PREVALENCE OF TOXOPLASMOSIS IN THIKA REGION, KENYA AND THE IMMUNOPATHOLOGY ASSOCIATED WITH *Toxoplasma gondii* INFECTION IN BALB/c MICE

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(Molecular Medicine)

JOMO KENYATTA UNIVERSITY OF

AGRICULTURE AND TECHNOLOGY

2018

Prevalence of Toxoplasmosis in Thika region, Kenya and the Immunopathology associated with *Toxoplasma gondii* infection in BALB/c mice

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A thesis submitted in partial fulfillment for the degree of Doctor of Philosophy in Molecular Medicine in the Jomo Kenyatta University of Agriculture and Technology

2018

DECLARATION

This thesis is my original work and has not been presented for a degree in any other university

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DEDICATION

This work is dedicated to my family, who encouraged and supported me all through to this level of education. Above all, to God, the creator of all beings, who provided strength, health and favor to enable me see this output.

ACKNOWLEDGEMENT

I thank the almighty God for the gift of life, good health, his inspiration and guidance in my life and for enabling me to successfully complete my studies. Firstly, I wish to thank RPE-JKUAT for funding the research. My gratitude goes to all those who in one way or another made this work a success. My deep appreciations goes to my University supervisors who are also part of the JKUAT Toxoplasmosis research team led by Prof. Simon Karanja (Principal Investigator), Prof. Naomi Maina, Dr John Kagira and Dr Maina Ngotho for their invaluable academic guidance and inspiration at every stage of this study. Even when the ideas and work were very raw, you always had time to look at it, make suggestions for improvement and necessary corrections. My sincere gratitude to Prof Karanja who ignited the challenge in me to study toxoplasmosis as an emerging protozoan zoonosis, even when i did not have an idea on what to further in my postgraduate studies and also for funding the research. To my fellow students; Dr David Kamau, Adele Njuguna, Edwin Ogendi and James Maina Ichagichu (Posthumous), a great thank you. Sincere gratitude goes to the Institute of Primate Research (IPR), National Museums of Kenya, for allowing me to carry out my research using their laboratories and animal resources. I sincerely thank the staff of Institute of Tropical Medicine and Infectious Diseases (ITROMID), and Jomo Kenyatta University of Agriculture and Technology (JKUAT) and IPR for facilitating my graduate program. Special thanks go to the staff of Animal Sciences and Tropical Infectious

Diseases Departments of IPR for invaluable support during this study. Finally, I am grateful to my family for their moral support and for being a source of encouragement and inspiration to me.

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ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
ALA	Alkaline phosphatase
Bp	Base pair
CD	Cluster designation
CDC	Center for disease control
CFT	Complement Fixation Test
CNS	Central nervous system
CSF	Cerebrospinal fluid
DEX	Dexamethasone
DNA	Deoxyribonucleic acid
Dpi	Days post infection
ELISA	Enzyme Linked Immunosorbent Assay
FDC	Follicular Dendritic Cells
Flc	Follicles
G.O.K	Government of Kenya
Gc	Germinal centers

IFA	Immunofluorescence Assay
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IPR	Institute of Primate Research
IRC	Institutional Review committee
JKUAT	Jomo Kenyatta University of Agricultural and Technology
KNBS	Kenya National Bureau of Statistics
LAMP	Loop Mediated Isothermal Amplification
MKU	Mount Kenya University
ML	Molecular ladder
NK	Natural killer
OD	Optical density
p.i	post infection
PBSP	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
Pg	Picograms

SCID	Severe combined Immunodefficiency
SDG's	Strategic Development Goals
T.E	Toxoplasmic encephalitis
T.gondii	*Toxoplasma gondii*
Th	T-helper cell
TNF	Tumour Necrosis factor
Mg	Milligram
µl	Microliter

ABSTRACT

Toxoplasma gondii is an important food and waterborne opportunistic pathogen that causes severe disease in immunocompromised individuals. The detection of *T. gondii* in free range chickens is a good indicator of environmental contamination with *T. gondii* and possible risk to human beings. The pathology and immune responses associated with the ensuing disease has not been well described in strains from different parts of Kenya. The present study aimed at determining the prevalence of toxoplasmosis in Thika region and development of a neurological mouse model in BALB/c mice to assess the changes of IgG, Ig M, cytokines and organ pathology. In order to accomplish this, 105 free range chicken were collected; deoxyribonucleic acid extracted from brain and analyzed using PCR to detect presence of *T. gondii*. For experimental infections, BALB/c mice were infected intraperitoneally with 15 cysts of a *T. gondii* isolate; monitored for 42 days and euthanized at different time points. Another group of mice were orally treated with dexamethasone [DEX: 2.66mg/kg daily (Group 1); 5.32mg/kg daily (Group 2)] at 42 days after infection and followed by monitoring for a further 42 days. The control groups consisted of infected and untreated (positive control, Group 3) and uninfected and untreated (negative control, Group 4) mice. After euthanasia, blood was collected for serum and assessed for IgG, IgM, IL-10 and IFN-γ levels. The brain tissue was also collected and divided in halves; one half was used for cyst enumeration, while the other half was used for histology. The liver, heart and spleen were also collected and processed for histopathology. Results indicated that the overall prevalence of *T. gondii* infection in chicken was 79.0% (95% CI: 70.0-86.4%). A mortality rate of 15% and 28.6% was observed in mice given 2.66mg/kg/day and 5.32mg/kg/day of dexamethasone, respectively. The number of cysts in the brain of DEX treated mice increased up to twofold compared with chronically infected untreated mice. During the acute and chronic phases, there was

an increase in both IgM and IgG levels but following dexamethasone treatment, IgM levels declined but IgG levels continued to rise peaking on 42 days post-treatment. Elevated levels of interferon-gamma (IFN-γ) ($P<0.05$) and IL-10 ($P<0.01$) were observed between 7 and 42 days post infection (dpi). After dexamethasone treatment, IFN-γ and IL-10 levels declined significantly ($P<0.05$) at time points between 42 and 84 dpi. The decrease was higher ($P>0.05$) in Group 2 compared to Group 1, mice. Mice in the infected non-treated group had significantly ($P<0.01$) elevated IFN-γ and IL-10 levels compared to the dexamethasone treated mice at all time points. The brain of toxoplasmosis infected mice showed inflammatory infiltrations, neuronal necrosis, and cuffing. The severity of pathology was higher in mice treated with dexamethasone compared to the positive control groups. In conclusion, the study showed that *T.gondii* infection is highly endemic in free range chicken from Thika region. Further studies should be carried out to determine the possible role of roaming chickens in the epidemiology of the disease among humans in the area. The findings of this study also demonstrate that dexamethasone-induced reactivation of chronic toxoplasmosis may be a useful development of laboratory animal model in outbred mice used for *in vivo* studies. The results of this study also demonstrate that toxoplasmosis in mice was associated with elevated IFN-γ and IL-10 levels, and myocarditis, hepatitis characterized by mononuclear inflammatory cellular infiltration. Further work on other cytokines involved in the pathology should be carried out. Research to investigate the potential of IFN-γ and IL-10 as diagnostic and therapeutic tools should be undertaken.

CHAPTER ONE

INTRODUCTION

1.1. Background of the study

Toxoplasmosis is a zoonosis of increasing concern in both developed and developing countries. It is caused by a protozoan parasite *Toxoplasma gondii* whose main definitive host is the domestic cat while all warm blooded animals are the intermediate hosts (Smith & Reduck, 2000). The parasite causes Toxoplasmosis which is the most widely reported parasite zoonosis leading to high worldwide prevalence and distribution (Liesenfeld, 2002). It is estimated that nearly two billion people in the world are infected with toxoplasmosis (Dubey & Beattie, 1998). In Kenya, the prevalence of toxoplasmosis in human ranges between 23% and 60% (Bowry *et al.*, 1986).

The infection may be acquired by oral ingestion of food or water contaminated with oocysts present in the feces of members of the cat family, the definitive hosts for *T. gondii*. Other routes of infections include ingestion of tissue cysts found in undercooked meat and congenitally by transplacental transmission (Hill & Dubey, 2002). Environmental contamination with *T. gondii* oocysts expelled from cat, the definitive host varies among countries (Dubey, 2010). Identification of *T. gondii* in free range chickens can be one of the effective indicators of contamination because they are infected by feeding from the environment polluted with oocysts (Ogendi *et al*., 2013).

In Kenya, most of the chickens are reared under extensive production system. Free range poultry meat is popular in Kenya because it is assumed to be healthier than that of caged birds. Anecdotally, some communities recommend the meat to pregnant women in order to deliver healthy babies (Ogendi *et al*., 2013). The

keeping of poultry in highly populated areas increases the risk for transmission of zoonoses and a study involving farmers in Thika region, Kenya has established the risk factors associated with the transmission of toxoplasmosis (Ogendi *et al.,* 2013). Poultry that feed directly from the ground, such as free range chickens are exposed to toxoplasma oocysts infection and may serve as indicators of the presence of the parasite in the environment and as a source of contamination for other animals including man (Thiong'o *et al.,* 2016). In Thika region, farmers indicated that they disposed cat faeces in areas accessible by free-range chickens (Ogendi *et al.,* 2013). In the same region, a higher prevalence (39%) of toxoplasmosis was observed in chicken slaughter house workers compared to workers in other types of slaughter houses (Thiong'o *et al.,* 2016) and this shows that chicken meat and offals could be a source of the Toxoplasma-derived tissue cysts for man.

Toxoplasmosis in immunocompetent individuals is asymptomatic with a few cases being clinical. The parasite forms tissue cysts containing bradyzoites in a variety of organs, particularly the brain, heart, and skeletal muscle. Following infection, there is a brief acute stage characterized by the proliferative tachyzoite stage of the parasite, but thereafter the parasite undergoes latency, characterized by slowly growing bradyzoites within tissue cysts which remain viable presumably during the life of the host. However, in patients with immunodeficiency, such as in the case of Acquired Immune Deficiency Syndrome (AIDS), organ transplantation and radiotherapy (Denkers & Gazzinelli, 1998), the bradyzoite gets reactivated and gets transformed to tachyzoites which principally results in life-threatening toxoplasmic encephalitis and associated neuropsychiatric manifestations (Olgica & Vladimir, 2001; Gaddi & Yap, 2007). These classes of patients present different challenges to health professionals in terms of diagnosis, case management and drug treatment. In other organs, the tachyzoites cause severe pathology such as infiltration of leucocytes in the heart and liver, hepatocyte necrosis and disorganization of the germinal centers in the spleen.

During infections, the parasite invades a variety of immune cells and is subsequently disseminated throughout the body, traversing biological barriers to reach immunologically privileged sites such as the brain where it can cause severe pathologies (Barragan & Sibley, 2002; Bowry *et al.,* 1986).

Both humoral and cell mediated immunity performs a role in resistance against *T. gondii* (Dupont *et al*., 2012). Changes in the levels of antibodies have been documented following the reactivation of *T. gondii* infection. In one study (Kang *et al.,* 2006), the specific IgG and IgM titers of the toxoplasmosis/dexamethasone-treated mice were depressed significantly after dexamethasone treatment, but whether these changes are diagnostic have not been well ascertained.

Cellular immunity plays a key role in the host's immune reaction against toxoplasmosis **(**Lindberg & Frenkel, 1977). The macrophages and natural killer cells (NK) cells exert their function via a cytotoxic activity and or the secretion of cytokines involved in the regulation of immune response (Hunter *et al.,* 1994). *In vivo* studies indicate that IFN-γ is a major cytokine, which is produced by T-helper and T-cytotoxic cells which mediates resistance against *T. gondii* infection (Brinkmann *et al.,* 1993). IFN-γ is the main type one cytokine involved in toxoplamosis, although other cytokines such as TNF-α, IL-18, IL-22, and the macrophage migration inhibitory factor (MIF) have also been reported in mediating pathology (Vossenkamper *et al.,* 2004). As the disease progresses, some studies have reported that IL-10 counters the harmful effect of an exaggerated type-1 inflammatory response based on the high production of TNF-α, IFN-γ and Nitric oxide (NO) associated with the intestinal proliferation of *T. gondii* (Liesenfeld, 1999). From the foregoing, it is clear that the development of a strong cellular immune response is associated with *T. gondii* infections in the intermediate hosts.

1.2. Statement of the problem

Toxoplasmosis is the most widely reported parasite zoonosis leading to high worldwide prevalence and distribution (Liesenfeld, 2002). It is a leading cause of infectious reproductive failure in humans and animals. The disease is characterized by an asymptomatic acute phase followed by a chronic stage characterized by tissue cysts containing bradyzoites (Tenter *et al.*, 2000). Although in immunocompetent individuals the infection with the parasite causes little or no overt signs of disease, *Toxoplasma gondii* infection may lead to serious illness when the organism is contracted during pregnancy or when it is reactivated in immune-suppressed persons e.g. AIDS patients, organ transplantation and radiotherapy (Robert-Gangneux *et al.,* 2009; Denkers & Gazzinelli, 1998). Infection during pregnancy may cause blindness, central nervous system (CNS) abnormalities and, if untreated, death in children.

Cerebral toxoplasmosis is the most common AIDS related opportunistic infection of the central nervous system and the most common cause of focal deficits in human immunodeficiency virus (HIV)-positive patients (Vidal *et al.*, 2013). It has been suggested that the neurological involvement of HIV leads to an imbalance of the immune response and, as consequence, the reactivation of the latent infection occurs leading to Toxoplasmic encephalitis (TE), a neurologic disease with lethal outcome (Montoya & Remington, 1995). The prevalence of HIV in Kenya is currently at 6% with more than 1.6 million persons living with HIV/AIDS and about 900,000 stands a high risk of developing toxoplasmic encephalitis (Aspinall *et al.,* 2002). Thika region is known for having the highest number of cases of AIDS in Kenya and accordingly more people at greater risk of developing toxoplasmosis (NASCOP, 2008).

Currently, little information is available regarding the immune responses inherent to reactivated toxoplasmosis, particularly the changes in immunoglobulins and the production patterns of T-helper cells type 1&2 (Th1/2) cytokines. The existing mice murine models have used *T. gondii* strains from other continents with none describing the pathogenesis of strains isolated from Kenya.

1.3. Justification of the study

Toxoplasmosis is an important zoonotic disease associated with poverty and poor sanitation conditions which are highly prevalent in most parts of Kenya including the study area (Ogendi *et al.,* 2013). Few studies on the prevalence of toxoplasmosis have been done in different parts of Kenya. Therefore, this study intended to show the burden of *T. gondii* infection in free range chickens in the different sub-counties in Thika region of Kenya. The prevalence of Toxoplasmosis in free range chickens is a good indicator of environmental contamination with *T. gondii* and as a possible risk to human beings.

There has been increasing clinical cases of Toxoplasmosis in immunocompromised patients and those with congenital toxoplasmosis. Experimental evidence has shown that cytokines play a major role in the pathogenesis of *Toxoplasma gondii* and manipulation of these cytokines can present a beneficial or damaging effect on the host and thus modulate the disease pathology.

When produced in excess, pro - inflammatory cytokines may lead to tissue damage and thus, a delicate balance between pro and anti - inflammatory cytokines is necessary for the effective control of the parasite and survival of the host. Inspite of the foregoing, the pathology and immune responses associated with the ensuing disease has not been well described using isolates from different parts of Kenya (Sarcion & Gherardi, 2000; Miller *et al.,* 2009).

The use of an appropriate animal model to study important gaps in our knowledge of toxoplasmosis is recommended in view of the significance of this disease in human population especially, in immunocompromised individuals. The study therefore sought to develop an experimental mouse model of chronic toxoplasmosis mimicking the course of toxoplasmosis in immunosuppressed humans to determine its value as a model for disease reactivation studies. The model will help in determining the organ pathology and levels of IFN-γ which is aTh-1 proinflammatory cytokine and IL-10, the anti-inflammatory cytokine both associated with the ensuing disease with isolates from Kenya. The information will be essential as a springboard for investigation of the disease as it occurs during the immunosuppressive stage of HIV infection. Information obtained will also help in ensuring healthy lives and promote well-being for the people, one of the strategic development goals (SDG) of the Vision 2030, considering that it is a neglected and re-emerging disease of poverty.

1.4. Research Questions

1. What is the prevalence of toxoplasmosis in free range chicken in the greater Thika region?
2. What are the levels of IgG and IgM antibodies in serum from *T. gondii* infected and in immunosuppressed BALB/c mice?
3. What are the levels of IL-10 and IFN-γ cytokines in serum from *T. gondii* infected and in immunosuppressed BALB/c mice?
4. What are the histopathological changes in BALB/c mice infected with *T. gondii*?

1.5. Objectives

1.5.1 Main objective

To determine the prevalence of toxoplasmosis in free range chicken in Thika region, Kenya and the immunopathology associated with *T.gondii i*nfection in immunocompetent and immunosuppressed BALB/c mice.

1.5.2 . Specific objectives

1. To determine the prevalence of *T. gondii* in chicken from different sub-counties of the greater Thika region.
2. To determine the levels of IgG and IgM in *T. gondii* infected and in immunosuppressed BALB/c mice.
3. To determine the profiles of IFN-γ and IL-10 in *T. gondii* infected and in immunosuppressed BALB/c mice.
4. To determine the histopathological changes in *T. gondii* infected and in immunosuppressed BALB/c mice.

CHAPTER TWO

LITERATURE REVIEW

2.1. Biology of *Toxoplasma gondii*

The causative agent of toxoplasmosis is *Toxoplasma gondii*, which is a coccidian universal parasite. The genus name '*Toxoplasma*' is derived from the Greek word 'toxon' meaning bow, which describes the crescentric shape of the tachyzoite stage, and the species name '*gondii*' from the rodent from which it was first isolated (Michael & John, 2000).

There are three infective stages of *T. gondii*: a rapidly dividing invasive tachyzoite, a slowly dividing bradyzoite in tissue cysts, and an environmental stage, the sporozoite, protected inside an oocyst (Robert & Dardé, 2012). Tachyzoites are responsible for acute infection; they divide rapidly in any cell of the intermediate host and non-intestinal epithelial cells of the definitive hosts and spread through the tissues of the body. Tachyzoites are crescent-shaped cells, approximately 5μm long and 2μm wide, with a pointed apical end and a rounded posterior end. They are limited by a complex membrane, named the pellicle, closely associated with a cytoskeleton involved in the structural integrity and motility of the cell. Tachyzoites are the dissemination form. They are able to invade virtually all vertebrate cell types, where they multiply in a parasitophorous vacuole.

After proliferating, tachyzoites convert into bradyzoites ("*brady"*= slow in Greek), which take the form of latent intracellular tissue cysts that form mainly in the tissues of the muscles and brain. These cysts are more or less spheroid in brain cells or elongated in muscular cells. They vary in size from 10μm for the younger cysts, containing only two bradyzoites, to up to 100μm for the older ones, containing hundreds or thousands of densely packed bradyzoites. The transformation into cysts

is in part triggered by the pressure of the host immune system (Miller *et al*., 2009). The bradyzoites, once formed, can remain in the tissues for the lifespan of the host. In a healthy host, if some bradyzoites convert back into active tachyzoites, the immune system will quickly destroy them. However, in immunocompromised individuals, or in fetuses, which lack a developed immune system, bradyzoites can be released from these cysts to form tachyzoites again, causing a reactivated infection in these hosts (Figure 2.1); (Montoya & Liesenfeld, 2004).

Sporozoites are located in mature oocysts. Oocysts from infected cats are excreted unsporulated and uninfective when passed in feces. Sporulation occurs in the environment after 1-5 days (dependent on temperature and moisture; e.g. 1 day at 24-25ºC, 5 days at 15ºC and 21 days at 11ºC) (Jones *et al.*, 1999). Oocysts are 12- to 13-µm ovoid structures that after sporulation contain two sporocysts, each containing four sporozoites. The oocyst wall is an extremely robust multilayer structure protecting the parasite from mechanical and chemical damages. It enables the parasite to survive for long periods, up to more than a year, in a moist environment (Luft, 1983).

2.2. Life Cycle of *T. gondii*

Toxoplasma gondii is a tissue cyst forming coccidium functioning in a prey-predator system that alternates between definitive (sexual reproduction) and intermediate (asexual replication) hosts. It is unique among this group because it can be transmitted not only between intermediate and definitive hosts (sexual cycle) but also between intermediate hosts via carnivorism (asexual cycle) or even between definitive hosts.

The sexual phase or entero-epithelial cycle occurs in the cat's intestine after ingestion of cysts present in tissues of an intermediate host which culminates in the cyst wall destruction by gastric enzymes (Figure 2.1). Bradyzoites penetrate the

epithelial cells of the small intestine and initiate a sequence of numerous generations (termed types A to E) (Dubey, 1979) where after a variable number of schizogonies, macrogametocytes and microgametocytes develop from ingested bradyzoites and fuse to form zygotes. After fertilization, oocysts formed within enterocytes are liberated by the disruption of the cell and excreted as unsporulated forms in cat feces. The shedding of oocysts begins 3 to 7 days after the ingestion of tissue cysts and may continue for up to 20 days. Sporulated oocysts are more resistant to the environmental and chemical conditions than are unsporulated oocysts; their thermic tolerance range being – 4 to 55ºC. During a primary infection, the cat can excrete millions of oocysts daily for 1-3 weeks. The oocysts are very strong and may remain infectious for more than one year in warm humid environments. In liquid medium they can survive for several years (Dubey, 1998).

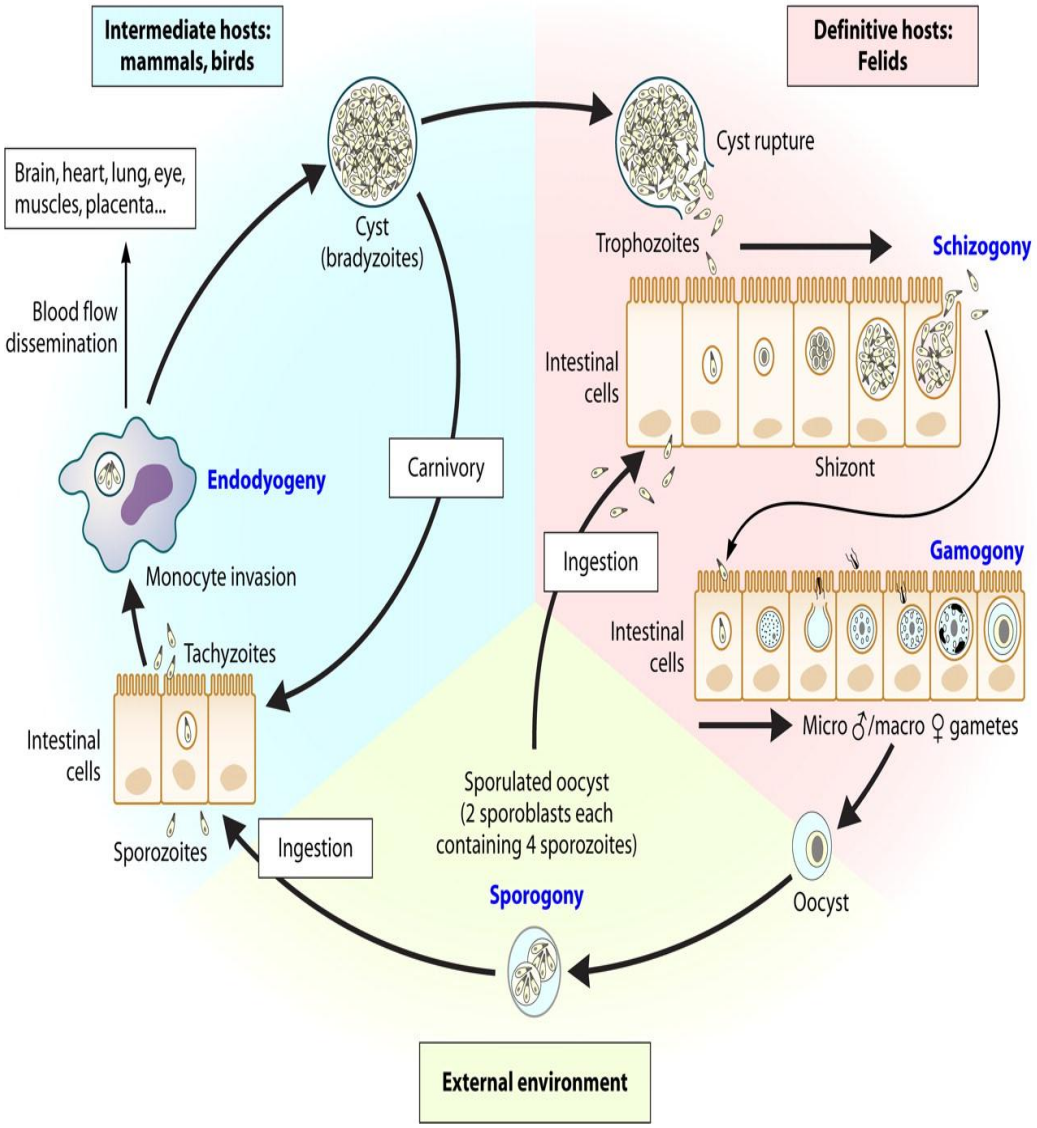

Figure 2.1: Life cycle of *Toxoplasma gondii*. Shown are the biology, infection, and replication of the three infective stages of the parasites in their respective hosts (Dubey *et al.*, 1998).

The asexual phase or extra-intestinal cycle occurs in any warm blooded animal after infection by any infectious stage. Once ingested by an intermediate host, the outer walls of cysts or oocysts are disrupted by enzymatic degradation, releasing either bradyzoites or sporozoites into the intestinal lumen (Bhopale, 2003). These then actively invade surrounding cells and transform into tachyzoites. Tachyzoites are spread via the blood system in lymphocytes, macrophages and free in the plasma where they multiply and lead to cell destruction and production of necrotic foci surrounded by inflammation.

Tachyzoites are capable of crossing tissue boundaries, such as the blood-brain barrier and the placenta (Carruthers, 2002). They are able to infect almost any type of tissue, especially those in the eye, central nervous system, heart, placenta and skeletal muscle (Montoya & Liesenfeld, 2004). Parasite invasion is driven by actin-based motility, generating a parasitophorous vacuole derived from invagination of the host cell plasma membrane and secretion of parasite proteins (Sibley *et al*., 2007). Within the parasitophorous vacuole, tachyzoites divide every 6-9h by a process called endodyogeny, in which daughter cells form internally within the mother cell (Morrisete & Sibley, 2002). Rupture of the host cell leads to emergence of parasites that infect new host cells.

2.3. Epidemiology of toxoplasmosis

Toxoplasmosis is an important parasitic infection of man and animals. The infection is usually asymptomatic and mild, while the prevalence of exposure increases with age and the lifestyle. Toxoplasmosis is more common in populations with lower socioeconomic status. Approximately 25% of the global human population is thought to be infected (Hammond *et al.*, 2014).

For women of child bearing age, the areas of highest prevalence are within Latin America (about 50–80%), parts of Eastern and Central Europe (about 20–60%), the

Middle East (about 30-50%), parts of Southeast Asia (about 20–60%), and parts of Africa (about 20–55%) (Pappas *et al*., 2009). Toxoplasmosis infection is endemic in the farms and rural areas where there is poverty and low hygiene.

Toxoplasmosis has become an increasing problem worldwide due to the AIDS epidemic. In individuals with HIV infection, the seropositivity rate to *T. gondii* is approximately 50-78% in certain areas of Western Europe and Africa. Approximately two-thirds of all people living with HIV are in sub-Saharan Africa. Approximately 5.7 million South Africans were living with HIV at the end of 2007.

Cases of toxoplasmosis have been reported in Kenya where the earliest study was documented in 1968 as reported by Mas *et al.* (1968). Since then, *T. gondii* has been detected in the general Kenyan population as well as in groups with reduced immunity. A serological survey done in Nairobi, Kenya involving 127 children revealed a significant rise of prevalence of the *T. gondii* specific antibodies from 35% in pre-school to 60% in the early school age group (Bowry *et al.*, 1986). The findings suggested that poor sanitary habits and conditions as well as water shortage in primary schools may cause parasitic infection through contact between children. 322 samples of sera from blood donors in four areas of Kenya were screened for *Toxoplasma gondii* antibodies by haemagglutination and 54% proved positive.

Chunge and colleagues investigated the prevalence of antibodies to *T. gondii* in serum samples from pregnant women and cord blood at Kenyatta National Hospital, Nairobi (Chunge *et al.*, 1989). This was the first documented study on *T. gondii* infection during pregnancy in Kenya. In another study, screening results of 322 samples of sera from blood donors in Kenya indicated high seroprevalence (Griffin& & Williams, 1983). Fifty four percent (54%) of HIV positive patients attending Kenyatta National Hospital, Nairobi had *Toxoplasma* specific IgG in contrast to 1% of the HIV negative group (Brindle *et al.*, 1991). A clinical case

report of toxoplasmosis was documented in a patient with HIV infection (Lodenyo *et al.,* 2007). About 12.7% of hospitalised HIV positive patients with neurological complications at a private hospital in Nairobi, had *T. gondii* infection (Jowi *et al*., 2007).

Co-infection of *T. gondii* and other parasites such as *Toxocara canis* has been investigated using samples from patients in Kenya. *Toxoplasma gondii* was detected in five of seven *T. canis* positive sera from Maasai land (Wiseman *et al*., 1970). In another study carried out by Adele *et al.* (2017) to establish the prevalence of gastrointestinal tract parasites in fecal samples of 103 cats kept by households in Thika region, *Toxoplasma gondii* was detected in 7.8% of the samples collected. Such publications and clinical case reports show that there are cases of multiple infections involving *T. gondii* in Kenya. However there are no recent statistics of *T. gondii* infection in animals and man in Thika region.

In a published review, Dubey and colleagues (2012), showed that there is high prevalence of toxoplasmosis in both humans in Ethiopia. In another study, a community based cross-sectional study revealed a high seroprevalence of *T. gondii* (83.6%) and significant presence of associated risk factors among pregnant women in Jimma Town, South West Ethiopia (Zemene *et al.,* 2012).

Uganda has one of the highest HIV prevalence rates in sub-Saharan Africa (6.5%) (UNAIDS, 2012). Unsurprisingly, seroprevalence of toxoplasmosis among HIV-positive Ugandans varies from 34% to 54%. Reactivated infection has been detected in 23% of these patients. Further, almost one in four of these patients who have focal neurology suffer from toxoplasmosis caused by reactivated parasites.

Livestock farming is important in Tanzania for economic purposes as well as a source of food. Therefore, food-borne parasitic diseases, such as toxoplasmosis pose a serious threat to economic wellbeing and food. A cross-sectional study was

conducted to investigate seroprevalence and risk factors of *T. gondii* seropositivity in apparently healthy, unvaccinated dairy goat flocks reared under mixed smallholders, northern Tanzania (Swai & Kaaya, 2012). Seroprevalence of *T. gondii* was found to be 19.3 % of goats and 45.17 % of flocks. Risk factors for infection in goats were identified as sex (infection was significantly higher in females than males), crossbreeding and district where farm is located.

In a separate study, *T. gondii* was detected in 13% of 130 randomly selected farms and seroprevalence was 3.6% in a total of 655 cattle (Schoonman *et al.,* 2010). Risk factors for cattle included herd size and type of farming practice. These data suggest that toxoplasmosis may be posing a significant animal and human health risk and consumption livestock products may play a role in the transmission of the disease to humans.

In another study**,** the burden of *T. gondii* was studied in the population of Nyamisati village, Tanzania. The seropositivity rate was 4% (19/450) among the subjects of Nyamisati origin and 47% (15/32) among immigrants from other areas of Tanzania (Gille *et al.,* 1992).The generally low transmission in this mainly Muslim village appeared to be related to sparse consumption of contaminated food and low prevalence of oocysts due to scarcity of felines. A bigger serological survey carried out in Tanga district of north-eastern Tanzania aimed to assess *T. gondii* infection rates among occupationally-exposed groups including abattoir workers, livestock keepers and animal health workers (Swai & Schoonman, 2009). Antibodies to *T. gondii* were detected in 91 (46%) of the 199 individuals studied. The seroprevalence of toxoplasma antibodies was significantly higher amongst individuals who keep livestock (52.2%) and abattoir workers (46.3%). These results strengthen previous findings that consumption of raw or undercooked meat and keeping pets especially cats presents more of the risk factors than occupational groups.

Results generated from a study conducted over 30 years ago alleged that toxoplasmosis was less common than other parasitic infections in Gezira Province, Sudan (Omer *et al.,* 1981). About 10 years later, seroprevalence of toxoplasmosis in the same province was shown to be 41.7% (Abdel, 1991). Females within child-bearing age (20-49 years) had a significantly higher prevalence rate than males of the same age. The known food habits of the study population suggest ingestion of *T. gondii* cysts in meat is the main mode of transmission of the disease.

A survey of toxoplasmosis was conducted on pregnant women attending antenatal clinics in Khartoum and Omdurman Maternity Hospitals (Elnahas *et al*., 2003). Results disclosed seroprevalence levels of 34.1%. Risk factors for IgG anti-toxoplasma seropositivity were; Southern ethnic origin and consumption of raw meat. Thirty (18.1%) out of 166 women who were IgG anti-toxoplasma seropositive gave history of intrauterine fetal death, while 31 (9.7%) out of 321 women who were sero-negative gave history of intrauterine fetal death (Elnahas *et al*., 2003). In another study**,** Toxoplasmosis prevalence levels ranging from 12% to 31% was recorded for two rural populations of Rwanda. *Toxoplasma gondii* was identified as a causative agent in AIDS patients with nervous system complications (Gascon *et al.,* 1989).

2.4. Transmission of *T.gondii* and associated risk factors

Transmission of the parasite can occur in several ways, as illustrated in Figure 2.2. Definitive hosts can be infected by ingestion of oocysts from the environment or cysts in prey. In the warm-blooded animals, the intermediate hosts, transmission mainly occurs through the mouth by consuming food or drink contaminated by oocysts evacuated from felids and tissue cysts from other intermediate hosts when accidentally ingested (Montoya & Liesenfeld, 2009). This could involve ingestion of raw or partly cooked meat, especially pork containing *Toxoplasma* cysts. Tissue

cysts may also be ingested during hand-to-mouth contact after handling undercooked meat, or from using knives, utensils, or cutting boards contaminated by raw meat.

The transmission can also occur by ingestion of unwashed fruits or vegetables that have been in contact with contaminated soil containing infected cat feces (Jones *et al.,* 2012) and Ingestion of contaminated cat feces: This can occur through hand-to-mouth contact following gardening, cleaning a cat's litter box, contact with children's sandpits; the parasite can survive in the environment for over a year (Hill & Dubey, 2002). In addition to felids, intermediate hosts carried with tachyzoites or tissue cysts are also responsible for the spread of *T. gondii*.

The transmission may also occur through receiving an infected organ transplant or infected blood via transfusion, though this is rare. Congenital transmittance from the mother to fetus can also occur (Figure 2.2). However the pathogenicity varies with the age and species involved in infection and the mode of transmission of *T. gondii* (Assadi *et al*., 1995; Dubey, 2010).

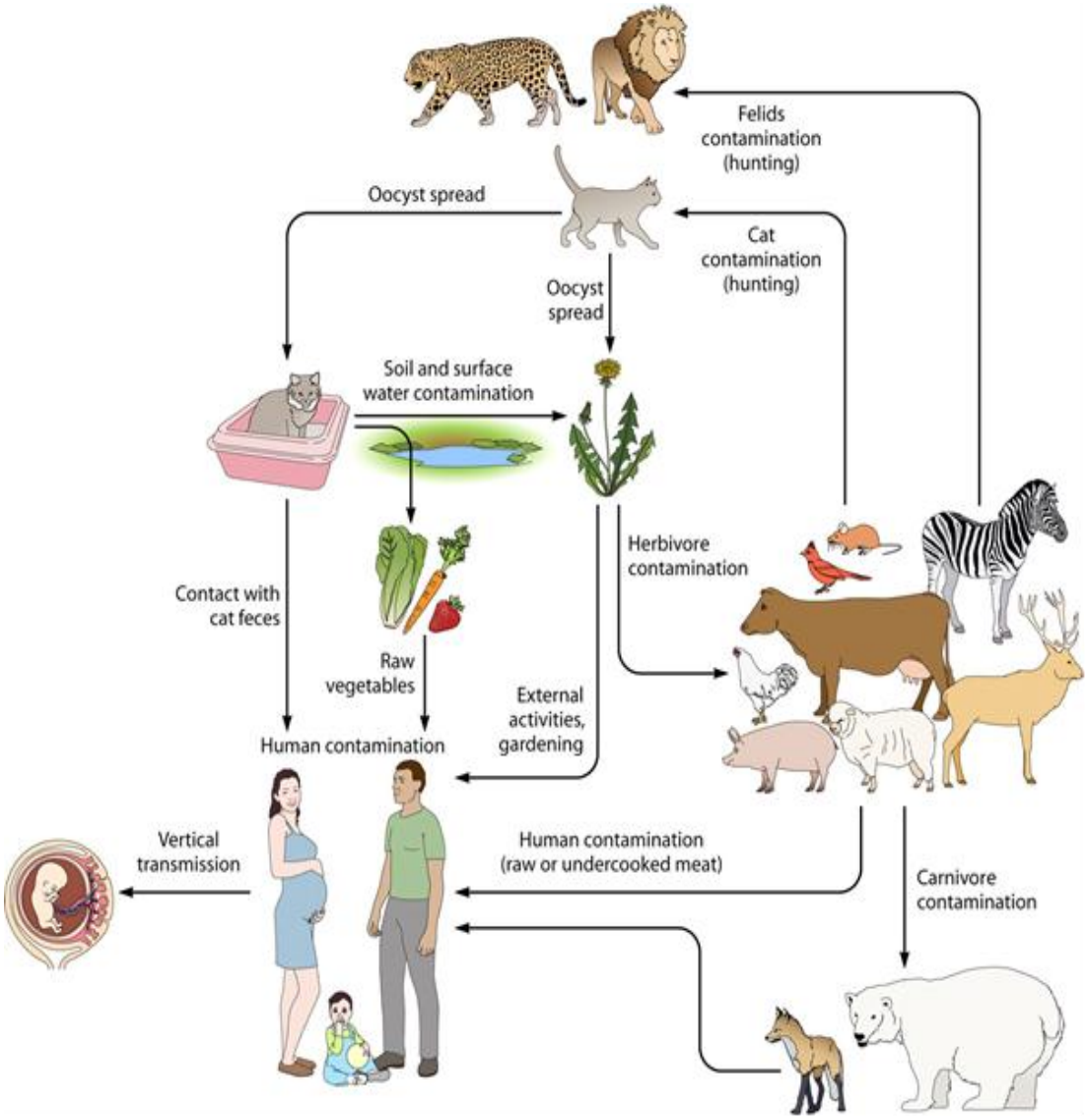

Figure 2.2: Sources of *T.gondii* infection in humans. The various sources of food-borne and environmental contamination of humans are represented (Florence *et al*., 2012).

In one study carried out in Mexico, water, sewage, and soil were found to be potential sources of infection for *T. gondii*. The study had sought to determine *T. gondii* infection and associated characteristics in 61 plumbers, 203 construction workers, and 168 gardeners in Durango City, Mexico. In the total population, the prevalence of *T. gondii* infection was significantly higher in workers living in suburban areas, without education, workers that consumed chorizo, and those who suffered from any disease ($P < 0.05$). In gardeners, prevalence of infection was significantly higher in those with blood transfusion, and memory impairment ($P < 0.05$). This may suggest a likelihood of transmission by this procedure. The study also showed that *T. gondii* infection was positively associated with consumption of unwashed fruits (adjusted odds ratio [OR] 5 2.41; 95% confidence interval [CI]: 1.13–5.13), and with raising animals (adjusted OR 5 2.53; 95% CI: 1.00–6.37). Previous studies have observed an association of *T. gondii* infection and consumption of unwashed fruits in psychiatric patients (Alvarado-Esquivel *et al*., 2010).

In a similar study, the risk factors of 963 pregnant women attending an obstetric hospital in Fortaleza, Brazil was studied. Results showed that consumption of homemade water ice (adjusted OR = 1.49, 95% CI = 1.09–2.04) and vegetables washed with untreated water (adjusted OR = 1.43, 95% CI = 1.05–1.94), were factors associated with *T. gondii* infection. Young women in northeastern Brazil living under poor socioeconomic conditions are at highest risk for acquiring infection with *T. gondii.* Transmission through drinking contaminated water has been considered uncommon by other studies. However, the widespread infection of marine mammals indicates that contaminated water may be a potential source of infection (Dubey, 2004). Toxoplasmosis outbreaks associated with contaminated water supplies provide further evidence for this (Dubey, 2004).

Some factors associated with toxoplasmosis in pregnancy include, eating undercooked or cured meat (meat with preservatives such as salt, nitrates or sugar added (Cook *et al*., 2000; Baril *et al.,* 1999; Jones *et al.,* 2001). In some case-control studies, undercooked meat was shown to be the largest risk factor for *T. gondii* infection. An infection rate of between 30-60% was recorded in one of these studies in pregnant women and this was associated with the consumption of undercooked contaminated meat (Kijlstra & Jongert, 2008). Similarly, pork has been considered a major source of infection in Europe and the USA, but recent studies involving pigs in the Netherlands, Austria and Germany have shown that *T. gondii* infections in pigs have dropped to less than 1% over the last ten years (Tenter *et al*., 2000). This could be due to modern and more hygienic farming systems, as well as the increasing use of frozen meat (Kijlstra & Jongert, 2008).

While advocates of protection for cats claim that they have negligible impacts on the environment, there is a growing body of scientific evidence about cats and their potential to contaminate the environment with *T. gondii* oocysts (Jessup, 2004). Feral and owned free-roaming cats also help spread *T. gondii* to humans and livestock (Lehmann *et al*., 2003). In a study conducted by Weigel *et al*. (1995), it was found out that *T. gondii* seroprevalence in pigs increased when more seropositive juvenile cats were present on the farm, suggesting that oocysts from cats were disseminated into the surrounding habitat.

2.5. Immunology of toxoplasmosis

2.5.1. Humoral immune responses to *T. gondii*

Infection evokes a strong adaptive immune response that control parasite replication but do not eliminate the infection. Under the pressure of the adaptive immune response, *Toxoplasma gondii* parasite transforms from tachyzoite to bradyzoite forms.The bradyzoite live in cystic structures that persist for the life of the

individual, occasionally releasing bradyzoite that are able to infect other cells and transform into tachyzoites for a brief time, considering the immediate pressure from the adaptive immune system. (Remington *et al.,* 2004). Tachyzoites trigger a strong immune response responsible for the clinical symptoms of toxoplasmosis during the acute or reactivation of the latent infection.

Antibodies can mediate their protective effects through a variety of mechanisms. In immunocompetent individuals free tachyzoites provoke a strong immune system and *In vitro* studies have found that they can opsonize parasites for phagocytosis, block invasion, and also activate the classical complement pathway. The tachyzoites are killed by serial activation of complement associated antibodies, reactive oxygen and nitrogen radicals, osmotic fluctuations, and intracellular acidification. (Hammouda *et al.,* 1995).

The onset of humoral immune response against *T. gondii* is initiated by the production of specific *Toxoplasma gondii* antibodies. Immunoglobulin M (IgM) antibody response occurs as an early event, and disappears within a few weeks or months, thus its presence in plasma indicates recent infection. Immunoglobulin G (IgG) production peaks within 1 to 2 months after infection but remains elevated for life (Erbe *et al.,* 1991).

The antibodies are responsible for lysing extracellular tachyzoites and due to their structure, they enable excellent agglutination and have a high level of cytotoxicity. However, some tachyzoites attach to cells and invade intracellular milieu and parasitophorous vacuole to avoid the innate and adaptive immune mechanisms. Tissue cysts primarily form in muscles, heart, brain, and retina, are responsible for the chronic infection.

2.5.2. Cellular immune responses against *Toxoplasma gondii*

Tissue cysts which consists of aggregations of slow multiplying bradyzoites primarily form in muscles, heart and retina are responsible for the chronic infection.Cellular immunity is the key component of the host's immune reaction in the event of attack by Toxoplasma. This type of immunity mediated by macrophages, natural killer cells, CD4+ T cells (Th1) and CD8+ T cells is necessary in promoting a self-limiting infection, ensuring the survival of its host and thus itself (Carruthers, 2002; Miller *et al.*, 2009; Amodio *et al*., 2013; Muzzio *et al.*, 2014).During the early phase of the infection, most nonspecific effector mechanisms which are not restricted to the MHC are exerted as aresult of the combined and synergetic action of the NK cells and the macrophages, activated by IFN-γ. At this stage, monocyte macrophage lineage cells differentiate into antigen-presenting cells (APC).

Neutrophils, eosinophils and mast cells infiltrate at the site of infection and are involved in setting up a non-specific early immune response (Bliss *et al.*, 2000). These effector cells exert their function via a cytotoxic activity and/or the secretion of cytokines involved in the regulation of immune response (Subauste & Wessendarp, 2000).

T-helper (CD4+) and T-Cytotoxic (CD8+ TL) cells are the main players involved in resistance of the host to Toxoplasma infection (Suzuki & Remington (1988). In mice, mature CD4+ TL cells are required for the development of resistance during the early phase of the infection (Araujo, 1991).They are divided into two sub-populations including, Th1 which produce IL-2 and IFN-γ; and type 2 (Th2) cells which produce IL-4, IL-5, IL-6 and IL-10. During the active phase of Toxoplasma infection, the CD8+ TL appear to play asignificant role and enable aprotective immunity to be passed on (Subauste *et al.*, 1991). These cells activated by the IL-2

secreted by the CD4+ TL, exert a cytotoxic activity against tachyzoites or cells infected with *T. gondii* (Yano *et al*., 1989).

2.5.2.1. Protective cytokines

Interferon γ (IFN-γ) has numerous biological activities, including: the secretion of INF-γ which increases the phagocyte activity of macrophages and the cytotoxic activity of CD8+ TL and the restriction of tachyzoite replication not only of macrophages, but also of non-phagocytic cells (Ely *et al.*, 199).

Another cytokine, Interleukin 12 (IL-12), which is secreted by the macrophages and the dendritic cells during *T.gondii* infection, appears to play a major anti-Toxoplasma role during the acute phase of the infection. In human models of *T. gondii* infection, CD154 (expressed primarily on activated CD4 T- cells) acts by triggering dendritic cells and macrophages to secrete IL12, which in turn enhances production of IFN-γ by T cells (Subauste & Wessendarp, 2000). IL-12 is also responsible for maintaining a long-term immune response during the chronic phase of the infection. TNF-alpha is another cytokine essential for control of chronic infection with *T. gondii* (Yap *et al*., 1998). This cytokine, produced by monocyte macrophages, TL and basophil mastocytes exerts an early protective effect by increasing the microbicidal capacities of the macrophages and inducing the secretion of IFN-γ by the NK cell and this protective action is exerted in mice in both the acute and chronic phase of the disease (Chang *et al*.,1990).

Interleukin 6 (IL-6), another important cytokine produced mainly by monocyte macrophages and endothelial cells increases the cytotoxic activity of NK cells. It is later involved in the differentiation of B-Lymphocytes into antibody secreting cells; differentiation of cytotoxic T-Lymphocytes and in murine toxoplasmosis, an increase in serum IL-6 correlates with clinical signs (Lyons *et al.*, 2001). Another cytokine, Interleukin 5 (IL-5), mainly produced by TL and eosinophils, triggers the

growth, differentiation, activation and chemotaxis of eosinophils (Zhang & Denkers, 1999).

There are other important cytokines involved in protection including; Interleukin 15 (IL-15) which is produced by macrophages which induces the maturation of NK cells and the proliferation of CD8+ T-Lymphocytes (Khan & Casciotti, 1999); Interleukin 18 (IL-18), has the capacity to increase the activity of NK cells in experimentally-induced toxoplasmosis (Cai *et al.*, 2000); Interleukin 2 (IL-2) which is produced exclusively by the CD4+ TL has been shown to increase survival of the animals and a reduction in the number of cysts present in the brain of murine models is sensitive toToxoplasma infection (Sharma *et al.*, 1985).

2.5.2.2. Regulatory cytokines

Interleukin 10 (IL10) is one of the regulatory cytokines which is secreted by type-2 CD4+T-Lymphocytes, macrophages and B-Lymphocytes. This cytokine play a anumber of roles during *T. gondii* infection.It has been shown to inhibit the proliferation of type-Th1 CD4+ cells along with the secretion of cytokines by these same cells. It also inhibits the production of nitrate and oxygenated derivatives and of pro-inflammatory cytokines (IL-1, IL-6, TNF-α) by monocyte macrophages (Gazzinelli *et al.*, 1992).

Interleukin 4 (IL-4) and Transforming growth factor-β (TGF-β) are other two cytokines involved in regulating *T.gondii* infection. Interleukin 4 (IL-4) which is secreted by type-2 CD4+ T-Lymphocytes, increases the expression of class-II MHC antigens and triggers IgE isotype switching.

Transforming growth factor-β (TGF-β) which is considered to be an antagonist of TNF-α, IFN-γ, TNF-β and IL-2, is well known for its immunosuppressant action on leukocyte cell lines (Langermans *et al.*, 2001). It is possible that different cytokines

production initiates the extent of the infection in individuals. Hence, Toxoplasmosis may specifically impact levels of circulating cytokines, and results in differences in the clinical severity of toxoplasmosis (Pernas *et al.*, 2014). Tissue-specific immune responses, such as that in the central nervous system are being currently elucidated.

2.5.3. Toxoplasmosis in immunocompromised hosts

Most cases of toxoplasmosis in immune ocompromised patients are a consequence of latent infection and reactivation and dissemination of the toxoplasmosis. Among immunodeficient individuals, toxoplasmosis most often occurs in those with defects of T-cell–mediated immunity, such as those with hematologic malignancies, bone marrow and solid organ transplants, Hodgkin's disease or those undergoing immunosuppressive therapy or acquired immunodeficiency syndrome (AIDS) and it can be can be life threatening (Tenter *et al*., 2000).

The mechanisms by which HIV induces susceptibility to opportunistic infections such as toxoplasmosis are likely multiple. These include depletion of CD4 T cells; impaired production of IL-2, IL-12, and IFN-γ; and impaired cytotoxic T-lymphocyte activity (Okoye & Picker, 2013; Vijay *et al.,* 2011). Cells from HIV-infected patients exhibit decreased invitro production of IL-12 and IFN-γ, and decreased expression of CD154 in response to *T gondii* (Murray *et al.,* 1984; Subauste *et al.,* 2001; Subauste *et al.,* 2004).These deficiencies may play a role in the development of toxoplasmosis associated with HIV infection. In patients with AIDS, *T. gondii* tissue cysts can reactivate with CD4 counts of less than 200 cells/μl; with counts of less than 100 cells/μl, clinical disease becomes more likely. Without adequate prophylaxis or restoration of immune function, patients with CD4 counts of less than 100 cells/μl who are *T gondii* IgG-antibody positive have a 30% risk of eventually developing reactivation disease.

2.5.4. Clinico-pathological manifestations of toxoplasmosis

The clinical manifestations of toxoplasmosis may vary depending on parasite characteristics such as virulence of the strain and inoculum size, as well as host factors such as genetic background and immune status (Montaya & Liesenfeld, 2004).While asymptomatic infection with *T. gondii* resulting in a latent infection with tissue cysts is common in humans, symptomatic infection, is seen much less frequently. Specific groups of patients including congenitally infected fetuses and newborns, and immunologically impaired hosts are, however, at high risk for severe infection due to this parasite (Tenter *et al*., 2000).

In congenital infection and immunosuppressed individuals, several forms of the disease may occur. Toxoplasmosis commonly presents in the form of lymphadenitis with 3-7% causing clinically significant lymphadenopathy, characterized by single or multiple non-matted lymph nodes that are generally not tender and firmness of the lymph nodes is variable. This may be accompanied by a number of nonspecific symptoms in a portion of individuals, such as headache, fever, malaise, fatigue and sore throat (Bhopale, 2003; Hill *et al*., 2005; McCabe *et al.,* 1987). However, in more severe cases, retinochoroiditis, myocarditis and pulmonary necrosis may occur in individuals with depressed immune system (Bhopale, 2003) and toxic encephalitis is the most common clinically significant disease manifested in this group of individuals (Hill *et al.,* 2005). In congenital toxoplasmosis, the classic signs in live-born infants include hydrocephalus or microcephalus, cerebral calcifications, and retinochoroiditis (Dubey & Jones, 2008). Ocular disease is the most common manifestation of congenital toxoplasmosis, and retinochoroiditis may occur later in life in an otherwise healthy child who was exposed to *T. gondii* in utero (Butler *et al*., 2013).

2.6. Molecular features which facilitate parasite entry into the mammalian host cells

Toxoplasma gondii invades host cells and gain entry by active penetration resuling to the parasite residing within a non-fusogenic vacuole (Sibley, 2011). This process helps the pareasite to gain access to nutrients; interact with host cell signaling pathways and also helps its detection by pathogenic recognition systems.

During invasion, three proteins are secreted from parasite organelles, called the micronemes, dense granules and rhoptries, into the host cell. Their functions are not fully known but probably associated with host cell penetration and creation of an intracellular environment suitable for parasite growth and development.They have also been associated with other two key functions including; alteration of the host cell function and inhibition of the immune responsedirected towards the parasite (Lim *et al.,* 2012).

2.7. *T.gondii* subtypes

As is the case for many pathogens, the outcome of infection with *T. gondii* is highly dependent on the interplay of host and microbial factors.The genus *Toxoplasma* has only one species, known as *gondii* comprised of three clonal types (type I, II and III); Howe & Sibley, 1995; Binas & Johnson, 1998). These differ in virulence and epidemiological pattern of occurrence (Montoya & Liesenfeld, 2004; Literac *et al.*, 1998; Howe *et al*., 1997). Studies using murine models show that the type I strain is highly virulent and has a lethal dose of a single parasite regardless of the genetic background of the mouse. Type II and III strains have a 50% lethal dose of more than 103 parasites and the outcome is dependent on the genotype of the host (Mordue *et al.,* 2001). Type I and II strains have been reported in human cases; with type I often associated with severe congenital and ocular disease, suggesting

that it is pathogenic in humans. Type III has been shown to be more common in animals (Montoya & Liesenfeld, 2004).

2.8. Identification of *T.gondii* using molecular techniques

Detection of *T.gondii* in biological samples can be done by molecular techniques in order to determine its genetic material. The methods can be used for more appropriate detection of Toxoplasmosis especially in cases in which inadequacy of conventional methods is confronted with deteriorating and potentially severe clinical outcome especially in cases of congenital toxoplasmosis and immunosuppression (Slawska *et al*., 2005; Nimri *et al.,* 2004; Ivovic *et al.,* 2012). This can be done by amplifying a specific fragment of the DNA genome and subsequent visualization of the PCR amplification products on agarose gel following staining or on an automated sequencer by laser detection. Many different applications of PCR have been described by Rappelli *et al.,* (2001). Polymerase Chain Reaction has been used to detect DNA of *T. gondii* in various biological samples and has showed higher sensitivity in diagnosis compared to serological tests (Olariu *et al.*, 2014; Burg *et al.,* 1989; Cermakova *et al*., 2005; Murat *et al.*, 2013; Dupouy-Camet *et al.*, 1993).

The sensitivity and specificity of PCR-based methods depend on an appropriate technique for isolation of genetic material from samples and the method is usually independent of the immune response. The results may also be affected by the characteristics of the DNA sequence chosen for amplification, and the parameters of the amplification reaction itself.

Molecular methods based on polymerase chain reaction (PCR) have been classified into two groups. The techniques in the first group used to detect *T. gondii* DNA in biological and clinical samples Include; conventional PCR, nested PCR and real-time PCR. The second group consisting of molecular methods such as PCR-RFLP,

microsatellite analysis and multilocus sequence typing of a single copy *T. gondii* DNA, are mainly used for strain typing (Su *et al*., 2010).

Molecular detection of toxoplasmosis using different assays and protocols is usually based on the detection of specific DNA sequences, from highly conserved regions such as the B1 gene repeated 35 times in the genome (Burg *et al.,* 1989), 529bp repetitive element with about 200-300 copies in the genome (Homan *et al.,* 2000), ITS-1 (internal transcribed spacer) that exists in 110 copies and 18S rDNA gene sequences. When compared, the methods using the B1 and 529-bp sequences with real-time PCR revealed a ten-fold improvement in sensitivity when the 529-bp sequence was used (Reischl *et al.,* 2003). Several other single-copy sequences, including the SAG1, SAG2, SAG3, SAG4 and GRA4 genes, have been used as PCR targets in other research laboratories (Howe *et al.,* 1997; Meisel *et al.,* 1996).

2.9. Diagnosis of toxoplasmosis

Toxoplasmosis is frequently asymptomatic and clinical manifestations, when present, are usually non-specific and mimic other infections, making definitive clinical diagnosis very difficult (Hill & Dubey, 2002; Kompalic *et al.,* 2004). Diagnosis is usually made by immunological testing, histological identification, isolation in tissue culture, recovery of the parasite DNA by the polymerase chain reaction (PCR) or by a combination of these techniques. Cerebral toxoplasmosis can also be diagnosed using computerized tomography and magnetic resonance imaging (Hill & Dubey, 2002; Sukthana, 2006).

Serological tests are most widely used, yet they have the greatest limitations as they often provide ambiguous results (Kompalic *et al.*, 2004). Examples of these tests include the Sabin-Feldman dye test, which is the traditional gold standard, indirect fluorescent antibody assay (IFA), complement fixation test (CFT) and the enzyme-linked immunosorbent assay (ELISA) (Hill & Dubey, 2002). In a primary *T. gondii*

infection, IgM appears a few weeks after infection, followed by IgA and IgE. These acute phase immunoglobulins peak after about two months and are usually undetectable by serological tests by six to nine months but can persist for longer periods of time (Montoya & Rosso, 2005; Sukthana, 2006). IgG, for example, which appears after IgM, peaks after four months and persists at low levels throughout the duration of the host's life.

A problem with serological tests is that the detection of antibodies in immunocompromised individuals may be difficult due to the deterioration of the immune system (Schneider *et al.,* 1992). A further problem is that IgM may persist for longer than expected periods and discrimination between recent and older infections may therefore be a problem (Ho-Yen *et al.,* 1992; Remington *et al.*, 2004). This is an important factor when diagnosing toxoplasmosis in immunocompromised individuals as the presence of IgG indicates a risk for the reactivation of a latent infection, and IgM indicates the possibility of an acute infection. In pregnant women, positive IgM results indicate the likely acquisition of infection during gestation and a positive IgG and negative IgM result indicates a previous infection (Montoya & Rosso, 2005).

Avidity tests have helped to overcome this problem as they help differentiate between recently and distantly acquired infections (Lappalainen & Hedman, 2004). Avidity tests are based on the fact that during acute infections, IgG antibodies bind antigen relatively weakly and therefore have a low avidity. Chronic infections, however, have more strongly-binding antibodies and therefore have a high avidity (Lappalainen & Hedman, 2004; Montoya & Rosso, 2005).

Some of these problems can be overcome with the use of PCR. This method has both advantages and disadvantages. Advantages are that the detection of nucleic acid is not affected by the condition of the immune system; it is generally more

sensitive and rapid than serological tests and diagnosis can be made from biopsies, blood, cerebrospinal fluid (CSF) and amniotic fluid. Disadvantages are that, false positive results due to contamination may occur; it may be too sensitive in detecting nonviable *T. gondii* remnants that do not cause disease, and may yield false negative results due to inhibition (Johnson *et al.*, 1993). These problems with PCR can, however, be overcome and more rapid and sensitive methods are regularly being developed. These advances in PCR techniques are helping to make it an invaluable diagnostic tool.

2.10. Prevention and Treatment of toxoplasmosis

Toxoplasmosis is a curable but potentially fatal disease. To prevent infections in humans, a number of measures can be taken. Individuals should practice good hygiene. Hands should be washed thoroughly after handling meat or soil before beginning any other tasks. All fruit and vegetables must also be washed before they are to be consumed. All meat should be well cooked to a minimum temperature of 67ºC before consuming so as to kill tissue cysts. Tissue cysts can also be killed by cooling to -13ºC. Pregnant women should be especially careful, and should limit contact with cats, cat litter, soil and raw meat (Tenter *et al*., 2000; Hill & Dubey, 2002).

Treatment for immunocompetent individuals is usually not necessary. Sulfadiazine plus pyrimethamine is the most commonly recommended therapy for congenital and ocular toxoplasmosis, as well as infection in immunocompromised individuals (Hill & Dubey, 2002; Montoya & Liesenfeld, 2004). Alternative treatments for patients intolerant to sulphonamides are also used. These include clindamycin plus pyrimethamine, clarithromycin plus pyrimethamine, and atovaquone (Arens *et al.*, 2007). There are no vaccines currently available for *T. gondii***,** although several are in early development (Liu *et al*., 2012). In patients with a high frequency of

recurrence and in immunocompromised patients, maintenance treatment has been successfully used (Silveira *et al*., 2002).

2.11. Models used to study genetic principles and human diseases

Animal models have greatly improved our understanding of the cause and progression of human genetic diseases and have proven to be a useful tool for discovering targets for therapeutic drugs. Rodents are the most common type of mammal employed in experimental studies, and extensive research has been conducted using rats, mice, gerbils, guinea pigs, and hamsters (Hardouin & Nagy, 2000).

When animal models are employed in the study of human disease, they are frequently selected because of their similarity to humans in terms of genetics, anatomy, and physiology. Animal models are often preferable for experimental disease research because of their unlimited supply, high reproductive rates, and relatively low cost of use and ease of manipulation (Nadia & Brown, 2007). For example, to obtain scientifically valid research, the conditions associated with an experiment must be closely controlled. This often means manipulating only one variable while keeping others constant, and then observing the consequences of that change. In addition, to test hypotheses about how a disease develops an adequate number of subjects must be used to statistically test the results of the experiment. Therefore, scientists cannot conduct research on just one animal or human, and it is easier for scientists to use sufficiently large numbers of animals (rather than people) to attain significant results.

Nonetheless, despite promising results with certain preclinical treatments in animal models, the same treatments do not always translate to human clinical trials (Yu & Bradley, 2001). As a result, many diseases are still incurable. Most available animal models are made in mice, and they recreate some aspects of the particular disease.

However, few, if any, replicate all the symptoms. This statement is particularly true for neurodegenerative diseases, most of which involve cognitive deficits.

In animal models, dexamethasone is the most commonly used drug for immunosuppression (Munck *et al.*, 1984; Wilkens & De Rijk, 1997). Dexamethasone just like other glucocorticoids, act by binding to the glucocorticoid receptor (GR), which blocks the expression of proinflammatory cytokines and adhesion molecules. Repression of many pro-inflammatory genes occurs by inhibition of transcription factors including nuclear factor kappaB (NFkappaB) and activator protein-1 (AP-1). This drug inhibit the vasodilation and increased vascular permeability that occurs following inflammatory insult and they decrease leukocyte emigration into inflamed sites (Perretti *et al.,* 2000; Ashwel *et al.,* 2000). Dexamethasone also affect the function of immune cells by altering the function differentiation programmes of progenitor cells (McEwen *et al.,* 1997).Thus, during chronically stressful conditions such as Dexamethasone pharmacotherapy may alter immune cell differentiation and indeed, probably shape the immune response as it develops (Munck *et al.,* 1984). Through immunosuppression, the drug can be used to mimic the effect of HIV in human host.

In a commonly used SCID mouse model of *T. gondii*, sulfadiazine treatment has been shown to suppress infection thus allowing chronicity to develop, and its withdrawal leads to disease relapse (Johnson *et al.,* 1992). SCID mice have some limitations such as non-availability in most laboratories and cumbersome to work with because of their severely impaired immune systems and the requirement for them to be kept in sterile conditions to prevent opportunistic infections (Johnson *et al.,* 1992). On the other hand mice such as BALB/c mice are widely available and are used for a variety of disease models (Beermann *et al.,* 2004). In the current study, dexamethasone was used as an immunosppresant to imitate the immunosupression which occurs following HIV/AIDS disease in humans.

CHAPTER THREE

MATERIALS AND METHODS

3.1. Study area

Thika region (1°4' 60 S) (37° 4' 60 E) is located in Central Kenya and occupies an area of 1,960.2 Km² (Figure 3.1). It has an estimated human population of 864,509 (KNBS, 2009). Originally, Thika region was made up of 5 administrative areas including Thika and Ruiru municipalities, Gatundu, Kakuzi, and Gatanga divisions. In the current study, the sub-counties included in the study were Thika municipality, Gatanga and Kakuzi and they have a tropical climate with an annual rainfall ranging between 500-1500mm while the mean temperature is 19.8^{o}C (Government of Kenya, 2005). Majority of farmers in Thika are small holder-farmers, practicing mixed agriculture, including livestock production, food and cash crops. Most households in the region have cats kept as pets and for control of rodents. In addition, 60% of the farmers keep free-range chickens (Ogendi *et al.*, 2013). Specimens from chickens were processed and analyzed in laboratories within the Jomo Kenyatta University of Agriculture and Technology (JKUAT) and the Institute of Primate Research (IPR) laboratories.

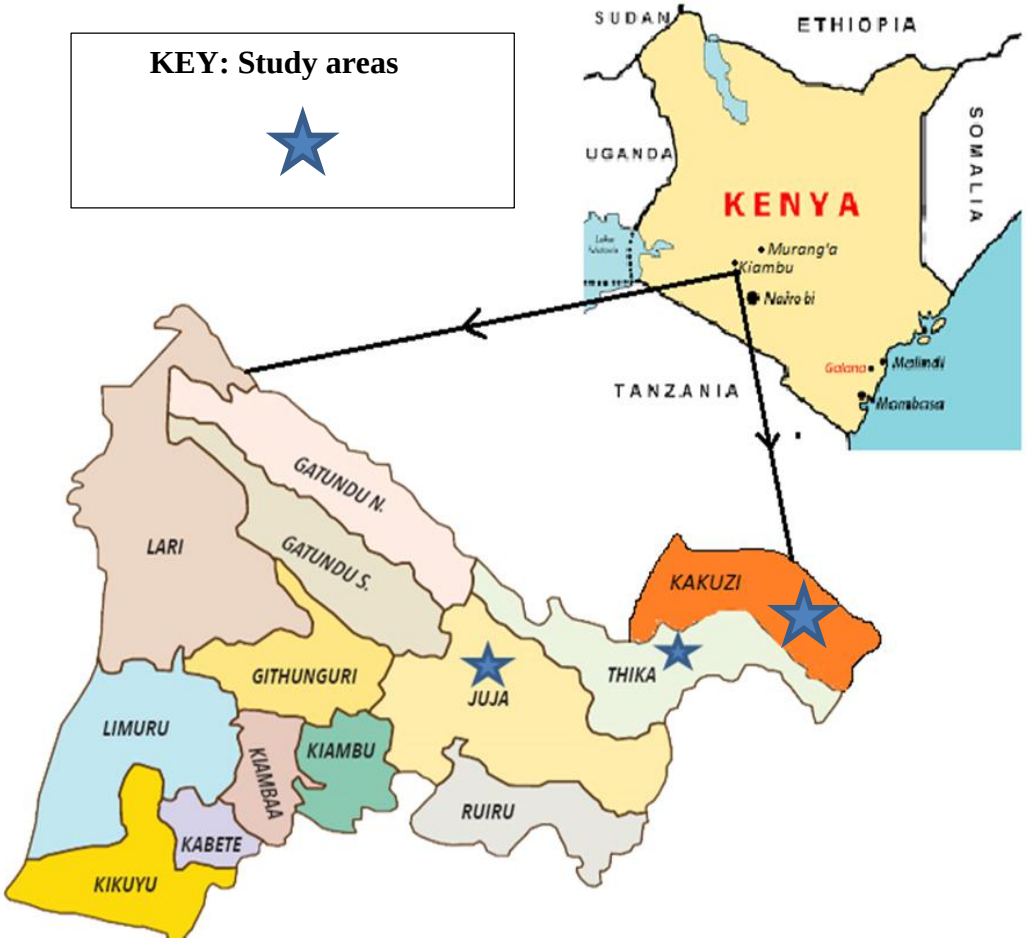

Figure 3.1: Current map of the larger Thika region showing some of the study sites (star) covered in the study (Source:http://www.kiambu.go.ke/about/administrative-political-units).

3.2. Study designs

Both descriptive cross-sectional and laboratory experimental study designs were employed in this study. The descriptive cross-sectional study was used to determine the prevalence of toxoplasmosis in chicken. This study was aimed at observing, describing, and documenting aspects of toxoplasmosis as it occurred naturally and

to serve as a starting point for hypothesis generation and theory development. This design was appropriate for the study as it involved a description of the current situation of toxoplasmosis in Thika region. A laboratory experimental study design was used to determine the immunopathological changes associated with *T. gondii* infection in immunocompetent and immunocompromised BALB/c mice.

3.3. Sample Size Determination

3.3.1. Sample size determination for the prevalence study

According to Cochran (1977), the total sample size was arrived at through calculation as follows:

$$\mathbf{N= \{Z^2 (pq)\}/ e^2}$$

Where; **N=** is the sample size,

Z= is the selected critical value of desired confidence level,

P= is the estimated proportion of an attribute that is present in the population,

q = 1− *p* and

e = is the desired level of precision

Since there is no known estimate available of the proportion in the target population assumed to have the characteristic of interest, the maximum variability, which is equal to 50% (p =0.5) and taking 95% confidence level with ±5% precision, the calculation

for required sample size will be as follows:

$$N = \frac{1.96^2 (0.5)(1-0.5)}{(0.05)^2} = 384$$

Due to financial constraints, a response rate of 27.3% (n=105) was reached. Since the required size of the population was not attained, a final estimate (n_f) was calculated using the correction formula described by Cochran,(1977):

$$n_f = \mathbf{n/\{1+ (n-1/N)\}}$$

Where: n_f= desired final sample size

n = desired sample size

N = estimate of the population size

Hence the final sample estimate (n_f) was:

$n_f = 105/\{1+(105-1/384\} = 82$

Thus, the sample was picked as a proportion of 105 chickens using proportionate stratified sampling technique. From the three study areas (Thika municipality, Kakuzi and Gatanga) a total proportionate sample size of a minimum of 82 chickens was to be selected. However, in this study, all the chickens collected were used to exclude any likely experimental errors.

3.3.2. Sampling and sample size determination for the experimental studies

The experimental mice were assigned to the experimental groups through simple random sampling technique. The sample size of the study subjects was estimated based on a previous study that yielded statistically analyzable (conclusive) results

(Nicoll *et al.,* 1997). A total of 80 BALB/c mice were intraperitoneally administered with approximately 15 *T. gondii* cysts in a 200µl inoculum (Weiss & Kim, 2000).

3.4. Collection and processing of brain samples from chicken

A cross-sectional study was undertaken in Thika region between February and April 2014. A total of 105 free range chickens aged between one and 4 years (estimated by looking at the size of the spurs (Brisbin *et al.,* 2007) and the information provided by the farmers) were purchased from households within Kakuzi Sub-County (55), Gatanga Sub-County (20) and Thika Municipality Sub-County (30) of Thika region. Gatanga and Kakuzi sub-counties are rural while Thika municipality has an urban and a semi-urban transition. Stratified random sampling was employed to collect the one hundred and five chickens from the households (one chicken per house) in three divisions (strata) within Thika where free range chickens are commonly reared.

The purchased free range clinically healthy chickens of both sexes were kept by farmers as a source of meat and eggs. They were sacrificed by a registered veterinary laboratory technician by cervical dislocation as described by Beaver *et al,* (2001). The samples of the head portion of each chicken were placed in separate DNase free nylon bags, marked appropriately and later they were transported in a cool box to the laboratory. Whole brain was obtained from each chicken under sterile conditions. Half of the portion kept at -20^{0}C for DNA extraction while the other half was kept at 4^{O}C for *T. gondii* expansion.

3.5. Extraction of DNA from chicken brain samples

Genomic DNA was extracted from chicken brain samples using commercial DNA extraction kit (ZymoResearch Quick-gDNA^{TM} Miniprep kit, USA; Cat. No. D3025). Briefly, the DNA from chicken brain tissues was extracted according to the

following instruction: 25mg of frozen brain tissue was mechanically homogenized in 500µl of Genomic Lysis buffer [SDS 1% and 10µl of proteinase K (100µg/ml)] and incubated at 55ºC for 1 hour. The lysate was centrifuged at top speed (10,000Xg) for five minutes and by making sure not to disturb the debris, the supernatant was transferred to a Zymo-spin column in a collection tube and then centrifuged at 10,000xg for one minute. The collection tube was discarded with the flow through and then the column transferred to a new collection tube; 200µl of DNA pre-wash buffer added to the spin column and centrifuged at 10,000xg for one minute. 500µl of g-DNA wash buffer was added to the spin column, centrifuged at 10,000xg for one minute and then the spin column was transferred to a clean microcentrifuge tube and 100µl DNA elution buffer added to the spin column. Incubation was done for 5 minutes at room temperature and the centrifuged at top speed for 30 seconds to elute the DNA. From each sample, 100µl of DNA was eluted and stored at -20°C in DNase-free Eppendorf tubes until use. Reference *T. gondii* (RH) DNA was kindly donated by the Friedrich-Loeffler-Institut Bundesforschungsinstitut für Tiergesundheit in Germany, through the assistance of Dr Gereon Schares.

3.6. Nested Polymerase chain reaction for detection of *Toxoplasma gondii* from chicken brain samples

Nested PCR reaction targeting a repetitive 529 bp DNA fragment sequence (GenBank Accession number AF146527) was performed in chicken brain samples as previously described (Kong *et al.*, 2012). The first round of 529 bp nested PCR amplification contained 12.5 μL 2x Taq PCR Master Mix (0.1U Taq Polymerase, 500 μMDNTPS each, 20mMTris-HCl, pH 8.3, 100mMKCl, and 3Mm MgCl2), 0.5 μL of the 5μM primers NF1 and NR1 (Table 3.1), 1 μL of extracted DNA from chicken brain samples, and 12.5 μL nuclease-free water. Reactions were cycled 30 times by initial denaturation at 94◦C for 2min, denaturation at 94◦C for 1min,

followed by annealing at 58◦C for 30 s, an extension step at 72◦C for 40s, and a final extension step at 72◦C for 5 minutes. The first round product was diluted 1: 100. The second round of PCR mixtures contained 1μL diluted product, 12.5μL 2x Taq PCR MasterMix, 0.5 μLof each 5μMprimerNF2 and primer NR2 (Table 3.1), and 10.5 μL nuclease-free water. The second round PCR was cycled 31 times by initial denaturation at 94∘C for 2 minutes, denaturation at 94◦C for 1min, followed by annealing at 52◦C for 15s, and a final extension step at 72◦Cfor 20s. Amplifications were performed using a thermal cycler (Mastercycler Gradient Eppendorf Germany).

The reference *T. gondii* (RH) DNA was used as positive control, while PCR water was used as the negative control. A 100 bp DNA ladder (Invitrogen, Carlsbad, CA, USA) was used as a marker.The two controls were included in every amplification step. The product generated in the second amplification was run in 1.5% agarose gel prestained with 3μL of ethidium bromide (1μg/mL) and visualized under ultraviolet (UV) light.

Table 3.1: Sequence of nPCR primer sets targeting the *T. gondii* 529bp repeat element (Kong *et al.*, 2012).

Primer	Sequence (5' to 3')
1. NFI	TGACTCGGGCCCAGCTGCGT
2. NRI	CTCCTCCCTTCGTCCAAGCCTCC
3. NF2	AGGGACAGAAGTCGAAGGGG
4. NR2	GCAGCCAAGCCGGAAACATC

3.7. Agarose gel electrophoresis

The eluted DNA samples were run on agarose gel and visualized on a UV light box. Agarose gel (1.5% w/v) was prepared by dissolving 1.5g of agarose powder into 100 ml of 1XTBE buffer. The gel solution was stirred, brought to boil in a microwave for 3 min to completely dissolve the powder. The gel solution was poured in a casting tray having combs and left for 10min to polymerize. Ethidium bromide (3µl) was incorporated into the gel solution before it was set to facilitate visualisation of DNA under UV light. The DNA sample (5µl) was mixed with 3µl of loading dye (ethidium bromide (1µg/ml)) and loaded into the wells and subjected to electrophoresis at 80V for 45 min. The gel was viewed on a UV light box to visualize the DNA ladder pattern (Sambrook *et al*., 1989). DNA samples (5µl) from non-infected healthy mice were run alongside as controls.

3.8. *Toxoplasma gondii* isolate and expansion

The brains from free range chicken previously preserved for *T.gonii* expansion (see section 3.4) were ground in a mortar using pestle; 1mL of phosphate buffered saline (PBS, pH 7.2) was added and homogenized using tissue homogenizer (Subauste, 2012). Enumeration of cysts was done by transferring three aliquots of 20 μL of the brain suspensions onto microscopic slides; a coverslip was placed over each sample and the number of cysts counted in the entire sample under 20x magnifications directly without staining.The brain suspension was serially diluted with PBS to adjust to a desired final concentration of approximately 15 tissue cysts/200 μl (Ole, 2008).

To obtain cysts for experimental infections, three BALB/c mice were intraperitoneally injected each with approximately 15 tissue cysts to allow for expansion of *T. gondii* cysts for use in experimental infection described below. The mice were monitored for six weeks after infection and anesthetized using concentrated CO_2 gas before sacrifice and parasites were isolated as stated above. Confirmation of presence of *Toxoplasma gondii* in the chicken samples was done as described in sections 3.5 to 3.7.

3.9. Experimental studies

3.9.1. Laboratory animals

Female BALB/c white mice were obtained from the rodent breeding facility at the Institute of Primate Research, Nairobi, Kenya. They were 6–8 weeks old and weighed 20-30g. They were housed under standard laboratory conditions, in plastic cages (medium size cages; length 16.9 inches, width 10.5 inches, and height 5 inches) and were provided with wood shaving bedding and nesting material. Food (Mice Pellets®, Unga Feeds Ltd, Kenya), and drinking water was given *ad libitum*.

Care, use and disposal of animals were done according to the IPR guidelines on handling of experimental animals.

3.9.2. Immunosuppression of Balb/c mice

The immunosuppressive drug used in this study was Dexamethasone (DEX) (Decadron DexPak PHARMA Links, India). The drug was administered orally in drinking water at concentrations of 4mg/L and 8 mg/L. At these concentrations, the dosing is equivalent to 2.66 and 5.32 mg/kg daily, assuming that the average weight of a mouse is 30g and that the water consumption rate is 10mL daily (Kawedia *et al.,* 2012).

3.10. Experimental infections

3.10.1. Acute and chronic infection study

In the first part of the experiment, 32 mice in groups of four were randomly chosen and euthanized in batches of four by concentrated CO_2 inhalation on 3, 5 and 7 dpi for acute infection and 14, 21, 28, 35 and 42 dpi for chronic infection. Sixteen BALB/C mice were controls and not infected with *T. gondii* (Table 3.2).

After euthanasia, sampling for blood from the heart was done as previously described (Parasuraman *et al.,* 2010). The blood was kept in separate labelled microfuge tubes for each mouse, on the bench till a blood clot was formed, and then kept at 4◦C overnight. The clots were then disturbed using wooden splints and centrifuged in amicrofuge (Eppendorf Centrifuge Model 5415) at room temperature for 3 minutes at $200g$. The serum was transferred into other respective labelled tubes and stored at −20◦C to be used for detecting antibodies (IgG and IgM) by ELISA (Mabtech AB, Sweden), according to the manufacturer's instructions. The brain tissue was also collected and divided in halves; one half was processed to

prepare tissue homogenates for cyst enumeration, while the other half together with the surgical removal of peripheral organs (liver, heart and spleen) were preserved in 10% formalin and processed for histology as described below.

Table 3.2: Experimental design for the Acute and Chronic infections

Sampling Time-point Days post-infection (DPI)	**Acute and chronic infections**
Day 0	Sampling and intraperitoneal infection with 15 *T. gondii* cysts (Weiss & Kim, 2000)
3, 5 & 7 dpi	Clinical assessment as well as mortality rate and survival time. Sampling blood for serum and brain, liver, heart and spleen
14, 21, 28, 35 & 42 dpi	Clinical assessment as well as mortality rate and survival time. Sampling blood for serum and brain, liver, heart and spleen

KEY: dpi= days postinfection.

3.10.2. Determination of effect of Dexamethasone

In the second part of the experiment, 48 previously infected BALB/c mice were obtained at 42 dpi and assigned into three groups (Table 3.3). Sixteen mice per group were each administered with dosages of 2.66 mg/kg/day (Group 1) and 5.32 mg/kg (Group 2) daily of dexamethasone (DEX) in drinking water over a period of 6 weeks (Nicoll *et al.,* 1997). Another sixteen infected nontreated mice were used as controls (Group 3). Sixteen uninfected control mice were given untreated water

(Group 4).The mice were monitored daily over six weeks for survival analysis and any clinical signs and mortalities were recorded. After every two weeks for six weeks after treatment, four mice from each group were serially euthanized using concentrated carbon dioxide and sampling done as previously described in Section 3.10.1. Mice that showed any severe clinical signs of toxoplasmosis were anesthetized immediately using concentrated carbon dioxide and sampling of blood and brain was also done.

Table 3.3: Experimental design for the immunosuppressed mice

Sampling Time point dpi	Immunosuppressed mice (Dexamethasone)			
	Infected mice Treated with Dexamethasone		Controls	
	Group 1 (2.66mg/kg/day)	Group 2 (5.32mg/kg/day)	Group 3 Infected control	Group 4 (Non infected control)
Day 0	Infection	Infection	Infection	No infection
7-42 days p.i	Clinical assessment (as well as mortality rate and survival time)			
42-84 days p.i	Treatment with dexamethasone, Sampling at 56, 70 & 84 dpi	Treatment with dexamethasone, Sampling at 56, 70 & 84 dpi	Sampling at 56, 70 & 84 dpi	Untreated water, Sampling at 42, 56, 70 & 84 dpi

KEY: dpi= days postinfection.

3.11. Serum preparation for antibody ELISA

Four mice from each treatment group were euthanized by concentrated CO_2 inhalation at various time points. The mice were bled via cardiac puncture and their blood kept in separate labelled microfuge tubes for each mouse, on the bench till a blood clot was formed, and then kept at 4°C overnight. The clots were then disturbed using wooden splints and centrifuged in a microfuge (Eppendorf Centrifuge Model 5415) at room temperature for 3 minutes at 200g. The serum was transferred into other respective labelled tubes and stored at -20°C to be used for antibody and cytokine ELISA, (Mabtech, Sweden) according to the manufacturer's instructions.

3.12. Quantification of brain cysts

As described earlier in section 3.10.1, one half of the brain from each mouse in the experimental infections was used for evaluation of cyst burden. The number of cysts was determined by placing three aliquots of 20μl of the brain suspensions onto microscopic slides and counting was done as previously described (Ole, 2008). Using the average of the counts, the concentration of cysts per ml of brain suspension was calculated.

3.13. Measurement of cytokine levels

Cytokine production was evaluated using commercial ELISA kits according to the manufacturer's instructions (MABTECH AB, Augustendalsvagen 19, Sweden). Briefly, each well of a 96-well high protein binding microtiter plate was coated with 100µl/well of the respective monoclonal antibody diluted in PBS, pH 7.4 and incubated overnight at 4-8°C. The plates were washed twice with PBS (200 µl/well) and blocked by adding 200µl/well of PBS with 0.05% Tween 20 containing 0.1% BSA (incubation buffer) and incubated for 1 hour at room temperature. Serum

samples or recombinant mouse IFN-γ and IL-10 standards were then applied to the plates, and incubated for 2 hrs at 37°C. After washing, the respective biotinylated monoclonal antibody for IFN-γ and IL-10 was added and the plates incubated for an additional 1hr at 37°C. One hundred microliters of Streptavidin-ALP was then added to each microtiter well and incubated for 1hr at 37°C. After washing, 100µl of p-nitrophenyl phosphate substrate was added to each well and the optical density measured at 405nm for pNPP in an ELISA reader after suitable developing time. Cytokine concentrations were determined by reference to standard curves generated with murine recombinant cytokines. The sensitivity limits of the assays were 20pg/ml for IL-10 and 4pg/ml for IFN-γ as per the instructions of the manufacturer.

3.14. Determination of O.D levels of anti-IgG and anti-IgM antibodies

Serum was prepared from the blood collected from the heart. Antibody levels for IgG and IgM were estimated using commercial ELISA kits (Mabtech AB, Augustendalsvagen 19, Sweden). Each well of a high protein binding 96-well plate (Maxisorp) was coated with 100μl/well of anti-IgG or IgM antibody diluted to 1 μg/ml in PBS and incubated overnight at 4◦C. The plates were washed twice with PBS (200 μl/well) and blocked by adding 200 μl/well of PBS with 0.05% Tween 20 containing 0.1% BSA (incubation buffer) and incubated for 1 hour at room temperature. After washing five times with PBS-Tween, 100 μl of serum samples or recombinant mouse IgG and IgM standards was then added to the plates and incubated for 2 hrs at 37◦C. After washing, 100 μl per well of anti-IgG-ALP diluted 1 : 1000 and anti-IgM-ALP diluted 1 : 500 in incubation buffer was added, respectively, and incubated for 1 hour at room temperature. After washing, 100 μl of p-nitrophenyl phosphate substrate was added to each and after a suitable developing time, the optical density (OD) of duplicated samples was measured at 405 nm for pNPP using a spectrophotometer (Titertek Multiscan ELISA reader, Helsinki, Finland) and compensated by comparing the OD of a standard positive

serum in each plate. The cut off values were determined as per the manufacturer instructions.

3.15. Histological analysis

Brain, liver, spleen and heart tissues were fixed in 10% buffered formalin and processed for paraffin embedding and sectioning. To verify the histological changes, tissue sections were stained with haematoxylin and eosin and observed under light microscope. The severity of the histopathological lesions in brain was evaluated by grading the lesions using a random scale (Table 3.4) as described by Tanaka *et al*. (2013). All of the histological analysis was done using a 40x objective. The scores for all lesions were added for each mouse, and the total pathological score for each mouse was used for data analysis.

Table 3.4: Brain histopathological inflammatory scores (Tanaka *et al*., 2013).

Inflammatory score	**Histopathological lesions**
A0	No lesion
A1	Minimal lesion limited to localized perivascular cuffs with slight mononuclear cell infiltration in the meninges
A2	Mild lesion, including perivascular cuffs, meningitis, and local glial cell infiltration.
A3	Moderate lesion, including perivascular cuffs, meningitis, glial cell activation, focal necrosis, and rarefaction of the neuropil with occasional macrophage infiltration.
A4	Severe lesion, including perivascular cuffs, meningitis, glial cell activation, and focally extensive necrosis

In the peripheral organs, the total numbers of focal or diffuse inflammatory foci was counted per tissue section to determine inflammatory infiltrate score (Table 3.5). In the heart tissue, an inflammatory infiltrate was considered when 30 or more leukocytes were detected in each inflammatory focus (Dong *et al.*, 1992). They were graded on a three-point scale ranging from 0 to 3+ based on the degree of cellular infiltration and myocardial cell necrosis.

In the liver, the inflammatory foci scattered in the parenchyma were quantified as described by Ishak *et al.*, (1995). Based on the degree of lymphocyte infiltration and hepatocyte necrosis, the level of inflammation was classified from A0 to A3, with a higher score indicating more severe inflammation. Segments of spleen were scored for the enlargement of lymphocyte infiltrated areas and for the increased numbers of macrophages, necrotic cells and presence of pigments (0, absent; and 1, present; Evangelos *et al.*, 2006). In these organs, the inflammatory changes were examined in two non-continuous sections (40µ distance between them) from each mouse in 25 microscopic fields using a 20x objective. A total inflammation score was determined from the summed scores of each mouse from each group or sampling time point and used for data analysis.

Table 3.5: Inflammatory score of the peripheral organs

Inflammatory Score	**HEART** (Dong *et al.,* 1992**)**	**LIVER** (Ishak *et al.,* 1995)	**SPLEEN** (Evangelos *et al.,* 2006**)**
A0	Normal appearance with no detectable lesions	No focal necrosis; no portal inflammation	No enlargement of lymphocyte areas
A1	Focal heart muscle necrosis ± inflammation (<50 fibers affected	One focus or less per 10x objective; mild in some or all portal areas	Slight enlargement of lymphocyte areas
A2	Multifocal heart muscle necrosis ± inflammation (50–100 fibers affected).	Two to four foci per 10x objective; moderate in some or all portal areas	Moderately enlarged lymphocyte areas
A3	Severe diffuse heart muscle necrosis ± inflammation (>100 fibers affected).	Five to ten foci per 10x objective; moderate to marked, all portal areas	Pronounced enlargement of lymphocyte areas

3.16. Ethical consideration

Prior to commencement of the study, all protocols and procedures used were reviewed and approved by the Institute of Primate Research Institutional Animal Care and Use committee (Approval number: IRC/21/11) [Appendix 2].

3.17. Statistical analysis

Statistical determination of the differences between mean values to every other mean obtained for the treatment groups was done by analysis of variance (ANOVA). The survival of mice on different drug regimens was evaluated by the

Kaplan-Meier product limit method. The difference between the curves obtained was analysed by the Wilcoxon rank sum test. Difference of histological analysis among groups of animals, were compared by using Mann–Whitney U test.

For quantitative variables, descriptive statistics were used and frequencies and proportions computed and their corresponding 95% confidence intervals (95% CI) calculated (Collett, 2002). Chi-square test was applied to determine whether there were significant difference between occurrence of the disease and area of origin, sex and age of the chickens. Statistical analysis and graphs were performed using GraphPad prism version 5.0 (GraphPad Software, USA).Values of $P < 0.05$ were considered statistically significant.

CHAPTER FOUR

RESULTS

4.1. Prevalence of *T. gondii* in chicken using nPCR

The overall mean prevalence of *T. gondii* in all the three areas was 79.0% (95% CI: 70.0–86.4%). Results of the spatial distribution of *T. gondii* showed that 83.3% (95% CI: 65.3–94.4%) of the chicken brain tissues from Thika Municipality were positive for *T. gondii* (Table 4.1). The *T. gondii* prevalence for Kakuzi and Gatanga sub-counties was 80.0% (95% CI: 67.0–89.6%) and 70.0% (95% CI: 45.7–88.1%), respectively (Table 4.1). However, there was no significant (P = 0.5088; $\chi 2$= 1.354) difference in prevalence of *T. gondii* infection among chicken from the three sub-counties.

The chickens sampled in the study were grouped into three groups (>1 year but <1.5 years; ≥1.5 years but <2 years; and ≥2 years) based upon the age (Table 4.1). Chickens aged 1 year but <1.5 years showed the lowest prevalence (40%) followed by those in age groups ≥1.5 years but <2 years and ≥2 years, respectively. Statistical analysis indicated that the prevalence of *T. gondii* was significantly (P = 0.003; $\chi 2$ = 11.87) associated with age of the bird. The prevalence of *T. gondii* was apparently higher in females (79.4%) compared to males (78.6%), though the differences were not significant (P = 0.922; $\chi 2$ = 0.01).

Table 4.1: Prevalence of *Toxoplasma gondii* in Thika, region, Kenya based on areas of origin, sex and age of chicken.

Risk factor	Positive/total samples	Prevalence (%)	*P* and $\chi 2$ Values
Sub county of origin			
Thika Municipality	25/30	83.3	
Kakuzi	44/55	80	$P = 0.5088$
Gatanga	14/20	70	$\chi 2 = 1.354$
Overall	83/105	79	2df
Age of chicken			
>1 year <1.5 years	4/10	40	$P = 0.003$
≥1.5 years but <2 years	40/45	89	$\chi 2 = 11.87$
≥2 years	39/50	78	2df
Sex of chicken			
Male	33/42	78.6	$P = 0.922$
Female	50/63	79.4	$\chi 2 = 0.01$
			2df

In this study, presence of *T. gondii* was investigated in a total of 105 chicken brain tissues obtained from Thika region in Kenya, by testing for detection of the 529 bp repeat element. The secondary amplification products clearly showed the predicted amplicon size of 164bp as shown in Plate 4.1.

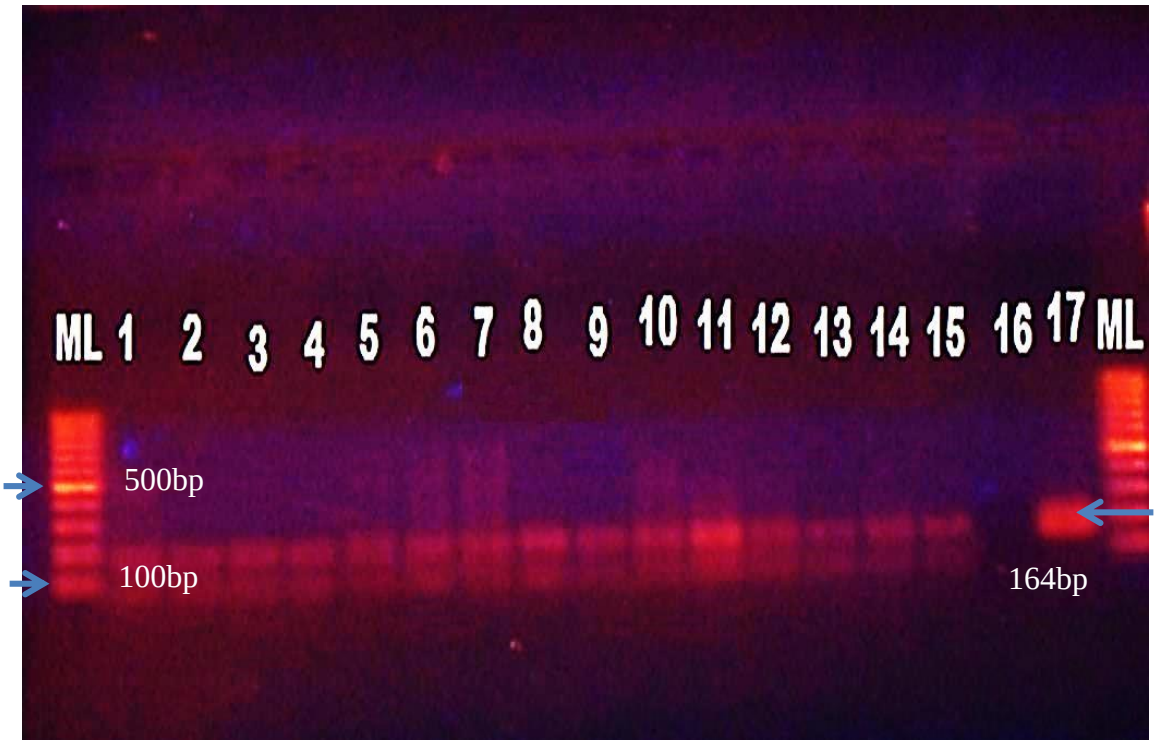

Plate 4.1: Gel photograph for secondary PCR amplification products of *T. gondii* in brain tissues of chicken from Kakuzi, Kenya. Lanes 1-15: Chicken samples, Lane 16: negative control, Lane 17: positive control, ML: 100bp DNA ladder.

4.2. Clinical signs and cumulative survival

The infected mice treated with dexamethasone [2.66mg/kg/day and 5.32mg/kg/day] showed various clinical signs which increased with increase in dosage level. They included tottering gait with decreased activity coupled with piloerection, tachypnea, hunched appearance and evidence of emaciation. The mice which were infected and not treated did not show any clinical signs. The uninfected control mice did not manifest any clinical signs.

Results of the cumulative survival of mice evaluated using the Kaplan-Meier method showed that all (100%) of both non- infected and those infected and not treated survived during the whole experimental period (Figure 4.1) However, a mortality rate of 15% and 28.6% was recorded in mice infected and treated with 2.66mg/kg/day and 5.32mg/kg/day, respectively. Treatment with 5.32mg/kg daily of dexamethasone significantly ($P<0.05$) increased the mortality rate, as compared to infected untreated mice. In this study, 85% of mice treated with 2.66mg/kg/day of dexamethasone survived after the fourth week after treatment, while the mice treated with 5.32mg/kg/day of dexamethasone showed 85% survival at week 3 after treatment, decreasing to 71.4% survival at week 9 after treatment. The percentage survival time in days of the Toxoplasma infected treated groups was significantly ($P<0.05$) lower with increase in dosage level.

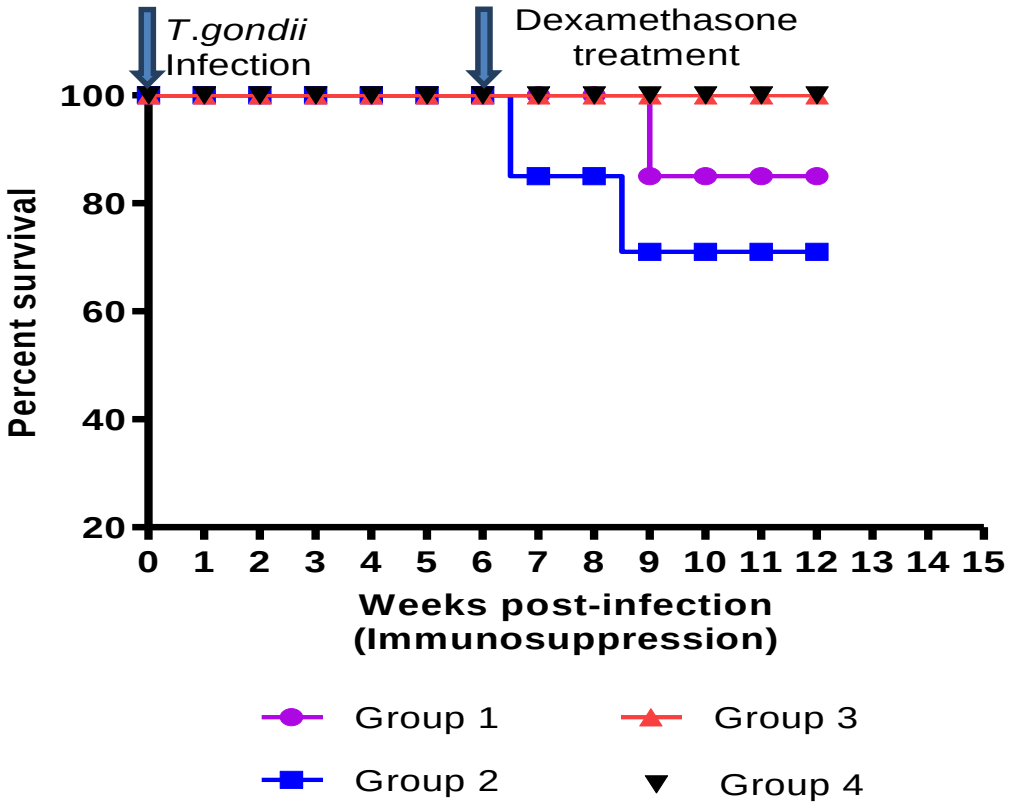

Figure 4.1: Effect of dexamethasone on survival (Kaplan-Meier estimates) of mice infected with *T. gondii* infection and treated with dexamethasone :Group 1= Mice infected with *T. gondii* and treated with dexamethasone at 2.66mg/kg/day; Group 2= Mice infected with *T. gondii* and treated with dexamethasone at 5.32mg/kg/day; Group 3= Mice infected with *T. gondii* and not treated with dexamethasone; Group 4 =Non infected control .

4.3. Brain tissue cysts burden

The presence of toxoplasma tissue cysts in the brain of infected non treated mice was observed from 14 dpi and was more evident as the disease progressed. The mean cyst numbers ranged from (Mean ± SD) 63.5 ± 6.77 at 14 dpi and increased to 115.7±12.58; 192.73±47.24; 354.69±56.23 and 413.53±36.053 [P<0.05] at 21, 28, 35 and 42 dpi respectively (Figure 4.2).

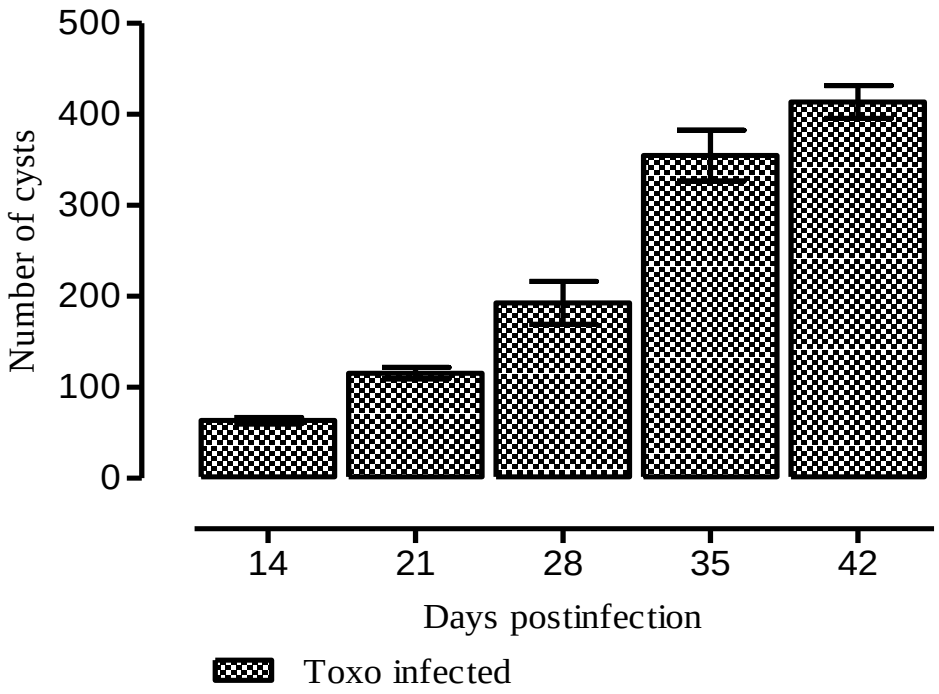

Figure 4.2: Numbers of cysts per ml of brain tissue (mean ± SD) in infected mice with established chronic Toxoplasmosis.

After treatment with dexamethasone, a progressively increasing number of tissue cysts were observed in the brains of mice in various treatment groups as compared to those in chronically infected untreated mice (Figure 4.3). In the mice treated with 2.66mg/kg/day, the mean number of tissue cysts per ml of brain sample at 14, 28, and 42 days post treatment (dpt) was 687.99 ± 35.43, 770.495 ± 12.722, and 845.88 ± 15.43, respectively. For the mice treated with 5.32mg/kg/day, there was significant increase in cyst numbers, with the mean numbers of cysts per ml of brain sample at 14, 28, and 42 dpt being 740.17 ± 34.6, 940.78 ± 54.7, and 1047.14± 103.76 respectively. The mean number of tissue cysts per ml of brain sample in the infected non-treated mice at 14, 28, and 42 dpt was 468.91 ± 11.35, 559.42 ± 30.41, and 647.39 ± 32.12, respectively. The tissue cyst numbers per ml of brain sample in the mice treated with 2.66mg/kg/day and 5.32mg/kg/day were significantly ($P = 0.001$) higher than those from the infected non-treated mice. However, the differences in numbers of cysts in the group treated with 2.66mg/kg/day and 5.32mg/kg/day were not significant ($P > 0.05$).

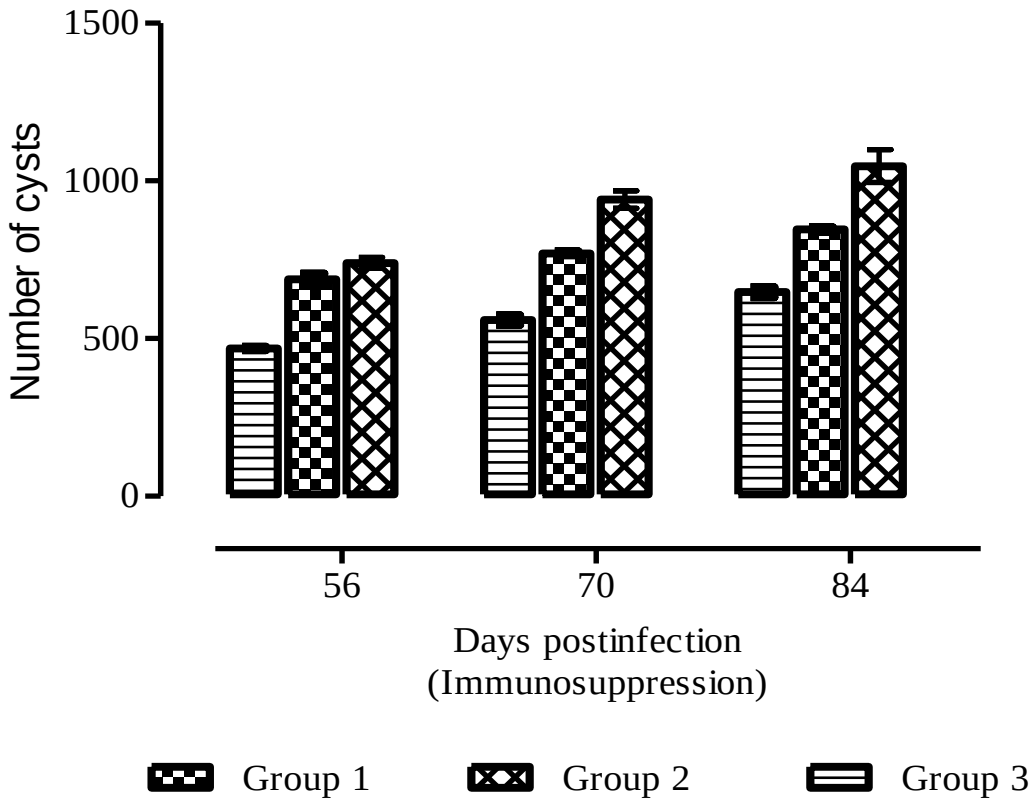

Figure 4.3: Numbers of cysts per ml of brain tissue (mean ± SD) in mice with established chronic Toxoplasmosis after treatment with 2.66mg/kg/day and 5.32mg/kg/day)of dexamethasone : Group 1= Mice infected with *T. gondii* and treated with dexamethasone at 2.66mg/kg/day; Group 2= Mice infected with *T. gondii* and treated with dexamethasone at 5.32mg/kg/day;Group 3= Mice infected with *T. gondii* and not treated with dexamethasone.

Toxoplasma gondii cysts were observed in the H&E stained histological sections. These cysts were round in shape, were variable in size, and were widely dispersed throughout the tissue (Plate 4.2). The proportion of mice having *T. gondii* tissue cysts in the mice treated with 2.66mg/kg/day and 5.32mg/kg/day of DEX was 42.8% and 57.1%, respectively. The infected non-treated mice recorded similar results as the mice treated with 2.66mg/kg/day (Plate 4.2).

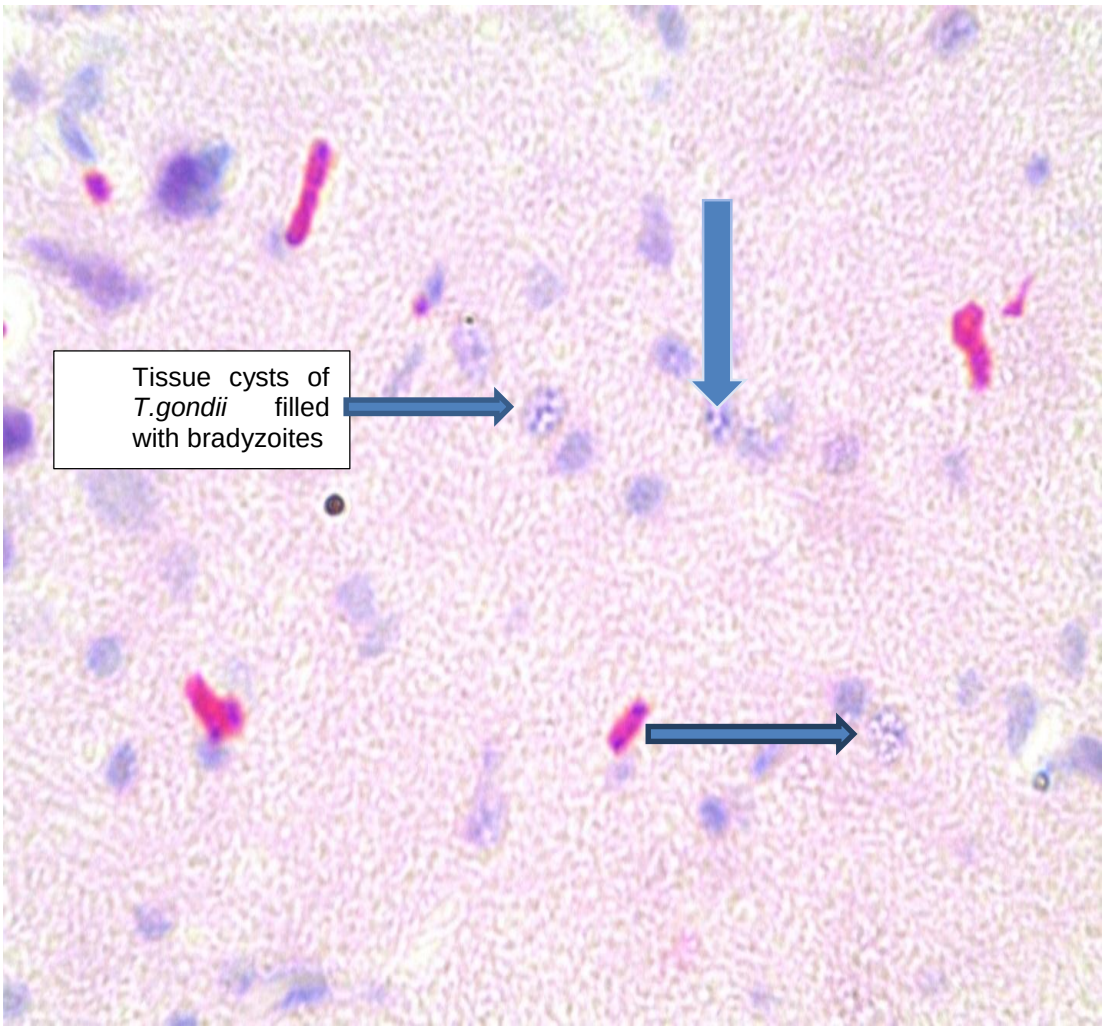

Plate 4.2: *Toxoplasma gondii* cysts (arrows) in the brains of chronically infected BALB/c mice

4.4. Mean O.D levels of anti *T.gondii* IgM and IgG antibodies

The mean O.D levels of anti-*T.gondii* specific IgM and IgG antibody responses are shown in figures 4.4 and 4.5, respectively. After *T. gondii* infection, the OD of IgM significantly ($P < 0.05$) increased from 0.12 (95%; CI: 0.089–0.15) at 7 dpi and peaked at 0.69 (95%; CI: 0.68–0.71) at 35 dpi [Appendix 3]. There was no

corresponding increase in IgM OD levels in the noninfected nontreated mice. On the other hand, there was a significant ($P < 0.05$) increase in the OD of IgG rising from 0.27 (95%; CI: 0.26–0.28) at 7 dpi to 1.302 (95%; CI: 0.59–2.02) at 35 dpi [Appendix 4]. There was no corresponding increase in IgG OD levels in the non-infected non-treated mice.

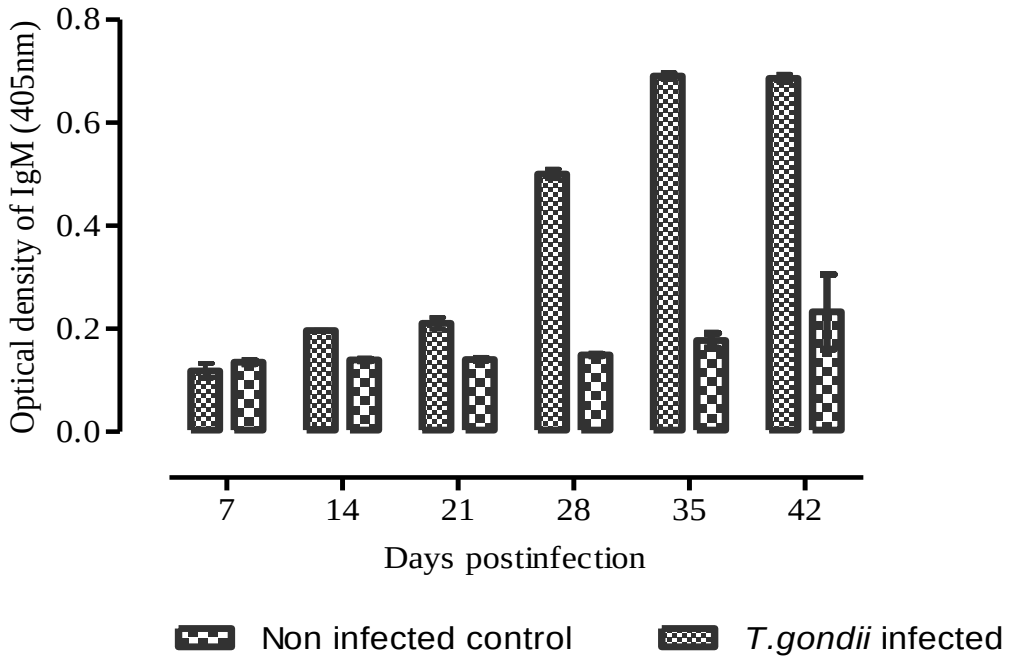

Figure 4.4:Mean OD levels of anti-IgM antibodies in sera from mice intraperitoneally infected with *T. gondii*. Data represent the mean and standard deviations obtained from four mice per each time point

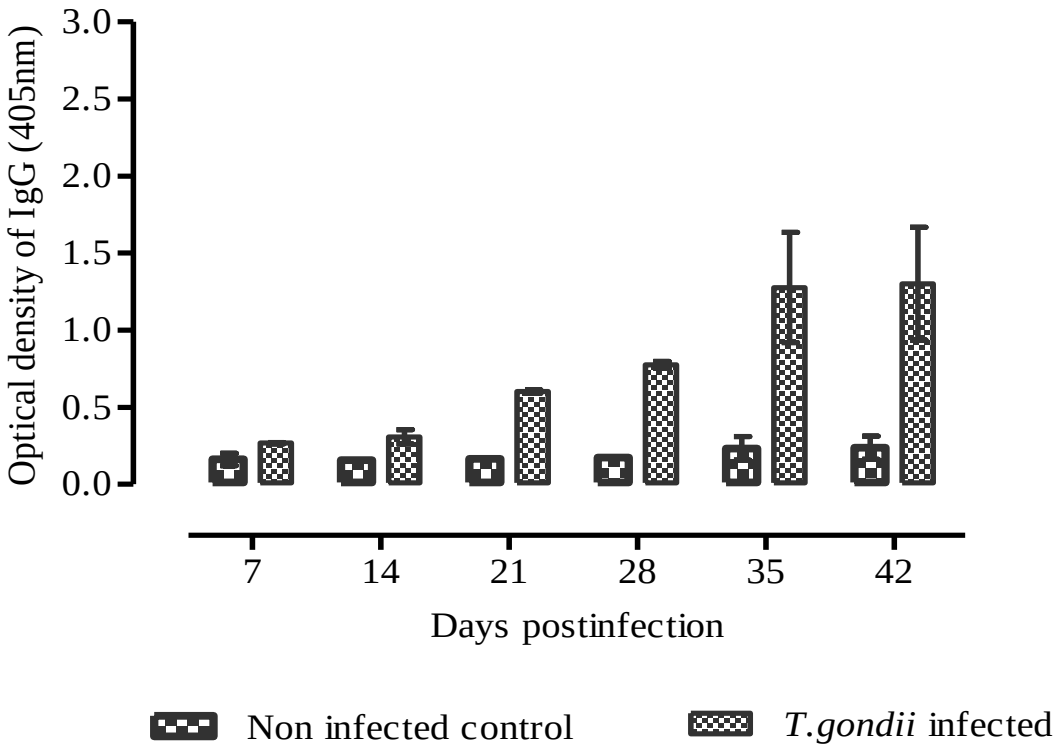

Figure 4.5: Mean OD levels of anti-IgG antibody in sera from mice intraperitoneally infected with *T. gondii.* Data represent the mean and standard deviations obtained from four mice per each time point.

In the dexamethasone-treated mice, there was a significant (P <0.001) decrease in the mean OD levels of *T.gondii* anti-IgM levels at time points between 42 and 84 days after infection (Figure 4.6) [Appendix 5]. The decline in the mice treated with 2.66mg/kg/day was from 0.55OD (95%; CI: 0.503–0.599) (42 dpi) to 0.41OD (95%; CI: 0.29–0.42) (84 dpi) while the decline in the mice treated with 5.32mg/kg/day was from0.503OD (95%; CI: 0.49–0.52) (42 dpi) to 0.34OD (95%; CI: 0.29–0.38) (84 dpi).This shows that IgM levels decrease was associated with increased dose, although the differences were not significant (P>0.05).

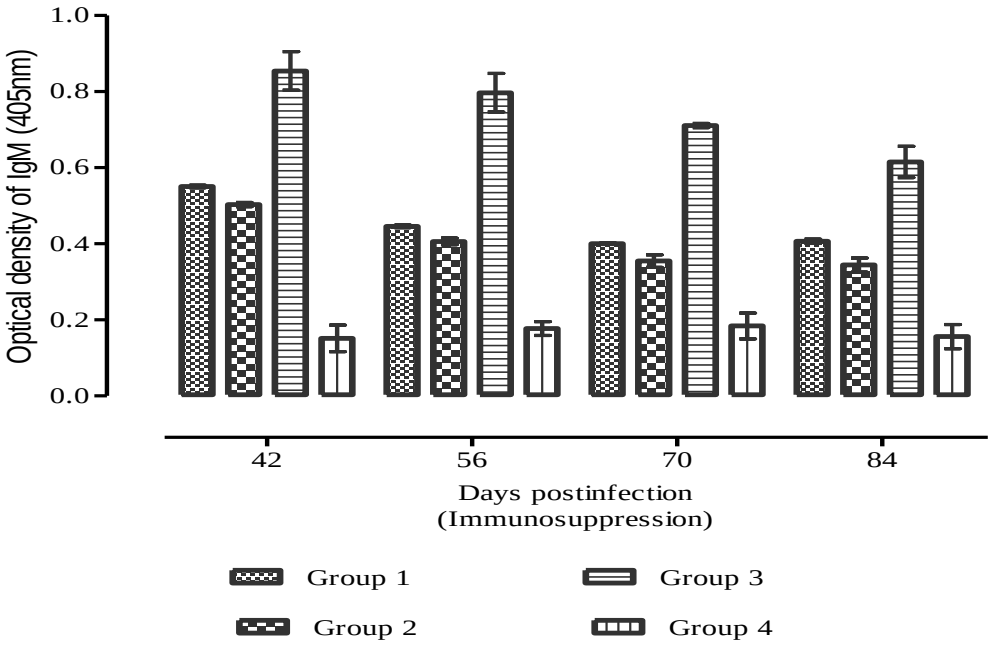

Figure 4.6: Mean OD levels of anti-IgM antibodies in sera from mice intraperitoneally infected with *T. gondii* and later (42 dpi) administered with dexamethasone. Data represent the mean and standard deviations obtained from four mice per each time point: Group 1 = mice infected with *T. gondii* and treated with dexamethasone at 2.66mg/kg/day; Group 2 = mice infected with *T. gondii* and treated with dexamethasone at 5.32/kg/day; Group 3 = mice infected with *T. gondii* and not treated with dexamethasone; Group 4 = noninfected control.

Following dexamethasone treatment, the mean OD levels of *T.gondii* anti-IgG maintained an upward trend in mice in all the groups (Figure 4.7). The OD level of IgG in the mice treated with 2.66mg/kg/day was 0.96 (95%; CI: 0.93–0.99) at 42 dpi and increased to 2.31OD (95%; CI: 2.26–2.36) at 84 dpi.

The OD level of IgG in the mice treated with 5.32mg/kg/day was 0.95 (95%; CI: 0.93–0.97) at 42 dpi and increased to 2.004O.D (95%; CI: 1.47–1.53) at 84 dpi. Significantly ($P < 0.001$) decreased levels of IgG were recorded in the treated mice compared to the infected nontreated at all time points after treatment [Appendix 6]. There was significant ($P < 0.05$) decrease in IgG antibody levels between treated mice with increase in dosage level. Overall, IgG levels were significantly ($P < 0.05$) higher compared to IgM at all post infection time points.

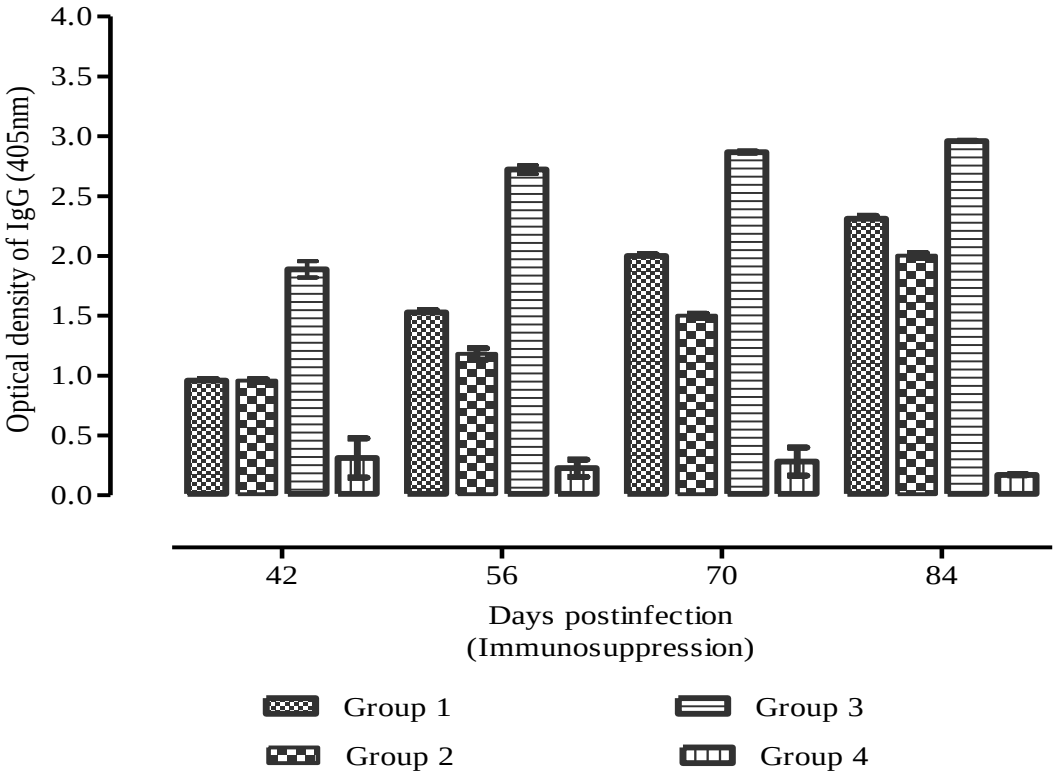

Figure 4.7: Mean OD levels of the anti- IgG antibodies in sera from mice intraperitoneally infected with *T. gondii* and later (42 dpi) treated with dexamethasone. Data represent the mean and standard deviations obtained from four mice per each time point: Group 1 = mice infected with *T. gondii* and treated with dexamethasone at 2.66mg/kg/day; Group 2 = mice infected with *T. gondii* and treated with dexamethasone at 5.32/kg/day; Group 3 = mice infected with *T. gondii* and not treated with dexamethasone; Group 4 = noninfected control.

4.5. Results of Cytokines levels

4.5.1. IFN-γ Levels

The mean IFN-γ cytokine changes before treatment are shown in Figure 4.8. In the infected non-treated mice, IFN-gamma levels increased from 3.5pg/ml (95%; CI: 2.93-4.07 at 0 dpi and reached 7.34pg/ml (95% CI: 3.61-10.40) on 7dpi, and this was followed by a decline to 3.94pg/ml (95% CI: 2.66-5.22) on 14 dpi. Thereafter, there was a progressively significant ($p<0.001$) increase in IFN-γ reaching 10.59pg/ml, (95% CI: 9.03-12.15) at 35 dpi [Appendix 7]. (Figure 4.8).

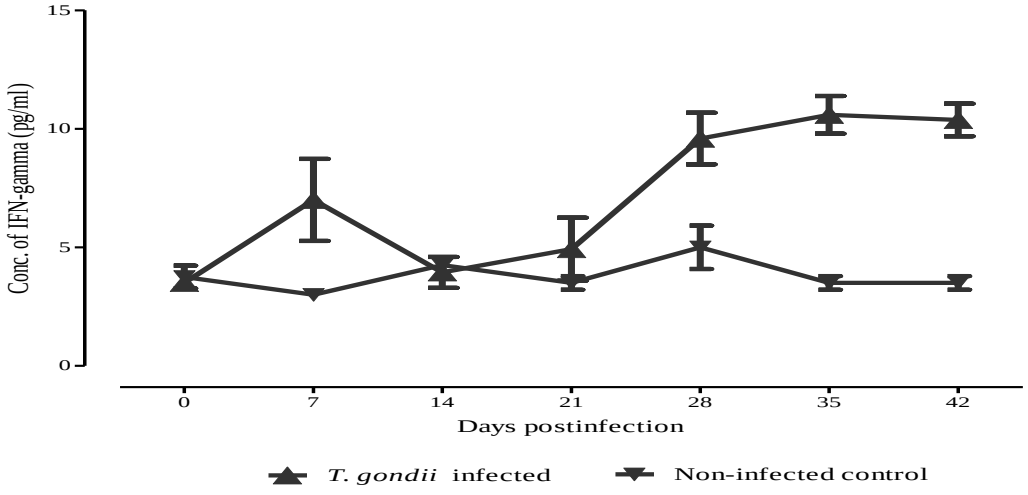

Figure 4.8: Mean levels of IFN-γ in serum of BALB/c infected with *T. gondii* during the early [7-14dpi] and late stages [21-35dpi] of infection before treatment. The data are expressed as the means ± SEM.

After the mice were treated with dexamethasone, IFN-γ productions levels progressively declined at time points between 42 and 84 dpi. The decline in the 2.66mg/kg/day of dexamethasone treated mice (Group 1) was from 17.84pg/ml (95% CI: 1.60-34.08) at 42 dpi to 4.54pg/ml (95% CI: 3.58-5.49) and 10.02pg/ml (95% CI: 2.98-17.07) at 70 dpi and 84 dpi respectively (Figure 4.9).

The corresponding decline in the 5.32mg/kg/day of dexamethasone treated mice (Group 2) was from 15.51pg/ml (95% CI:-0.64-31.66) at 42 dpi to 2.78pg/ml (95%; CI: 1.067-4.497) and 7.89pg/ml (95%; CI: 3.02-12.73.50) at 70 dpi and 84dpi, respectively. The decrease in IFN-γ levels was associated with increased dose, although the difference in IFN-γ levels between the 2 doses were not significant ($P>0.05$); Appendix 8). The IFN-γ levels in the infected non-treated mice (Group 3) increased from 21.48pg/ml (95%CI: 10.59-32.38) at 42 dpi to 26.38pg/ml (95% CI: 20.01-32.75) at 56 dpi and thereafter, a progressive decline in IFN-γ levels reaching 13.53pg/ml (95% CI: 0.42-26.64) and 11.03pg/ml (95% CI: 5.43-16.64) at 70 dpi and 84 dpi, respectively. Mice in the infected non-treated group (Group 3) maintained significantly ($P<0.001$) higher levels of IFN-γ compared to the infected treated mice (Figure 4.9). However, there were no significant ($P>0.05$) differences in reduced detectable IFN-γ levels between the groups.

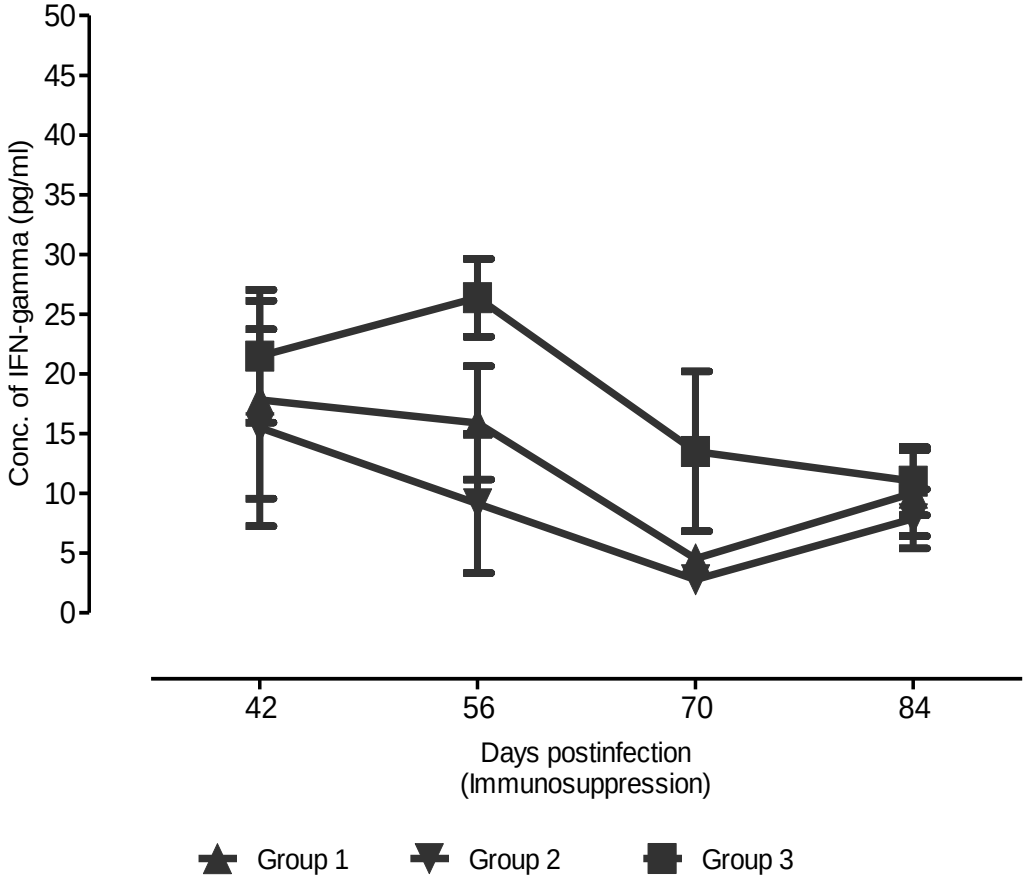

Figure 4.9: Mean levels of IFN-γ in serum in BALB/c infected with *T. gondii* and after dexamethasone treatment. The results are expressed as the means ± SEM of 4 mice. Group 1= *T. gondii* infected dexamethasone treated (2.66mg/kg/day); Group 2= *T. gondii* infected dexamethasone treated (5.32mg/kg/day); Group 3= *T.gondii* infected;Group 4=Non infected control.

4.5.2. Interleukin 10 (IL-10) Levels

The levels of IL-10 also increased following *T. gondii* infection. The levels significantly (P< 0.001) increased from 3.5pg/ml (95%; CI: 2.93-4.07) at day 0 post-infection reaching 99.6pg/ml (95% CI: 83.62-115.58) at 7dpi and remained elevated up to day 35dpi (119.6pg/ml; 95%; CI: 106.27-124.45) (Figure 4.10; Appendix 9).

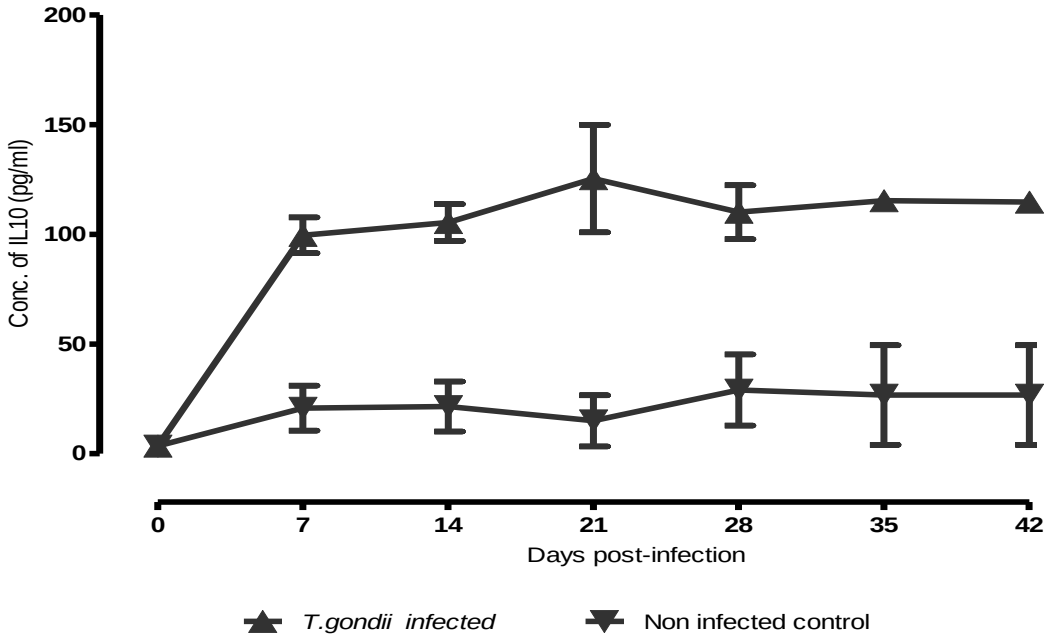

Figure 4.10: Mean levels of IL10 in serum of BALB/c infected with *T. gondii* during the early [7-14dpi] and late stages [21-35dpi] of infection before treatment. The data are expressed as the means ± SEM.

Following dexamethasone treatment, the levels of IL-10 maintained a downward trend in mice in the treated groups (Figure 4.11). The levels of IL-10 in the 2.66mg/kg/day of dexamethasone treated mice (Group 1) was 135.66pg/ml (95% CI: 82.79-188.54) at 42 dpi and dropped to 120.52pg/ml (95% CI: 89.38-151.66) and 71.73pg/ml (95% CI: 45.67-97.79) at 56 and 84 dpi, respectively. The IL-10 levels in mice treated with 5.32mg/kg/day (Group 2) was 116.92pg/ml (95% CI: 89.69-144.15) at 42 dpi and dropped to 71.73pg/ml (95% CI: 53.91-89.55); 67.392pg/ml (95% CI: 27.16-107.62) and 55.59pg/ml (95% CI: 40.77-70.43) at 56, 70 and 84 dpi respectively.

The IL-10 levels for mice in the infected non treated group (Group 3) increased from 141.97pg/ml (95% CI: 134.26-149.68) to 159.56pg/ml (95% CI: 81.29-237.82) at 42dpi and 56 dpi respectively. This was followed by a decline to 99.71pg/ml (95% CI: 77.16-122.27) at 84 dpi. Mice in the infected non-treated Group recorded significantly ($P<0.01$) higher IL-10 levels compared to the treated Groups at all time points [Appendix 10].

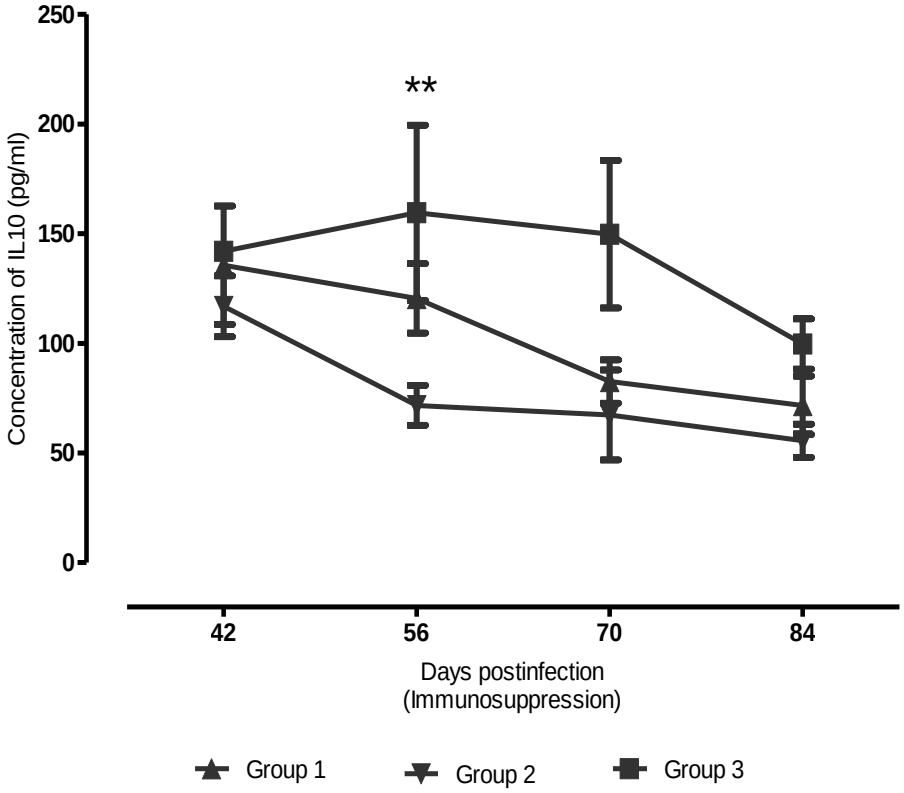

Figure 4.11: Levels of IL10 in serum from BALB/c mice infected with *T. gondii* and after dexamethasone treatment. The data are expressed as the means ± SEM of 4 mice. The numbers in parenthesis indicate weeks post-treatment: Group 1= *T. gondii* infected dexamethasone treated (2.66mg/kg/day); Group 2= *T. gondii* infected dexamethasone treated (5.32mg/kg/day); Group 3= *T. gondii* infected.

4.6. Clinical and Histological changes in the brains of *T. gondii* infected mice treated with dexamethasone.

The clinical signs which were observed during the infection included tottering gait leading to decreased activity of the mice, emaciation, piloerction, hunched appearance and tachypnea. As shown in plate 4.3, the lesions in the various groups of mice were characterized by diffuse infiltrates of mononuclear cells, glial nodules, vascular cuffing by lymphocytes and focal mononucleated cell infiltrates in the meninges.

During the early stages of the infection (0–42 dpi) before DEX treatment, inflammatory changes in the brain were observed with the development of infection. From 7 dpi, the brain tissues from the infected mice presented with inflammatory lesions characterized mainly by lymphocyte infiltration into the brain meninges. This infiltration increased and, by 42 dpi, perivascular cuffing by lymphocytes was pronounced (Figure 4.12).

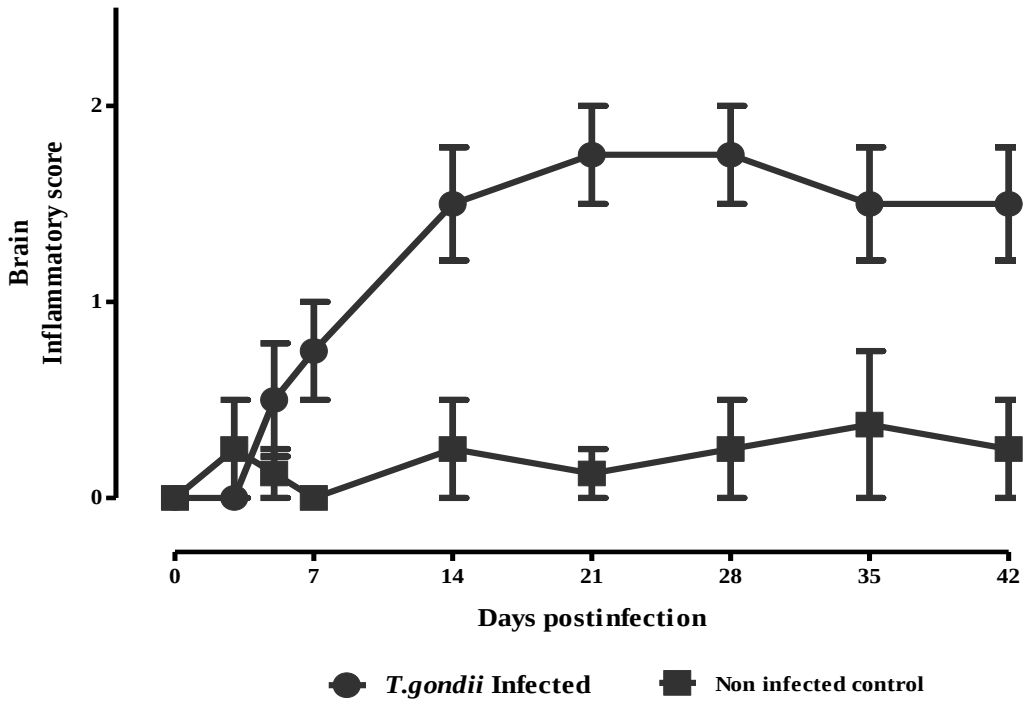

Figure 4.12: Inflammatory score in the brain of BALB/c mice infected with *T. gondii* before treatment.

After dexamethasone administration, there was an increased inflammatory response with increase in dosage level (Figure 4.13). Mice in the group treated with 2.66mg/kg/day and 5.32mg/kg/day of DEX had a minimal to moderate inflammatory changes including perivascular cuffs, meningitis, glial cell activation and focal necrosis, and macrophage infiltration at 4 and 6 weeks after treatment.

Proportionately, brain tissue necrosis was observed in 14.3% and 28.5% of mice treated with 2.66mg/kg/day and 5.32 mg/kg/day, respectively (Plate 4.3D). No necrotic alterations were observed in the other groups. Gliosis was observed in

71.4% and 57.2% of brain tissues from mice treated with 2.66mg/kg/day and 5.32mg/kg/day, respectively, and all (100%) mice in the infected nontreated mice.

The proportion of mice with lymphocyte infiltration in the group treated with 2.66mg/kg/day and 5.32mg/kg/day and the infected non-treated group was 28.5%, 71.4%, and 100%, respectively (Plate 4.3C). Numerous perivascular and leptomeningeal infiltrations of lymphoid cells and macrophages were observed, most often on the surface of the cerebral cortex and in the thalamus. Other types of pathology observed in the mice were formation of microglial nodules with surrounding astrocytosis. Increased intake of dexamethasone in mice showed a slight reduction in the severity of microgliosis and astrocytosis.

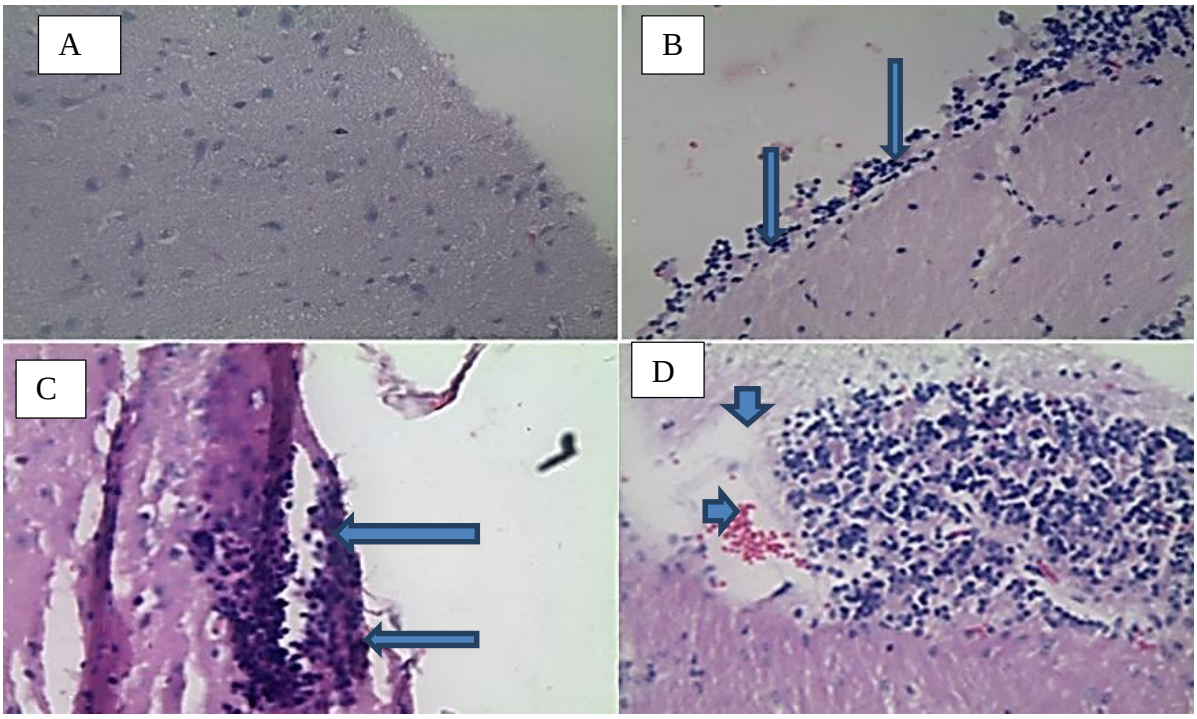

Plate 4.3: Histopathological findings in the brain of mice infected *T. gondii.* [A]: Brain of non-infected mice. There are no alterations throughout the tissue. [B and C]: Brain of infected mice. Increased number of lymphoid cells can be seen in the meninges [B] (arrows), suggesting the development of meningitis. [C] Perivascular cuffing by lymphoid infiltrations (arrows) along the meninges. [D] Lymphoid inflammation with zones of necrosis (arrow heads) in the infected treated groups.

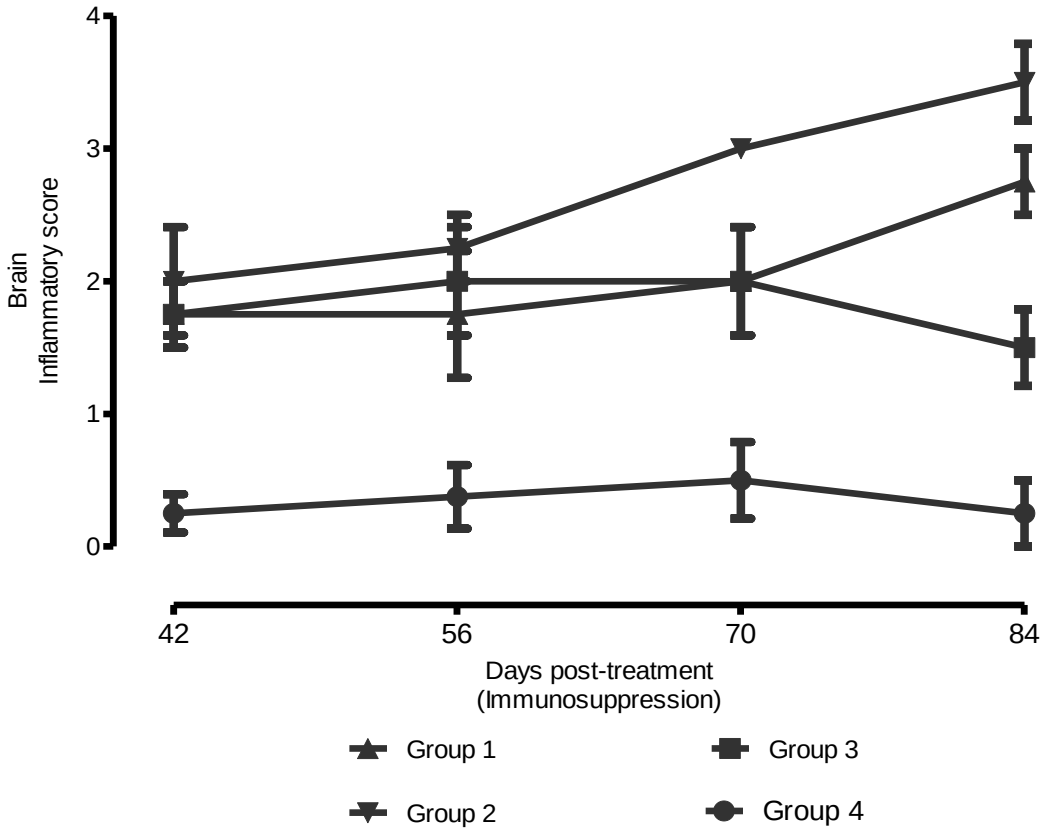

Figure 4.13: Inflammatory score in the brain of BALB/c mice infected with *T. gondii* after treatment with dexamethasone: Group 1= Mice infected *with T. gondii* and treated with dexamethasone at 2.66mg/kg/day; Group 2= Mice infected with *T. gondii* and treated with dexamethasone at 5.32mg/kg/day; Group 3= Mice infected with *T. gondii* and not treated with dexamethasone; Group 4 =Non infected control.

4.7. Histological changes in the heart, spleen and liver of BALB/c mice infected with *T.gondii*

In general, the histopathological changes in the liver, heart and spleen of infected mice consisted of mild-to-moderate congestion, focal polymorphonuclear inflammatory infiltrate and multifocal or focal mononuclear inflammatory infiltrate. Between 3dpi and 14dpi, the liver showed increased pathology characterized by hepatic necrosis, infiltration of lymphocytes and monocytes scattered in parenchyma and portal triad areas

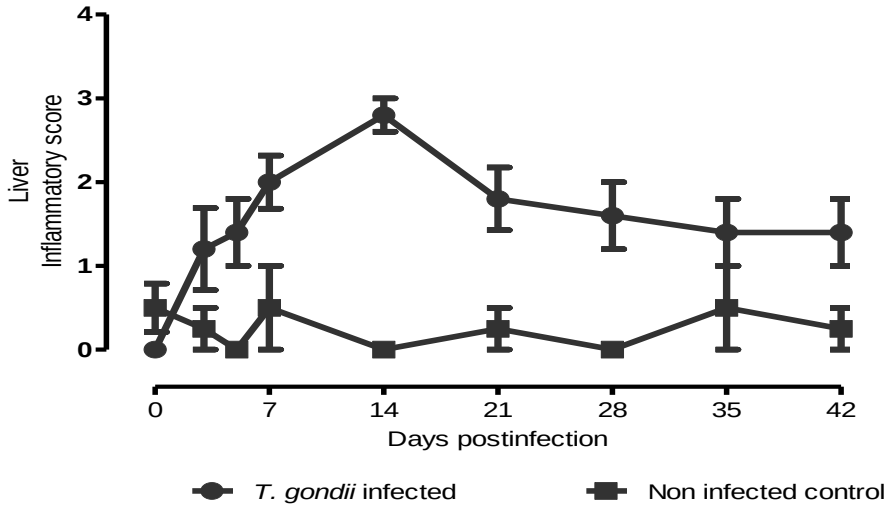

Figure 4.14: Inflammatory scores in the liver of BALB/c mice infected with *T. gondii* before treatment with dexamethasone. The data was obtained by analyzing 40 microscopic fields per section on four sections using a 20x objective from each mouse and from four mice per group.

Following dexamethasone treatment, the mice treated with 2.66mg/kg/day (Group 1) and 5.32mg/kg/day (Group 2) of dexamethasone showed varied degrees of inflammatory responses (Plate 4.4; Figure 4.15). For the mice treated with 2.66mg/kg/day of dexamethasone, an inflammatory score of 1.4 (±0.245) and 2.0 (±0.00) was observed between 56dpi and 84 dpi, respectively, while the mice treated with 5.32mg/kg/day (Group 2) of dexamethasone recorded an inflammatory score of 1.6 (±0.245) and 2.6 (±0.25) at 56 and 84 dpi, respectively. On the other hand, the infected non-treated mice presented an inflammatory score of 0.6 (±0.245) at 42 dpi but did not significantly ($P>0.05$ change with the progression of the infection maintaining at 0.8 (±0.2) at 56, 70 and 84 dpi. Although the treated mice presented with progressively increased inflammatory scores there was no significant difference ($P>0.05$) in the liver inflammatory response between the same groups.

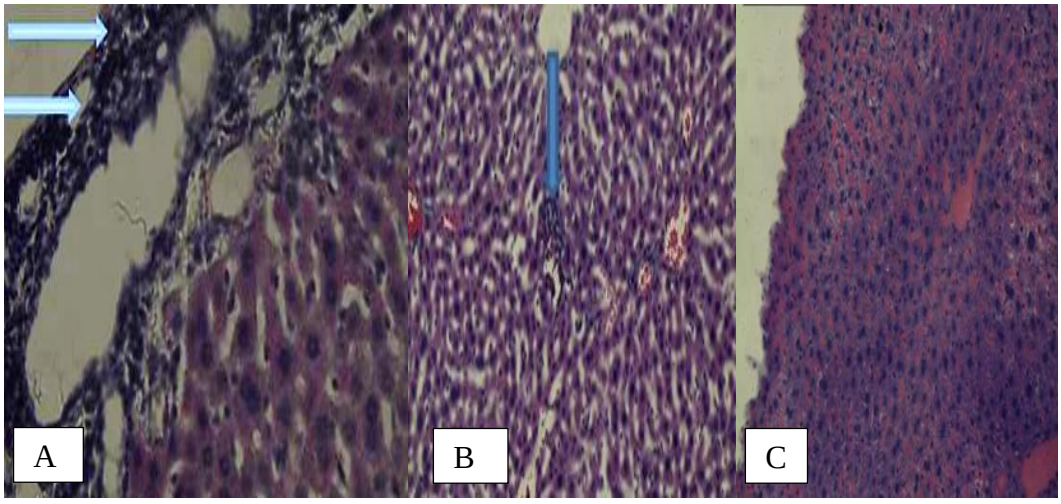

Plate 4.4. Liver of infected and treated mice (on the 56th day of infection) showing dense granulomas, irregularly distributed (arrows) (A and B). Liver of uninfected mice (C).

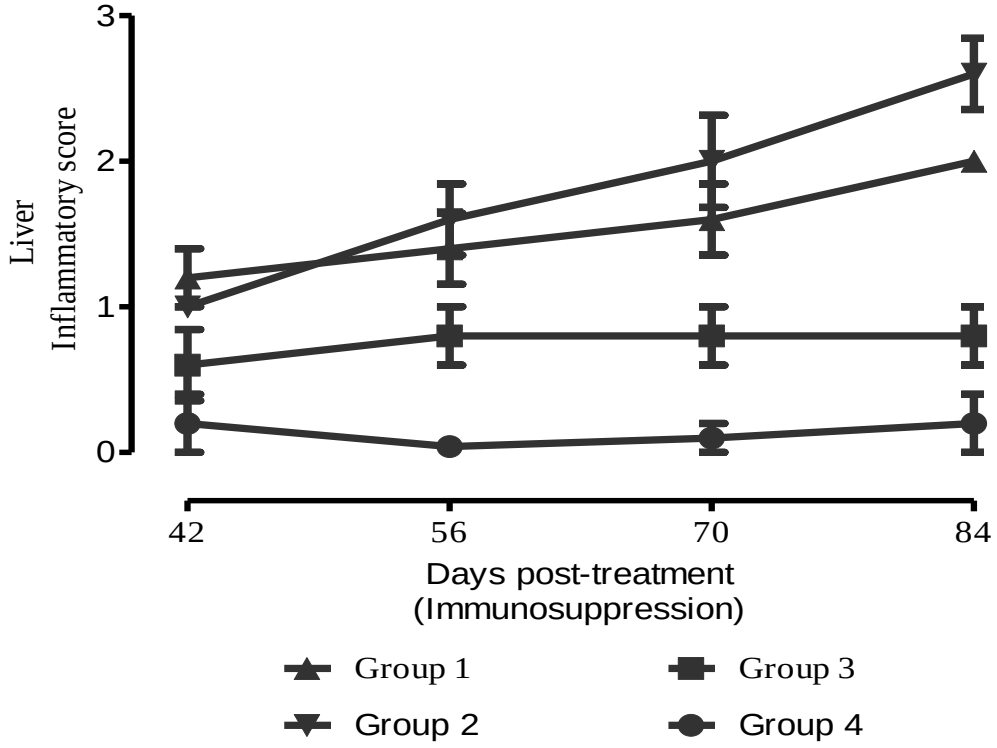

Figure 4.15: Inflammatory scores in the liver of BALB/c mice infected *with T. gondii* after treatment with dexamethasone. The data was obtained by analyzing 40 microscopic fields per section on four sections using a 20x objective from each mouse and from four mice per group: Group 1= *T. gondii* infected dexamethasone treated (2.66mg/kg/day): Group 2= *T. gondii* infected dexamethasone treated (5.32mg/kg/day); Group 3= *T.gondii* infected; Group 4 =Non infected control.

In the heart of infected treated mice, the histopathological lesions were relatively fewer compared to those in liver and were characterized by inflammatory infiltrates of lymphocytes and monocytes (Plate 4.5). The inflammatory score at 7dpi was 1.75±0.25 and this was followed by a significant ($P<0.001$) decrease reaching the lowest inflammatory score of 1.25±0.25 at 35dpi (Figure 4.16).

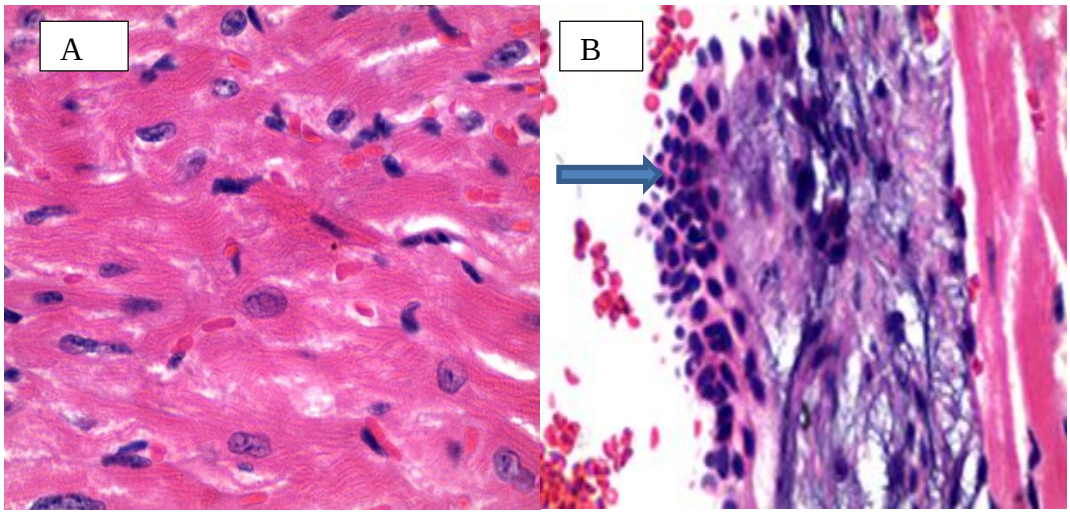

Plate 4.5. Normal tissue structure can be seen (A). Treatment with dexamethasone (5.32mg/kg/day of DEX) at 70dpi (B), markedly increased the severity and number of myocardial lesions. BALB/c mice displayed intense inflammatory lesions in the heart, characterized by inflammatory infiltrates of lymphocytes and monocytes (arrows).

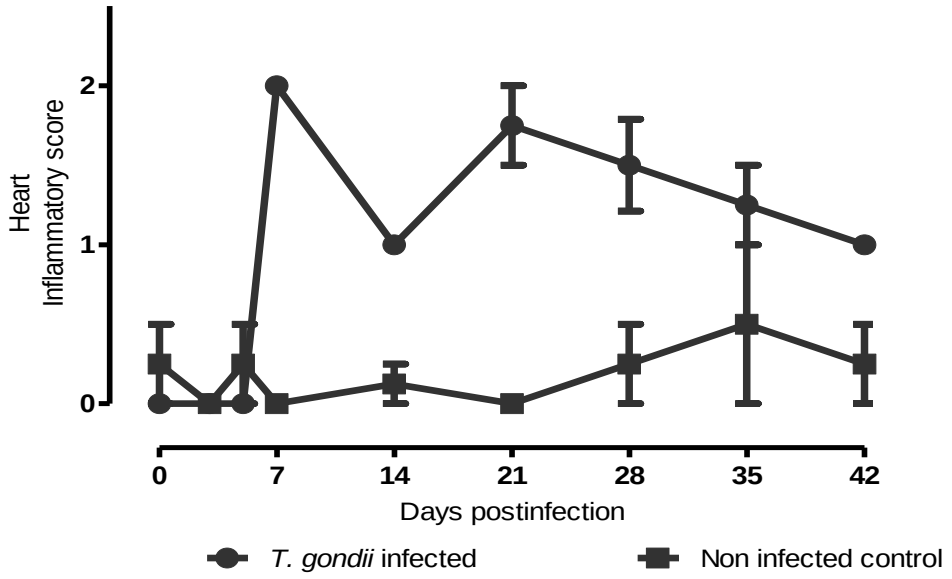

Figure 4.16: Inflammatory score in the heart of BALB/c mice infected with *T. gondii* before dexamethasone treatment. The data was obtained by analyzing 40 microscopic fields per section on four sections using a 40 objective from each mouse and from four mice per group.

As shown in Figure 4.17, treatment with dexamethasone markedly increased the severity and number of myocardial lesions in these infected animals. The toxoplasma infected group (Group 3) presented with higher inflammatory lesions at the time of treatment (day 42 dpi; $P<0.01$). However at 56, 70 and 84 dpi, an increasing inflammatory score was noted although there was no significant difference ($P>0.05$). All the heart tissues of mice from Groups 1 recorded an inflammatory score of 1.25±0.25 at 56, 70 and 84 dpi ($P>0.051$) while Group 2 recorded a significant ($p<0.01$) inflammatory score of 1.25±0.25; 1.5±0.289 and 2.5±0.289 at 56, 70 and 84 dpi, respectively. The uninfected control group (groups 4) showed no myocardial lesions at any time.

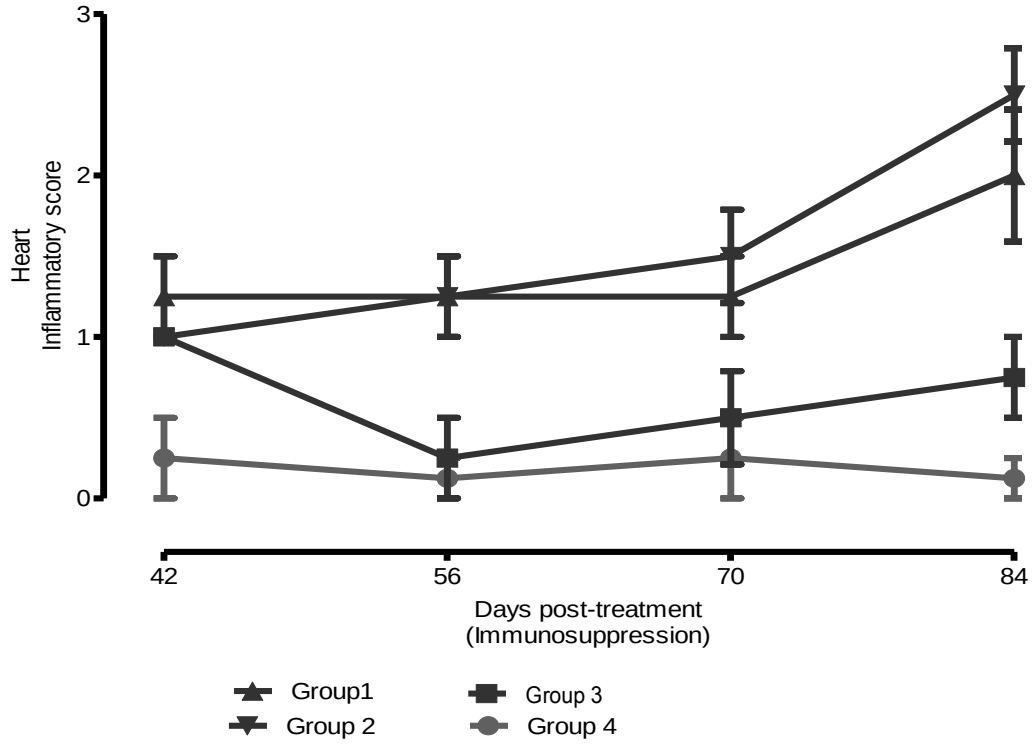

Figure 4.17: Inflammatory score in the heart of BALB/c mice infected with *T. gondii* after dexamethasone treatment. The data was obtained by analyzing 40 microscopic fields per section on four sections using a 40 objective from each mouse and from four mice per group: Group 1= *T. gondii* infected dexamethasone treated (2.66mg/kg/day); Group 2= *T. gondii* infected dexamethasone treated (5.32mg/kg/day); Group 3= *T.gondii* infected; Group 4 =Non infected control.

The spleen was also affected by *T. gondii* but unlike the liver, the inflammatory response started from 5 dpi. The infected mice spleens from the infected treated mice presented general disorganization of the germinal centers at 70 dpi. The marginal zone disappeared and the limits between the disorganized germinal center and the red pulp were blurred (Plate 4.6 B). The spleens of non-infected mice did not exhibit any change in the organizational of the germinal centers (plate 4.6A)

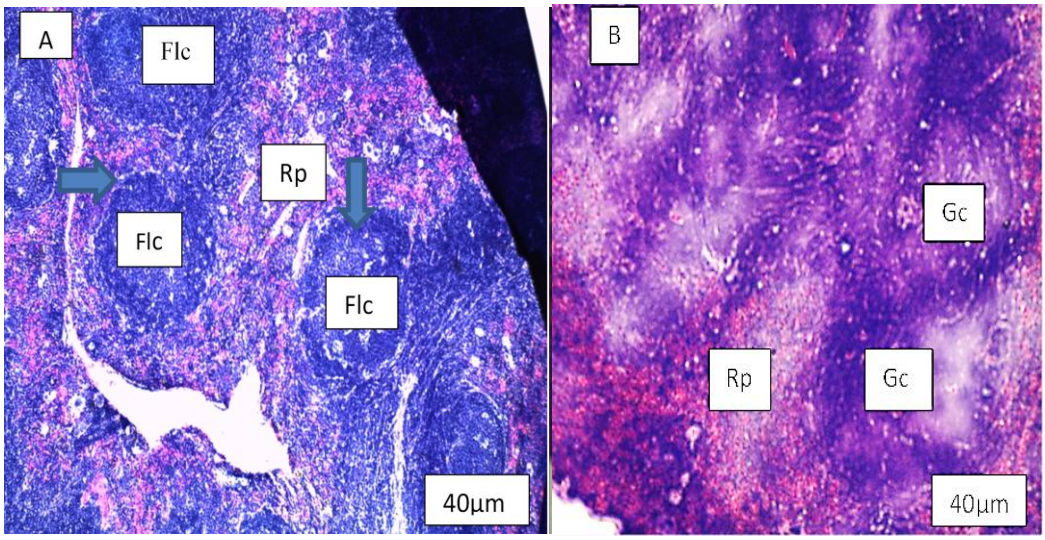

Plate 4.6: A section of spleen from non-infected mice at 70 DPI showing no histological abnormalities with white and red pulps (Rp) with well-defined limits. B cell resting follicles (Flc) with surrounding thick marginal zones (A); A section of spleen from mice treated with 5.32mg/kg/day of DEX at 70 DPI. Blurred limits in the border between disorganized germinal center (Gc) and red pulp (Rp), absent marginal zone (X40 objective).

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1.Discussion

5.1.1. Prevalence of *Toxoplasma gondii* in chicken

The study was undertaken in an area which was previously shown to have high prevalence of *T. gondii* amongst the slaughterhouse workers (Thiong'o *et al*., 2016). The high (79%) prevalence of *T. gondii* in all the 3 sub-counties showed that the free range chickens are a major reservoir for *T. gondii* parasites. The free range chicken in the study area had free access to habitats around homesteads where they scavenged for feed which mainly included left overs, grass and insects. As observed by Ogendi *et al.,* (2013), most of the homesteads have free range cats which defecate in the vegetation where the chicken feed on. The prevalence of *T.gondii* in cats in Thika region was 7.8% (Adele *et al.,* 2017). This could explain the high prevalence of *T.gondii* in chicken observed in the study area. In most developing countries, the free range chickens are slaughtered at home or in unsupervised slaughter houses and their viscera such as heads are left for scavengers that can include cats and other chicken (Dubey *et al.,* 2012). The latter allows the lifecycle to be completed in both chicken (intermediate hosts) and cats (definitive hosts). Chickens have been identified as important sources of *T. gondii* in other countries (Dubey, *et al.,* 2005) and they serve as important indicator of the potential risk of transmission to human.

Results of this study are similar to those of a study done in Brazil where *T. gondii* DNA was amplified in 84.6% of the chicken brain tissue samples and 80.8% of heart samples evaluated (Aigner, 2010). However, the prevalence was higher than

that reported in chicken from Bahia State, Brazil (42%) and Egypt (47%); (Goncalves, 2012; El-Massry, 2000).

In the current study, the differences in the prevalence according to area of origin of the chicken may be related to differences in cat densities, the number of chickens examined and sanitation condition in these areas (Ogendi *et al.,* 2013). The higher prevalence of toxoplasmosis in Thika municipality could be as a result of urban area and peri-urban livestock keeping and characterized by clustered dwellings, high population density and poor sewerage system. Kakuzi on the other hand has reported high number of cats around the farms, which is very important, as cats are reservoirs for animal and human toxoplasmosis (Ogendi *et al*., 2013).

A significant relationship between the prevalence of *T. gondii* and the different age groups was detected. The highest prevalence was detected in older chicken (>2years); whereas, the group of relatively younger chicken (>1<1.5 years) revealed lowest prevalence. This direct correlation between the prevalence of *T. gondii* with age of the chicken might be related to the fact that as birds became older, their cumulative likelihood for exposure increased or older birds have had more opportunities to get infected than the younger ones (Zhao, 2012).

Female chickens had significantly higher prevalence than males and these results agree with those reported by Akhtar *et al.,* (2014). The variation in prevalence could be attributed to the fact that female animals are reported to be more susceptible to protozoan parasites as compared to males (Alexander & Stimson, 1988). A previous report had shown female mice to be more sensitive to pathogenic symptoms of toxoplasmosis than male (Roberts *et al.,* 1996). However, other reports have reported a higher prevalence rate in males as compared to females (Tasawar *et al.,* 2012). The differences in the hormonal profiles of males and females may play an important role in determining the susceptibility to parasitic infections (Miller, 1990;

Roberts *et al.,* 2001). Estrogen has been shown to enhance antibody production but immunity can be broken down by various factors including nutrition, age, reproductive and environmental factors (Tasawar *et al*., 2012).

For a better understanding of the epidemiology and dynamics of *T. gondii* transmission among the various host population, the specific diagnosis of *T. gondii* infections in chickens is important. Previous studies have used serological methods which are characterized by long and laborious test procedures and low sensitivity due to low antibody levels (Sensini, 2006). Mouse bioassays take longer to aid in diagnosis and require ethical considerations (Aigner *et al*., 2010). In this study, detection of *T. gondii* DNA in brain samples was done by nPCR, based on the multilocus 529bp repeat element which gives increased diagnostic sensitivity and accuracy than can be obtained when targeting the B1 gene that exists in 35 copies/genome (Kong *et al.,* 2002; Edvinsson, *et al.,* 2006). However, the sensitivity of PCR may be limited by the random distribution of the parasite and varying parasite densities in affected tissue (Thiong'o *et al*., 2016; Hill *et al.,* 2006). The use of PCR may also be limited by the need for thermocycler, expensive reagents and skilled manpower. It would be important to investigate the feasibility of using other less expensive, field friendly molecular techniques such as LAMP.

5.1.2. Dexamethasone treatment of mice chronically infected with *T. gondii* interferes with *T. gondii* anti-IgG and IgM levels

Serum OD's for anti-IgG and IgM in the present study increased immediately after *T. gondii* infection and these findings are similar to those reported by Kang *et al*. (2006). The antibodies have the ability to kill the parasite by the attachment of the parasite to the host cell receptors, or mediate parasite lysis by activation of the complement system which can lyse and therefore clear extracellular tachyzoites

(Sibley *et al*., 1993; Kang *et al*., 2000; Bhopale, 2003). Indeed, antibodies levels are often used in diagnosis of toxoplasmosis (Ferreira & Camargo, 1990).

Following dexamethasone administration, serum OD's for anti-IgM levels of the experimental mice were depressed significantly whereas serum OD's for anti-IgG levels were not significantly altered [Appendix 6]. Dexamethasone administration alters antibody production and modulate levels of cell surface markers (Emilie *et al.,* 1987). Previous studies have shown that sera from mice acutely infected with toxoplasmosis had higher IgM levels than those from chronically infected mice (Singh *et al.,* 2010). Dexamethasone has been shown to cause immunosuppression by affecting the function of immune cells. It alters the differentiation programmes of progenitor cells by shaping the immune response as it develops (Munck *et al.,* 1984; McEwen *et al.,* 1997). Dexamethasone has been known to induce a loss of progenitor cells in bone marrow (Igarashi *et al.,* 2005).

In humans, it has been shown that elevated Toxoplasma IgG antibody levels are associated with onset of schizophrenia and epilepsy (Stommel *et al.,* 2001;Torrey and Yolken, 2003).These observations may suggest that reactivation of chronic *T. gondii* infection has occurred during the chronic stage of infection as aconsequence of proliferation of tachyzoites in the brain and that such reactivation of infection might be involved in the etiology of these diseases (Leweke *et al*., 2004).

5.1.3. Cytokines levels in the experimental mice

In the present study, BALB/c mice infected with *T. gondii* showed that IFN-γ productions were markedly increased after *T. gondii* infection. This observation is consistent with previous studies in mice by Gazzinelli *et al.* (1992), where equally, IFN-γ levels were exceedingly elevated at the disease onset.

Once released, IFNγ binds to the IFNγ receptor (IFN-γR), which eventually leads to the activation of IFN-γ signals "signal transducer and activator of transcription 1" (STAT1); (Kim *et al.,* 2007). These factors acts on macrophages and monocytes inducing the transcription of various genes involved in anti-parasitic responses including production of toxic reactive-oxygen species (ROS); (Arsenijevic *et al.,* 2001).

The immune response during the acute phase of infection limits the parasite replication. While replicating parasites may have a role, the immune-mediated (particularly $CD4^+$ cells) bystander effect is to the greatest extent the culprit (Mordue *et al.,* 2001). The high levels of IFN-γ production levels are suggestive of its early involvement in parasite clearance (Lee *et al.,* 2000). The secretion of IFN-γ increases the phagocyte activity of macrophages and also triggers the conversion of tachyzoites into bradyzoites leading to chronicity (Bohne *et al*., 1993; Ely *et al.,* 1999; Nijhawan *et al*., 2013). The cytokine also prevents bradyzoite rupture, allows long specific protection against new parasite infections and is hence responsible for regulation of *T. gondii* load and distribution in the tissues (Capron & Dessaint, 1988).

The IFN-γ levels were significantly depressed in the dexamethasone-treated *T. gondii* infected mice (Gazzinelli *et al*., 1994). Dexamethasone administration induce programmed cell death in developing thymocytes and T cells. Harold *et al.* (2006) has shown that it is a potent suppressor of cytokine production in T cells (Herold *et al.,* 2006). This drug, just like other glucocorticoids, act by binding to the glucocorticoid receptor (GR), which blocks the expression of proinflammatory cytokines and adhesion molecules. Previous early studies done by Hunter *et al*. (1994) showed that mice lacking T cells do not survive latent infection while depletion in T cells during the chronic phase or as a result of immuosuppression re-activates the disease (Gazzinelli *et al.,* 1992).

Although IFN-γ-dependent pro - inflammatory cytokines are essential for resistance to *T. gondii* infection, an over-production of inflammatory cytokine, IFN-γ can result in serious tissue damage (Mordue *et al.,* 20011). Therefore, the intensity of the immune responses mounted against *T. gondii* just like any other infection must be regulated to avoid exaggerated immune-pathologic effects due to excessive inflammation.

In this study, the IL-10 levels were also elevated during the acute and chronic infection and there was also a decline in immunosuppressed mice (42-84 dpi). This anti-inflammatory cytokine, has the ability to antagonize T helper 1 (Th1) responses (Fiorentino *et al*., 1989). IL-10 is considered to be an inhibitor of Th1 and Th2 immune responses (Moore *et al.,* 2001, Lieberman & Hunter 2002, O'Garra & Vieira 2007).Therefore, the role of IL-10 cytokines secreted by macrophages, monocytes, DC, B cells, and CD4+ and CD8+ T cells during both the acute and the chronic phases of infection in both immunocompetent and immunosuppressed mice is to acts broadly on accessory cells and adaptive cells responses to downregulate or limit the consequences of an exaggerated inflammatory response and major histocompatibility complex (MHC) and costimulatory molecule expression (Hunter *et al*., 1994; Moore *et al.,* 2001; Hall *et al.,* 2012; Gerard *et al*., 2008).

This cytokine also prevents tissue immune destruction through immunomodulation (Gaddi & Yap, 2007). It has also been identified as a factor induced by *T. gondii* infection (Gazzinelli *et al.,* 1992; Burke *et al*., 1994) that can contribute to the suppression of T cell function (Candolfi *et al.,* 1995; Khan *et al.,* 1995).

5.1.4. Histopathology

5.1.4.1. Brain tissue burden and cumulative survival of mice

The results of this study show that BALB/c mice can be used to develop a good model for toxoplasmosis. During the acute stage, the disease is characterized by tachyzoite proliferation, followed by a chronic stage, mainly characterized by the appearance of latent cysts within the central nervous system (Yap & Sher, 1999). The clinical manifestations and the ensuing pathology are mainly influenced by strain of the parasite and immune status of the host (Suzuki & Remington, 1993).The present study sought to determine the effect of administration of dexamethasone (DEX), an immunosuppressive drug, on pathogenesis of the disease in mice.

In this study, treated mice exhibited varied degrees of survival rates with higher mortalities being observed in mice given 5.32mg/kg/day of DEX possibly due to elevated parasite burden in the brain. Higher tissue cysts numbers correlated with dose of DEX and the results are similar to that of a previous study (Vijay *et al*., 2011). The infected mice had signs of toxoplasmosis which has been reported earlier (Olgica & Vladimir, 2001). Clinical disease with typical locomotor signs of cerebral toxoplasmosis in DEX treated animals was associated with significantly shorter survival. It has been shown that immunosuppression of chronically infected mice results in the reactivation of a latent infection, leading to proliferation of tachyzoites (Silva *et al*., 2010).

5.1.4.2. Histological changes in the brain

The current study showed that mice infected with *T. gondii* developed pathology which was associated with diffuse infiltration of lymphocytes, glial nodules, vascular cuffing by lymphocytes, and gliosis. Other studies have shown that

lymphocytes and plasma cells are the predominant cells in brains of patients having coinfection of HIV and toxoplasmosis (Falangola, 1994). In the current study, increased intake of dexamethasone resulted in increase in the extent of brain cellular inflammation and necrosis. This has also been observed in patients having clinical toxoplasmosis characterized by inflammation, to which microglia respond by forming nodules in attempts to contain the infection (Däubener *et al.*, 2001). This was evident in this study and often times this containment effort is simply ineffective due to the immunosuppressed nature of the host. Human toxoplasmosis is normally associated with coagulative necrosis of infected cells and around the necrotic areas; there are inflammatory cells, newly formed capillaries, edema, reactive astrocytes, and microglia (Chimelli, 2011). The clinical effects of this uncontrolled infection are often dire.

The study showed that there was a decrease in the number of lymphoid cells in the brain, following treatment with DEX, suggesting the diminished immune response. Most infections by *T. gondii* are asymptomatic, but the risk of brain infection increases drastically in immunocompromised individuals. Immunosuppression of the host as in the case of immunosuppression caused by increasing DEX treatment results in uncontrolled release of bradyzoites during rupture of cysts in the brain of latently infected individuals. Subsequently, released bradyzoites convert into rapidly proliferating tachyzoites that may cause severe brain damage if left untreated (Dellacasa *et al.*, 2007).

Dexamethasone like the other corticosteroid drugs have long been known to cause immunosuppression through lymphocytopenia, monocytopenia, and shrinkage of the spleen (Antopol, 1950). In these immunosuppressed hosts, the infection in the central nervous system can lead to severe pathogenesis which is associated with toxoplasmosis as was the case in this study. A comparison done with other studies involving immunosuppressed humans has shown that cerebral toxoplasmosis is

among the most frequent CNS pathologies and as many as one-third of all *T. gondii*-infected HIV-positive patients not treated with antiretroviral therapy may develop toxoplasmic encephalitis (Grant *et al.,* 1990).

5.1.4.3. Histological changes in the heart, liver and spleen

Toxoplasma gondii infection caused different histological manifestations as shown in this study. In the early infection, BALB/c mice displayed intense inflammatory lesions in the liver, heart as well as disorganization of the germinal centers of the spleen, suggesting a strong immune response in the pathogenesis of the disease. In the spleen, the white pulp appeared enlarged due to cellular proliferation and its limit with the red pulp started to disappear. The detectable changes in the splenic architecture of the structures in the spleen of dexamethasone treated mice have been associated with a decreased ability to mount an immune response against the toxoplasma parasites (Odermatt *et al*., 1991). Multiple mechanisms have been implicated in splenic disorganization, including $CD8^+$ T cell-mediated cytolysis of infected stromal cells or follicular dendritic cells (FDCs) and marginal-zone macrophages (Mueller *et al*., 2007).

The results of the present study showed that chronically infected non treated mice had an increase in mononuclear cells upon infection. The recruitment of inflammatory cells as was the case in these organs, is one of the most important immune mechanisms induced by IFN-γ and is geared towards control of parasite multiplication. These cells could also be responsible for the higher levels of cytokines observed in the initial stage of *T.gondii* infection observed in the study. However, although there was a decline in the cytokine levels in the immunosuppressed mice, there was marked infiltration of mononuclear cells in the organs, resulting in myocarditis and hepatitis. This could be a reflection of

reactivation and spread of toxoplasma parasites following decline in inflammatory response hindering the control and proliferation of the parasite (Kaplan *et al.*, 2009).

5.2 Conclusions

1. The results of this study indicated a 79% prevalence of toxoplasmosis infection in free range chicken in Kenya and this could indicate environmental contamination with *T. gondii* oocysts. This occurrence in chicken is central to a better understanding of epidemiology and dynamics of transmission among the various host populations.
2. The results of this study indicate that immunological and pathological features of *T. gondii* in immunosuppressed BALB/c mice mimic toxoplasmosis in immunosuppressed humans. The model should facilitate investigation of the pathobiology, diagnosis and treatment of toxoplasmosis, although the anti-inflammatory effects of the drug must be put into consideration.
3. The IgM and IgG levels increased immediately after *T. gondii* infection.The antibodies have the ability to kill the parasite.
4. Dexamethasone administration led to significantly decreased levels of IgM in the experimental mice whereas serum IgG levels were not significantly altered. Dexamethasone has been shown to cause immunosuppression by affecting the function of immune cells. It alters the differentiation programmes of progenitor cells by shaping the immune response as it develops
5. The manipulation of IFN-γ cytokine can put forth a beneficial or damaging effect on the host and thus modulate the disease pathology.
6. The cytokine IL-10 protects the infected mice from an exaggerated cellular immune response by inhibiting the production of pro - inflammatory cytokines, IFN-γ.
7. In both immunocompetent and immunosuppressed mice, the pathological signs evident in the study were myocarditis and hepatitis characterized by

mononuclear cell infiltration. Splenic exhaustion characterized by loss of normal spleen architecture also characterized the infection.

8. Histopathological analysis of the tissues demonstrated signs of disseminated toxoplasmosis as a result of reactivation of infection.

5..3. Recommendations

1. Strain genotyping investigations should be carried to determine the circulating genotypes in chicken in the study area and their zoonotic significance.The generated information will be important for planning an effective optimal prevention and control programs for toxoplasmosis.
2. An elaborate epidemiological study should be carried out in the study area and other parts of the county to determine the prevalence and risk factors of toxoplasmosis.
3. *Toxoplasma gondii* infection in both immunocompetent and immunosuppressed hosts may specifically impact levels of circulating cytokines and result in differences in the clinical severity of toxoplasmosis. Therefore further work on other protective and regulatory cytokines involved in the pathology should be carried out.

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APPENDICES

Appendix 1: Papers published/submitted in Peer reviewed Journals

a) https://www.hindawi.com/journals/bmri/2016/7589278/

Hindawi Publishing Corporation
BioMed Research International
Volume 2016, Article ID 7589278, 5 pages
http://dx.doi.org/10.1155/2016/7589278

Research Article

Detection of Natural *Toxoplasma gondii* Infection in Chicken in Thika Region of Kenya Using Nested Polymerase Chain Reaction

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Received 3 August 2016; Accepted 19 September 2016

Academic Editor: Jose Vasconcelos

The detection of *Toxoplasma gondii* in free-range chickens is a good indicator of possible risk to human beings. The aim of this study was to investigate the occurrence of *T. gondii* in free-range chicken using polymerase chain reaction (PCR). Brain samples from 105 free-range chickens from three administrative areas in Thika region, Kenya, were collected, DNA-extracted, and analyzed using PCR to detect presence of *T. gondii*. The overall prevalence of *T. gondii* in all the three areas was 79.0% (95% CI: 70.0–86.4%) and the prevalence across the three areas was not significantly different ($P = 0.5088$; $\chi^2 = 1.354$). Female chickens had higher (79.4%) prevalence than males (78.6%), although the difference was not significant ($P = 0.922$, $\chi^2 = 0.01$). However, chickens that were more than 2 years old had significantly ($P = 0.003$; $\chi^2 = 11.87$) higher prevalence compared to younger ones. The study indicates that there was a high occurrence of *T. gondii* infection in free-range chickens from Thika region and that the infection rate is age dependent. Further studies should be carried out to determine the possible role of roaming chickens in the epidemiology of the disease among humans in the area.

1. Introduction

Toxoplasmosis is a zoonosis of increasing importance in both developed and developing countries. It is caused by a protozoan parasite *Toxoplasma gondii* whose main definitive host is the domestic cat while all warm blooded animals are the intermediate hosts. The disease accounts for the highest human prevalence among the parasitic zoonoses [1, 2]. In Kenya, the prevalence of toxoplasmosis in human ranges from 23% to 60% [3, 4]. Humans become infected postnatally by ingesting tissue cysts from undercooked meat, consuming food or drink contaminated with oocysts, or accidentally ingesting oocysts from the environment [5]. *Toxoplasma gondii* is known to cause congenital disease and has been implicated as leading cause of meningoencephalitis in patients having HIV/AIDS [6].

In Kenya, most of the chickens are reared under the extensive production system. Free-range poultry meat is popular in Kenya because it is assumed to be healthier than that of caged birds. Anecdotally, some communities recommend the meat to pregnant women in order to deliver healthy babies [7]. The keeping of poultry in highly populated areas increases the risk for transmission of zoonoses and a recent study involving farmers in Thika region, Kenya, has established the risk factors associated with the transmission of toxoplasmosis [7]. Poultry that feed directly from the ground, such as free-range chickens, are exposed to contamination and may serve as indicators of the presence of the parasite in the environment

b) https://www.hindawi.com/journals/pri/2017/4302459/

Hindawi
Pathology Research International
Volume 2017, Article ID 4302459, 8 pages
https://doi.org/10.1155/2017/4302459

Research Article

Development of Neurological Mouse Model for Toxoplasmosis Using *Toxoplasma gondii* Isolated from Chicken in Kenya

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Received 25 January 2017; Accepted 19 April 2017; Published 24 May 2017

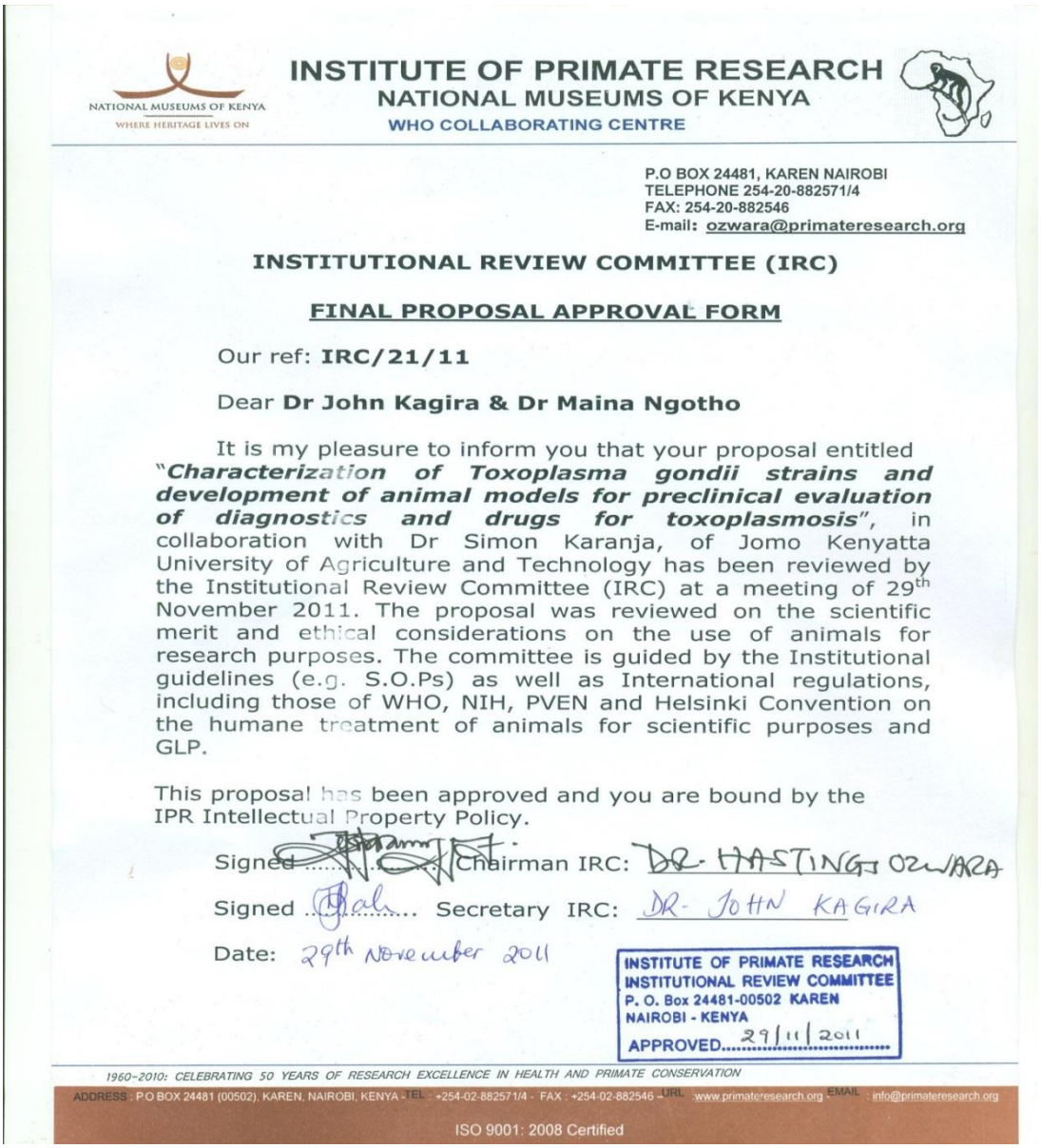
Academic Editor: Shahid Pervez

Animal models for the toxoplasmosis are scarce and have limitations. In this study, a neurological mouse model was developed in BALB/c mice infected intraperitoneally with 15 cysts of a *Toxoplasma gondii* isolate. The mice were monitored for 42 days and euthanized at different time points. Another group of mice were orally treated with dexamethasone (DXM: 2.66 mg/kg daily, 5.32 mg/kg daily) at 42 days after infection and monitored for a further 42 days. A mortality rate of 15% and 28.6% was observed in mice given 2.66 mg/kg/day and 5.32 mg/kg/day of DXM, respectively. The mean cyst numbers in the brain of DXM treated mice increased up to twofold compared with chronically infected untreated mice. Infections up to 42 days were associated with an increase in both IgM and IgG levels but following dexamethasone treatment, IgM levels declined but IgG levels continued on rising. The brain of toxoplasmosis infected mice showed mononuclear cellular infiltrations, neuronal necrosis, and cuffing. The severity of pathology was higher in mice treated with dexamethasone compared to the positive control groups. The findings of this study demonstrate that DXM-induced reactivation of chronic toxoplasmosis may be a useful development of laboratory animal model in outbred mice used for *in vivo* studies.

1. Introduction

Toxoplasmosis, caused by *Toxoplasma gondii,* is one of the most common parasitic infections of man and other warm-blooded animals [1]. It is estimated that between 500 million to 2 billion people worldwide are chronically infected with this parasite [2]. In Kenya, a prevalence between 23% and 60% has been described in the local human population [3]. Toxoplasmosis in immunocompetent individuals are asymptomatic with a few cases being clinical. However, the parasite can cause severe disease in people with immunodeficiencies and in fetuses infected in utero [2]. These two classes of patients present different challenges to health professionals in terms of diagnosis, case management, and drug treatment. Following infection, there is brief acute stage characterized by the proliferative tachyzoite stage of the parasite, but thereafter the parasite undergoes latency, characterized by slowly growing bradyzoites within tissue cysts. The tissue cysts remain viable presumably during the life of the host. During infections, the parasite invades a variety of immune cells and is subsequently disseminated throughout the body, traversing biological barriers to reach immunologically privileged sites

Appendix 2: Ethical approval for animal studie



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INSTITUTIONAL REVIEW COMMITTEE (IRC)

FINAL PROPOSAL APPROVAL FORM

Our ref: **IRC/21/11**

Dear **Dr John Kagira & Dr Maina Ngotho**

It is my pleasure to inform you that your proposal entitled "***Characterization of Toxoplasma gondii strains and development of animal models for preclinical evaluation of diagnostics and drugs for toxoplasmosis***", in collaboration with Dr Simon Karanja, of Jomo Kenyatta University of Agriculture and Technology has been reviewed by the Institutional Review Committee (IRC) at a meeting of 29th November 2011. The proposal was reviewed on the scientific merit and ethical considerations on the use of animals for research purposes. The committee is guided by the Institutional guidelines (e.g. S.O.Ps) as well as International regulations, including those of WHO, NIH, PVEN and Helsinki Convention on the humane treatment of animals for scientific purposes and GLP.

This proposal has been approved and you are bound by the IPR Intellectual Property Policy.

Signed ..... Chairman IRC: DR. HASTINGS OZWARA

Signed ..... Secretary IRC: DR. JOHN KAGIRA

Date: 29th November 2011

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Appendix 3: IgM levels analysis between day 0-35 post infection with *T. gondii*

Table Analyzed IgM

Two-way ANOVA

Source of Variation	% of total variation	P value
Interaction	31.76	P<0.0001
Column Factor	29.01	P<0.0001
Row Factor	38.50	P<0.0001

Source of Variation	P value summary	Significant?
Interaction	***	Yes
Column Factor	***	Yes
Row Factor	***	Yes

Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	4	0.6390	0.1597	542.8
Column Factor	1	0.5837	0.5837	1983
Row Factor	4	0.7747	0.1937	658.1
Residual	50	0.01471	0.0002943	

Number of missing values 0

Bonferroni posttests

T.Gondii infected vs Non infected control

Row Factor	*T.Gondii infected*	Non infected control	Difference	95% CI of diff.
7	0.1184	0.1353	0.01692	-0.009605 to 0.04344
14	0.1966	0.1400	-0.0566	-0.08312 to -0.03008
21	0.2109	0.1407	-0.07021	-0.09673 to -0.04369
28	0.5004	0.1490	-0.3514	-0.3779 to -0.3249
35	0.6908	0.1657	-0.5250	-0.5516 to -0.4985

Row Factor	Difference	t	P value	Summary
7	0.01692	1.708	P > 0.05	ns
14	-0.0566	5.715	P<0.001	***
21	-0.07021	7.089	P<0.001	***

28	-0.3514	35.48	P<0.001	***
35	-0.5250	53.01	P<0.001	***

Appendix 4 : IgG levels analysis between day 0-35 post infection with *T. gondii*

Table Analyzed	IgG graph 2
Two-way ANOVA	

Source of Variation	% of total variation	P value
Interaction	17.75	0.0026
Column Factor	32.79	P<0.0001
Row Factor	24.02	0.0004

Source of Variation	P value summary	Significant?
Interaction	**	Yes
Column Factor	***	Yes
Row Factor	***	Yes

Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	4	1.208	0.3019	5.232
Column Factor	1	2.230	2.230	38.65
Row Factor	4	1.634	0.4085	7.080
Residual	30	1.731	0.05770	

Number of missing values	0
Bonferroni posttests	
Non infected control vs *T.gondii* infected	

Row Factor	Non infected control	*T.gondii* infected	Difference	95% CI of diff.
7	0.1618	0.2675	0.1057	-0.3614 to 0.5728
14	0.1564	0.3088	0.1524	-0.3147 to 0.6195
21	0.1643	0.6034	0.4391	-0.02805 to 0.9062
28	0.1742	0.7750	0.6008	0.1337 to 1.068
35	0.2387	1.302	1.063	0.5963 to 1.531

Row Factor	Difference	t	P value	Summary
7	0.1057	0.6223	P > 0.05	ns
14	0.1524	0.8971	P > 0.05	ns
21	0.4391	2.585	P > 0.05	ns
28	0.6008	3.537	P<0.01	**
35	1.063	6.261	P<0.001	***

Appendix 5: IgM levels analysis between days 42-84 post infection with *T. gondii* after treatment with Dexamethasone

Table Analyzed	Data IgM			
Two-way ANOVA				
Source of Variation	% of total variation	P value		
Interaction	2.73	P<0.0001		
Column Factor	87.86	P<0.0001		
Row Factor	4.89	P<0.0001		
Source of Variation	P value summary	Significant?		
Interaction	***	Yes		
Column Factor	***	Yes		
Row Factor	***	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	9	0.1383	0.01537	5.382
Column Factor	3	4.447	1.482	519.1
Row Factor	3	0.2474	0.08248	28.89
Residual	80	0.2284	0.002855	
Number of missing values	24			
Bonferroni posttests				
Group 1 vs Group 2				
Row Factor	Group 1	Group 2	Difference	95% CI of diff.
42(0)	0.5508	0.5028	-0.04794	-0.1512 to 0.05529
56(2)	0.4457	0.4063	-0.03942	-0.1426 to 0.06382
70(4)	0.4006	0.3546	-0.0460	-0.1492 to 0.05723
84(6)	0.4068	0.3441	-0.06274	-0.1660 to 0.04049
Row Factor	Difference	t	P value	Summary
42(0)	-0.04794	1.554	P > 0.05	ns
56(2)	-0.03942	1.278	P > 0.05	ns
70(4)	-0.0460	1.491	P > 0.05	ns
84(6)	-0.06274	2.034	P > 0.05	ns
Group 1 vs Group 3				
Row Factor	Group 1	Group 3	Difference	95% CI of diff.
42(0)	0.5508	0.8545	0.3037	0.2005 to 0.4070
56(2)	0.4457	0.7971	0.3514	0.2482 to 0.4546
70(4)	0.4006	0.7110	0.3104	0.2072 to 0.4136
84(6)	0.4068	0.6151	0.2083	0.1050 to 0.3115

Row Factor	Difference	t	P value	Summary
42(0)	0.3037	9.845	P<0.001	***
56(2)	0.3514	11.39	P<0.001	***
70(4)	0.3104	10.06	P<0.001	***
84(6)	0.2083	6.750	P<0.001	***

Group 1 vs Group 4

Row Factor	Group 1	Group 4	Difference	95% CI of diff.
42(0)	0.5508	0.1180	-0.4327	-0.5359 to -0.3295
56(2)	0.4457	0.1607	-0.2850	-0.3882 to -0.1818
70(4)	0.4006	0.1507	-0.2499	-0.3531 to -0.1467
84(6)	0.4068	0.1226	-0.2842	-0.3874 to -0.1810

Row Factor	Difference	t	P value	Summary
42(0)	-0.4327	14.03	P<0.001	***
56(2)	-0.2850	9.238	P<0.001	***
70(4)	-0.2499	8.101	P<0.001	***
84(6)	-0.2842	9.212	P<0.001	***

Group 2 vs Group 3

Row Factor	Group 2	Group 3	Difference	95% CI of diff.
42(0)	0.5028	0.8545	0.3517	0.2484 to 0.4549
56(2)	0.4063	0.7971	0.3908	0.2876 to 0.4941
70(4)	0.3546	0.7110	0.3564	0.2532 to 0.4596
84(6)	0.3441	0.6151	0.2710	0.1678 to 0.3742

Row Factor	Difference	t	P value	Summary
42(0)	0.3517	11.40	P<0.001	***
56(2)	0.3908	12.67	P<0.001	***
70(4)	0.3564	11.55	P<0.001	***
84(6)	0.2710	8.784	P<0.001	***

Group 2 vs Group 4

Row Factor	Group 2	Group 4	Difference	95% CI of diff.
42(0)	0.5028	0.1180	-0.3848	-0.4880 to -0.2815
56(2)	0.4063	0.1607	-0.2456	-0.3488 to -0.1424
70(4)	0.3546	0.1507	-0.2039	-0.3071 to -0.1007
84(6)	0.3441	0.1226	-0.2215	-0.3247 to -0.1182

Row Factor	Difference	t	P value	Summary
42(0)	-0.3848	12.47	P<0.001	***
56(2)	-0.2456	7.960	P<0.001	***
70(4)	-0.2039	6.610	P<0.001	***
84(6)	-0.2215	7.179	P<0.001	***

Group 3 vs Group 4

Row Factor	Group 3	Group 4	Difference	95% CI of diff.

42(0)	0.8545	0.1180	-0.7364	-0.8397 to -0.6332
56(2)	0.7971	0.1607	-0.6364	-0.7396 to -0.5332
70(4)	0.7110	0.1507	-0.5603	-0.6636 to -0.4571
84(6)	0.6151	0.1226	-0.4925	-0.5957 to -0.3892

Row Factor	Difference	t	P value	Summary
42(0)	-0.7364	23.87	P<0.001	***
56(2)	-0.6364	20.63	P<0.001	***
70(4)	-0.5603	18.16	P<0.001	***
84(6)	-0.4925	15.96	P<0.001	***

Appendix 6: IgG levels analysis between days 42-84 post infection with *T. gondii* after treatment with Dexamethasone

Table Analyzed	IgG Graph 1			
Two-way ANOVA				
Source of Variation	% of total variation	P value		
Interaction	4.82	P<0.0001		
Column Factor	83.43	P<0.0001		
Row Factor	11.36	P<0.0001		
Source of Variation	P value summary	Significant?		
Interaction	***	Yes		
Column Factor	***	Yes		
Row Factor	***	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	9	4.259	0.4732	110.0
Column Factor	3	73.66	24.55	5709
Row Factor	3	10.03	3.343	777.2
Residual	80	0.3441	0.004301	
Number of missing values	24			
Bonferroni posttests				
Group 1 vs Group 2				
Row Factor	Group 1	Group 2	Difference	95% CI of diff.
42(0)	0.9581	0.9584	0.0003583	-0.1263 to 0.1271
56(2)	1.529	1.181	-0.3473	-0.4740 to -0.2206
70(4)	2.000	1.501	-0.4987	-0.6254 to -0.3720
84(6)	2.310	2.004	-0.3060	-0.4327 to -0.1793
Row Factor	Difference	t	P value	Summary
42(0)	0.0003583	0.009462	P > 0.05	ns
56(2)	-0.3473	9.172	P<0.001	***
70(4)	-0.4987	13.17	P<0.001	***
84(6)	-0.3060	8.082	P<0.001	***

Group 1 vs Group 3

Row Factor	Group 1	Group 3	Difference	95% CI of diff.
42(0)	0.9581	1.889	0.9306	0.8039 to 1.057
56(2)	1.529	2.721	1.193	1.066 to 1.319
70(4)	2.000	2.868	0.8678	0.7411 to 0.9945
84(6)	2.310	2.960	0.6502	0.5235 to 0.7769

Row Factor	Difference	t	P value	Summary
42(0)	0.9306	24.58	P<0.001	***
56(2)	1.193	31.50	P<0.001	***
70(4)	0.8678	22.92	P<0.001	***
84(6)	0.6502	17.17	P<0.001	***

Group 1 vs Group 4

Row Factor	Group 1	Group 4	Difference	95% CI of diff.
42(0)	0.9581	0.1454	-0.8127	-0.9394 to -0.6860
56(2)	1.529	0.1601	-1.368	-1.495 to -1.242
70(4)	2.000	0.1649	-1.835	-1.962 to -1.709
84(6)	2.310	0.1718	-2.138	-2.265 to -2.011

Row Factor	Difference	t	P value	Summary
42(0)	-0.8127	21.46	P<0.001	***
56(2)	-1.368	36.14	P<0.001	***
70(4)	-1.835	48.47	P<0.001	***
84(6)	-2.138	56.47	P<0.001	***

Group 2 vs Group 3

Row Factor	Group 2	Group 3	Difference	95% CI of diff.
42(0)	0.9584	1.889	0.9302	0.8035 to 1.057
56(2)	1.181	2.721	1.540	1.413 to 1.667
70(4)	1.501	2.868	1.367	1.240 to 1.493
84(6)	2.004	2.960	0.9562	0.8295 to 1.083

Row Factor	Difference	t	P value	Summary
42(0)	0.9302	24.57	P<0.001	***
56(2)	1.540	40.67	P<0.001	***
70(4)	1.367	36.09	P<0.001	***
84(6)	0.9562	25.25	P<0.001	***

Group 2 vs Group 4

Row Factor	Group 2	Group 4	Difference	95% CI of diff.
42(0)	0.9584	0.1454	-0.8131	-0.9398 to -0.6864
56(2)	1.181	0.1601	-1.021	-1.148 to -0.8945
70(4)	1.501	0.1649	-1.337	-1.463 to -1.210
84(6)	2.004	0.1718	-1.832	-1.959 to -1.705

Row Factor	Difference	t	P value	Summary
42(0)	-0.8131	21.47	P<0.001	***
56(2)	-1.021	26.97	P<0.001	***
70(4)	-1.337	35.30	P<0.001	***
84(6)	-1.832	48.39	P<0.001	***

Group 3 vs Group 4

Row Factor	Group 3	Group 4	Difference	95% CI of diff.
42(0)	1.889	0.1454	-1.743	-1.870 to -1.617
56(2)	2.721	0.1601	-2.561	-2.688 to -2.434
70(4)	2.868	0.1649	-2.703	-2.830 to -2.576
84(6)	2.960	0.1718	-2.788	-2.915 to -2.662

Row Factor	Difference	t	P value	Summary
42(0)	-1.743	46.04	P<0.001	***
56(2)	-2.561	67.64	P<0.001	***
70(4)	-2.703	71.39	P<0.001	***
84(6)	-2.788	73.64	P<0.001	***

Appendix 7: IFN-γ levels analysis between day 0-35 post infection with *T. gondii*

Table Analyzed	IFN-gamma 3			
Two-way ANOVA				
Source of Variation	% of total variation	P value		
Interaction	20.46	0.0002		
Column Factor	32.75	P<0.0001		
Row Factor	24.48	P<0.0001		
Source of Variation	P value summary	Significant?		
Interaction	***	Yes		
Column Factor	***	Yes		
Row Factor	***	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	5	81.59	16.32	6.603
Column Factor	1	130.6	130.6	52.85
Row Factor	5	97.62	19.52	7.900
Residual	36	88.97	2.471	
Number of missing values	0			
Bonferroni posttests				
T. gondii infected vs Non-infected control				
Row Factor	*T. gondii* infected	Non-infected control	Difference	95% CI of diff.
0	3.500	3.000	-0.5000	-3.604 to 2.604
7	7.000	3.000	-4.000	-7.104 to -0.8964
14	3.942	3.250	-0.6916	-3.795 to 2.412
21	4.924	3.500	-1.424	-4.528 to 1.679
28	9.590	3.500	-6.090	-9.193 to -2.986
35	10.59	3.500	-7.090	-10.19 to -3.986
Row Factor	Difference	t	P value	Summary
0	-0.5000	0.4498	P > 0.05	ns
7	-4.000	3.598	P<0.01	**
14	-0.6916	0.6222	P > 0.05	ns

21	-1.424	1.281	P > 0.05	ns
28	-6.090	5.478	P<0.001	***
35	-7.090	6.378	P<0.001	***

Appendix 8: IFN-γ levels analysis between days 42-84 post infection with *T. gondii* after treatment with Dexamethasone

Table Analyzed	IFN-gamma graph			
Two-way ANOVA				
Source of Variation	% of total variation	P value		
Interaction	4.39	0.8667		
Column Factor	12.29	0.0423		
Row Factor	19.33	0.0220		
Source of Variation	P value summary	Significant?		
Interaction	ns	No		
Column Factor	*	Yes		
Row Factor	*	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	6	253.0	42.16	0.4112
Column Factor	2	708.8	354.4	3.457
Row Factor	3	1115	371.7	3.626
Residual	36	3691	102.5	
Number of missing values	16			
Bonferroni posttests				
Group 3 vs Group 2				
Row Factor	Group 3	Group 2	Difference	95% CI of diff.
42(0)	21.48	15.51	-5.976	-29.73 to 17.78
56(2)	26.38	9.152	-17.23	-40.98 to 6.527
70(4)	13.53	2.782	-10.74	-34.50 to 13.01
84(6)	11.03	7.879	-3.154	-26.91 to 20.60
Row Factor	Difference	t	P value	Summary
42(0)	-5.976	0.8346	P > 0.05	ns

56(2)	-17.23	2.406	P > 0.05	ns
70(4)	-10.74	1.501	P > 0.05	ns
84(6)	-3.154	0.4406	P > 0.05	ns

Group 3 vs Group 1

Row Factor	Group 3	Group 1	Difference	95% CI of diff.
42(0)	21.48	17.84	-3.640	-27.39 to 20.11
56(2)	26.38	15.91	-10.47	-34.23 to 13.28
70(4)	13.53	4.535	-8.991	-32.74 to 14.76
84(6)	11.03	10.02	-1.009	-24.76 to 22.74

Row Factor	Difference	t	P value	Summary
42(0)	-3.640	0.5085	P > 0.05	ns
56(2)	-10.47	1.463	P > 0.05	ns
70(4)	-8.991	1.256	P > 0.05	ns
84(6)	-1.009	0.1410	P > 0.05	ns

Group 2 vs Group 1

Row Factor	Group 2	Group 1	Difference	95% CI of diff.
42(0)	15.51	17.84	2.335	-21.42 to 26.09
56(2)	9.152	15.91	6.754	-17.00 to 30.51
70(4)	2.782	4.535	1.753	-22.00 to 25.51
84(6)	7.879	10.02	2.145	-21.61 to 25.90

Row Factor	Difference	t	P value	Summary
42(0)	2.335	0.3262	P > 0.05	ns
56(2)	6.754	0.9433	P > 0.05	ns

70(4)	1.753	0.2448	P > 0.05	ns
84(6)	2.145	0.2996	P > 0.05	ns

Appendix 9: IL 10 levels analysis between day 0-35 post infection with *T. gondii*

Table Analyzed	IL 10			
Two-way ANOVA				
Source of Variation	% of total variation	P value		
Interaction	15.20	P<0.0001		
Column Factor	68.89	P<0.0001		
Row Factor	15.08	P<0.0001		
Source of Variation	P value summary	Significant?		
Interaction	***	Yes		
Column Factor	***	Yes		
Row Factor	***	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	5	19770	3954	21.74
Column Factor	1	89620	89620	492.7
Row Factor	5	19620	3924	21.57
Residual	33	6002	181.9	
Number of missing values	3			
Bonferroni posttests				
T.gondii infected vs Non infected control				
Row Factor	*T.gondii* infected	Non infected control	Difference	95% CI of diff.
0.0000	3.500	3.500	0.0000	-26.77 to 26.77

7.000	99.60	3.250	-96.35	-125.3 to -67.44
14.00	105.4	3.000	-102.4	-129.2 to -75.67
21.00	125.4	3.000	-122.4	-151.3 to -93.51
28.00	110.1	3.000	-107.1	-136.0 to -78.20
35.00	115.4	4.000	-111.4	-138.1 to -84.60

Row Factor	Difference	t	P value	Summary
0.0000	0.0000	0.0000	P > 0.05	ns
7.000	-96.35	9.354	P<0.001	***
14.00	-102.4	10.74	P<0.001	***
21.00	-122.4	11.88	P<0.001	***
28.00	-107.1	10.40	P<0.001	***
35.00	-111.4	11.68	P<0.001	***

Appendix 10: IL10 levels analysis between days 42-84 post infection with *T. gondii* after treatment with Dexamethasone

Table Analyzed	IL10 Final graph			
Two-way ANOVA				
Source of Variation	% of total variation	P value		
Interaction	6.29	0.6173		
Column Factor	25.03	0.0007		
Row Factor	18.00	0.0113		
Source of Variation	P value summary	Significant?		
Interaction	ns	No		
Column Factor	***	Yes		
Row Factor	*	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	6	7277	1213	0.7448
Column Factor	2	28950	14470	8.889
Row Factor	3	20810	6937	4.261
Residual	36	58620	1628	
Number of missing values	48			
Bonferroni posttests				
Group 3 vs Group 2				
Row Factor	Group 3	Group 2	Difference	95% CI of diff.
42(0)	142.0	116.9	-25.05	-129.1 to 79.00
56(2)	159.6	71.73	-87.83	-191.9 to 16.22
70(4)	149.8	67.39	-82.41	-186.5 to 21.64
84(6)	99.71	55.60	-44.11	-148.2 to 59.94

Row Factor	Difference	t	P value	Summary
42(0)	-25.05	0.8781	P > 0.05	ns
56(2)	-87.83	3.078	P < 0.05	*
70(4)	-82.41	2.888	P < 0.05	*
84(6)	-44.11	1.546	P > 0.05	ns

Group 3 vs Group 1

Row Factor	Group 3	Group 1	Difference	95% CI of diff.
42(0)	142.0	135.7	-6.309	-110.4 to 97.74
56(2)	159.6	120.5	-39.04	-143.1 to 65.01
70(4)	149.8	82.57	-67.23	-171.3 to 36.82
84(6)	99.71	71.73	-27.98	-132.0 to 76.07

Row Factor	Difference	t	P value	Summary
42(0)	-6.309	0.2211	P > 0.05	ns
56(2)	-39.04	1.368	P > 0.05	ns
70(4)	-67.23	2.356	P > 0.05	ns
84(6)	-27.98	0.9807	P > 0.05	ns

Group 2 vs Group 1

Row Factor	Group 2	Group 1	Difference	95% CI of diff.
42(0)	116.9	135.7	18.74	-85.31 to 122.8
56(2)	71.73	120.5	48.79	-55.26 to 152.8
70(4)	67.39	82.57	15.18	-88.87 to 119.2
84(6)	55.60	71.73	16.13	-87.92 to 120.2

Row Factor	Difference	t	P value	Summary
42(0)	18.74	0.6569	P > 0.05	ns
56(2)	48.79	1.710	P > 0.05	ns
70(4)	15.18	0.5320	P > 0.05	ns

84(6)	16.13	0.5653	P > 0.05	ns