CYTOKINE AND HAEMATOLOGICAL PROFILES IN SPRAGUE-DAWLEY RATS EXPERIMENTALLY INFECTED WITH *TRICHINELLA ZIMBABWENSIS AND PLASMODIUM BERGHEI* ANKA

By

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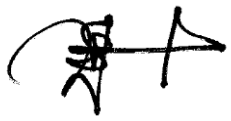
PREFACE

The research contained in this thesis was completed by the candidate, from June 2016 to June 2017, while based in the Discipline of Parasitology, School of Life Sciences of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Westville Campus, South Africa, under the supervision of Prof. S. Mukaratirwa and Dr. P. Murambiwa.

The content of this work has not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.



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DECLARATION: PLAGIARISM

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6. Haematological data from this study have been shared among other two MSc students as part of a larger project on *Plasmodium berghei* ANKA/*Trichinella zimbabwensis* co-infection in Sprague-Dawley rats.

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ABSTRACT

Human co-infections of helminths and malaria parasites are a common phenomenon in areas where malaria is endemic, more so in Sub-Saharan Africa were tissue-dwelling helminths, such as *Trichinella spp* are also endemic. However, the immune-pathogenesis of the diseases when there is co-infection remains obscure, hence there is a need to understand the host immune responses induced during co-infection. This study therefore aimed at investigating the host cytokine and haematological profiles in Sprague-Dawley rats experimentally co-infected with *T. zimbabwensis* and *Plasmodium berghei*. A 42 day study was done, where 168 male Sprague-Dawley rats (90-150g) were divided into four separate experimental groups, control (n=42); malaria infected (n=42); *Trichinella* infected (n=42); and co-infected group (n=42). *Trichinella* induction using *Trichinella zimbabwensis* muscle larvae (500) *per os* was done on day 0, while malaria induction using *Plasmodium berghei* parasitized RBCs 1 x 10^5 was injected intraperitonally on day 28 of the study. Animals were sacrificed at day 0, 7, 14, 21, 35 and 42 post-infection for blood and sera collection. *Plasmodium berghei* parasitaemia, *Trichinella* parasite load, haematology parameters and serum levels of TNF-α, IL-4, IL-6 and IL-10 were measured. Results for % parasitaemia showed that the *P. berghei* (MI) group reached the peak at day 6 with 66.77% ± 4.63% while the *P. berghei* and *T. zimbabwensis* (CI) reached its peak on day 7 with 69.17% ± 3.80%.). *Trichinella* parasite load results showed that the adult worms in the *T. zimbabwensis* group (MI) had a mean of 18.50% ± 8.06% on day 7 p.i. while the co-infected with *P. berghei* and *T. zimbabwensis* (CI) develop a mean of 15.00% ± 6.44%. On day 42, the muscle larvae (ML) in the *T. zimbabwensis* group (MI) increased exponentially with a mean of 97.37% ± 20.6% compare to the *P. berghei* and *T. zimbabwensis* group (CI) 71.63% ± 17.11%. Cytokines levels in the serum showed significant concentrations (P<0.001) of tumour necrosis factor-alpha (TNF-α) on day 7 p.i. in the *P. berghei* group (MI), and significant concentrations (P<0.001) of interleukin-10 (IL-10) was also observed on day 7 p.i. in the *P. berghei* group (MI) respectively. IL-4 and IL-6 did not yield result as the serum concentrations were below the lowest standard of the assay protocol, these could be due to some other components of the assays such as the proprietary monoclonal antibody pairs that makes them less sensitive.

In conclusion, we observed that *P. berghei* and *T. zimbabwensis* (CI) group showed rapid development and increased parasitaemia than rats in the *P. berghei* (MI) group. Therefore, rats co-

infected with *P. berghei* and *T. zimbabwensis* may aggravate the course of disease in the host by increasing parasitaemia, but elevated IL-10 plays a crucial role in the suppression of hepatic pathology in the host. Moreover, malaria conferred protection to *T. zimbabwensis* infection, as demonstrated by reduced worm counts in the co-infected (CI) group.

Key words: cytokines, co-infection, parasitaemia, Male Sprague-Dawley rats, *Trichinella zimbabwensis, Plasmodium berghei.*

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LIST OF ABBREVIATIONS

µl – Microliter

ACT – Artemisinin-based combination therapy

ANOVA – Analysis of variance

BRU – Biomedical research unit

BW – Body weight

CNS – Central nervous system

ECM – Experimental cerebral malaria

EDTA – Ethylenediaminetetraacetic acid

ELISA – Enzyme-linked immunosorbent assay

FSH – Follicle-stimulating hormone

H_2O – Water

HGF – Hemopoietic growth factor

IFAT – Immunofluoroscence antibody test

IFN-γ – Interferon gamma

IgE – Immunoglobulin E

IL – Interleukin

IRS – Indoor residual spraying

LED – Light emitting diode

LLINs – Long lasting insecticidal nets

LPG – Larvae per gram

NBL – New born larvae

NK – Natural killer

NK – Natural killer cell

NO – Nitric oxide

PBMC – Peripheral blood mononuclear cells

PCR – Polymerase chain reaction

PI – Post-infection

pRBCs – Parasitized red blood cells

RBC – Red blood cell

RDT – Rapid diagnostic tests

SEM – Standard error of mean

SPP. – Species

TGF-β – Transforming growth factor beta

Th – T-helper cell

TNF-α – Tumor necrosis factor alpha

UKZN – University of KwaZulu-Natal

WBC – White blood cell

WHO – World health organization

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1.0 CHAPTER 1: INTRODUCTION

1.1 Background

According to the World Health Organization (WHO), malaria is one of the most serious infectious diseases of humans with over 250 million clinical cases every year worldwide (Murray et al., 2012). It is the major cause of mortality and morbidity especially in developing countries due to the absence of vaccine, parasite resistance to the available anti-malarial drugs, anopheline mosquitoes resistance to insecticide spraying and the poor socio-economic situation (Ter Kuile et al., 2003). Due to the increasing parasite drug-resistance and mosquito insecticide-resistance over the past years, malaria still remains endemic in many parts of the world (Onkoba et al., 2015a). The burden of malaria is aggravated by the high number of malaria co-infections with other infectious diseases (Onkoba et al., 2015a; Ademola and Odeniran, 2016). In 2010, it was estimated that about 3.3 billion people were at risk of malaria and about 1,238,000 deaths were recorded that year (Murray et al., 2012). In areas where malaria is endemic, it is the norm that *Plasmodium*-infected people also suffer from a concurrent helminth infection which has been shown repeatedly to modulate the immune system of their host in order to survive (Ateba-Ngoa et al., 2015).

Trichinellosis is an emerging and re-emerging zoonotic disease caused by a parasitic tissue-dwelling nematode of the genus *Trichinella*. The parasite is acquired by the ingestion of raw or inadequately cooked meat products containing muscle larvae of the *Trichinella* parasite (Murrell and Pozio, 2011). Geographical distribution is worldwide and *Trichinella* spp have been reported to occur in 66 countries and infects an estimated 11 million people (Yang et al., 2010a). The geographical distribution of which overlaps with malaria in endemic areas of Tanzania, Uganda, Kenya, Ethiopia, Zimbabwe, South Africa and Mozambique (Onkoba et al., 2015a). According to Pozio (2007), it has also been reported that *Trichinella spp*. infections in animals and in humans are found in ten sub-Sahara Africa countries (Pozio, 2007). From the period of 1986–2009, about 65 818 human trichinellosis cases and 42 deaths were reported worldwide and during that period only one sub-Sahara African country reported human trichinellosis, contributing 0.04% and 3.6%

of human trichinellosis and mortality worldwide, respectively (Murrell and Pozio, 2011; Mukaratirwa et al., 2013).

The disease is manifested with targeted clinical signs and symptoms of variable intensity, depending upon the extent of invasion (Kociecka, 2000). *Trichinella* species exhibit a cosmopolitan distribution infecting mammals exceptionally with scavenging and cannibalistic behavior (Matenga et al., 2006). *Trichinella zimbabwensis*, a non-encapsulated species, is the most prevalent *Trichinella spp* in southern Africa (Mukaratirwa et al., 2013). *Trichinella zimbabwensis* complete its life cycle independently of whether or not the host is warm-blooded or cold-blooded (Mitreva and Jasmer, 2006).

The production of cytokine (tumor necrosis element alpha [TNF-α] and gamma interferon [IFN-γ]) in malaria infections appears to be indispensable for the inhibition of parasitaemia and stimulation of phagocytosis to increase clearance of parasitized erythrocytes (Lyke et al., 2004). High plasma concentrations of pro-inflammatory cytokines corresponding to TNF-α and IFN-γ and a reduction of anti- inflammatory cytokine, IL-10, may be accountable for pathophysiological and clinical outcomes of malaria (Clark and Cowden, 2003). Pro-inflammatory cytokines are secreted following the release of parasite antigens in the course of the bursting of parasitized red blood cells (pRBCs) (Clark et al., 2003).

Epidemiological studies have proven that the largest burden of malaria infections is felt by communities living in poor regions of developing countries (Shankarkumar et al., 2011). In these areas, the excessive prevalence of soil-transmitted helminthic infections has also been documented (Onkoba et al., 2015a). This results in co-infections, multi-parasitism or polyparasitism (Supali et al., 2010). Co-infection with distinctive pathogens is a common occurrence which is able to alter the development of diseases and these relationships are complicated, because the immune response can control survival and proliferation of the pathogen and be accountable for tissue damage (Furze et al., 2006). Also, co-infections with malaria often complicate and increase the severity of parasitic diseases (Brooker et al., 2006a; Ademola and Odeniran, 2016). The effect of helminths on concurrent infection with malaria is of designated interest, given their prevalence in human populations (Furze et al., 2006). However, the immune pathogenesis of the diseases when there is co-infection remains obscure despite the need to understand the host immune responses induced during co- infection.

1.2 Aim and Objectives

Therefore, this study was aimed at investigating the cytokine responses in experimental animal paradigms co- infected with *Trichinella zimbabwensis and Plasmodium berghei*.

The specific objectives of the study are to:

a. Determine the host cytokine responses induced during co-infection with *Trichinella zimbabwensis* and *Plasmodium berghei* in Sprague-Dawley rats
b. Determine the heamatological profiles during co-infection with *Trichinella zimbabwensis* and *Plasmodium berghei* in Sprague-Dawley rats
c. Determine the level of parasitaemia in Sprague-Dawley rats co-infected with *Trichinella zimbabwensis* and *Plasmodium berghei*

Research question

a. What is the level of host cytokine response in Sprague-Dawley rats during co-infection with *Trichinella zimbabwensis* and *Plasmodium berghei*?
b. What is the level of heamatological profiles during co-infection with *Trichinella zimbabwensis* and *Plasmodium berghei* in Sprague-Dawley rats?
c. What is the level of parasitaemia load in Sprague-Dawley rats during co-infection with *Trichinella zimbabwensis* and *Plasmodium berghei*?

Hypotheses

a. Co-infection of *Trichinella zimbabwensis* and *Plasmodium berghei* in Sprague-Dawley rats does not influence the level of cytokine.
b. Co-infection of *Trichinella zimbabwensis* and *Plasmodium berghei* in Sprague-Dawley rats does not influence the level of heamatological profiles.
c. Co-infection of *Trichinella zimbabwensis* and *Plasmodium berghei* in Sprague-Dawley rats does not influence the level of parasitaemia load.

2.0 CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Malaria remains one of the most widespread infectious ailments of our time (Delves et al., 2012). The five recognized *Plasmodium* species that cause human malaria are *Plasmodium vivax, Plasmodium ovale, Plasmodium falciparum, Plasmodium malariae* and *Plasmodium knowlesi* (Lopes et al., 2014), although *P. falciparum* is the most common in Sub-Saharan Africa, whereas *P. malariae* is sporadic worldwide (Pow, 2012). Female mosquitoes of the genus *Anopheles* transmit single-cell eukaryotic plasmodium parasites, which are causative agent of malaria (Hammerschmidt-Kamper, 2012). Malaria parasites cause anaemia via haemolysis and elevated splenic clearance of infected and uninfected red blood cells (Marcelline, 2015). In humans, the malaria parasites develop and multiply first within the liver cells and then within the red blood cells (RBCs), the stages of the parasite during the erythrocytic cycle are those that cause the clinical symptoms of malaria (Sahu et al., 2016a).

2.1.1 The *Plasmodium* life cycle

The plasmodium life cycle is relatively complex and it involves a number of highly specific interactions with well-defined types of host cell which is found both in the mosquito vector, which is the primary host, and a place of sexual reproduction (gametogony) and in the vertebrate host, where the asexual replication (schizogony) takes place (Blandine et al., 2004). Malaria is acquired through a bite of an infected female *Anopheles* mosquito, which injects the sporozoites into the host's dermis. The sporozoites are then carried in the blood stream to the hepatocytes in the liver. They mature to tissue schizonts, which later release merozoites into liver sinusoids. The erythrocytic cycle starts when the mature tissue schizonts invade the red blood cells (RBC) leading to the formation of ring forms or trophozoites. However, in case of *P. vivax* and *P. ovale*, some sporozoites may go into hibernation, (the *cryptobiotic phase)*, giving rise to *hypnozoites.* The hypnozoites can lie dormant for months or years and upon reactivation they cause clinical relapse (Clark and Schofield, 2000; Mackintosh et al., 2004). The trophozoites formed subsequently develop into schizonts that later release a new generation of merozoites. The

parasitized red blood cells eventually rupture to release a new generation of merozoites into the blood stream to repeat the same cycle. The sexual cycle in the vector is completed by differentiation of some merozoites into male and female gametocytes (Mackintosh et al., 2004). The malaria parasite has been shown to have complex metabolic processes such as utilization of amino acids derived from haemoglobin and detoxification of haeme. The enzyme plasmodium aldolase has been identified as one of the enzymes of parasite anaerobic glycolysis (Clark and Cowden, 2003). Reports indicate that the parasites increase the permeability of red blood cell to acquire nutrients, yet maintaining the red blood cell structure intact for at least 48 hours (Mackintosh et al., 2004). The parasite ingests haemoglobin from RBCs to form a food vacuole where haemoglobin is degraded, releasing haem moeity. The toxic haeme is in turn detoxified by haeme polymerase and sequestrated as haemozoin (malaria pigment). Many of the antimalarial drugs act by inhibiting haeme polymerase thereby causing accumulation of toxic haeme moeity (Mackintosh et al., 2004). Malaria manifestations vary and depend on age and the acquisition of immunity, host and parasite genetic polymorphisms and *Plasmodium* spp. (Lyke et al., 2004). Variations in human cytokine responses and their link to malaria disease manifestations is subject of much debate (Lyke et al., 2004), although a common imbalance in the secretion of pro-inflammatory and anti-inflammatory cytokines based on malaria precipitated hypoxia has been observed (Clark and Cowden, 2003). The basic life cycle of all the *Plasmodium* species is similar as shown in Figure 2.

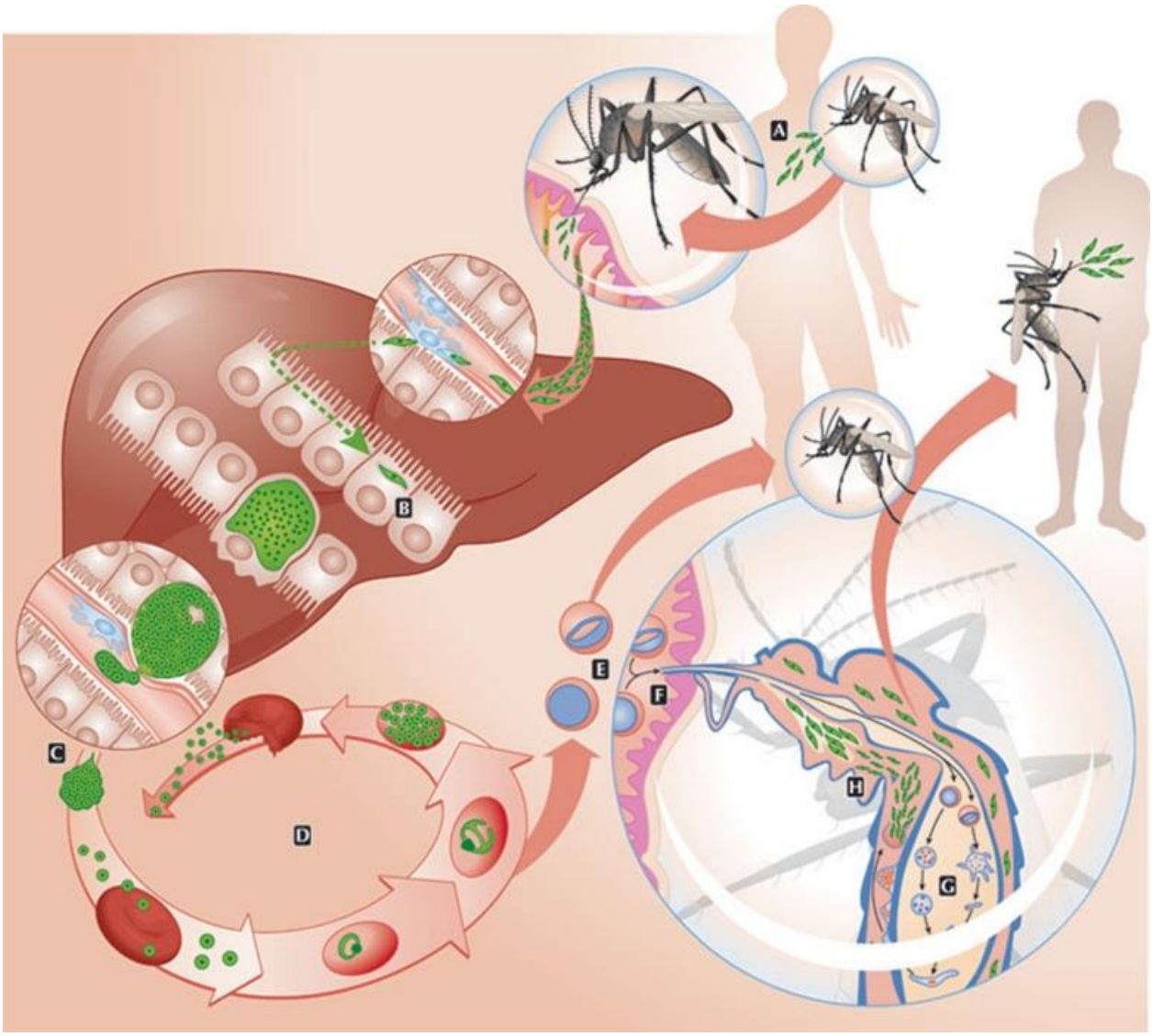

Figure 1: The *Plasmodium* life cycle (Portugal et al., 2011).

2.1.2 Pathogenesis of malaria

The pathogenesis of malaria is complex and entails immunologic and non-immunologic mechanisms (Miller et al., 2002). Although it is generally accepted that severe malaria is the outcome of alterations in various tissues and organs. These alterations often lead to metabolic acidosis and localized ischemia (Angulo and Fresno, 2002). Previous studies observed that parasite factors can contribute to the severity of disorder, as is apparent from their ability to result in production of pro-inflammatory cytokines (Chotivanich et al., 2000). More so, much evidence has

been collected that points to glycosylphosphatidylinositols from *Plasmodium* as essential pathogenic compounds because of their ability to induce TNF-α and IL-1. This view is strongly supported by means of the fact that toxicity of malaria parasite extracts can be neutralized by monoclonal antibodies in experimental units (Angulo and Fresno, 2002). It is important to note that the presence of antiglycosylphosphatidylinositol antibodies in the serum of patients may only provide safety in opposition to clinical signs of malaria (Clark and Schofield, 2000). IL-12 and/or IFN-γ link to involvement with the pathogenesis of CM (celebral malaria) comes from studies with knockout mice infected with *P. berghei* ANKA and IRF-$1^{-/-}$ mice infected with *P. berghei* (Senaldi et al., 1999). Although in cases where IL-12 and IFN-γ show a dominant pathological role, a protective outcome has been verified for IL-10 (Angulo and Fresno, 2002). It is important to also note that the administration of murine IL-1, which is a pro-inflammatory cytokine, also helps in the protection of CM, although various pro-inflammatory cytokines that obviously play a role in CM could also be redundant, making it difficult to unequivocally assign to them a pathogenic function in all clinical instances (Alghasham and Rasheed, 2014).

2.1.3 Global epidemiology/Economic impact of malaria

Malaria has a global distribution and consequential health burden. The spatial limits of its distribution and seasonal activity are sensitive to climate factors, as well as the local capacity to control the disease (Caminade et al., 2014). Malaria disease is present in several regions of the world like Africa, South-East-Asia and South-America and people living in Sub-Saharan Africa have the highest risk of getting infected by the disease (Hammerschmidt-Kamper, 2012). In total, 104 countries are endemic to malaria disease with substantial geographic disparities (Bhutta et al., 2014). It is estimated that 81% of reported cases and 91% of deaths due to malaria in 2010 occurred in the Sub-Saharan Africa region, and about 86% of malaria incidence involved children under the age of five years and pregnant women particularly in the first pregnancies are probably the most severely affected ones (Bhutta et al., 2014).

Malaria is caused by a protozoan parasite from the genus *Plasmodium,* which is transmitted via the bite of a female *Anopheles* mosquito. There are four species of *Plasmodium* causing human malaria. *Plasmodium falciparum* occurs predominantly in sub-Saharan Africa and is responsible for the majority of mortality and burden due to malaria. *Plasmodium vivax* is the second most

important species, and its occurrence is particularly prominent in Asia. The other two species are *Plasmodium malariae and Plasmodium ovale* (Matthys, 2006). The severity of *P. falciparum* malaria is as a result of organism's ability to invade young and old red blood cells, which is not characteristic of the other *Plasmodium* species (Menkir and Lema, 2014). Moreover, lack and absence of malaria prevention and control services, health services deterioration, population movement, desert fringes and highlands have contributed to the huge malaria cases and deaths (Menkir and Lema, 2014). In addition, ideal climatic conditions which are exacerbated by some of the world's most efficient malaria vectors, such as *Anopheles gambiae*, *Anopheles arabiensis* and *Anopheles funestus* and natural disasters such as El-Nino and tsunamis (Menkir and Lema, 2014).

It is not a debate that nations with high malaria incidence exhibit low levels of economic development (Barofsky et al., 2011). Moreover, to severe health consequences, nations with high malaria incidence also exhibit low levels of economic development, malaria affects human capital and income in several ways, for example, infection during childhood causes cognitive impairment and can reduce educational outcomes or labour productivity (Barofsky et al., 2011). Malaria in the community or household can reduce overall investment and income available for schooling. In addition to the immediate effects of malaria on health, there is evidence that malaria exposure during infancy and childhood can have detrimental effects on long-term cognitive development (Barofsky et al., 2011). A 2006 systematic review of 18 studies concluded that *P. falciparum* malaria in childhood affects short- and long-term neurocognitive performance, including attention, memory, visio-spacial skills, and language functioning (Kihara et al., 2006). Aside from the population health impact of malaria reduction, there is a large body of work that looked at the effects of malaria reduction on income and human capital attainment (Barofsky et al., 2011).

Figure 2: Geographical distribution of malaria and areas at risk of transmission (WHO 2011).

2.1.4 Diagnosis of malaria

Diagnosis of malaria is an essential component for effective disease management and in decreasing mortality and morbidity (Bronzan et al., 2008). Although diagnosis is complicated by the fact that acquired immunity to malaria can result in asymptomatic infections as no existing malaria diagnostic test can distinguished malaria illness from parasitaemia with concomitant fever of another cause (Bronzan et al., 2008). Several methods are used in detecting malaria parasites within peripheral blood since diagnosis of malaria is situated on clinical criteria (Williams et al., 2008). Clinical diagnosis based on clinical symptoms is the most commonly used method, and it is the basis for self-treatment and least expensive. However, the overlapping of malaria signs with different tropical ailments impairs its specificity and therefore encourages the indiscriminate use of anti-malarials for managing febrile conditions in endemic areas (Wongsrichanalai et al., 2007). This practice was in the past inexpensive and well-tolerated when anti-malarials were still effective (Biritwum et al., 2000).

There is a variation in the accuracy of clinical diagnosis depending on the level of endemicity, malaria season, and age group and no single clinical algorithm is a universal predictor (Mwangi et al., 2005). Only in children in hyper-endemic areas can clinical diagnosis determine the medication resolution (Chandramohan et al., 2002). In this situation, majority of the populations are chronically parasitemic, and could also be concomitant with other febrile causing illnesses (Wongsrichanalai et al., 2007). Accurate diagnosis can be done traditionally with the aid of using microscopic examination of blood smear for *Plasmodium* spp. In 1904, Gustav Giemsa introduced Microscopic examination of Giemsa-stained blood smears which has subsequently become the gold standard of malaria diagnosis (Wongsrichanalai et al., 2007). Giemsa staining and microscopic examination of blood smeared slides is known to be the most suitable diagnostic instrument for malaria control considering the fact that it is inexpensive to perform, and it has the capability to differentiate malaria species, and quantify parasites. In the age of high-quality light emitting diode (LED) illumination and solar battery chargers, microscopy has turn out to be more viable in remote areas. Improving diagnostic accuracy in malaria control systems can be both technically and financially challenging (Biritwum et al., 2000). Current rapid diagnostic tests (RDT) promote ease-of-use and safety in comparison to the earlier assays of the early and mid-1990s. RDT use, especially in developing countries, has escalated for the past few years. Most commonly used RDTs only detect *P. falciparum*, however, RDTs that distinguish *P. falciparum* from the three non-falciparum species are available. Several factors in the manufacturing process as well as environmental conditions may affect RDT performance (Bell et al., 2006; Mboera et al., 2013). Also other methods like the antigen detection and polymerase chain reaction (PCR) are being used in both endemic and non-endemic arrears depending on the type of infection and the severity of the infection (Bronzan et al., 2008). Although, in malaria endemic areas, reliably establishing parasitaemia and excluding other causes of severe disease can be difficult (Drakeley and Reyburn, 2009).

2.1.5 Control and treatment of malaria

Malaria is the world's most established parasitic disease and against which effective control measures are urgently needed (Angulo and Fresno, 2002). The control and prevention method of malaria includes use of long lasting insecticidal nets (LLINs) to individuals with risk of infection and indoor residual spraying (IRS), and use of anti-malarial drugs (Fairhurst et al., 2012).

Artemisinin-based combination therapy (ACT) is recommended for areas with drug resistance or more deadly malaria strains (Organization and Control, 2010). National malaria programs recorded that ACT distribution increased from 2 million in 2004 to 73 million in 2008 (Aregawi et al., 2009). But less than 15% of children in Africa received ACT in 2008 and access to other types of anti- malarial therapy is also limited (Aregawi et al., 2009). Intermittent preventative method (IPT) is recommended for population groups in areas of excessive transmission who are particularly vulnerable to *Plasmodium* infection and its consequences, particularly pregnant women and infants. The goals postulated by the Global Malaria Action Plan (Malaria, 2008) were that the interventions should reduce the numbers of malaria cases and deaths per capita by 50% or more between 2000 and 2010 and by 75% or more between 2000 and 2015. Currently there are five ACTs recommended for use; artemether-lumefantrine, artesunate-amodiaquine, artesunate-mefloquine, artesunate-sulfadoxine pyrimethamine and dihydroartemisinin-piperaquin of which only one (artemether-lumefantrine) is available as a child-friendly taste-masked formulation (Hemingway et al., 2016). The primary hindrance to the control of malaria transmission through administration of effective anti-malarial drugs in Sub-Saharan Africa is that medical facilities are limited, poorly equipped and poorly funded, despite being the epicentre of malaria infection (WHO, 2008).

2.1.6 The immune system and *Plasmodium* spp interaction

Efforts have been devoted to the study of the characteristics of immune responses against *Plasmodium* spp. It is now generally accepted that innate immunity plays a crucial role in clearing *Plasmodium spp* infection from parasitized hosts. There is growing evidence that the increased production of pro-inflammatory cytokines during immune or inflammatory responses can exert powerful influences on the central nervous system (CNS). Cytokines play a role in the pathogenesis of numerous disorders including infection, autoimmune disease, stroke, trauma, and neuro degenerative disease (Janssen et al., 2010). Most of the elimination of *Plasmodium* spp. often occurs in the spleen under normal circumstances, although the liver has been proven to function as an alternative clearing site (Angulo and Fresno, 2002). The splenic response is complicated and involves distinctive tissue changes that provoke alterations in blood flow through the organ (Angulo and Fresno, 2002). These changes prevent the access of infected erythrocytes to splenic tissues in which the immune response is going on until armed effector cells are produced.

Splenic marginal zone macrophages, a phagocytic subset of macrophages at the interface between red and white pulp, seem not to be involved in eradicating infected erythrocytes (Angulo and Fresno, 2002). This task is apparently assumed by macrophages of the red pulp (Angulo and Fresno, 2002). Recent work has shown that *P. chabaudi* infection in T-cell-deficient mice is exacerbated early and these results fit well with data ascribing early production of gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) both to splenic/T lymphocytes and to natural killer (NK) cells (Choudhury et al., 2000).

2.1.7 Production of cytokines and their roles in *Plasmodium* spp infection

Early production of IFN-γ is very crucial in resistance against malaria infection. In support of this view, analysis of IFN-γ mice infected with *P. chabaudi* showed a critical role of IFN-γ in immunity against this pathogen, although the production of parasite-specific immunoglobulins was not affected (Angulo and Fresno, 2002). According to reports, it has been reported that IFN-γ responsive factor (IRF-1) in mice infected with *P. berghei* showed was related to lower mortality in wild-type mice, these results are therefore an indication that IFN-γ and/or other molecules under the control of IRF-1 play a role in the pathological outcome of the diseases. IL-12 appears to be critically linked to or to act through IFN-γ production, thereby allowing an early and sustained Th1 response (Angulo and Fresno, 2002).

In addition to its role in resolving primary infection, IL-12 has been shown to be required for the production of a protective immunoglobulin G2a (IgG2a) antibody (Su and Stevenson, 2002). A similar picture to that depicted for IFN-γ emerged for the role of TNF-α in early responses against *Plasmodium*. Treatment with anti-TNF-α monoclonal antibody resulted in a tendency toward longer times for parasite clearance. Interestingly, this effect is associated with reduced levels of IFN-γ, supporting the general view of an association between the ability to produce high levels of TNF-α and an accelerated cure and improved prognosis in humans (Angulo and Fresno, 2002).

2.1.8 Impact of IL-10 on the regulation of host responses during malaria

Increased levels of pro-inflammatory cytokines such as IFN-γ, IL-1, TNF-α and IFN-γ -induced protein (IP)-10 (CXCL10) form part of an early host response to the parasite and these cytokines have also been associated with severe anaemia, hyperglycemia and cerebral malaria in *P. falciparum* infection (Armah et al., 2007; do Rosario and Langhorne, 2012). In order to limit excessive damage to the host, control of this inflammatory response is of a necessity. One way of controlling or down-regulating host immune responses is through the cytokine IL-10 which is a key cytokine that has been proven to have an essential regulatory function in establishing this balance in malaria (O'Garra et al., 2008). IL-10 has recently been described as the strongest predictor of disease in endemic regions (Sinha et al., 2010) and considered as a biomarker for inflammatory placental malaria (do Rosario and Langhorne, 2012). Recently, it has been proven in an area of low malaria endemicity that even though the magnitude of IFN-γ response reduces enormously with time, the IL-10 response of peripheral blood mononuclear cells (PBMC) was stably maintained without a tremendous decline over a minimum of 6 years (Wipasa et al., 2011). High production of IL-10 has been found in patients protected from severe anaemia (Ouma et al., 2008; do Rosario and Langhorne, 2012) and in sera of women with pregnancy suffering from asymptomatic malaria (Wilson et al., 2010). Nevertheless, not all of the studies are in line with the idea that IL-10 suppresses or controls severe malaria, as excess levels of IL-10 have also been associated with high disease severity in humans, including cerebral malaria, anaemia and respiratory distress (Ong'echa et al., 2008; Ayimba et al., 2011). Again, IL-10 induced through co-infecting non-lethal malaria parasite in conjunction with *P. berghei* ANKA can hinder experimental cerebral malaria (ECM) (Niikura et al., 2010).

In similar way, infection of non-human primates with *P. cynomolgi*, the highest degree of anaemia was associated with robust pro-inflammatory responses and the quantity of IL-10 correlated with higher haematocrit values (Praba-Egge et al., 2002; do Rosario and Langhorne, 2012). IL-10 appears to hinder parasite clearance, at the same time down-regulating pro-inflammatory response and avert hepatic pathology (Couper et al., 2008). Additionally, IL-10 has also been known to promote hyper-parasitaemia of *P. chabaudi adami* (Weidanz et al., 2005). Furthermore, $CD11b^{high}Ly6C^{+}$ inflammatory monocytes that migrate from the bone marrow to the spleen in the

course of *P. chabaudi* infection produce not only pro-inflammatory cytokines such as IL-6 and TNF-α, but also IL-10 (Sponaas et al., 2009).

2.2 Trichinellosis

Nematodes of the genus *Trichinella* are some of the most widespread zoonotic pathogens in the world and the impact of parasitic zoonosis on health, the economy and society has increased in recent years (Kurdova-Mintcheva et al., 2009). Despite concerted efforts to control trichinellosis, the disease remains a threat in many countries especially developing countries (Murrell and Pozio, 2011; Mukaratirwa et al., 2013). Infection has been detected in domestic and wild animals of all continents, aside from Antarctica, which is one of the places where there is not any report of the parasite (Pozio, 2007). The risk of future human *T. zimbabwensis* infection in sub-Saharan Africa is increasing due to poverty, food insecurity, climate change and failure of veterinary controls and surveillance (Gottstein et al., 2009; Mukaratirwa et al., 2013; Onkoba et al., 2015b). Moreso, globalization has exacerbated the risk through increased movement of people, wildlife and livestock in and out of *T. zimbabwensis* endemic zones of southern Africa (Mukaratirwa et al., 2013; Onkoba et al., 2015b). *Trichinella* spp. plays a vital role in the suppression of the host immune response and other immune-related pathologies (Bai et al., 2012).

2.2.1 Life cycle of *Trichinella* spp

The life cycle of *Trichinella* spp involves mammalian hosts, together with humans and is known to complete all stages of development in one host (Wu et al., 2009). Although only humans and non-human primates become clinically affected, transmission from one host to another can only occur by consumption or ingestion of contaminated muscle which is infected with the encysted larval stage of parasite (Wu et al., 2009). The ingested larvae invade the small intestine, where they undergo development into adult worms within 1-2 weeks in the intestine causing irritation and mild abdominal cramping or even diarrhea (Wu et al., 2009). The adult male and female mate and produce newborn larvae (NBL) which leave the intestine and migrate through the circulatory system and penetrate the striated muscle tissue and reside in the nurse-cells causing muscle pains, fever, periorbital edema, eosinophilia, occasional CNS or cardiac damage, hypoglycaemia and acute renal failure (Gottstein et al., 2009). In the muscle, *Trichinella* larvae mature to become

infective to another host and adult worms continue to produce larvae in the intestines for several weeks before they are expelled (Gottstein et al., 2009). Once larvae encyst in musculature, they can remain alive and infective for years (Gottstein et al., 2009). The number of larvae ingested determines the severity of the disease (Bruschi and Murrell, 2002). *Trichinella* spp maintain their life cycle only when another animal ingest muscle tissue containing the encapsulated or non-encapsulated larvae (Bruschi and Murrell, 2002).

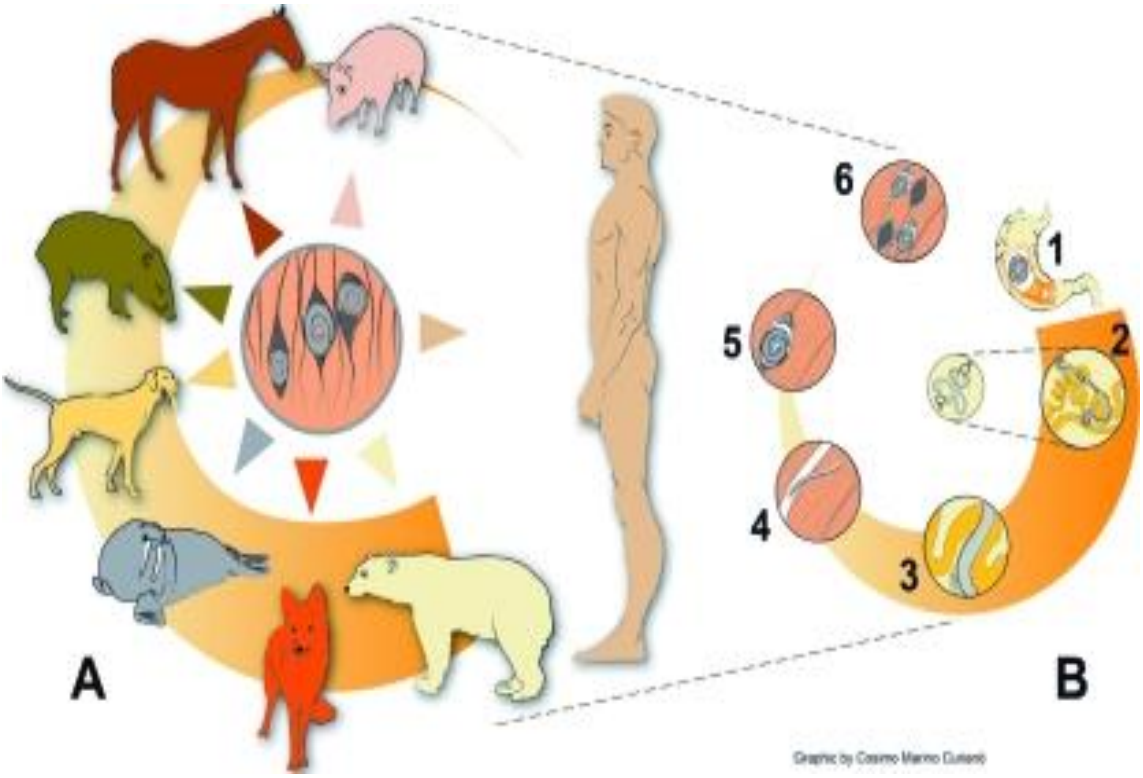

Figure 3: *Trichinella sp*. life cycle (Gottstein et al., 2009).

2.2.2 Immune response during *Trichinella* infection

During *Trichinella* infection, the intestinal mucosa is the first barrier to encounter parasites, and mucosal immunity is prone to be essential in protecting the host against *Trichinella* infection. There is evidence that the intestinal mucosa of infected animals produces antibodies specifically against *T. spiralis*, and the production of mucosal IgA mediates the protective immunity (Yang et al., 2010b). Studies have also shown that attenuated *Salmonella typhimurium* is a strong vector for oral delivery of heterologous antigens to the immune system, resulting in long-lasting mucosal and systemic responses and thus providing an effective platform technology for the design of novel

vaccination strategies (Yang et al., 2010b). Helminthic parasite infection could elicit Th2 responses against the host, particularly at the larval stage. *Trichinella spiralis* can also activate host IL-4 and IL-5 cytokines, IgE levels, and eosinophilia (Park et al., 2011). Studies regarding the immune evasion mechanisms of *Trichinella* infection have been previously reported, in particular, IL-10 operates as an inflammation regulator in cases of intestinal and muscle trichinellosis in mice (Park et al., 2011).

The essential features of immune responses to *Trichinella* are IgE production and eosinophilia (Watanabe et al., 2005). The IgE production has been described with the aid of induction of T helper (Th)2 cells that produce cytokines such as interleukin (IL)-4 and IL-13, which are key molecules for identifying B-cell differentiation to IgE-producing cells (Watanabe et al., 2005). The IgE antibody is effective in the intestinal lumen which is transported from plasma and the suppression of IgE production was developed in rats by means of treatment with anti-IgE and the IgE-suppressed rats were more vulnerable to major infection with *Trichinella* (Watanabe et al., 2005). Although there has been a contrasting result previously reported that IgE antibodies were accountable for the protection against muscle larvae using IgE gene knockout mice with a BALB/c background (Watanabe et al., 2005). In rats, rapid expulsion from the intestine is considered to be IgE-mediated protection against *Trichinella* spp and the muscle larvae are expelled immediately from the intestine of immunized animals after oral infection. Rapid expulsion in rats is induced by IgE antibodies and $CD4^{+}OX22^{-}$ T cells (Watanabe et al., 2005). The IgE antibody binds to *Trichinella* spp to prevent invasion (Watanabe et al., 2005). A selective suppression of IgE production was developed in rats by treatment with anti-IgE. The IgE suppressed rats were more susceptible to primary infection with *Trichinella* spp (Watanabe et al., 2005).

2.2.3 Global epidemiology and economic impact of *Trichinella* spp infection

The etiological agents of human trichinellosis exhibit essentially global distribution in domestic and wild animals, aside from Antarctica, where there is no documentation of the parasite (Pozio, 2007). This global distribution of *Trichinella* and ranging cultural consuming habits signify the primary factors favouring human infections in industrialized and non-industrialized countries. Human trichinellosis has been documented in 55 (27.8%) nations around the globe (Pozio, 2007). In a number of these countries, however, trichinellosis impacts mainly the ethnic minorities and

tourists due to the fact that the native inhabitants do not consume raw meat or meat of some animal species (Pozio, 2007). *Trichinella* spp. have been documented in domestic animals (in general pigs) and in wildlife of 43 (21.9%) and 66 (33.3%) countries respectively and out of the 198 nations of the world, approximately 40 (20%) are small islands with distance from the major continents, or metropolis–states where *Trichinella* spp. cannot circulate among animals for lack of nearby fauna (Pozio, 2007). Nevertheless, concrete information on the incidence of *Trichinella* spp in domestic and/or wild animals is still limited in 92 countries (Pozio and Murrell, 2006).

Trichinellosis not only is a public health hazard by affecting human patients but also represents an economic challenge in porcine animal production and food safety (Gottstein et al., 2009). Trichinellosis has been an important, though often unrecognized, disease for thousands of years (Bruschi and Murrell, 2002). Species of *Trichniella* responsible for the infection are widely distributed, including the Arctic, temperate lands, and the tropics, humans appear to be especially prone to developing clinical disease. Infection in wild animal is far more common than is generally recognized (Bruschi and Murrell, 2002).

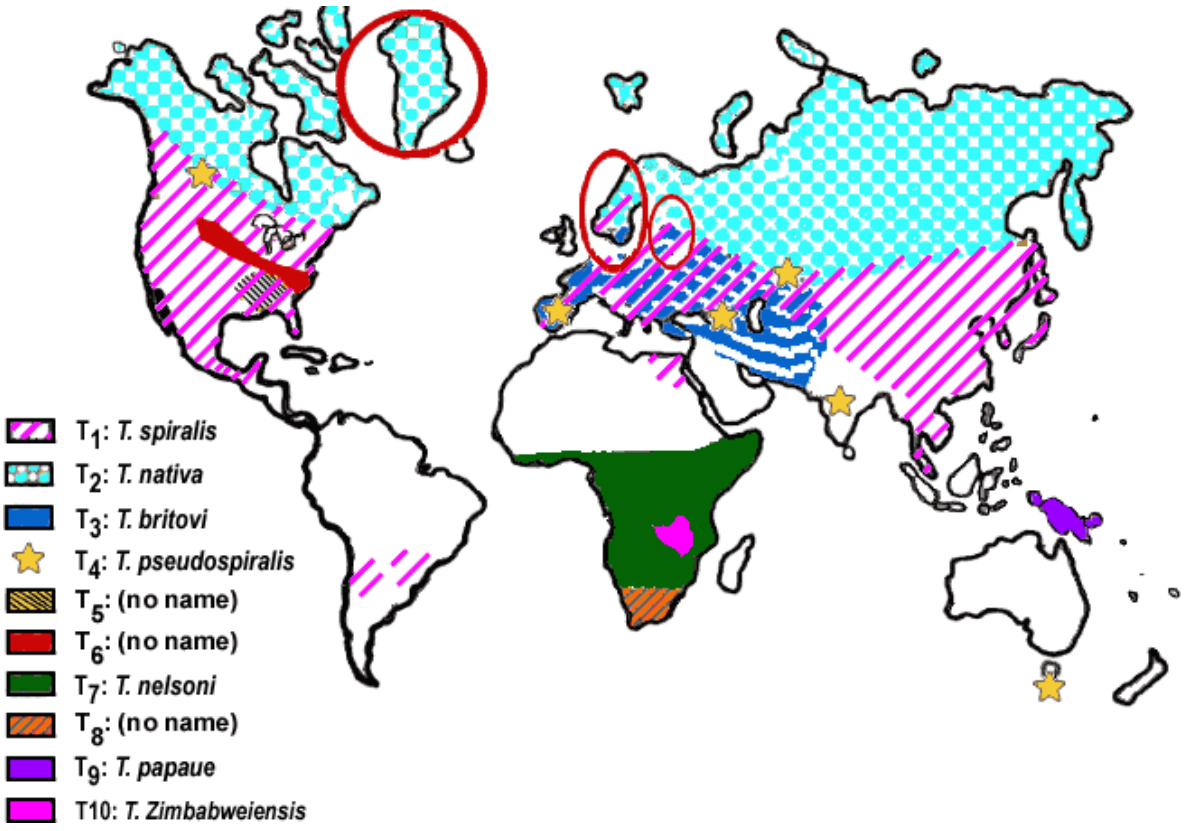

Figure 4: World map showing the distribution areas of *Trichinella spiralis, Trichinella nativa, Trichinella britovi, Trichinella pseudospiralis, Trichinella nelsoni, Trichinella papuae, and Trichinella zimbabwensis* (Pozio et al., 2002).

2.2.4 Diagnosis of *Trichinella*

The early clinical diagnosis of trichinellosis is difficult because of the absence of pathognomonic signs and symptoms, however, epidemiological data are very vital in the diagnosis of *Trichinella* infection, since the acute phase of this infection is associated with facial oedema and myalgia which might lead to complication of and thromboembolic and encephalitis disease (Dupouy-Camet and Murrell, 2007). Also, the chronic phase of the disease is not usually easy to diagnose. However, a delay in diagnosis and treatment in turns favours the establishment of larvae in muscle tissue and the development of a collagen capsule for the encapsulated species, which leaves the larvae difficult to treat with current antihelminthics (Pozio et al., 2001; Gottstein et al., 2009). Diagnosis of trichinellosis is basically based on three main criteria; the clinical symptoms, the laboratory findings and epidemiological investigation (Kociecka, 2000). Detection of circulating antigens with the aid of immunoassay methods may also be valuable for *Trichinella* diagnosis during the period of the parenteral phase, when the standard serological test designed to detect certain antibodies have yet to become positive, despite the fact that detection of circulating antigen perhaps might be a valuable confirmatory test although circulating antigen is not detected in every patient and its detection is consequently of limited value to the clinician (Dupouy-Camet and Murrell, 2007). Several studies have proven that eosinophilia is correlated with the degree of myalgia and is drastically higher in people with neurological problems, apart from moderately elevated white blood cell counts. Eosinophilia appears early prior to the development of clinical symptoms and signs, between the second and the fifth week of illness in different degrees, practically in each case of trichinellosis with only few exceptions (Gottstein et al., 2009). During the acute stage of infection, a tremendous decrease in eosinophil levels in patients with severe trichinellosis will also be viewed to be a predictor for a severe outcome. The mechanism underlying this decrease has not been absolutely understood, though it could be related to modifications in the levels of certain cytokines and to the massive exit of eosinophils from the vascular system, leading to huge tissue infiltrates (Dupouy-Camet and Murrell, 2007).

More so, trichinellosis cannot be exempted if this antibody class is not available, hence, IgE is not considered to show diagnostic relevance for routine diagnosis and again, IgA and IgM exhibit no advantage to conventional IgG detection (Noeckler et al., 2001). Furthermore, IgG antibodies can probably be verified between 12 to 60 days after infection, the time factor of seroconversion is dependent on a number of factors such as the number of ingested larvae, the *Trichinella* species involved, and the individual immune response (Gottstein et al., 2009). Out of conventional sero-diagnostic methods, ELISA is essentially the most commonly used method for the detection of *Trichinella* infection in humans and the validation procedure of an ELISA to detect anti-*Trichinella* IgG in human sera has revealed a large number of cross-reactions with sera from persons affected by other diseases, most often if these persons originate from developing countries where a high number of parasitic diseases occur (Gómez-Morales et al., 2008). The direct detection of muscle-stage larvae centered upon appropriate examination of muscle biopsies etiologically proves the diagnosis (Gottstein et al., 2009).

2.2.5 Control and treatment of *Trichinella* infection

As a result of the predominantly zoonotic importance of infection, most developed countries have focused on the prevention and elimination of *Trichinella* from the food chain (Gottstein et al., 2009). Also, there are areas of uncertainty in the management of human trichinellosis because there have been very few perspective, controlled clinical trials of treatment for this infection (Dupouy-Camet and Murrell, 2007). Nevertheless, on an empiric basis, most specialists endorse the use of anthelminthics and corticosteroids (Gottstein et al., 2009). Anthelmintics used for the treatment of trichinellosis comprise primarily of albendazole (Zentel; Smith-Kline Beecham) and mebendazole (Vermox; Janssen Pharmaceutica, Beerse, Belgium) (Dupouy-Camet and Murrell, 2007). Albendazole has the advantage that most patients reach requested plasma levels and for this reason do not require monitoring, meanwhile mebendazole plasma levels can fluctuate considerably among different patients and would require individual monitoring and dosing (Kadry et al., 2005). The application of anthelmintics at the stage of intestinal invasion aims particularly at the removal of intestinal forms of *Trichinella* spp. from the lumen of the gastrointestinal tract and such treatment is of primary significance for an early and effective therapy, mainly within the first three days following the infection (Kociecka, 2000). If applied, such remedy prevents future muscular invasion and the spread of disease, moreso, in many instances; this option cannot be

acquired anymore (Gottstein et al., 2009). The sequel of anthelmintic therapy on the path of a more advanced stage of the infection, on lethality, and on already encysted larvae is obscured and poorly elucidated till present (Gottstein et al., 2009).

Despite therapy, lethality in instances with excessive disease intensity was up to 5%, but in milder cases, prognosis is excellent, and most patients show a disappearance of symptoms within 2 to 6 months whilst from time to time, chronic myalgia and rheumatalgia will persist (Gottstein et al., 2009). Basically, the effectiveness of chemotherapy using albendazole or mebendazole is dependent on the time of administration and early application produce excellent result, although no sound case control efficacy studies have been conducted to date (Gottstein et al., 2009). Nevertheless, anthelmintics may not be usefull against long-term sequelae and chronic trichinellosis and an attempt to increase the bioavailability of anthelmintics have been successfully accepted in a mouse model by adding 2-hydroxypropyl-cyclodextrin (Casulli et al., 2006). Cymetidine has produced similar results on human cystic echinococcosis, although, experience is lacking for human trichinellosis (Gottstein et al., 2009).

2.2.6 Co-infection of malaria and helminths

Parasites are more often studied taking into consideration each parasite separately, more so, field experience has shown that infections with the aid of two or more parasite species at the same time frequently occur and this could have impact on the pathogenesis of each infection (Ademola and Odeniran, 2016). Although parasite host interactions could occur depending on the mechanisms by which, co-infections cause either more damage on the host than the combined effect outcome of the infectious agents, or less damage than the combined effect outcome of the infectious agents (Alizon and van Baalen, 2008). The interaction of the parasites which may have an impact on the epidemiology of co-infecting parasites could consequently influence the host wellbeing (Ezenwa and Jolles, 2011). Co-infections with malaria are usually complex and increase the severity of parasitic infection (Brooker et al., 2006a). Multiple parasitic infections are prevalent in tropical region (Ademola and Odeniran, 2016). Recent studies in vertebrates have shown that interactions between co-infecting parasites can be pronounced and have relevant consequences for disease development, severity and transmission dynamics (Telfer et al., 2010; Knowles, 2011).

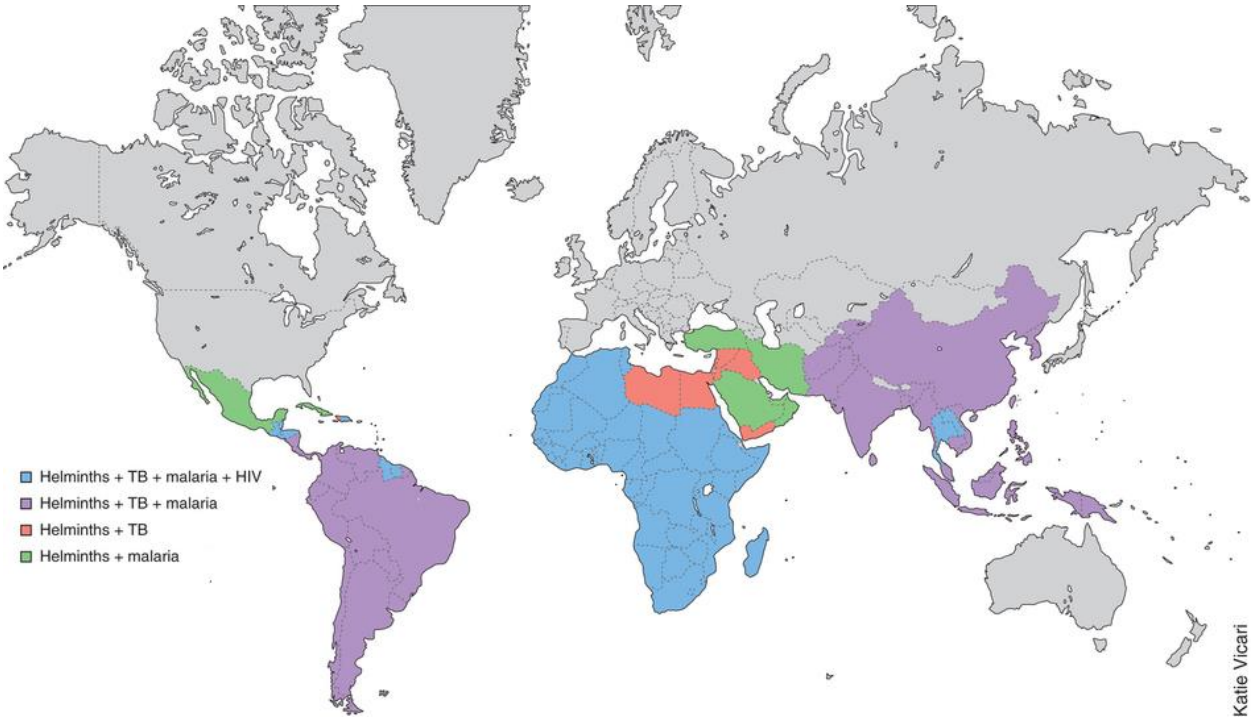

Figure 5: World map showing the geographic distribution of malaria co-infection with helminths in humans (Salgame et al., 2013).

2.2.7 The effect of helminths-malaria co-infection

Malaria and helminthiasis are the two types of the most prevalent of human infection in the developing world which overlap extensively in their epidemiological distributions and frequently co-infect the same individuals (Mazigo et al., 2010). The question of whether and how these two types of parasites might interact within co-infected hosts has attracted much curiosity and controversy (Knowles, 2011). Co- infecting parasites could interact both positively (facilitation) or negatively (competition) through a range of mechanisms including resource competition, immune-mediated interactions and direct interference (Knowles, 2011). Thus far, studies of helminth-malaria co-infection have focused mainly on immune-mediated mechanisms, without much doubt as a result of the known immunomodulatory effects of helminths (Grainger et al., 2010). For this reason, it has been suggested that by polarizing immune responses towards Th2-type effector mechanisms, helminths will reduce the pro-inflammatory Th1-type mechanisms needed to kill malaria parasites (Wammes et al., 2010). Secondly, helminths mostly elicit potent regulatory T cell (Treg) responses, which suppress all types of cellular effector mechanisms

including those against malaria parasites (Wammes et al., 2010). Several epidemiological studies have now sought evidence for effects of helminth co-infection on human malaria, with variable results, whilst some report high risk of clinical malaria in helminth co-infected individuals (Roussilhon et al., 2010), others find no effect or even suggest a protective effect (Knowles, 2011). Thus, firm conclusions about helminth-malaria interactions in humans and the likely effect of antihelminthic treatment on the burden of malarial disease are yet to be reached (Knowles, 2011).

3.0 CHAPTER 3: MATERIALS AND METHODS

3.1 Chemicals

Chemicals were sourced as indicated:

Giemsa stain (Sigma-Aldrich Chemical Company, Missouri, St Louis, USA); calcium chloride (CaCl2), potassium hydroxide (KOH), sodium sulphate (Na2SO4), sodium hydroxide (NaOH), Hydrochloric acid (Hcl).

3.2 Ethical consideration

Experimental protocols and procedures of the study were reviewed and approved by the Animals Ethics Committee of the University of KwaZulu-Natal (UKZN) (AREC/018/016 PD) in accordance with the South African national guidelines on animal care, handling and use for biomedical research.

3.3 Study Animals

Male Sprague-Dawley rats with body weight ranging from 90g-150g were used for the experiment. Animals were bred and housed in the Biomedical Resource Unit (BRU) of the University of KwaZulu-Natal, Westville campus and were maintained under standard laboratory conditions of constant temperature (22±2 °C), and CO_2 content of <5000 p.p.m., relative humidity of 55±5%, and illumination (12 h light/dark cycles) with free access to feed (sterilized rodent pellets chow from Meadows, Pietermaritzburg, South Africa) and clean water.

3.4 Experimental design

Rats were randomly selected into four groups of 4 animals as follows; control group (C); malaria infected group (M); *Trichinella* zimbabwensis-infected group (T); and co-infected group (MT).

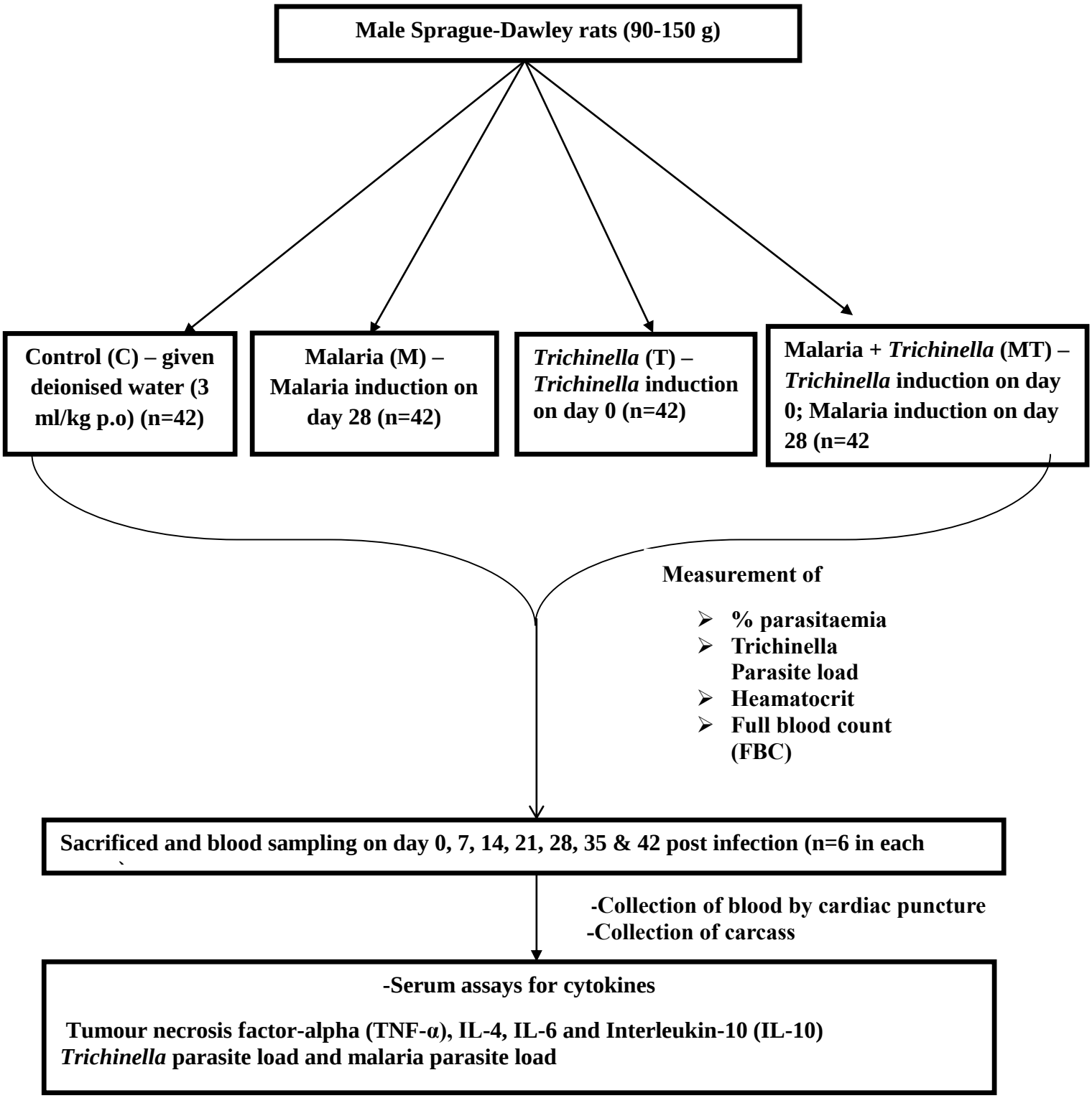

Figure 6: Diagram showing experimental design

For each group, animals were randomly selected into groups of six animals per cage (n=6 in each group) with free access to clean water and heat sterilized rodent pellets ad libitum (Meadows, Pietermaritzburg, South Africa). The rats were maintained on a 12 h dark/light cycle and temperature (22±2 °C). Cage bedding was changed every second day to ensure a clean and stable environment. Terminal studies were done on 6 animals from each group on day 0, 7, 14, 21, 28, 35 and 42 days and whole blood, sera and plasma collection and whole carcasses were digested for the *Trichinella*-infected groups on day 42. The serum and plasma samples were stored in a Bio Ultra freezer (Snijers Scientific, Tilburg, Netherlands) at -80 °C until assayed.

3.5 *Trichinella* induction

Forty-two (42) male Sprague-Dawley rats with body weight ranging from 90g-150g were used for the *Trichinella* group. The animals were housed at the Biomedical Resource Unit (BRU), University of KwaZulu-Natal. Animals were randomly selected into groups of six animals (n=6 in each group) with free access to clean water and standard heat sterilized rodent pellets ad libitum (Meadows, Pietermaritzburg, South Africa). The rats were maintained on a 12 h dark/light cycle and temperature (22±2 °C). Cage bedding was changed every second day to ensure a clean and stable environment. *T. zimbabwensis* infection was induced using a crocodile-derived *T. zimbabwensis* (ISS1209) parasite strain. The strain was maintained by a standardized serial infection of male Sprague-Dawley rats stock (150-250g). Muscle larvae were obtained from infected stock rats whole carcasses digested at 42 days post infection (dpi) following the method as previously described by (Pozio et al., 2002). The experimental animals were infected on day 0 with *T. zimbabwensis* larvae by oral gavage at a dose of 500 muscle larvae (ML) per animal using an 18 G curved oral dosing needle. Recovery of adult worms from the intestines was achieved using a modification of the protocol by (Mukaratirwa et al., 2003). The small intestines were immersed in 0.85% saline solution, split open longitudinally with scissors and washed with H_2O under 212μm sieve. After washing, the intestinal tissues were further incubated for 12hr at 37 ºC and then re-washed under a 212 μm. The washings were viewed under dissecting microscope at 20 x objective for adult worm counts; whereas larvae in muscles were recovered using the modified artificial digestion protocol as described by (Pozio et al., 2002). Terminal studies were done on 0, 7, 14, 21, 28, 35 and 42 days post infection.

3.6 Malaria induction

Malaria was induced in forty-two (42) male Sprague-Dawley rats with body weight ranging from 90g-150g. The animals were housed at the Biomedical Resource Unit (BRU), University of KwaZulu-Natal. Animals were randomly selected into groups of six animals per cage (n=6 in each group) with free access to clean water and standard heat sterilized rodent pellets ad libitum (Meadows, Pietermaritzburg, South Africa). The rats were maintained on a 12 h dark/light cycle and temperature (22±2 °C). Cage bedding was changed every second day to ensure a clean and stable environment. The rodent-susceptible *P. berghei* ANKA parasite was sourced from University of Cape Town, South Africa. Malaria induction was confirmed by microscopic examination of Giemsa stained thin blood smears from the rat tail (Andrews et al., 2006). The experimental animals were infected on day 28 by a single intra-peritoneal injection of *P. berghei* (10^5 parasitised RBC). Daily malaria parasite density was measured in *P berghei*-infected animals throughout the remaining 14 day experimental period using thin blood films stained by Giemsa method. The slides were observed using an Olympus microscope (Olympus cooperation, Tokyo, Japan) with a x50–x100 oil immersion objective and percentage parasitaemia was determined

3.6.1 *Plasmodium berghei* ANKA and *Trichinella zimbabwensis* co-infection induction

Forty-two (42) male Sprague-Dawley rats with body weight ranging from 90g-150g were used for the co-infection group. The animals were housed and maintained as previously described in the other groups. The experimental animals were infected on day 0 with *T. zimbabwensis* larvae by oral gavage at a dose of 500 muscle larvae (ML) per animal using an 18 G curved oral dosing needle and the same animals were co-infected with *P. berghei* on day 28 post-*T. zimbabwensis* infection by a single intra-peritoneal injection of *P. berghei* (10^5 parasitised RBC) (Gumede et al., 2003). *P, berghei* parasitaemia was measured daily throughout the remaining 14 days using thin blood films stained by Giemsa method. Recovery of adult *T. zimbabwensis* worms from the intestines was achieved using a modification of the protocol by (Mukaratirwa et al., 2003). Larvae in muscles were recovered using the modified artificial digestion protocol as described by (Pozio et al., 2002). Terminal studies were done on 0, 7, 14, 21, 28, 35 and 42days post infection.

3.6.2 *Plasmodium berghei* parasitaemia measurement

Parasitaemia was determined in two groups, the malaria infected (M) and co-infected (MT), after infecting the experimental animals with *P. berghei* on day 28. Daily *P. berghei* parasitaemia was measured in the two groups throughout the remaining 14day experimental period. Thin blood films stained were examined. Briefly, a small incision in the tip of the rat tail was made followed by placing a small drop of blood on a microscope slide. This was smoothly and uniformly smeared using a second microscope slide. The blood film on the slide was subsequently dried in a stream of warm air, fixed in methanol for 30 s and then stained with Giemsa stain for 30-45 min, thereafter the stained slide was washed gently with buffer (7.4), the blood film was air dried at room temperature and observed using an Olympus microscope (Olympus cooperation, Tokyo, Japan) with a x50–x100 oil immersion objective. Extreme caution was taken to distinguish parasites from inclusions in RBC such as Howell-Jolly bodies, platelets on top of RBC, or from precipitated stain. Parasitaemia count of 5 microscope fields was followed by calculation of the percentage of infected RBCs. The parasite determination was the same for the malaria infected (M) and co-infected (MT) groups.

$$\%\ \text{Parasitaemia} = \frac{\text{Total RBCs infected with } P.\,berghei}{\text{Total RBC counted (infected} + \text{non} - \text{infected)}} \times 100$$

3.6.3 Terminal studies

On days 0, 7, 14, 21, 28, 35 and 42 post-infection, six animals were sacrificed from each group. The sacrifice procedure was performed by isoform inhalation in an anaesthetic chamber for 3 minutes. Blood was collected from the rats on the days of sacrifice by cardiac puncture into 1 ml blood tubes containing clotting activator gel (dvac gel and clot activator tubes). The blood was subject to centrifugation using a Thermo scientific Heraeus Labofuge 200 microprocessor controlled table top centrifuge at 132 xg for 10 - 15 minutes at 4 °C. Following centrifugation, serum was collected into Eppendorf micro-centrifuge tubes. The plasma samples were stored in Bio Ultra freezer (Snijers Scientific, Tilburg, Netherlands) at -80 °C until assayed. Cytokines concentrations were measured using ELISA. Recovery of adult worms from the intestines was

done using a modification of the protocol described by Mukaratirwa *et al.*, (2003) and muscle larvae were recovered using the modified artificial digestion protocol as described by Pozio *et al*., (2002).

3.6.4 Haematological profile measurement

Blood sample for haematological analysis was collected from each rat through cardiac puncture. The blood samples were collected into sample bottles containing ethylene diamine–tetra-acetic acid (EDTA). The parameters analyzed included white blood cell count (WBC), red blood cell count (RBC), haemoglobin (HGB), haematocrit (HCT), eosinophils (EO), neutrophils (NE), lymphocytes (LY), monocytes (MO) and basophils (BA). These parameters were measured using A C.T 5diff Beckman coulter counter (BCC) made in USA.

3.6.5 Cytokines measurement

To determine the levels of cytokines in sera, Procartalplex Mix&Match Rat 7-plex was used to measure Tumor necrosis factor-alpha (TNF-α) and Interleukin-10 (IL-10) levels in serum according to manufacturer's instructions. Different antigen standard set vials were centrifuged at 2000 x g for 10 sec. 50 µl of sample type specific buffer was added into the vial and a Universal Assay Buffer(1x) was used to reconstitute the standard. The vial was vortex gently for 10 seconds and centrifuged at 2000 x g for another 10 seconds to collect the content at the bottom of the vials. The vial was incubated on ice for 10mins to ensure complete reconstitution. Each content in the vial was pooled into one vial and filled up with type specific buffer to a total volume of 250 µl. 4-fold serial dilution of the reconstitution standard was prepared using the PCR 8 tubes and was labeled as Std1, Std2, Std3, Std4, Std5, Std6 and Std7 respectively.

The Antibody Magnetic Beads were vortex for 30 sec, and 50 µL of the Antibody Magnetic Beads were added to each well. A Procarta 96-Well Flat Bottom Plate was inserted into the Hand-Held Magnetic Plate Washer so that the A1 location of the 96-Well Plate matches up with the A1 on the washer, the 96-Well Plate was locked in place by pushing the 2 securing tabs, located on each end of the washer, towards the 96-Well Plate until they overlap the skirt of the 96-Well Plate. The Antibody Magnetic Beads were allowed to accumulate on the bottom of each well for 2 min, and the liquid was removed in the wells by quickly inverting the Hand-Held Magnetic Plate Washer

and 96-Well Plate assembly over a sink, the inverted assembly was blot into several layers of paper towels to remove any residual solution, and 150 µL of 1X Wash Buffer was added into each well. The Antibody Magnetic Beads was allowed to accumulate on the bottom of each well for 30 sec, and the wash buffer in the wells was removed by quickly inverting the Hand-Held Magnetic Washer and 96-Well Plate assembly over a sink. 25 µL of Universal Assay Buffer was added into each well, 25 µL of standards were also added into dedicated wells and 25 µL of Universal Assay Buffer to the blank wells. The 96-Well Plate was sealed using a Plate Seal provided. Afterwards, the 96-Well Plate was removed from the Hand-Held Magnetic Plate Washer and covered with the black microplate lid that was provided in the kit in order to protect it from light. The 96-Well Plate was shaken at 500 rpm for 120 min at room temperature and the plate was transferred to 4 °C and store on a level surface. After incubation, the 96-Well Plate was removed from 4 °C and shaken again for 30 min at room temperature.

The plate was washed for a total of three washes following the previous method as described above. 25 µL of Detection Antibodies Mix (1x) was added into each well and sealed the 96-Well Plate with a new Plate seal. The 96-Well Plate was removed from the Hand-Held Magnetic Plate Washer, covered with the black microplate lid provided in the kit and shake at 500 rpm for 30 min at room temperature. The plate was washed for a total of three washes following the previous method as described above and 50 µL of SA-PE solution was added into each well, the 96-Well Plate was again sealed with a new Plate seal. The 96-Well Plate was removed from the Hand-Held Magnetic Plate Washer and the plate was covered with the black mic roplate lid provided in the kit and shaken at 500 rpm for 30 min at room temperature. The plate was washed again for a total of three washes following the previous method as described above and 120 µL of Reading Buffer was added into each well. The 96-Well Plate was sealed with a new Plate Seal, the 96-Well Plate was removed from the Hand-Held Magnetic Plate Washer, covered with the black microplate lid provided in the kit and shakes at 500 rpm for 5 min at room temperature. The plate seal was removed and the plate was run on the Luminex instrument.

3.6.6 Statistical analysis

All data were expressed as means ± standard error of means (SEM), the data was analysed using a two-way analysis of variance (ANOVA) followed by Turkey-Kramer multiple comparison test to establish statistical comparison between various groups control, malaria, *Trichinella*, and co-infection groups. Levels of significance were determined by Bonferroni post-hoc test analyses. All data were expressed as mean ± standard error of mean (SEM). Two-way analysis of variance (ANOVA) with accompanying Tukey's post-hoc analysis for parametric data was used to compare mean values of adult worms and mean values of muscle larvae using Graph pad PRISM version 5.04 for windows (Graphpad software, San Diego, California, USA) and the differences between groups were considered significant when P-value was less than 0.05.

4.0 CHAPTER 4: RESULTS

4.1 Percentage parasitaemia after *P. berghei* infection

All animals did not make it safely to their terminal stage, two animals died in the malaria infected group, it could be due to high parasitaemia. The malaria mean parasitaemia and standard error mean was determined in the present study, on day 2 after *P. berghei* infection, the *P. berghei* (MI) group developed mean parasitaemia of 20.88% ± 1.47% while the co-infected with *P. berghei* and *T. zimbabwensis* (CI) developed a mean parasitaemia of 23.02% ± 0.92%. On day 3, the *P. berghei* (MI) group means parasitaemia increased to 23.70% ± 2.55% ($p < 0.01$) and 41.25% ± 1.61% in the *P. berghei* and *T. zimbabwensis* (CI). On day 4, the mean parasitaemia increased to 28.63% ± 4.22% ($p < 0.001$) and 50.20% ± 5.38% respectively. The *P. berghei* (MI) group reached the peak at day 6 with 66.77% ± 4.63% while the *P. berghei* and *T. zimbabwensis* (CI) reached its peak on day 7 with 69.17% ± 3.80%. There was a gradual decrease of malaria mean parasitaemia in both *P. berghei* only (MI) and co-infected with *P. berghei* and *T. zimbabwensis* (CI) till day 14. However, infection of rats with *P. berghei* and *T. zimbabwensis* (CI) resulted in increased parasitaemia compared to *P. berghei* mono-infected rats. The effects of *P. berghei* and *T. zimbabwensis* co-infection on percentage parasitaemia are shown in (Figure 7).

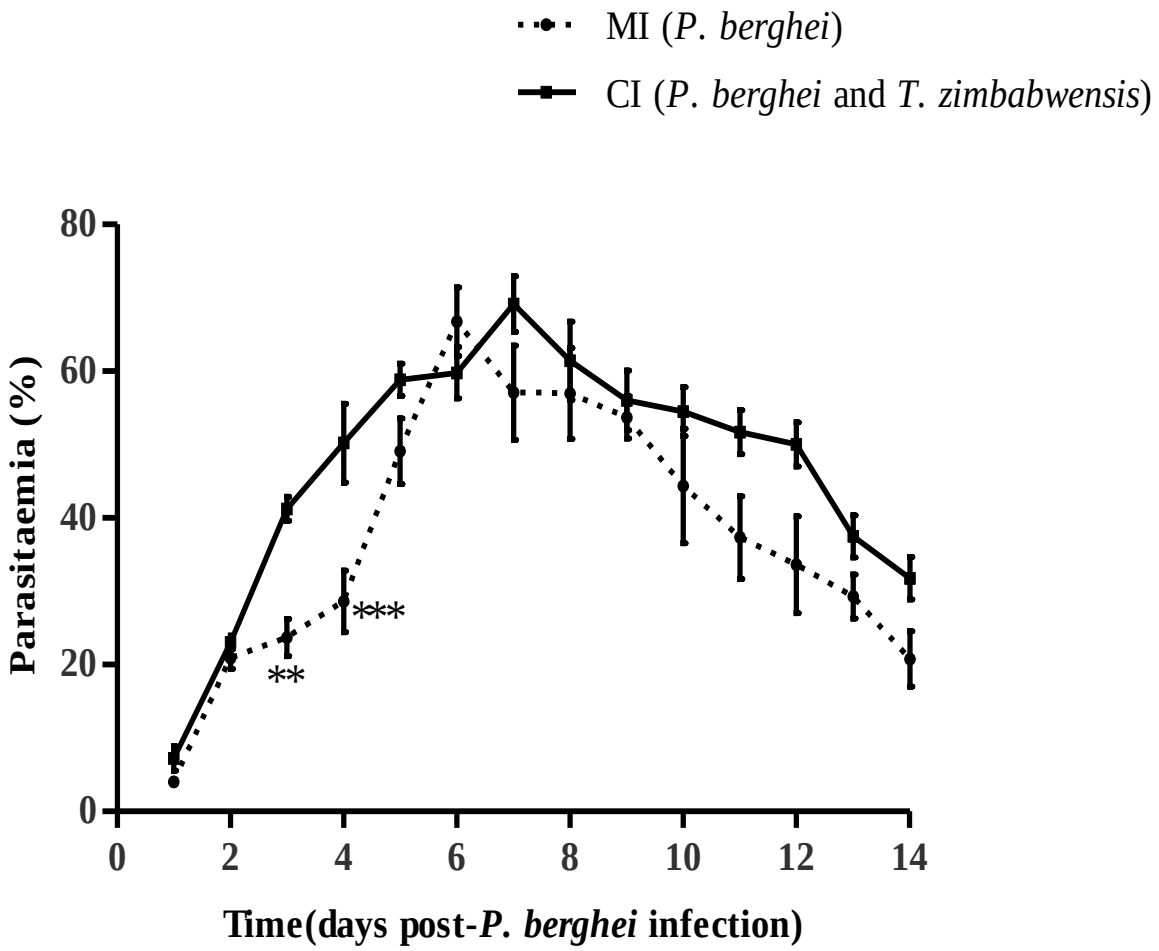

Figure 7: Percentage parasitaemia in male Sprague-Dawley rats infected with *Plasmodium berghei* only (MI) and co-infected with *P. berghei* and *T. zimbabwensis* (CI). Day 0 represents the day of *P. berghei* induction and 28 days post-infection with *T. zimbabwensis* when *Trichinella* larvae are now in the rat muscle). Values are presented as means and vertical bars indicate SEM (n=6 in each group). **P < 0.01, *** P<0.001.

4.2 *Trichinella zimbabwensis* parasite establishment

The adult worms (AW) of *T. zimbabwensis* in the intestine of male Sprague-Dawley rats were recovered in both the *T. zimbabwensis* group (MI) and the *P. berghei* and *T. zimbabwensis* group (CI) from day 7 to day 14 post infection (Fig. 8), the adult worms in the *T. zimbabwensis* group (MI) were more on day 7 with a mean of 18.50% ± 8.06% while the co-infected with *P. berghei* and *T. zimbabwensis* (CI) developed a mean of 15.00% ± 6.44%. There was a decrease on day 14 in both groups, the mean adult worm (AW) of *T. zimbabwensis* decreased to 3.00% ± 0.0% and 1.00% ± 0.0% respectively, at day 21 post infection, there was no adult worm recovered in the intestine in both groups. Muscle larvae were recovered from the muscle on the day 28, 35 and 42 post infection in both groups. On day 28, the *T. zimbabwensis* group (MI) had a mean of 41.50% ± 22.0%. larvae per gram (lpg) of muscle while the *P. berghei* and *T. zimbabwensis* group (CI) had a mean of 48.70% ± 23.14%. On day 35, the mean muscle larvae (ML) of *T. zimbabwensis* increased to 48.60% ± 7.49% in the *T. zimbabwensis* group (MI) and a slide decrease of 46.57% ± 11.2% in the *P. berghei* and *T. zimbabwensis* group (CI). On day 42, the *T. zimbabwensis* group (MI) increased exponentially with a mean of 97.37% ± 20.6% compare to the *P. berghei* and *T. zimbabwensis* group (CI) 71.63% ± 17.11%. The effects of *P. berghei* and *T. zimbabwensis* co-infection on *Trichinella* muscle larvae load is shown in Figure 8.

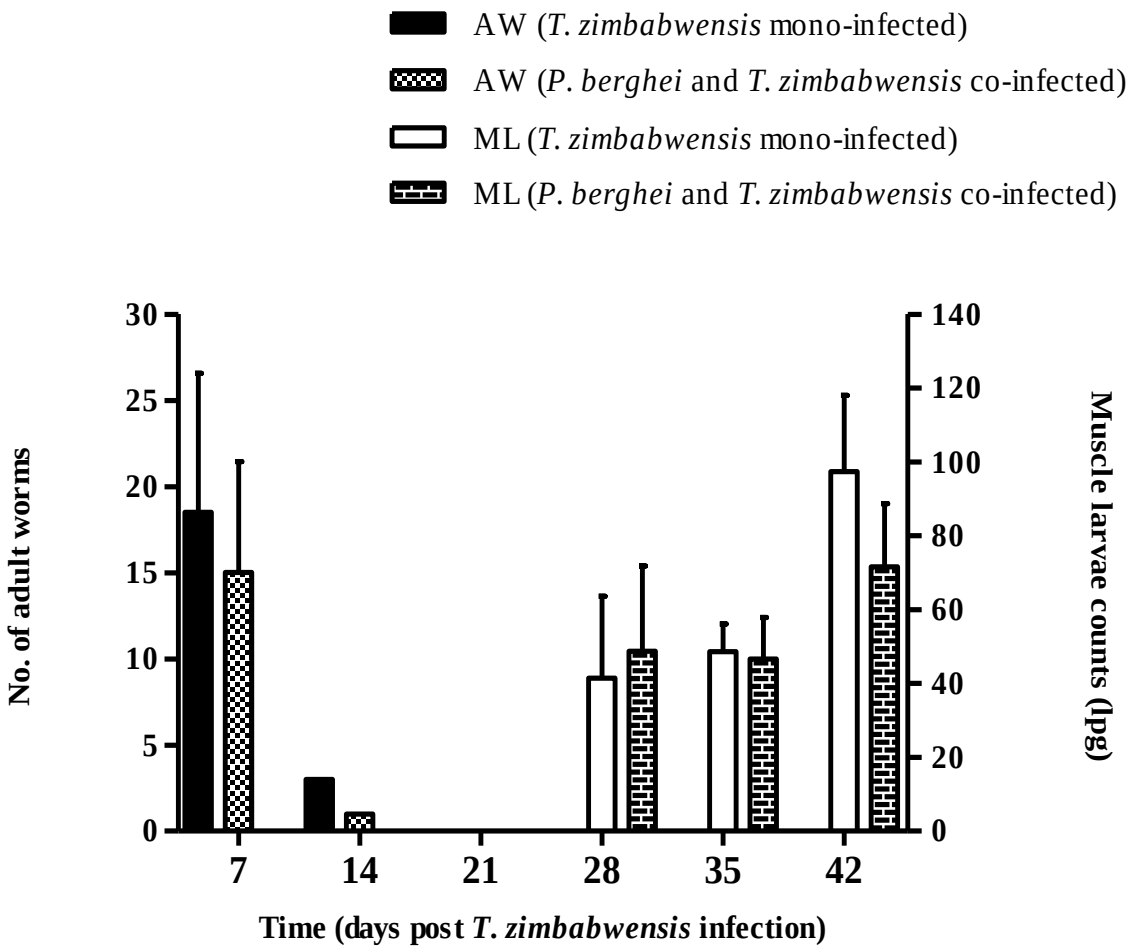

Figure 8: Mean number of intestinal adult worms (AW) and muscle larvae counts (ML) per gram of muscle (lpg) recovered from rats infected with *Trichinella zimbabwensis* only (MI) and the group co-infected with *Plasmodium berghei* (CI) on day 28 post-infection with *T. zimbabwensis*. Values are presented as means and vertical bars indicate SEM (n=6 in each group).

4.3 Haematology

Haematological changes were observed in different groups of the experiment, control (C), *Plasmodium berghei* (MI) group, *Trichinella zimbabwensis* (MI) and *P. berghei* and *T. zimbabwensis* (CI) group. In this study, there was a significant ($p < 0.001$) decrease in the RBC in rats infected with *T. zimbabwensis* (MI) and *P. berghei* and *T. zimbabwensis* (CI) group on day 7, a decrease was also observed in the *P. berghei* (MI) group on day 7 p.i. although it was not significant, a significant ($p < 0.01$) decrease in the *P. berghei* and *T. zimbabwensis* (CI) group was observed on day 14 p.i. respectively (Fig. 9). However, there was no significant difference in the WBC count among the *P. berghei* (MI), *T. zimbabwensis* (MI) and the co-infection (CI) group but a trend increase was observed in all the groups as compare to the control group (Fig. 10). Regarding haemoglobin counts, significantly ($p < 0.05$) high count was only observed on day 14 p.i in the *T. zimbabwensis* (MI) group (Fig. 11). A significant ($p < 0.05$) reduction (30.0% ± 4.0%) in haemotocrit counts was observed for *P. berghei* (MI), also a significant ($p < 0.001$) reduction (7.31.0% ± 4.2%) in *T. zimbabwensis* (MI), and a significant ($p < 0.001$) reduction (4.15% ± 1.5%) in *P. berghei* and *T. zimbabwensis* (CI) group was observed on day 7 p.i. (Fig. 12). A significant ($p < 0.001$) neutropaenia increase (22.88% ± 2.31%) was observed on day 7 p.i. in the *P. berghei* and *T. zimbabwensis* (CI) group compare to the control group (8.21% ± 1.31%), while the *P. berghei* (MI), *Trichinella zimbabwensis* (MI) and *P. berghei* and *T. zimbabwensis* (CI) groups presented almost normal neutrophil counts on Day 14 p.i. (Fig. 13). A significant ($p < 0.001$) reduction (38.12% ± 4.5%) in lymphocyte counts was observed for *T. zimbabwensis* (MI) group and a significant ($p < 0.01$) reduction (51.93% ± 7.31%) in the *P. berghei* and *T. zimbabwensis* (CI) group on day 7 p.i. compared to the *P. berghei* (MI), and control groups (Fig. 14). Significant ($p < 0.001$) monocytosis was observed in *P. berghei* (MI) group on day 7 and on day 14 p.i., although the *Trichinella zimbabwensis* (MI) and the *P. berghei* and *T. zimbabwensis* (CI) also showed gradual increase compare to the control group (Fig. 15). Also, rats infected with *T. zimbabwensis* (MI) and *P. berghei* and *T. zimbabwensis* (CI) showed significant ($p < 0.001$) increase of basophils on day 7 p.i compared with the uninfected (C), although there was a slight increase in the *P. berghei* (MI) group, on day 14 p.i, there was a drastic reduction in all the groups (Fig. 16).

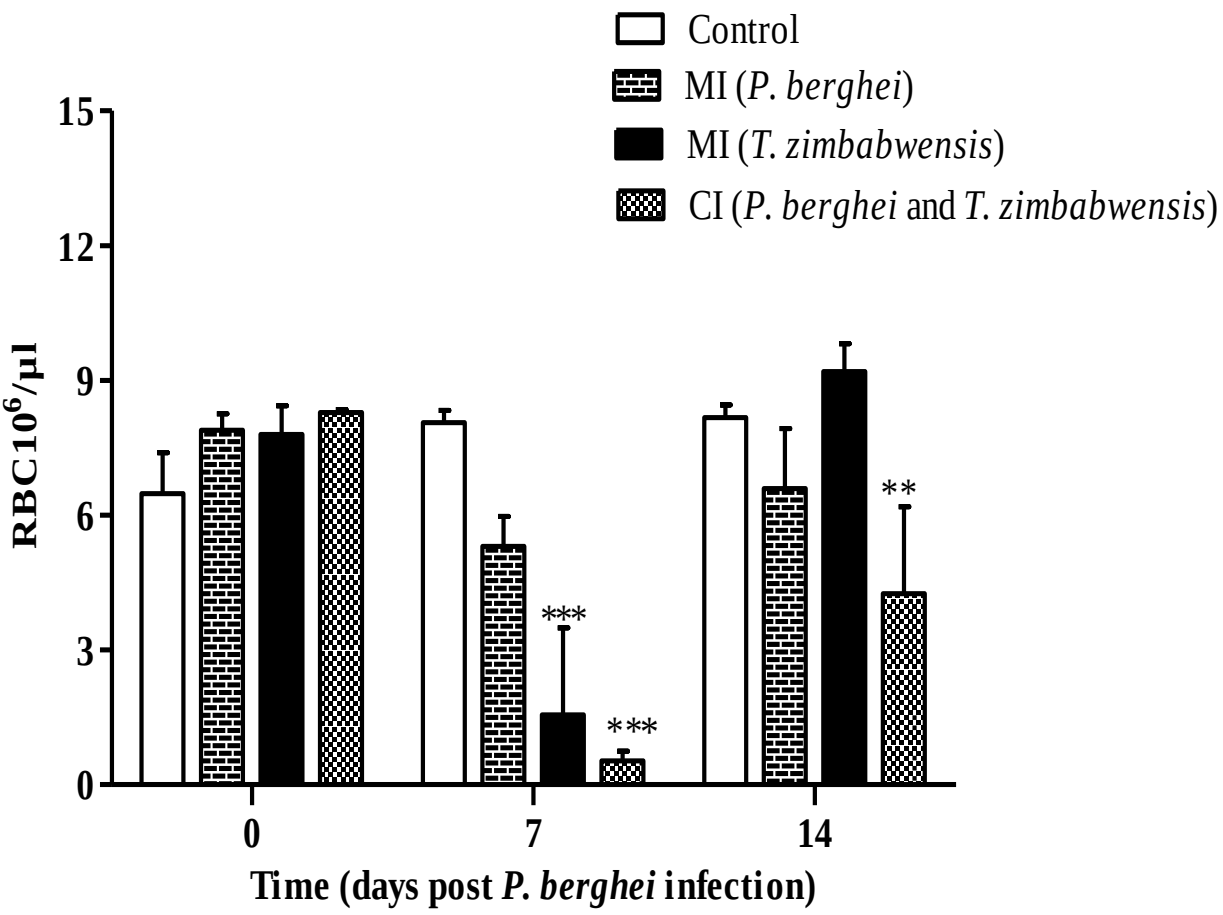

Figure 9: Comparison of the effects of *Plasmodium berghei* and *Trichinella zimbabwensis* mono-infection (MI) and co-infection (CI) on RBC concentration in male Sprague-Dawley rats. Day 0 represents the day of *P. berghei* when *Trichinella* muscle larvae is now in the rat muscle at day 28 post *Trichinella* infection. Values are presented as means and vertical bars indicate SEM (n=6 in each group). ** $P<0.01$, *** $P<0.001$.

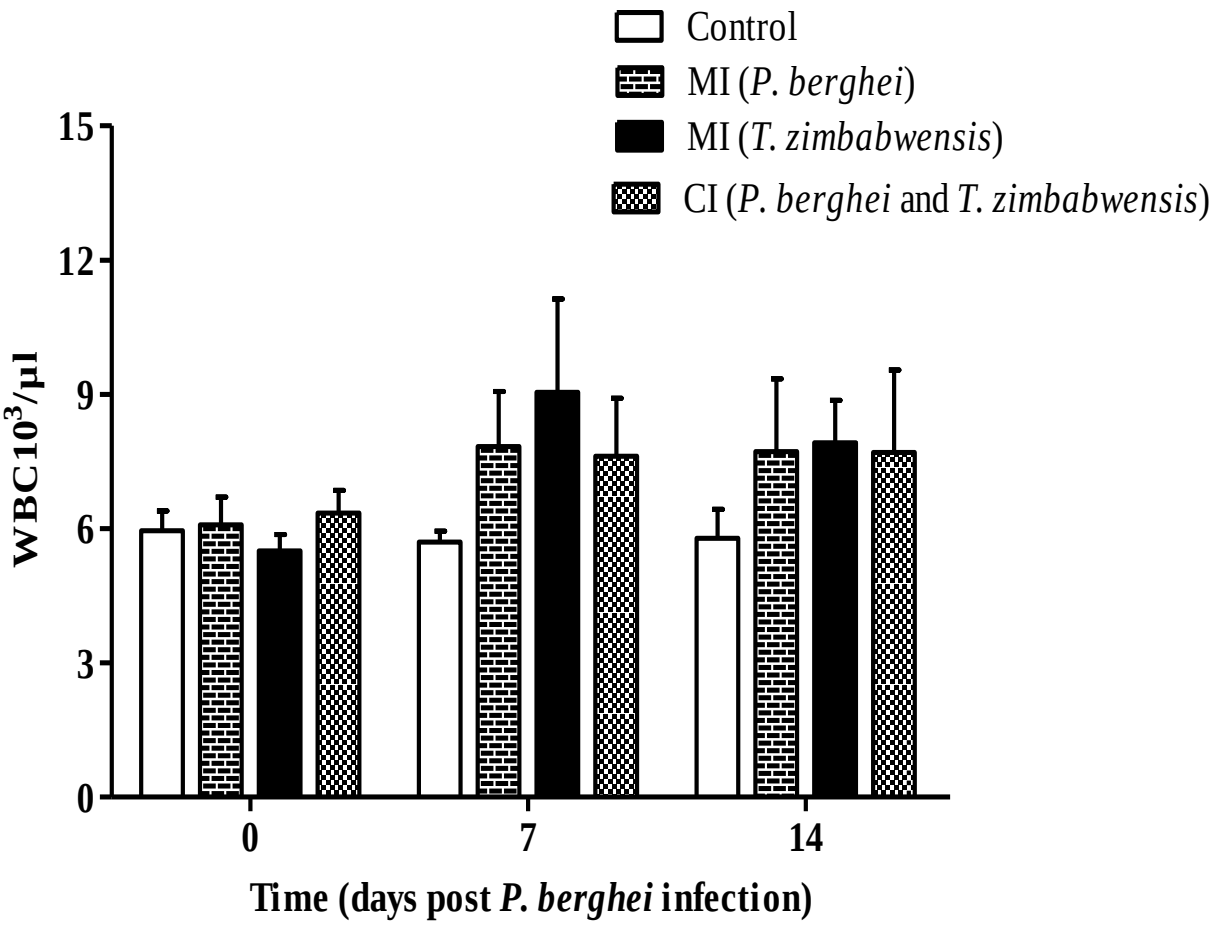

Figure 10: Comparison of the effects of *Plasmodium berghei* and *Trichinella zimbabwensis* mono-infection (MI) and co-infection (CI) on WBC concentration in male Sprague-Dawley rats. Day 0 represents the day of *P. berghei* when *Trichinella* muscle larvae is now in the rat muscle at day 28 post *Trichinella* infection. Values are presented as means and vertical bars indicate SEM (n=6 in each group).

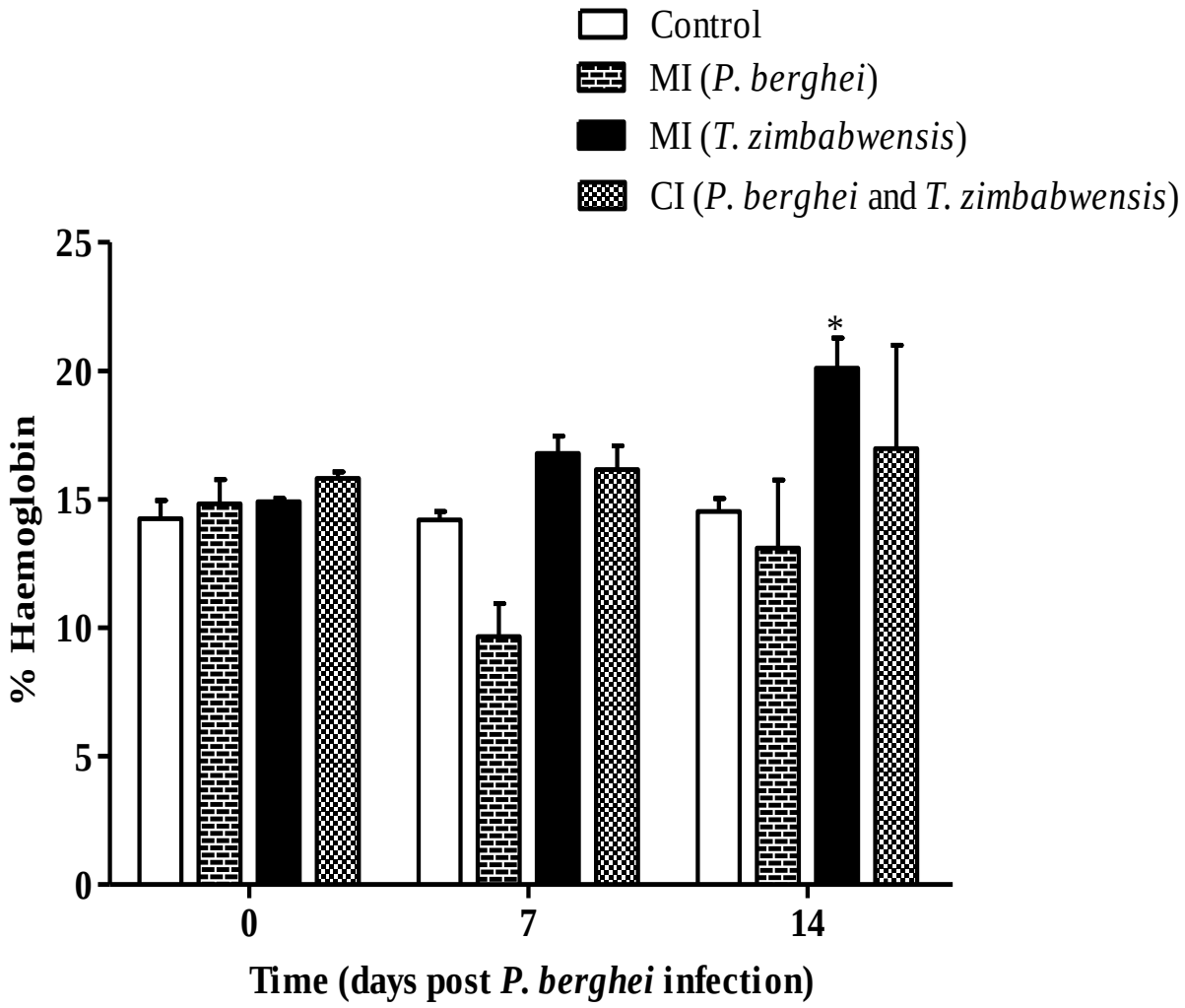

Figure 11: Comparison of the effects of *Plasmodium berghei* and *Trichinella zimbabwensis* mono-infection (MI) and co-infection (CI) on haemoglobin concentration in male Sprague-Dawley rats. Day 0 represents the day of *P. berghei* when *Trichinella* muscle larvae is now in the rat muscle at day 28 post *Trichinella* infection. Values are presented as means and vertical bars indicate SEM (n=6 in each group). * $P<0.05$

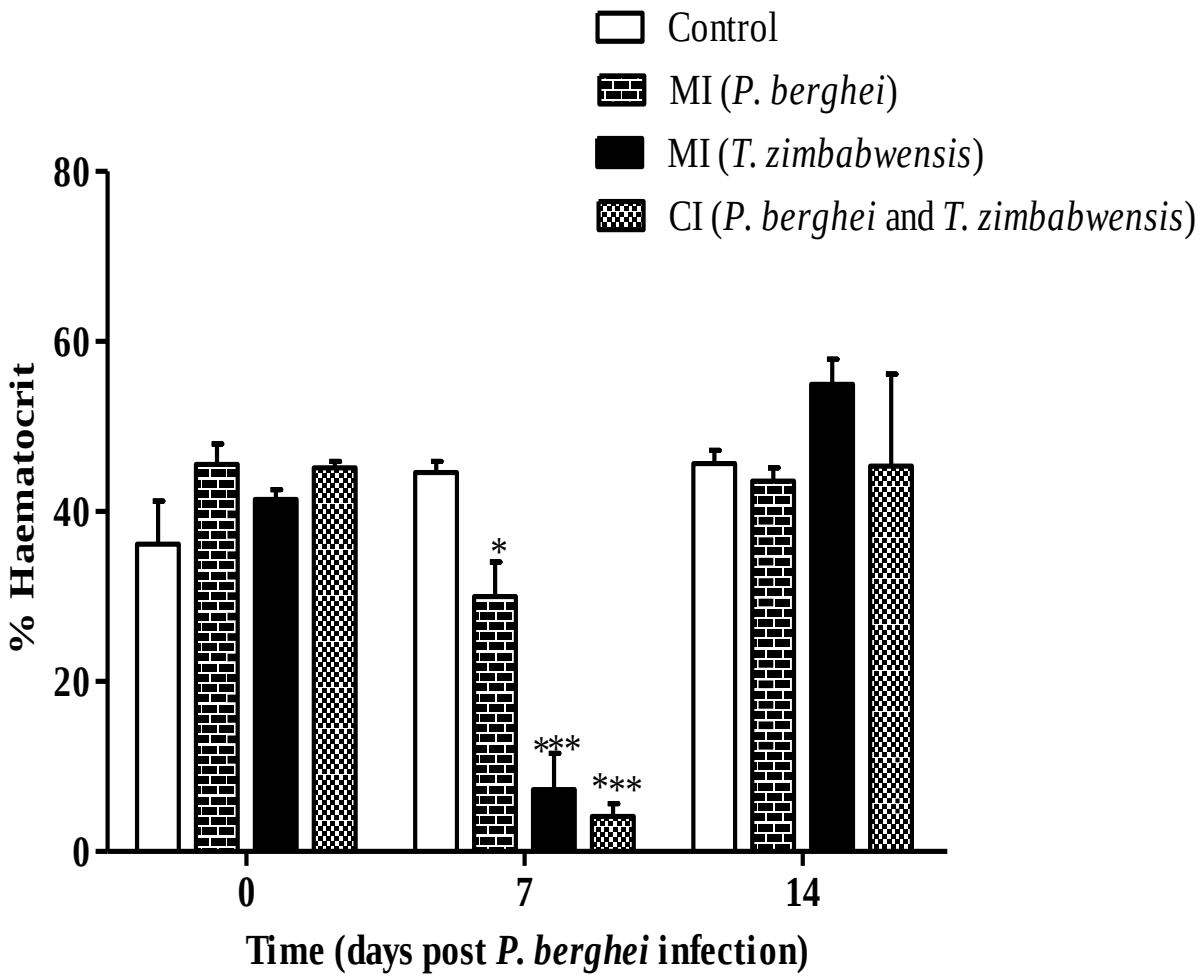

Figure 12: Comparison of the effects of *Plasmodium berghei* and *Trichinella zimbabwensis* mono-infection (MI) and co-infection (CI) on haematocrit concentration in male Sprague-Dawley rats. Day 0 represents the day of *P. berghei* when *Trichinella* muscle larvae is now in the rat muscle at day 28 post *Trichinella* infection. Values are presented as means and vertical bars indicate SEM (n=6 in each group). * $P<0.05$, *** $P<0.001$.

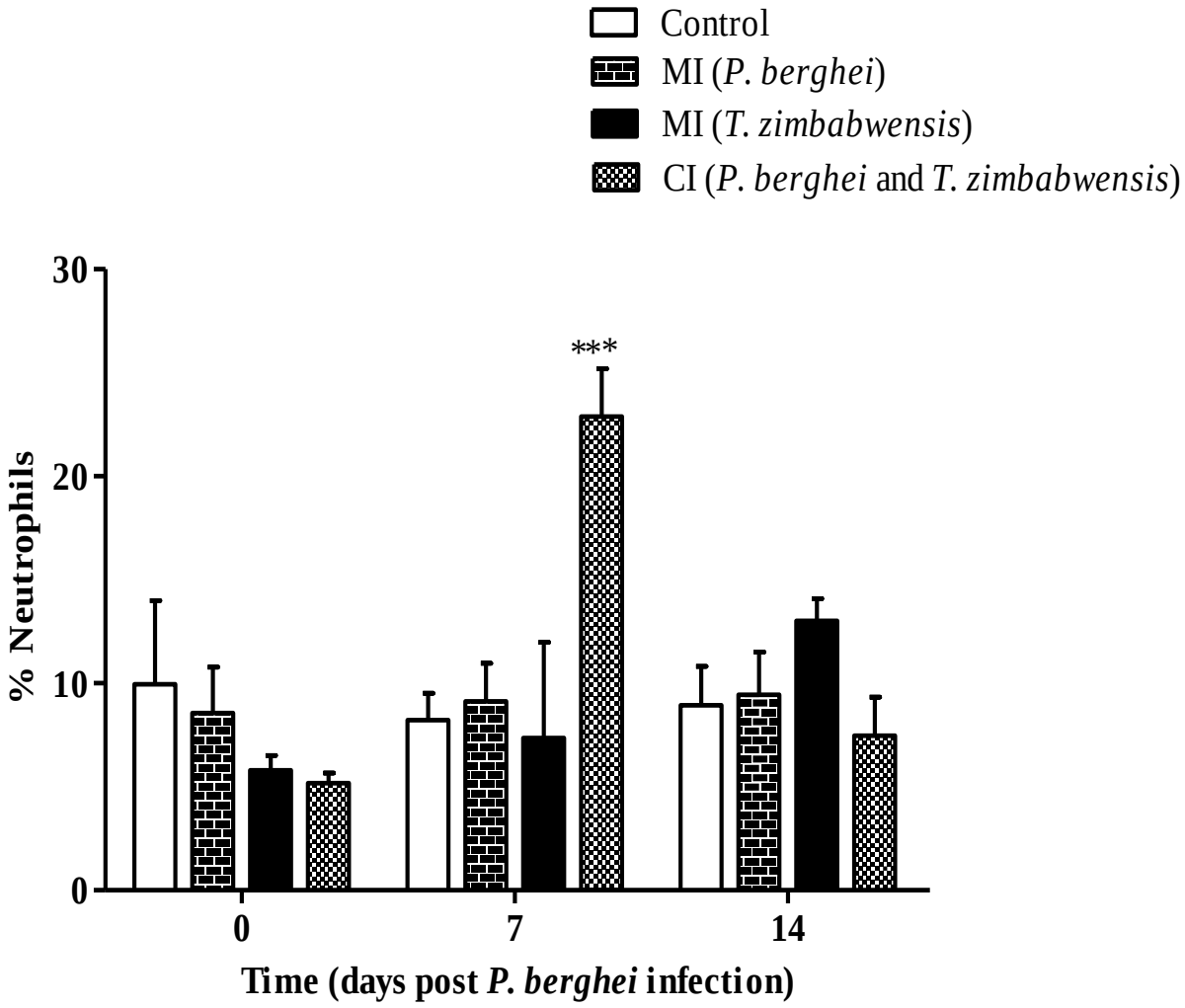

Figure 13: Comparison of the effects of *Plasmodium berghei* and *Trichinella zimbabwensis* mono-infection (MI) and co-infection (CI) on neutrophils concentration in male Sprague-Dawley rats. Day 0 represents the day of *P. berghei* when *Trichinella* muscle larvae is now in the rat muscle at day 28 post *Trichinella* infection. Values are presented as means and vertical bars indicate SEM (n=6 in each group). *** $P<0.001$.

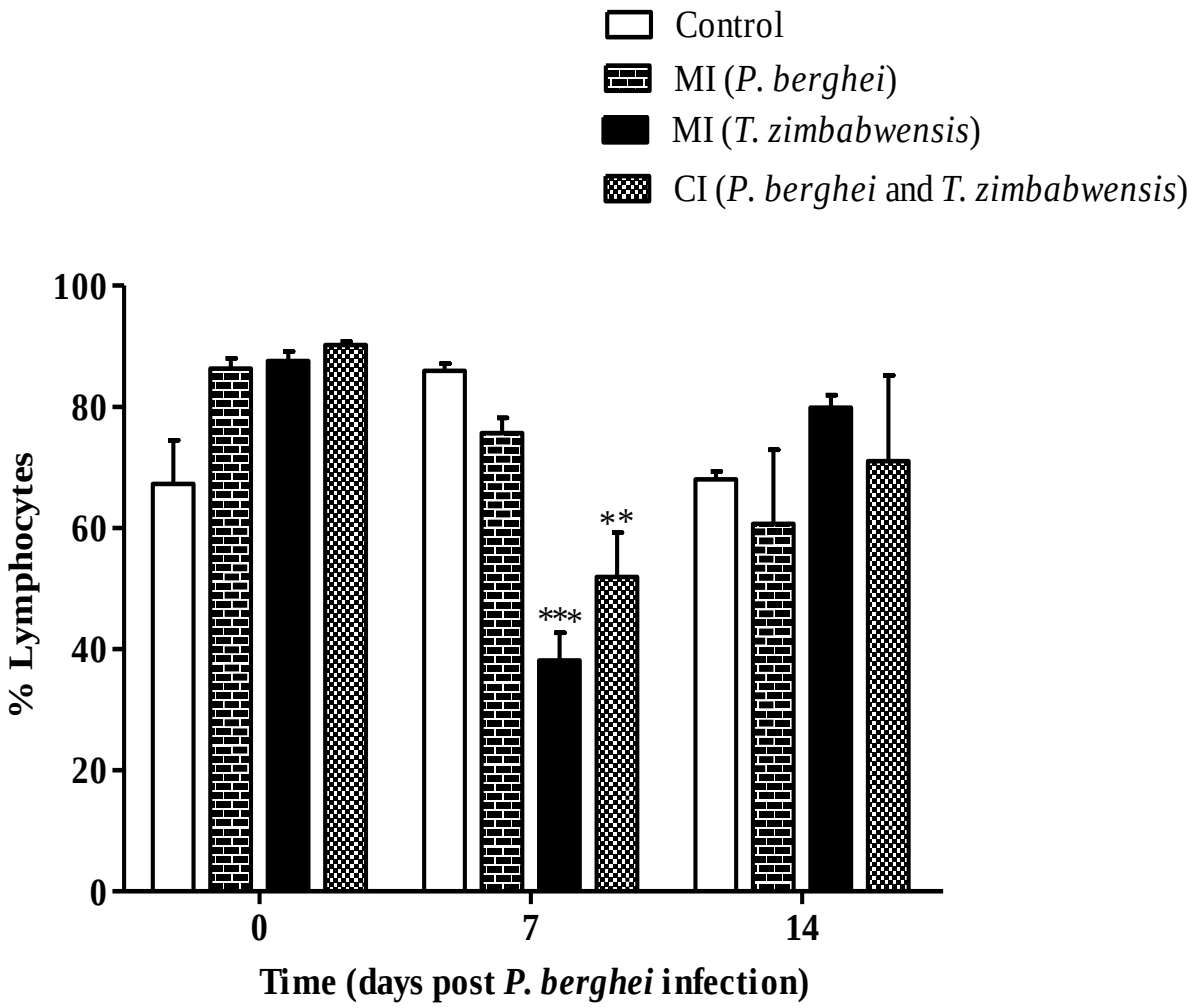

Figure 14: Comparison of the effects of *Plasmodium berghei* and *Trichinella zimbabwensis* mono-infection (MI) and co-infection (CI) on lymphocytes concentration in male Sprague-Dawley rats. Day 0 represents the day of *P. berghei* when *Trichinella* muscle larvae is now in the rat muscle at day 28 post *Trichinella* infection. Values are presented as means and vertical bars indicate SEM (n=6 in each group). * $P<0.05$, *** $P<0.001$.

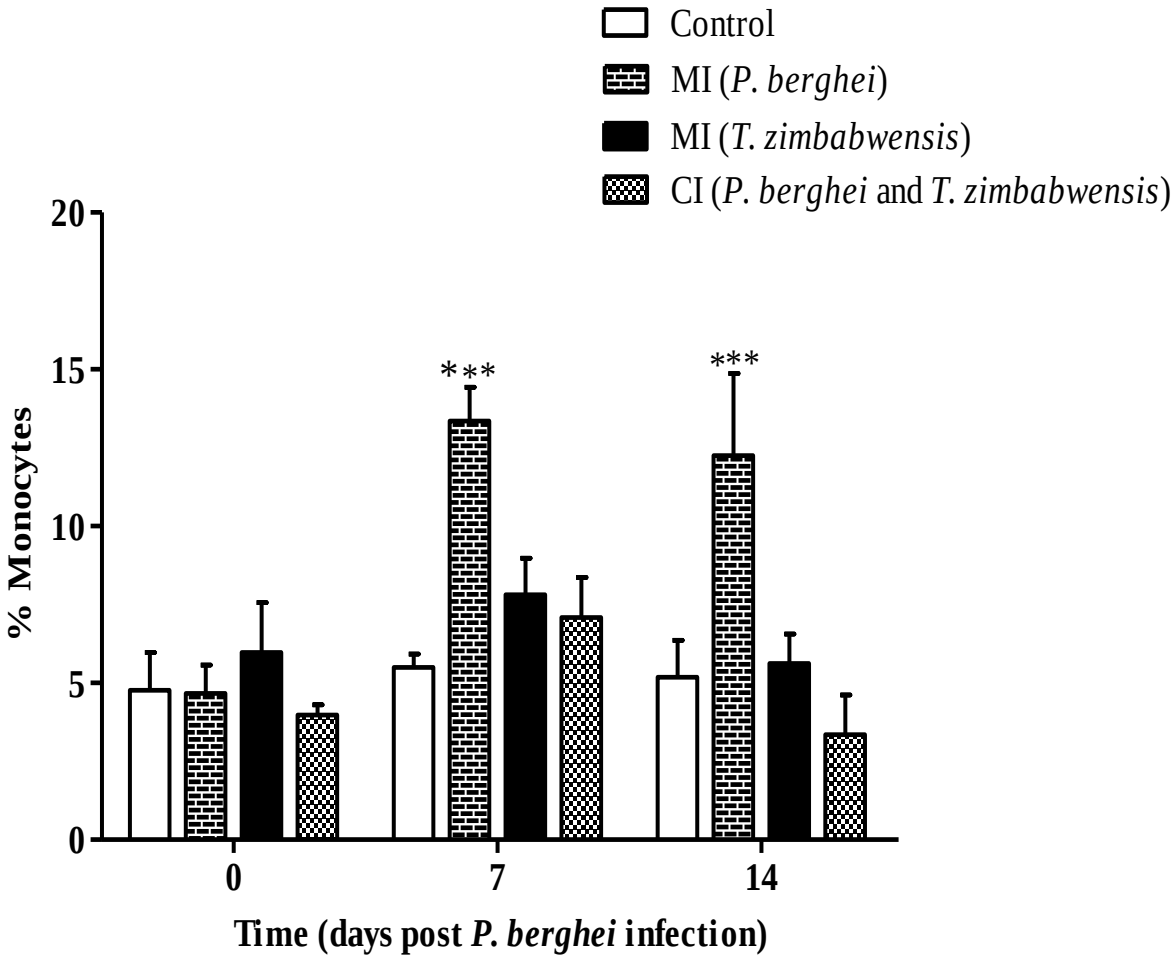

Figure 15: Comparison of the effects of *Plasmodium berghei* and *Trichinella zimbabwensis* mono-infection (MI) and co-infection (CI) on monocytes concentration in male Sprague-Dawley rats. Day 0 represents the day of *P. berghei* when *Trichinella* muscle larvae is now in the rat muscle at day 28 post *Trichinella* infection. Values are presented as means and vertical bars indicate SEM (n=6 in each group). *** $P<0.001$.

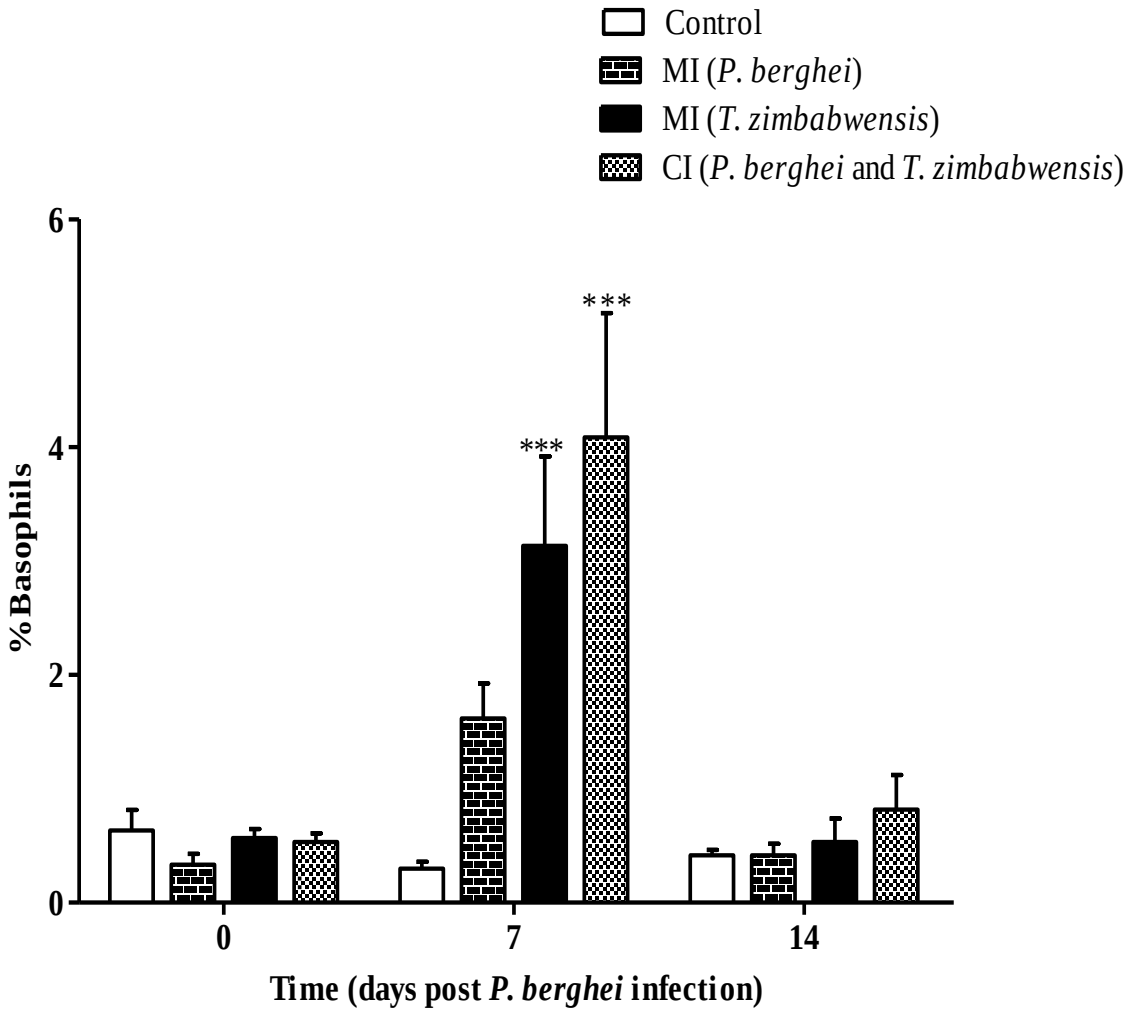

Figure 16: Comparison of the effects of *Plasmodium berghei* and *Trichinella zimbabwensis* mono-infection (MI) and co-infection (CI) on basophils concentration in male Sprague-Dawley rats. Day 0 represents the day of *P. berghei* when *Trichinella* muscle larvae is now in the rat muscle at day 28 post *Trichinella* infection. Values are presented as means and vertical bars indicate SEM (n=6 in each group). *** $P<0.001$.

4.4 Cytokines levels

TNF-α for the *P. berghei* (MI) group at day 7 post infection (535.6% ± 216.2%), was significantly different as compared to that of the control group (11.42% ± 4.06%). A decrease of (26.03% ± 11.75%) was also observed in the *T. zimbabwensis* (MI) group, however, *P. berghei* and *T. zimbabwensis* (CI) group decreased to (8.93% ± 1.50%). At day 14 p.i., decrease of (10.64% ± 1.68%), (8.16% ± 1.79%) and (6.74% ± 1.11%) was observed in all the groups *P. berghei* (MI), *T. zimbabwensis* (MI), and *P. berghei* and *T. zimbabwensis* (CI) group, although, these decrease was not statistically significant (P>0.05) (Fig. 17). At day 7 post infection, significant levels of Interleukin-10 (IL-10) concentrations was observed in the *P. berghei* (MI) group ($p < 0.001$) increase (3163% ± 14.8%) compare to the control (165.7% ± 27.27%), a slight increase of (169.1% ± 22.7%) was also observed in the *T. zimbabwensis* (MI) group, while a decrease of (156.9% ± 59.2%) was observed in the *P. berghei* and *T. zimbabwensis* (CI) group. At day 14 p.i., an increase of (322.8% ± 39.7%) and (192.4% ± 56.08%) was observed in the *P. berghei* (MI) group and *P. berghei* and *T. zimbabwensis* (CI) group respectively, while the *T. zimbabwensis* (MI) group decease to (145,6% ± 31.1%) compare to the control (165.7% ± 27.27%) (Fig.18). IL-4 and IL-6 did not yield result as the serum concentrations were below the lowest standard of the assay protocol.

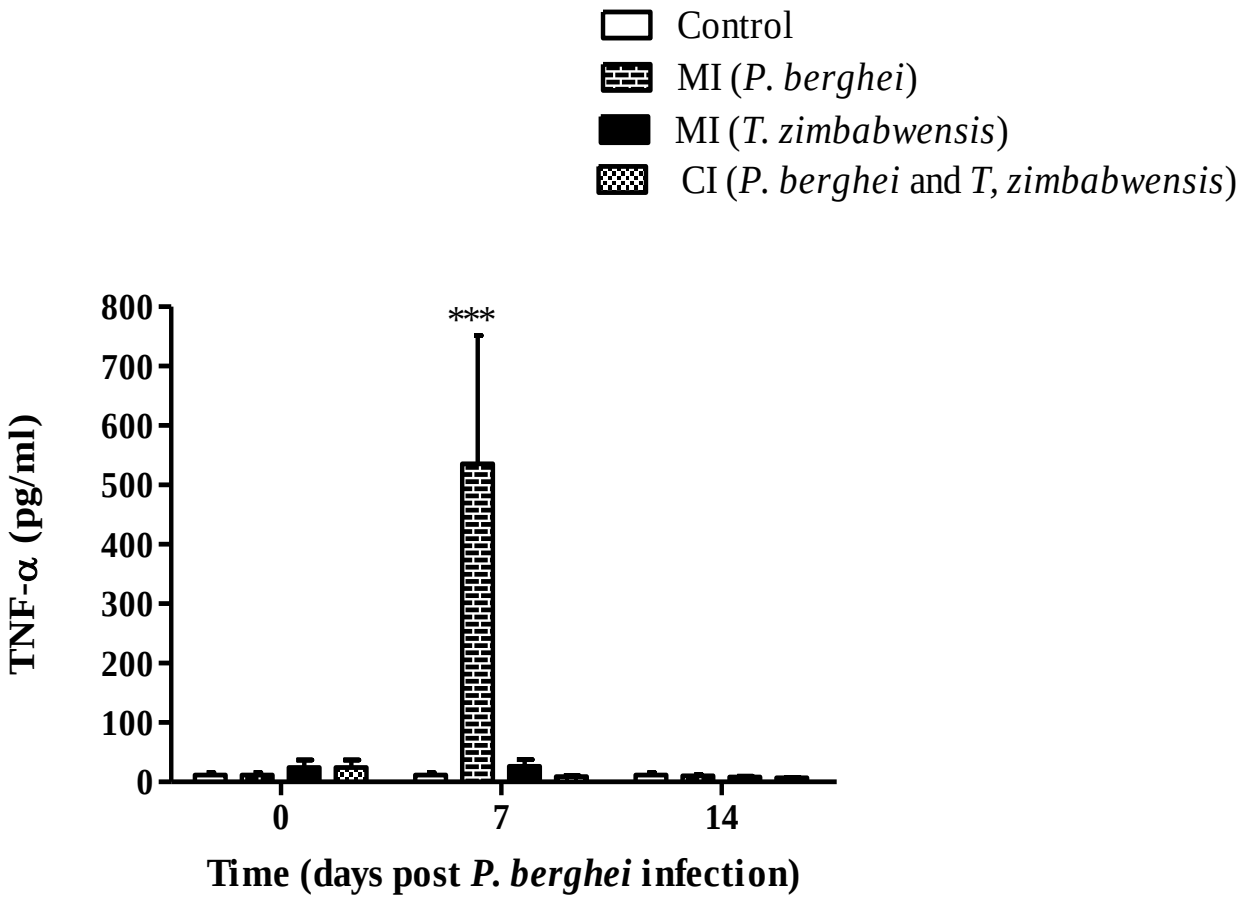

Figure 17: Comparison of the effects of *Plasmodium berghei* and *Trichinella zimbabwensis* mono-infection (MI) and co-infection (CI) on TNF-α concentration in male Sprague-Dawley rats. Day 0 represents the day of *P. berghei* when *Trichinella* muscle larvae is now in the rat muscle at day 28 post *Trichinella* infection. Values are presented as means and vertical bars indicate SEM (n=6 in each group). *** $P<0.001$.

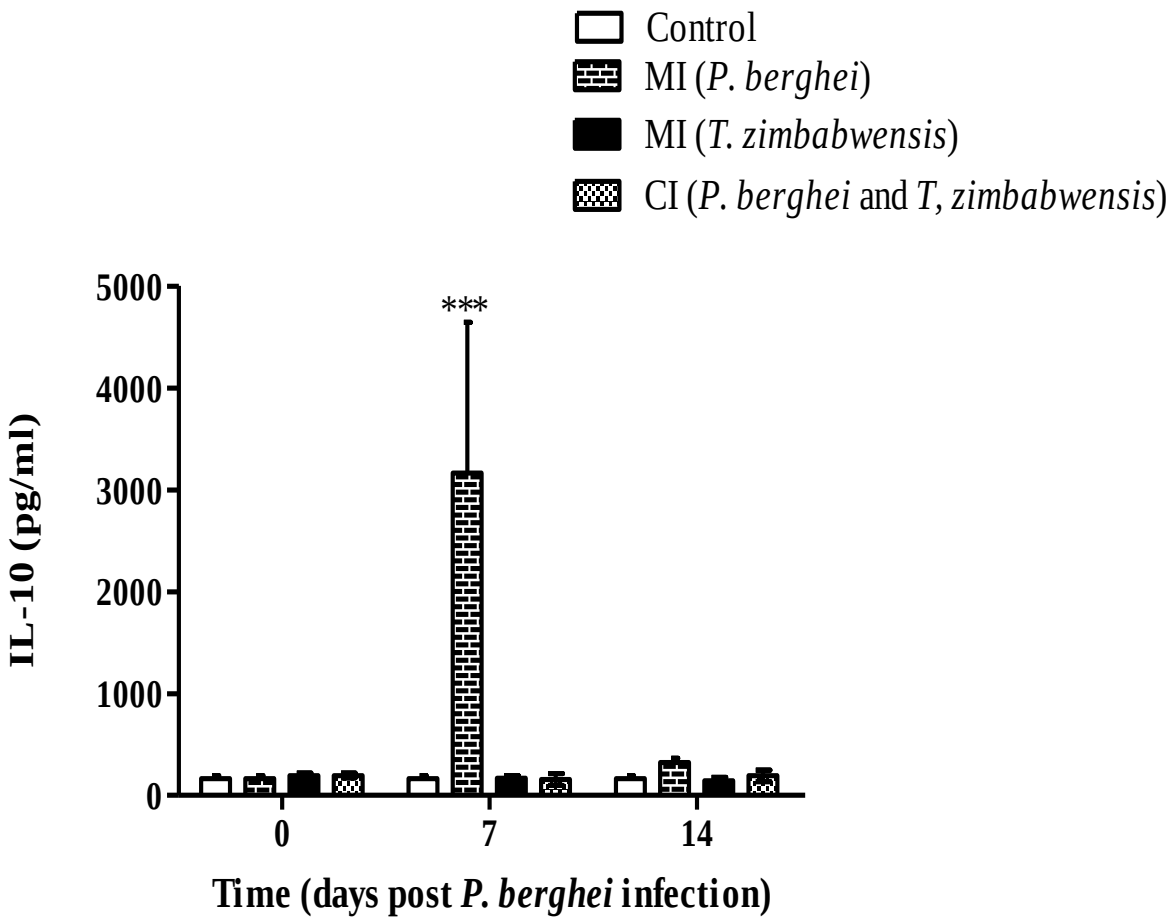

Figure 18: Comparison of the effects of *Plasmodium berghei* and *Trichinella zimbabwensis* mono-infection (MI) and co-infection (CI) on IL-10 concentration in male Sprague-Dawley rats. Day 0 represents the day of *P. berghei* when *Trichinella* muscle larvae is now in the rat muscle at day 28 post *Trichinella* infection. Values are presented as means and vertical bars indicate SEM (n=6 in each group). *** $P<0.001$.

5.0 CHAPTER 5: DISCUSSION

Helminths infections and malaria are common among populations in developing countries indicating that there may be interactions between parasites, as suggested by previous studies (Ademola and Odeniran, 2016). Over the years, most research has concentrated on single species parasite infections, recently there has been a resurgence of interest in contextual predictors of co-infcetions and an increased recognition of the global burden of disease attributable to neglected tropical diseases (NTDs). To the best of our knowledge, no studies have been carried out on the cytokine profile in Sprague-Dawley rats experimentally infected with *T. zimbabwensis and P. berghei* ANKA.

In the present study, we observed that *P. berghei* and *T. zimbabwensis* (CI) group showed rapid *P. berghei* development and increased parasitaemia than rats in the *P. berghei* (MI) group. A significant parasitaemia reduction was observed on day 3 and 4 in the *P. berghei* mono-infected group, and this demonstrates that depending on the existing immune interaction between a given host and malaria parasites, addition of a helminth co-infection may have contradicting effects on severity of malarial disease. This observation is in accordance with previous work (Bucher et al., 2011; Ademola and Odeniran, 2016) which also reported high parasitaemia during the early stages of malaria in chronic *S. mansoni* and *P. berghei* co-infected mice. The increase observed in parasitaemia in the co-infection group of rats may delay parasite clearance even after treatment (Legesse et al., 2004). The understanding of the mechanisms by which parasite interactions occur is still very limited. Antagonistic responses could aggravate the diseases pathologic effect of the infections, whereas, synergistic responses could decrease the diseases (Briand et al., 2005; Ademola and Odeniran, 2016; Babamale et al., 2017). In this present study, the antagonistic responses of the host to the parasite might have aggravated the infection in the *P. berghei* and *T. zimbabwensis* co-infected rats.

In resolving rodent malaria infections, blood parasitaemia is essentially controlled by an early and robust pro-inflammatory response and is kept under control by the anti-inflammatory cytokines IL-10 and TGF- β (Li et al., 2003). Increased inflammatory cytokines or decreased levels of anti-inflammatory cytokines have been found in severe malaria (Hunt and Grau, 2003; Sanni et al., 2004; Mitchell et al., 2005). The *Trichinella* dose and stage of infection could be one of key factors

influencing the size and direction of effect on malaria parasites in this study. There is also limited evidence from humans that dose effects may be very crucial in determining helminth-*Plasmodium* interactions (Nacher et al., 2002).

The results of the present study show that *T. zimbabwensis* adult worms persisted in the mono-infected and co-infected group in the small intestines from day 7 up to day 14 post infection. Our findings show that Th1-type cytokine and TNF-α were markedly produced as early as day 7 pi, which is not the case with *T. spiralis* infection (Fabre et al., 2009). No adult worms were recovered in both groups on day 21 post infection. *Trichinella zimbabwensis* muscle larvae (ML) were recovered from day 28 up to day 42 p.i. with an exponential increase in the *Trichinella* mono-infected group. This implies that the bulk of the larval migrations are mainly after day 21 p.i. Studies regarding the immune evasion mechanisms of *Trichinella* infection have been previously reported and in particular, IL-10 is reported to act as an inflammation regulator in cases of intestinal and muscle trichinellosis in mice (Park et al., 2011). Increased level of TNF-α cytokine was observed at day 7 p.i, which suggest that a protective immune response is established against *T. zimbabwensis* AW and NBL, which is geared towards AW expulsion, abrogation of inflammation caused by NBL or parasite killing (Harnett and Harnett, 2010) and eventual tissue healing (Maizels et al., 2009; Onkoba et al., 2015b). However, it is an established fact that IL-10 alone does not have effect on parasite survival, but in combination with TGF-β, parasite death occurs (Beiting et al., 2007). Helminth infections induce strong Th2 as well as regulatory T cell responses, and both of which are capable of dampening inflammatory responses (Maizels and Yazdanbakhsh, 2003; Maizels, 2009).

Significant decrease in the RBC in rats infected with *T. zimbabwensis* (MI) and *P. berghei* and *T. zimbabwensis* (CI) was observed on day 7 p.i, which is an indication of anaemia. This could be as a result of high destruction of erythrocytes and these results are in agreement with those reported earlier (Holy et al., 2015; Kanu et al., 2016). Blood parameters can be used as pathological and physiological indicators of animal health and one of the common pathological features observed in human as well as the experimental *T. zimbabwensis* mono-infected and co-infection groups is the loss of red blood cell resulting in anaemia and the degree of anaemia is an indicator of the disease severity (Ademola and Odeniran, 2016). Anaemia during trichinellosis might be due to either loss of RBC (Stijlemans et al., 2008). Anaemia is also one of the most common

complications in malaria infection in young children and pregnant women in hyperendemic areas (Ladhani et al., 2002; Maina et al., 2010). Decreased production of RBCs associated with malaria infection may also lead to development of anaemia through varied mechanisms (Fendel et al., 2010).

There was no significant difference in WBC count among the *P. berghei* and *T. zimbabwensis* mono-infection and co-infection groups. These results are comparable with other studies (Adedapo et al., 2007; Maina et al., 2010), which reported no significant difference in WBC between the *Plasmodium*-infected and non-infected groups. In contrast, other studies have demonstrated leukocytosis (Celik et al., 2009). Leukocytosis may be as a result of benign conditions such as inflammation-tissue necrosis, stress, hemolytic anaemia and infections (Kanu et al., 2016)**.** The present study demonstrates a significant low haemoglobin levels in the *P. berghei* (MI) group at day 7 and 14, and an increase in the *T. zimbabwensis* and co-infection groups. The decrease in the haemoglobin level is mostly associated with *Plasmodium* infection, and is caused by the destruction of infected RBCs and a decrease in cell production (Gansane et al., 2013). In sub-Saharan Africa, other factors which contribute to decreased haemoglobin levels in children include poor nutritional status, soil-transmitted helminths or other intestinal parasite infections and poor access to health care (Gansane et al., 2013). A significant decrease of haemotocrit was observed in the *P. berghei* (MI) group, *T. zimbabwensis* (MI) and *P. berghei* and *T. zimbabwensis* (CI) group as compared to the control group. This reduction in *P. berghei* is consistent with previous studies that have reported *Plasmodium*-induced anaemia (Menendez et al., 2000).

An increase in neutrophils at day 7 in the *P. berghei* and *T. zimbabwensis* (CI) group and a decrease in the co-infection group on day 14 was observed. The neutrophils are responsible for phagocytosis of pathogenic microorganisms and similarly, marked depression of precursor cells and marked phagocytosis of neutrophil precursor cell within the bone marrow and spleen may have been responsible for the severe drop in neutrophil number in the co-infection group on day 14 (Ademola and Odeniran, 2016). Cytokines and interleukins that are released after lysis of neutrophils plays crucial role in bone marrow stimulation to release neutrophils so that the production of neutrophils is increased (Maslachah and Sugihartuti, 2017). Neutrophils function as the first line of defense to eradicate *P. berghei* by phagocytizing parasites. This implies that infections of longer duration will elevate the percentage of parasitemia in the rats so that the increased numbers of parasites will

suppress the immune system (immunosupression), causing the immune system to become inadequate and unable to respond properly (Maslachah and Sugihartuti, 2017). High parasitemia rates will improve the mechanism of parasite invasion and suppress the host immune system (Onwuamah et al., 2010). Neutrophils play a crucial role in the immune response by modulating both cellular and humoral immunity through the synthesis and release of immunoregulatory cytokines (Maslachah and Sugihartuti, 2017).

Lynphocytes decreased at day 7 in the *T. zimbabwensis* mono-infection, co-infection groups and a decrease in the *P. berghei* group, at day 14 a gradual decrease was also observered in the *P. berghei* group. The decrease signifies lymphopaenia and suggests that *T. zimbabwensis* caused marked antigenic stimulation which leads to the acceleration and transformation of lymphocytes to plasma cells and transferred lymphocytes resulting to lymphopaenia. The decrease in lymphocytes counts associated with malaria observed in the study may be redistribution of lymphocytes with sequestration in the spleen (Erhart et al., 2004). Lymphocytes are involved in humoral and cellular immunity which is activated by T helper (Th) lymphocytes, which are a subset of T cells needed in the induction of immune responses against an invasion of foreign antigens (Maslachah and Sugihartuti, 2017). Moreover, an extremely severe course of trichinellosis may be accompanied by lymphopenia which is a manifestation of immunosuppression (Mukaratirwa et al., 2008). However, increase in the number of monocytes was observed in all the groups at day 7 compared to the control. These increases could be due to high demand for removal of particulate matter arising from severe pathology. The observation of increased monocytes counts in *P. berghei*, *T. zimbabwensis* mono-infection and co-infection groups from this study is in agreement with the earlier observations (Maina et al., 2010; Kotepui et al., 2014). Mononuclear cells are activated by *Plasmodium* during malarial infection and these cells produce inflammatory cytokines, such as interleukin-6 (IL6) and tumor necrosis factor (TNF). These cytokines help in the stimulation of the hepatic synthesis of acute phase inflammatory proteins, including CRP, which increase during malaria infection (Kotepui et al., 2014). Increased basophil was observed in *P. berghei, T. zimbabwensis* mono-infection and co-infection groups, the increase in basophils levels maybe as a result of hemolytic anaemia (Zainuddin, 2015). The increase in basophil count associated with malaria infection was in contrast with previous studies (Pelleau et al., 2012), which observed a decrease in the number of peripheral blood basophils that was related to the severity of symptoms. Such a decrease may be due to the recruitment and accumulation of these cells in the tissue.

TNF-α for the *P. berghei* mono-infected group at day 7 post infection increased significantly. The increased TNF-α levels suggest that TNF-α may be involved in the pathogenesis of malaria (Sahu et al., 2016b). A decrease in the levels of TNF-α was observed in *T. zimbabwensis* (MI) group, which is in agreement with the previous study (Onkoba et al., 2015b; Onkoba et al., 2016). This may imply that the parasite may be immune-modulating host immune system to initiate Th2 polarization that down-regulate production of pro-inflammatory cytokines (Harnett, 2014). Whereas, low levels of TNF-α and IFN-γ observed in the high transmission sites for malaria could be due to the reduction of T-cell subset (Ademolue et al., 2017). Previous studies have shown that prolonged stimulation of $CD4^+$ T-cells with increased level of antigens mediate adaptive peripheral tolerance, which is characterized by unresponsiveness to further stimulation, with an evident decrease in the secretion of TNF-α, IFN-γ, IL-2, and IL-6 (Mueller, 2010; Xing and Hogquist, 2012; Ademolue et al., 2017). The induction of a regulatory network of anti-inflammatory cytokines such as IL-10 and pro-inflammatory cytokine such as TNF-α might preclude pathology in the later stages of malaria infection. *Schistosoma haematobium* and *P. falciparum* co-infected patients had higher plasma levels of IFN-γ and similar levels of TNF-α, TGF-β and IL-10, compared to children infected with malaria only (Hartgers and Yazdanbakhsh, 2006). Increased levels of pro-inflammatory cytokines such as IFN-γ, IL-1, TNF-α and IFN-γ -induced protein (IP)-10 (CXCL10) form part of an early host response to the parasite and these cytokines have also been associated with severe anaemia, hyperglycemia and cerebral malaria in *P. falciparum* infection (Armah et al., 2007; do Rosario and Langhorne, 2012). Increased level of IL-10 was observed in the *P. berghei* (MI) group in this study. High levels of IL-10 have also been associated with increased disease severity in humans, including cerebral malaria, anaemia and respiratory distress (Prakash et al., 2006; Ong'echa et al., 2008; Ayimba et al., 2011; do Rosario and Langhorne, 2012). Studies regarding the immune evasion mechanisms of *Trichinella* infection have been previously reported, in particular, IL-10 operates as an inflammation regulator in cases of intestinal and muscle trichinellosis in mice (Park et al., 2011). According to previous studies, it has been demonstrated that $CD4^+CD25^+$ T cells producing IL-10 play a vital role during *Plasmodium* infection, possibly controlling the pro-inflammatory cytokines IFN-γ and TNF (Riley et al., 2006; Hojo-Souza et al., 2017). However, according to recent findings, IL-10 has been described as the strongest predictor of disease in endemic regions (Sinha et al., 2010) and

considered as a biomarker for inflammatory placental malaria (Kabyemela et al., 2008). This is in agreement with our data showing that at the point of peak parasitemia, the pro-inflammatory response is down-regulated and a concomitant increase in IL-10 is detected in the *P. berghei* mono-infected group. This finding may represent an attempt to balance the pro-inflammatory response and to prevent immune-mediated pathology.

IL-4 and IL-6 serum concentration were below the lowest standard of the assay protocol, these could be due to some other components of the assays such as the proprietary monoclonal antibody pairs that make them less sensitive. Another reason for not being able to detect IL-4 and IL-6 could be because of the presence of inhibitors that not only bind and inactivate the cytokines but also interfere with their detection. More importantly, there are inhibitors and antagonists in the plasma which often interfere with the immunoassays used to quantitate IL-4 and IL-6, this becomes very vital because the presence of these inhibitors or antagonists in the plasma may be used to document the presence of these pro-inflammatory cytokines indirectly.

6.0 CONCLUSIONS AND RECOMMENDATIONS

In conclusion, we observed that *P. berghei* and *T. zimbabwensis* (CI) group showed rapid development and increased parasitaemia than rats in the *P. berghei* (MI) group. Therefore, rats co-infected with *P. berghei* and *T. zimbabwensis* may aggravate the course of disease in the host by increasing parasitaemia, but elevated IL-10 plays a crucial role in the suppression of hepatic pathology in the host. Data presented here demonstrate that an early stage intestinal nematode infection can enhance malaria-associated pathology without necessarily altering immune reactivity, and larval infections may also alter disease progression in different ways. Increased IL-10 is essential in down-regulation of protective immunity against malaria parasites possibly due to its hematopoietic, immunoregulatory, and developmental properties.

Limitations

The main limitation of this study is that IL-4 and IL-6 did not produce result as concentrations were below the assay protocol. This could be due to some other components of the assays such as the proprietary monoclonal antibody pairs that make them less sensitive. The primary reason for not being able to detect IL-4 and IL-6 could also be due to the presence of inhibitors that not only bind and inactivate the cytokines but also interfere with their detection.

Recommendations

Future research studies are necessary to further investigate the impact of different *Trichinella* species on malaria infection, immunology, and pathology in order to understand the mechanism of immunoregulation during co-infection.

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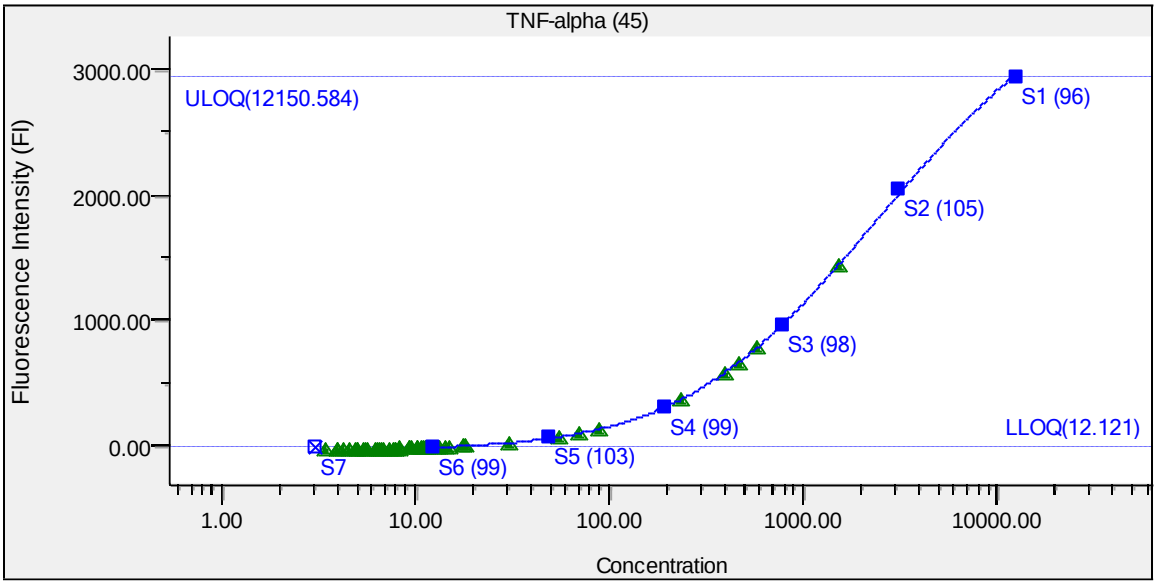
8.0 Appendices

8.1.1 TNF-α standard curve



Standard **Partial Outlier** **Outlier**

Unknown **Control**

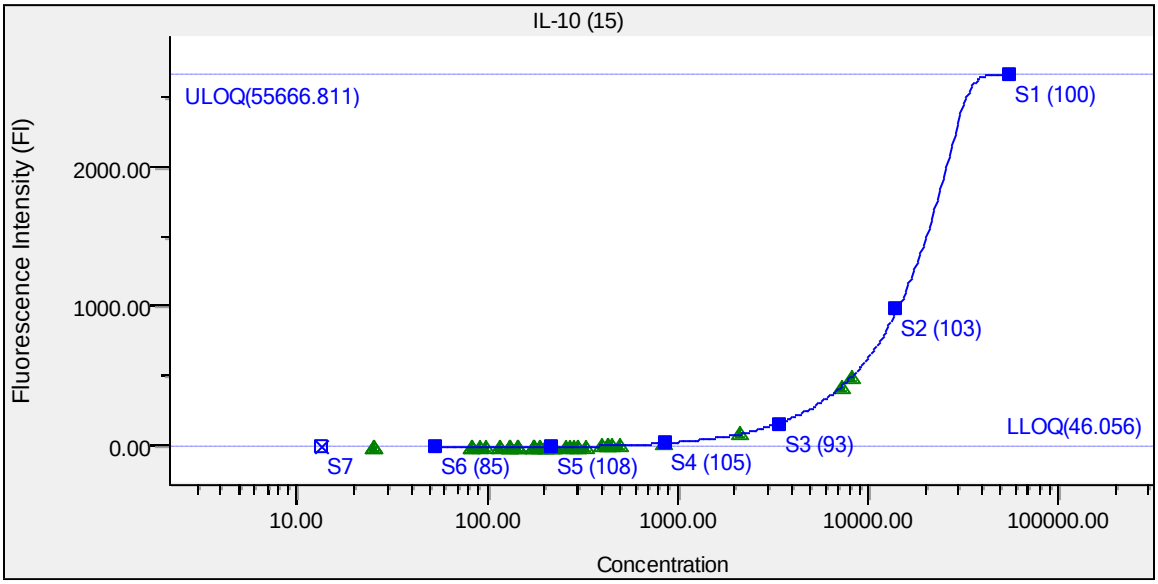
Regression Type: Logistic - 5PL

Std. Curve: FI = -24.4997 + (3917.24 + 24.4997) / ((1 + (Conc / 1370.96)^-0.72333))^1.49635

FitProb. = 0.4347, ResVar. = 0.6103

Figure 19: Standard curve showing the fluorescence intensity (FI) and concentration of TNF-α.

8.1.2 IL-10 standard curve



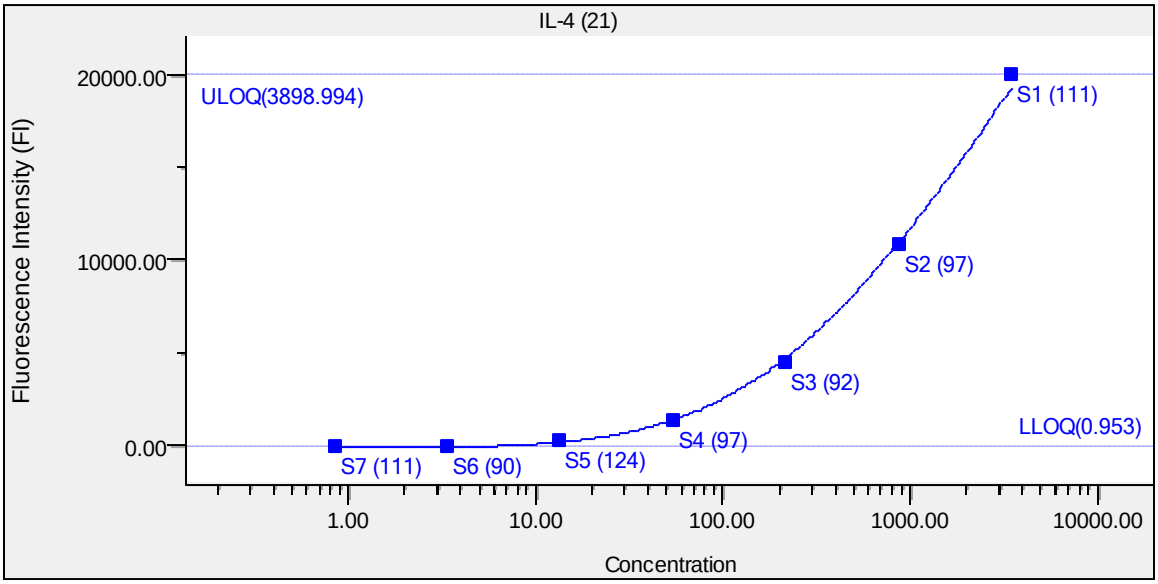
Regression Type: Logistic - 5PL

Std. Curve: FI = -8.98322 + (2683.92 + 8.98322) / ((1 + (Conc / 32578.4)^-12.0632))^0.1

FitProb. = 0.0335, ResVar. = 4.5211

Figure 20: Standard curve showing the fluorescence intensity (FI) and concentration of IL-10.

8.1.3 1L-4 standard curve



■ **Standard** □ **Partial Outlier** ☒ **Outlier**

▲ **Unknown** ▲ **Control**

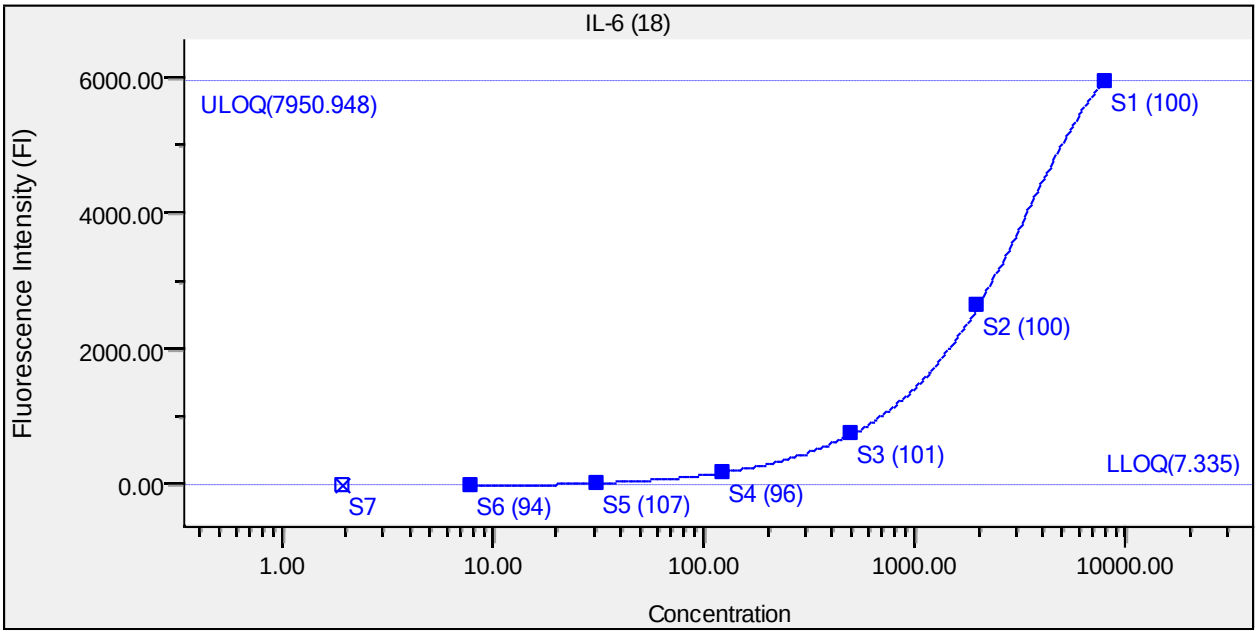
Regression Type: Logistic - 5PL

Std. Curve: FI = -8.4371 + (55447.2 + 8.4371) / ((1 + (Conc / 4.39396)^-0.32952))^10

FitProb. = 0.0000, ResVar. = 11.1243

Figure 21: Standard curve showing the fluorescence intensity (FI) and concentration of IL-4.

8.1.4 IL-6 standard curve



Regression Type: Logistic - 5PL

Std. Curve: FI = -14.4849 + (6713.82 + 14.4849) / ((1 + (Conc / 5247.42)^-2.4433))^0.37529

FitProb. = 0.4400, ResVar. = 0.5962

Figure 22: Standard curve showing the fluorescence intensity (FI) and concentration of IL-6.

8.1.5 UKZN ethical approval 2016

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

16 May 2016

Dr Pretty Murambiwa
School of Life Sciences
Westville Campus

Dear Dr Murambiwa,

Protocol reference number: AREC/018/016PD
Project title: Immune responses in experimental animal paradigms co-infected with *Trichinella. zimbabwensis* and *Plasmodium berghei*

Full Approval – Research Application

With regards to your revised application received on 05 May 2016. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 16 May 2017.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

.....
Dr Sanil Singh
Deputy Chair: Animal Research Ethics Committee

/ms

Cc Supervisor: Professor S Mukaratirwa
Cc Academic Leader Teaching & Learning: Professor A Olaniran
Cc Registrar: Mr Simon Mokoena
Cc NSPCA: Ms Jessica Light
Cc BRU – Dr Sanil Singh

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Website: http://research.ukzn.ac.za/Research-Ethics/Animal-Ethics.aspx

8.1.6 UKZN ethical approval 2017

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

12 June 2017

Dr Pretty Murambiwa (16834)
School of Life Sciences
Westville Campus

Dear Dr Murambiwa,

Protocol reference number: AREC/018/016PD
Project title: Immune responses in experimental animal paradigms co-infected with *Trichinella. zimbabwensis* and *Plasmodium berghei*

Full Approval – Renewal Application

With regards to your renewal application received on 31 May 2017. The documents submitted have been accepted by the Animal Research Ethics Committee and **APPROVAL** for the renewal has been granted.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 12 June 2018.

Attached to the Approval letter is a template of the Progress Report that is required at the end of the study, or when applying for Renewal (whichever comes first). An Adverse Event Reporting form has also been attached in the event of any unanticipated event involving the animals' health / wellbeing.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

.....

Dr S Islam, PhD
Chair: Animal Research Ethics Committee

/ms

Cc Supervisor: Professor S Mukaratirwa
Cc Dean & HoS: Professor A Olaniran
Cc Registrar: Mr Simon Mokoena
Cc NSPCA: Ms Stephanie Keulder
Cc BRU – Dr Sanil Singh

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