PCR has also been shown to be more efficient, more sensitive and specific when compared with other diagnostic methods (Padley *et al.,* 2003; Johnston *et al.,* 2006). This is supported by the fact that all samples that tested RDT positive were also positive by PCR. The prevalence of 18.5% *P. falciparum* raises important issues since the participants were not showing any febrile signs or disease symptoms at the time of sampling. Hence such persons may be asymptomatic patients and

serve as human reservoirs for parasite transmission. This raises public health concerns in the effort to eliminate malaria in Ghana

Toxoplasma gondii induces an extremely polarized Th1 type immune response (Gazzinelli *et al*., 1991, 1992), and an increased immunoregulation is needed to control the response and avoid severe immunopathology (Gazzinelli *et al*., 1996; Neyer *et al*., 1997; Suzuki *et al*., 2000). Cell mediated immune responses have been found to be essential for host control of intracellular infections (Yap and Sher, 1999). The activation of T cells by a stimulant (antigen or mitogen) could result in the boost in regulation of cytokine receptors and production and secretion of cytokines (Collins *et al.*, 1998). Cytokines have been found to play a major role in the regulation of several diseases. They are involved in mediating the differentiation of T helper (Th) cells to produce protective effectors and are also involved in mediating tissue damage (Zuezdanovic *et al.*, 2006) and in the occurrence of toxoplasmosis, the cytokines can be divided into either proinflammatory and anti-inlammatory cytokiness.

In this study, the levels of the pro-inflammatory (TNF-α and IFN-γ) and anti-inflammatory (IL10 and TGF-β) measured were significantly higher ($p < 0.005$) in the HIV seropositive than in the blood donor participants indicating a strong association between the HIV seropositivity and mean cytokine levels. This could be as a result of the higher susceptibility to opportunistic diseases in the immune-compromised group and thus an activated immune system.

The assessment of the cytokine profile of *Toxoplasma gondii* positive cases showed that all four cytokines measured were higher in the immune-compromised group; however, only TGF-β showed statistically significant difference. TGF-β showing significantly high levels contradicts studies which indicate that *T. gondii* Type II strains infected individuals usually have lower levels

of anti-inflammatory cytokines as compared to Type I strains (Xiao & Yolken, 2015). The higher levels of this cytokine may also be the reason for no febrile cases reported in the study participants. It is however not clear why the other anti-inflammatory cytokine (IL-10) did not show a similar profile compared to TGF-β.

Human Immuno Deficiency Virus, has been shown to suppress the activity of IFN-γ hence the comparatively lower levels to other cytokines (Gazzinelli *et al.*, 2016; Mihret *et al.*, 2014). This observation was made in this study and could also support the hypothesis that HIV infection causes a switch from Th1 to Th2 response in parasite immune regulation (Gazzinelli *et al.*, 2016). This trend seen in the blood donor participants may account for none of them showing any febrile symptoms.

The cytokine profile for Toxoplasma positive was similar to that of Malaria positive individuals in this study. This may be because both parasites belong to the same phylum and hence exhibit a similar mechanism of evasion in the body of host (Tan *et al.*, 2011). The comparison between Toxoplasma positives and Toxoplasma negatives within groups showed no significant difference indicating that infection with parasite had no significant influence on the cytokine profiles of the participants. Cytokine levels of the participant co-infected with both parasites were high as compared to singly infected participants however statistical inference cannot be made on only one sample; as such the relationship between *T. gondii* and *P. falciparum* co-infected individuals and cytokine profiles remain inconclusive.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

All the positive *T. gondii* study participants (19.5%) were of Type II clonal lineage. *T. gondii* positive and *T. gondii* negative participants displayed similar cytokine patterns. However, the significantly higher levels of cytokines in the immune compromised as compared to the immune competent group could be an indicator of the influence of other pathogens on their immune system. 18.5% of the participants showed DNA positivity for *P. falciparum* but only one participant had both *T. gondii* and *P. falciparum* co-infecting. This participant also displayed higher levels of cytokines as compared to those with single infections. The study showed that the participants had very little knowledge of Toxoplasmosis and the risks of transmission they are exposed to but revealed no significant association between *T. gondii* positivity and exposure to known risks factors.

6.2 Limitations

In this study, the influence of other pathogens on the cytokine profiles was not studied, that is the influence of other Th1/Th2 response inducing pathogens was not be assessed.

6.2 Recommendations

It is recommended that education about the disease be intensified within the general populace. It is also recommended that a study with a larger sample size should be carried out and other pathogens that elicit a Th1 or Th2 response be studied for their effect on cytokine levels.

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APENDICES

APPENDIX I

ETHICAL CLEARANCE FROM NOGUCHI ISTITUTIONAL REVIEW BOARD

NOGUCHI MEMORIAL INSTITUTE FOR MEDICAL RESEARCH
Established 1979
A Constituent of the College of Health Sciences
University of Ghana

INSTITUTIONAL REVIEW BOARD

Phone: +233-302-916438 (Direct)
+233-289-522574
Fax: +233-302-502182/513202
E-mail: nirb@noguchi.mimcom.org
Telex No: 2556 UGL GH

Post Office Box LG 581
Legon, Accra
Ghana

My Ref. No: DF.22
Your Ref. No:

4th March, 2015

ETHICAL CLEARANCE

FEDERALWIDE ASSURANCE FWA 00001824 **IRB 00001276**

NMIMR-IRB CPN 074/14-15 **IORG 0000908**

On 4th March 2015, the Noguchi Memorial Institute for Medical Research (NMIMR) Institutional Review Board (IRB) at a full board meeting reviewed and approved your protocol titled:

TITLE OF PROTOCOL : **Characterization of cellular responses to toxoplasma gondii infection in immune-competent and immune-compromised individauals in Ghana**

PRINCIPAL INVESTIGATOR : **Worlasi Debi Kartey-Attipoe, Mphil Cand.**

Please note that a final review report must be submitted to the Board at the completion of the study. Your research records may be audited at any time during or after the implementation.

Any modification of this research project must be submitted to the IRB for review and approval prior to implementation.

Please report all serious adverse events related to this study to NMIMR-IRB within seven days verbally and fourteen days in writing.

This certificate is valid till 3rd March, 2016. You are to submit annual reports for continuing review.

Signature of Chair: .....
Mrs. Chris Dadzie
(NMIMR – IRB, Chair)

cc: Professor Kwadwo Koram
Director, Noguchi Memorial Institute
for Medical Research, University of Ghana, Legon

APPENDIX II

ETHICAL CLEARANCE FROM NATIONAL BLOOD TRANSFUSION SERVICE

NATIONAL BLOOD TRANSFUSION SERVICE

Republic of Ghana

Post Office Box KB 78
Korle-Bu, Accra

Our Ref:.....NBTS/RES-76/RDAL-02

Your Ref:.....

Date:..... 20TH OCTOBER, 2015

E-mail: research@nbsghana.org

Ms. Worlasi Debi Kartey-Attipoe
Noguchi Memorial for Medical Research (NMIMR)
Department of Parasitology

Dear Ms. Worlasi Debi Kartey-Attipoe,

Re: Research Protocol (NBSGRD/150423/01) "Characterization of cellular responses to *toxoplasma gondii* infection in immune-competent and immune-compromised individuals in Ghana"

Thank you for your letter seeking approval to conduct research on the above topic at the Southern Area Blood Centre (SABC). You provided the following documents for consideration:

- Completed online registration form
- Abstract
- Ethical Clearance
- Introduction Letter
- Consent Form
- Questionnaire
- Clarification letter on samples to be collected

After consideration of these documents, the project has been approved for collection of 5ml blood sample from 50 blood donors who donate at the SABC between now and 31st December, 2015.

Approval is conditional upon:

- Continued adherence to NBSG approved operating procedures.
- Adherence to all ethical requirements.
- Provision of notification of when sample collection commences and ends.

You are required to submit a copy of the final report once the study is completed.

Yours sincerely,

Dr. Lucy Asamoah-Akuoko
Head, Research & Development
E-mail: lucyasamoah@yahoo.com

Cc: Head, SABC
Donor Services Manager
Head of Donor Care
Research Officer, R&D

Tel: 0302 - 663701-2 / 681281-2 / 666429 / 676443 **Email**: www.nbsghana.org / info@nbsghana.org

APPENDIX III

PARTICIPANT CONSENT FORM

NOGUCHI MEMORIAL INSTITUTE FOR MEDICAL RESEARCH
Established 1979 *A Constituent of the College of Health Sciences University of Ghana*

CONSENT FORM

Title: Characterization of cellular responses to *Toxoplasma gondii* infection in immune-competent and immune-compromised individuals in Ghana.

Principal Investigator: Worlasi Debi Kartey-Attipoe (MPhil, Student).

Address: 1. Department of Clinical microbiology, School of Medical Sciences, KNUST, Kumasi
2. Department of Parasitology, NMIMR, University of Ghana. Legon, Accra.

General Information about Research
Dear Sir/ Madam,
You are kindly invited to take part in this study voluntarily and you are at liberty to opt out without any consequence.

Purpose of study: To analyze the cells responsible for fighting *Toxoplasma gondii* and study how these cells interact with the germ within the body of different study groups in Ghana.
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 people and may show symptoms such as headache and fever. Toxoplasmosis is being described as uncommon because, many infected people develop natural protection to the disease soon after exposure. The germ can however become active in people with weak natural protection such as in pregnant women or people infected with HIV and cause very severe disease. The disease can be transmitted through eating undercooked meat, unwashed vegetables or accidentally, soil which has cat faeces. 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 which will provide us with your gender, age and other details. You will be required to give a blood sample (about a teaspoon full – 5 ml) to be taken from your arm vein. Blood will be taken by an expert using a new sterilized needle. Samples will be tested for the presence or absence of *Toxoplasma* and the test results will be communicated to you through the health practioner.

Possible Risks and Discomforts
This is a minimal risk study however you will experience temporary pain when blood is being drawn.

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

NMIMR INSTITUTIONAL REVIEW BOARD
VALID UNTIL
0 3 MAR 2016
APPROVED DOCUMENT

NOGUCHI MEMORIAL INSTITUTE FOR MEDICAL RESEARCH

Established 1979 *A Constituent of the College of Health Sciences University of Ghana*

CONSENT FORM

Title: Characterization of cellular responses to *Toxoplasma gondii* infection in immune-competent and immune-compromised individuals in Ghana.

Principal Investigator: Worlasi Debi Kartey-Attipoe (MPhil, Student).

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APPENDIX 1V

PARTICIPANT QUESTIONNAIRE FORM

NOGUCHI MEMORIAL INSTITUTE FOR MEDICAL RESEARCH

Established 1979 *A Constituent of the College of Health Sciences University of Ghana*

CHARACTERIZATION OF CELLULAR RESPONSES TO *TOXOPLASMA GONDII* INFECTION IN IMMUNE-COMPETENT AND IMMUNE-COMPROMISED INDIVIDUALS IN GHANA

QUESTIONNAIRE FORM

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)

Age (years): .....

Area of residence: .....

Duration at residence: .....

Contact address: .....
.....

Telephone No: .....

Educational background
1) No formal education
2) Elementary
3) Secondary/Vocational/ Tertiary (Polytechnic, University, etc)
4) Other (specify): .....

☐

Where do you work?
1) Office (bank, school, etc)
2) Hospital (OPD, labour ward, blood bank)
3) Slaughter House
4) Garden/ Farm
5) Market (Sell vegetables, raw meat)
6) Vocational Centre
7) Other (Specify).....

☐

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PART B: TOXOPLASMOSIS-RELATED INFORMATION

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

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

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

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

3. When was the test conducted?
a) 3 to 6 months ago
b) Up to a year ago
c) More than a year ago
d) Other (specify).....

4. What was the result? Positive Negative No idea

5. **If positive**, did you receive any treatment? Yes No

6. Do you own a cat or have cats in your house or your neighborhood?
Yes No

If "Yes" to ***Qu. 6****, please answer* ***Qu. 7****. If "No"* ***skip*** *Qu. 7*

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

8. Have you ever handled raw meat from pigs (pork)?
Yes No

9. Have you ever handled raw meat from sheep (mutton)?
Yes No

10. Have you ever handled raw meat from goats (chevon)?
Yes No

11. Have you ever handled raw meat from cows (beef)?
Yes No

12. Do you eat meat?
Yes No, (I am a vegetarian)

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*If your answer to **Qu. 12 is "No"** please skip the following questions*

13. In which form do you often eat your meat?
a) Cooked but tough
b) Cooked till soft
c) Cooked tough or soft

14. Which meat do you eat? (Please *choose as many as applicable.*)
a) Pork
b) Goat meat/mutton
c) Beef
d) Chicken
e) No idea
f) Others (specify).....

15. Do you eat khebabs? Yes No

16. If yes, what type do you enjoy eating most?
a) Pork
b) Goat meat/mutton
c) Beef
d) Chicken
e) No idea
f) Others (specify).....

17. Do you eat vegetables? Yes No

18. In what state do you prefer your vegetables when eating them?
a) Fresh and raw
b) Steamed
c) Cooked

Thank you very much