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SERUM BIOCHEMICAL PARAMETERS AND CYTOKINE PROFILES ASSOCIATED WITH ANIMAL AFRICAN TRYPANOSOMIASIS IN NATURALLY INFECTED CATTLE IN GHANA

BY

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THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF MPHIL MOLECULAR CELL BIOLOGY OF INFECTIOUS DISEASES DEGREE

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DECLARATION

I, Soale Majeed Bakari, hereby declare that this thesis is the outcome of my own research project under the supervision of Dr. Theresa Manful Gwira (Department of Biochemistry, Cell and Molecular Biology, University of Ghana) and Dr. Kwadwo Asamoah Kusi (Noguchi Memorial Institute for Medical Research, University of Ghana). To the best of my knowledge, no part of this thesis has either been presented for the award of any degree or previously published by another author, except where due citation is made in the text.

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ABSTRACT

Animal African Trypanosomiasis (AAT) greatly affects livestock production in sub-Saharan Africa. In Ghana prevalence of AAT is estimated to range between 5 – 50%. Studies have reported serum biochemical aberrations and variability in cytokine profiles in animals during infection. However, information regarding the biochemical parameters and cytokine profiles associated with natural infections are limited. This study was therefore aimed at establishing the levels of serum biochemical parameters and cytokine profiles in naturally infected cattle over a period of six months in 40 individual cattle at 4 – 5 weeks intervals. Nested Internal Transcribed Spacer (ITS)-based PCR and sequencing were used to characterize trypanosome infection in cattle at two areas (Adidome and Accra) of different endemicities. Levels of serum biochemical parameters (Creatinine, Cholesterol, Alkaline phosphatase (ALP), Alanine aminotransferase (ALT), Total bilirubin and Total protein) and cytokines (IL-10, IL-4, IFN-γ, TNF-α and IL-12) were measured in serum samples collected and then compared between infected cattle and uninfected controls.

The predominant trypanosome species detected in Accra (non-endemic) and Adidome (endemic) were Trypanosoma theileri and Trypanosoma vivax respectively. Serum biochemical parameters were similar between infected and uninfected cattle in Accra. Infected cattle at Adidome however had significantly higher levels of ALP (infected = 613.15U/L, uninfected = 342.5U/L, p = 0.02), Creatinine (infected = 101.90 µmol/L, uninfected = 77.75 µmol/L, p = 0.01), Total Protein (infected = 77.14 µmol/L, uninfected = 71.26 µmol/L, p = 0.01) and Total Bilirubin (infected = 2.45 µmol/L, uninfected = 1.96 µmol/L, p = 0.04) and significantly lower levels of cholesterol (infected = 2.45 mmol/L, uninfected = 3.15 mmol/L, p = 0.02) at specific time points.
At basal levels and during infection, significantly higher pro-inflammatory/anti-inflammatory (Th1/Th2) cytokine ratios were observed in cattle at Adidome compared to Accra, indicating a shift towards Th1 immune response in Adidome. Levels of IL-10 and TNF-α were however significantly elevated in infected cattle in Accra, suggesting high anti-inflammatory cytokine response in Accra. These results generally demonstrate that cattle in the non-endemic area, which were predominantly infected with non-pathogenic trypanosome species, have different biochemical profiles and immune responses compared to cattle in the endemic area with predominantly pathogenic trypanosome species.
DEDICATION

I dedicate this work to the memory of my late father Yapeiwura Joseph Bakar Soale who was keen on providing the best education for me and my siblings. I also dedicate this work to my lovely family.
ACKNOWLEDGEMENT

I give thanks to the Almighty God for his guidance, protection and the abundance of grace He bestowed on me throughout my period of study. I thank my able supervisors, Dr. Theresa Manful Gwira and Dr. Kwadwo Asamoah Kusi for their invaluable support, advice and suggestions towards the successful completion of this thesis. I acknowledge the West African Centre for Cell Biology of Infectious Pathogens (WACCBIP) for awarding me a fellowship that funded part of my research work. I also acknowledge the Leverhulme Trust and The Royal Society for funds awarded to Dr. Theresa Manful Gwira from which my project benefited immensely.

My Sincere thanks to all members of the TMG lab, most especially Ms. Jenifer Afua Ofori and Mr. Godwin Ametsi for their support. I also express my appreciation to Dr. Regina Appiah-Opong, Mr. Eric Kyei-Baafour, Mrs. Dorotheah Obiri, members of the Bakari family and all others who have offered diverse assistance towards successful completion of this work.
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<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>AAT</td>
<td>Animal African Trypanosomiasis</td>
</tr>
<tr>
<td>ApoL1</td>
<td>Apolipoprotein 1</td>
</tr>
<tr>
<td>ADAMSEL</td>
<td>Auditable Data Analysis and Management System for ELISA</td>
</tr>
<tr>
<td>CATT</td>
<td>Card Agglutination Test for Trypanosomiasis</td>
</tr>
<tr>
<td>CSIR</td>
<td>Council for Scientific and Industrial Research</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked Immunosorbent Assay</td>
</tr>
<tr>
<td>IFAT</td>
<td>Indirect Fluorescent Antibody Test</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IL-10</td>
<td>Interleukin 10</td>
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<td>IL-12</td>
<td>Interleukin 12</td>
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<td>Interleukin 13</td>
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<tr>
<td>IL-4</td>
<td>Interleukin 4</td>
</tr>
<tr>
<td>ITS</td>
<td>Internal Transcribed Spacer</td>
</tr>
<tr>
<td>LAMP</td>
<td>Loop-Mediated Isothermal Amplification</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
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</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>NASBA</td>
<td>Nucleic Acid sequence based amplification assay</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SRA</td>
<td>Serum resistance-associated</td>
</tr>
<tr>
<td>TbgsGP</td>
<td>\textit{T. b. gambiense-specific glycoprotein}</td>
</tr>
<tr>
<td>TLF</td>
<td>Trypanosome Lytic Factor</td>
</tr>
<tr>
<td>TNF-(\alpha)</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>VSG</td>
<td>Variable (Variant) surface glycoproteins</td>
</tr>
<tr>
<td>WASH</td>
<td>West African shorthorn</td>
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CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND

Animal trypanosomiasis remains a major constraint to livestock production in sub-Saharan Africa despite various strategies employed to control, treat and eradicate the disease. The disease is caused by several species of trypanosomes, and the species and strain of parasite can have great influence on the severity and course of infection, and also the epidemiology of host-parasite-vector relationships (Auty et al., 2015). The major pathogenic trypanosome species responsible for causing disease in animals are Trypanosoma congolense, T. vivax, T. brucei brucei and T. simiae (Adamu et al., 2009). These pathogenic species are the most commonly detected trypanosomes in Ghana. Data generation for epidemiological studies relevant for development of good treatment and control strategies to protect livestock against trypanosomes is dependent on correct detection and identification of causative species (Thumbi et al., 2008).

Although Polymerase Chain Reaction (PCR) based diagnostic methods have improved the detection of trypanosomes, some of these methods are relatively expensive and less sensitive compared to others. Nested Internal Transcribed Spacer (ITS) based PCR, a multi-species detection technique has been established to be less expensive, fast and accurate in detecting trypanosomes even in cases of low parasitaemia (Cox et al., 2005; Thumbi et al., 2008).

Trypanosome infection results from the bite of an infected tsetse fly and some other flying insects such as tabanids. The parasite survives and is free living in mammalian blood and other body tissues. Trypanosomes thrive in the host, evading the host immune system by changing
their surface antigenic coat, the variable surface glycoproteins (VSG) (Sow et al., 2014). Various pathological disorders result following trypanosome infection. Abnormalities in mammalian host physiology such as changes in blood pH, hormones, nutrients and levels of metabolites such as cholesterol and creatinine occur through the course of infection (Sanni et al., 2013; Sow et al., 2014). Infection is usually chronic, characterized by intermittent fever, anaemia, lymphadenopathy and weight loss (Itard, 1989; Osório et al., 2008). In a study carried out by Adamu et al. (2009), serum levels of cholesterol, triglycerides and high density lipoproteins were found to be significantly lower in Trypanosoma brucei infected pigs. Also, in a recent study by Sow et al. (2014), serum gamma-globulin and total protein levels were found to be elevated in trypanosome-infected goats. Other studies have revealed significant increases in levels of sodium, calcium, phosphate and blood urea nitrogen and a significant decline in levels of potassium and bicarbonate in the sera of rabbits experimentally infected with Trypanosoma congolense (Ameen et al., 2011). There are a few conflicting reports on serum biochemical changes in mammals due to trypanosome infection. However, the infection has been generally shown to exhibit haematological, pathological and serum biochemical aberrations (Takeet & Fagbemi, 2009). Serum biochemical and haematological parameters therefore constitute important indices for disease diagnosis and prognosis (Onasanya et al., 2015). Aside the effects of infections on the levels of serum biochemical parameters, genetic factors such as breed and genotype of animal, non-genetic factors such as age, sex, management system, medication and environmental factors such as nutrition and climate may also influence the levels of these serum biochemical and haematological parameters in animals (Onasanya et al., 2015).

In the blood stream of mammals, trypanosomes encounter innate immune response as a first and rapid line of defense and later adaptive immune responses. These immune response mechanisms
are mediated by various cytokines. A shift from the production of pro-inflammatory cytokines at the beginning of infection to anti-inflammatory cytokines in late or chronic infection is correlated with the ability of the host to reduce the parasitaemia and pathology, respectively (Baral, 2010). Namangala et al. (2001a) observed in mouse models that a balance between pro- and anti-inflammatory cytokines is central to the outcome of the disease. Previous studies have demonstrated the production of Interferon gamma (IFN-γ), Interleukin 12 (IL-12), Nitric Oxide (NO) and Tumor necrosis factor alpha (TNF-α) in early stages of infection and later Interleukin 13 (IL-13), Interleukin 4 (IL-4) and Interleukin 10 (IL-10) (Taylor & Mertens, 1999; Uzonna et al., 1999; Baral, 2010). IL-10 was shown to play a major role in maintaining the balance between pathogenic and protective immune responses during African trypanosomiasis (Namangala et al., 2001b). Severity and progression of the disease has not only been linked to parasite diversity but also to host related differences regulating immune responses (Kato et al., 2016). There is information on the prevalence of trypanosomiasis in Ghana but there is no reported literature on lifetime infection. Also, studies have not shown how serum biochemical parameters change over a period of infection and whether there are changes in the levels and types of cytokines as infection persist under natural conditions.

1.2 PROBLEM STATEMENT

During trypanosome infection, changes in serum biochemical, metabolic and cytokine profiles have been shown to occur. In previous studies, it has been shown that trypanosomiasis induces changes in the concentration of biochemical constituents in the blood of infected animals (Adamu et al., 2009; Takeet & Fagbemi, 2009; Ameen et al., 2011; Sazmand et al., 2011; Sanni et al., 2013; Sow et al., 2014). Also, immune response in the form of cytokine production has
been demonstrated (Darji et al., 1996; Taylor & Mertens, 1999; Uzonna et al., 1999; Baral, 2010; Namangala et al., 2001b). However, most of these studies were carried out on experimentally infected animals with only a single species of trypanosome and this does not reflect what happens in the field where animals are infected with different trypanosome species of varying pathogenicity including mixed infections. Studies carried out were also mainly cross sectional studies and, therefore, did not monitor the changes in cytokine profiles and serum metabolites/biochemical parameters over a period. Current knowledge of the serum biochemical levels and cytokine profiles during natural trypanosome infections in cattle is, therefore, limited.

1.3 JUSTIFICATION

Animal African trypanosomiasis has significant impact on livestock productivity. The disease causes huge economic losses due to reduced production of livestock for food, transportation and traction. Trypanosome infections are usually characterized by waves of parasitaemia, fever and anaemia leading to high morbidity and mortality. The parasites invade vital body organs such as kidneys, spleen and liver, leading to changes in biochemical profiles and cytokine production. These biochemical parameters and cytokine profiles constitute important physiological indices which have major health implications. There is, however, a gap in knowledge relating to the metabolic changes and cytokine profiles during natural trypanosome infections in cattle. Hence, the extent of damage caused by trypanosome infection in animals and the role of cytokines in immune response is not fully understood.

This study was, therefore, designed to enhance our understanding on the levels of serum biochemical parameters and cytokine profiles during the course of natural trypanosome infections. The research will thus provide insight into the pathophysiology and immune response
associated with pathogenesis of the disease in cattle, and this could lead to the identification of novel biomarkers. The research will also provide useful information on the different pathogenic species of trypanosomes prevalent in Ghana, thereby guiding policy on the procurement of chemotherapeutic agents in the country.

1.4 AIM OF STUDY

To characterise animal African trypanosomes and determine the associated levels of serum biochemical parameters and cytokine profiles in naturally infected cattle in Ghana over a 6 month period.

1.4.1 Specific Objectives

Specifically the study seeks to;

1. Detect and characterise trypanosome species from infected cattle using nested ITS-based PCR and sequencing.

2. Determine and compare levels of serum biochemical parameters among infected and non infected cattle at different times.

3. Determine the serum levels of IL-10, IL-4, IFN-gamma, TNF-alpha and IL-12 among the cattle and compare cytokine levels to respective infecting trypanosome species in infected cattle.
CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 TRYPANOSOMIASIS

Trypanosomiasis is a disease of humans and animals caused by several species of protozoan parasites, the trypanosomes, belonging to the genus *Trypanosoma* (Barrett *et al*., 2003). Trypanosomes are grouped into African and American trypanosomes based on their geographical distribution. African trypanosomiasis is mostly present in tropical countries in western, central and eastern Africa, extending to parts of southern Africa. The American trypanosomiasis on the other hand occurs in Mexico and in central and South America (WHO, 2015). During infection, trypanosomes persist for some time in the host bloodstream, evading immune responses and invading vital body organs such as liver, spleen and kidneys (Takeet & Fagbemi, 2009; Pays *et al*., 2014). This sometimes leads to serum biochemical aberrations (Takeet & Fagbemi, 2009). Some clinical manifestations of the disease include; listlessness, fever, emaciation, hair loss, discharge from the eyes, edema, anaemia and paralysis (Steverding, 2008). This leads to economic loss due to decreased productivity of the animals for food and transportation. Annual economic losses as a result of trypanosomiasis are estimated at 4.5 billion US dollars (Delespaux *et al*., 2010).

2.2. TRANSMISSION AND DISTRIBUTION OF AFRICAN TRYPANOSOMIASIS

The primary vector for *T. congolense*, *T. brucei* and *T. vivax* is the tsetse fly of the genus *Glossina*. The parasites have a developmental stage in the tsetse fly and are transmitted via the
saliva when an infected fly takes a blood meal. Trypanosomiasis is also mechanically transmitted by other biting insects such as tabanids.

The geographical distribution of trypanosomiasis in Africa largely corresponds to the distribution of tsetse flies (*Glossina spp.*) and covers an area of 8 million kilometers between latitude 14 degrees North and 20 degrees South (Figure 2.1) (Steverding, 2008). This area stretches from the southern part of the Sahara desert (lat. 15° N.) to Zimbabwe, Angola and Mozambique (lat. 20° S.) (Maré, 2004). In some instances however, the distribution of the vector and the disease do not correspond due to movement of the host and the presence of other modes of transmission (Desquesnes *et al.*, 2013). This is evident in the observance of *Trypanosoma vivax* in the western hemisphere (Caribbean, South and Central America) where tsetse flies are not present (Cadioli *et al.*, 2015).

![Figure 2.1: Distribution of tsetse flies in Africa. Dark portions of the map represents areas where trypanosomiasis is distributed. Adapted from www.fao.org/docrep.](http://ugspace.ug.edu.gh)
2.3. TRYPANOSOMIASIS IN GHANA

Ghana is located within the tsetse fly belt in sub-Saharan Africa and cases of trypanosomiasis have been detected in Ghana over some years now. No new cases of human African trypanosomiasis have been reported in Ghana in over a decade (WHO, 2015). An isolated case was, however, reported at Takoradi in 2013 (Cuypers et al., 2016). Animal African trypanosomiasis on the other hand is still widely distributed in Ghana and is a major constraint limiting livestock production in tsetse-infested areas in Ghana (Mahama et al., 2003). According to Mahama et al. (2003) in surveys carried out in Ghana over the years, Glossina palpalis s.l., G. tachinoides, G. morsitans s.l. and G. longipalpis have been found to be the prevalent economically important species of tsetse flies. The morsitans group was found to be linked with wild animals in open woodland savannah, the transitional forest zones had G. longipalpis and in the forested southern part of the country the fusca group of tsetse species were predominant (Adam et al., 2012). These tsetse species play a role in transmission of trypanosomiasis in Ghana since tsetse flies are the main vectors for transmission of the disease. Prevalence of animal trypanosomiasis as reported from surveys carried out between 1995-2001 ranges from 5% in low risk areas (Dangbe west and Nanton districts) to 50% in high risk areas (West Mamprusi and Damongo districts) (Mahama et al., 2003). Nakayima et al. (2012), in a study carried out in Koforidua and Adidome using molecular tools, estimated prevalence of trypanosomes to be 17.4%, 57.5% and 28.6% in tsetse flies, cattle and pigs, respectively. Trypanosoma congolense savannah and T. congolense forest were detected as the endemic subgroups of trypanosomes in these areas. A high prevalence of mixed infection of trypanosomes has also been reported in the Winneba District using serological methods (Turkson, 1993). In urban and suburban areas the
prevalence of trypanosomiasis has declined appreciably due to a decline in the number of tsetse flies, hence less contact between domestic animals and the vector (Mahama et al., 2003).

2.4. ANIMAL AFRICAN TRYPANOSOMES

Trypanosomes are unicellular eukaryotic parasites that vary in size from 8 to over 50 µm. These parasites are well adapted to residing and moving in the blood plasma or tissue fluid of the host. They infect several animals but appear to be pathogenic in mammals including humans (Fineile et al., 1983). The animal African trypanosomes are the animal infecting species of the parasite that cause the disease complex, animal African trypanosomiasis (AAT). Species of trypanosomes that causes AAT include; Trypanosoma congolense, T. vivax and T. brucei brucei found in several domestic animals (cattle, horses, goats, sheep, dogs and camels) and wild animals; T. simiae, and T. godfreyi mainly in domestic and wild pigs and T. suis in only wild pigs; T. evansi which is primarily a camel parasite but may also be found in horses, dogs, cattle, water buffaloes and elephants; T. equiperdum, which causes a transmissible disease (durine) in equines and the non-pathogenic T. theileri found in domestic and wild animals (Fineile et al., 1983; Uilenberg & Boyt, 1998). Trypanosoma congolense, T. vivax, T. simiae and T. brucei brucei are transmitted cyclically by tsetse flies. Trypanosoma evansi and T. vivax are mechanically transmitted by other biting flies such as tabanids. Trypanosoma equiperdum on the other hand is primarily transmitted sexually. The trypanosomes that cause AAT are grouped into three main subgenera, Duttonela (T. vivax T. congolense and T. uniforme), Nannomonas (T. congolense, T. simiae and T. godfreyi) and Trypanozoon (T. brucie brucei) (Uilenberg & Boyt, 1998).
2.4.1. *Trypanosoma congolense*

*Trypanosoma congolense* belongs to the sub-genus *Nannomonas*, and is the most important cause of AAT in cattle and other domestic animals (Maré, 2004). Although this species is small compared to other pathogenic trypanosomes, it is the most prevalent pathogenic African trypanosome (Coustou *et al.*, 2010). Various types or sub-divisions of *T. congolense* have been identified. They include; *T. congolense* savannah, *T. congolense* kilifi, *T. congolense* reverine/forest and *T. congolense* Tsavo.

2.4.2. *Trypanosoma brucei brucei*

*Trypanosoma brucei brucei* belongs to the sub-genus *Trypanozoon* and is a sub-species of *Trypanosoma brucei*. It is normally transmitted by the tsetse fly and is pathogenic to cattle and other animals. *T. brucei brucei* unlike the other two subspecies of *T. brucei* (*T. brucei gambiense* and *T. brucei rhodesiense*) cannot infect humans due to the presence of a serum innate immune factor called the Trypanosome Lytic Factor (TLF) in human blood (Alsford *et al.*, 2014). A component of the TLF, Apolipoprotein 1 (ApoL1) causes lysis of the trypanosome parasite upon uptake into acidic endosomes (Thomson & Finkelstein, 2015).

2.4.3. *Trypanosoma vivax*

*T. vivax* is the only pathogenic African trypanosome found outside Africa and belongs to the subgenus *Dutonella*. In Africa, *T. vivax* is a heteroxenous parasite present in tsetse fly (*Glossina*) populated regions; however outside the tsetse fly zones, such as in South America, the parasite is carried by other blood feeding flies where transmission is non-cyclical (Osório *et al.*, 2008).
Clinical manifestation as a result of *T. vivax* infection is similar to that of other pathogenic animal African trypanosomes.

### 2.4.4. *Trypanosoma theileri*

*Trypanosoma theileri* is considered as a non-pathogenic hemoparasite in cattle (Villa *et al.*, 2008). *T. theileri* infected cattle have however been described in some studies to exhibit clinical manifestations characteristic of trypanosomiasis (Doherty *et al.*, 1993; Villa *et al.*, 2008). It belongs to the subgenus *Megatrypanum* and is widely spread globally from the tropics to near the Arctic Circle, with higher prevalence in tropical and neo-tropical areas (Rodrigues *et al.*, 2006). Transmission is by ticks and biting insects belonging to the family tabanidae.

### 2.4.5. *Trypanosoma simiae*

*Trypanosoma simiae* belongs to the sub-genus *Nannomonas* and causes an acute rapidly fatal disease in pigs (Sturm *et al.*, 1998). Transmission is by cyclical means involving the insect vector, tsetse fly. Although *T. simiae* belongs to the same sub-genus as *T. congolense*, it is not known to cause disease in cattle.

### 2.4.6. *Trypanosoma evansi*

This trypanosome is primarily transmitted mechanically by biting flies and it is spread outside the tsetse belt in Africa, towards the Middle East and Southern Asia (Desquesnes *et al.*, 2013). *Trypanosoma evansi* has a wide host spectrum. In Africa, camels are the principal host, in Central and South America, the horse is mainly affected and in Asia cattle, buffaloes and pigs are important hosts (Eyob & Matios, 2013). The parasite also causes disease in mules, donkeys,
dogs and cats. Clinical manifestations of the disease due to *T. evansi* infection include; fever, anemia, weight loss, swelling of hind limbs, lethargy and hemostatic abnormalities (Mekata *et al.*, 2013). Aside the difference in transmission compared to other African trypanosomes, *T. evansi* also has the ability to invade host tissues (Desquesnes *et al.*, 2013).

2.5. LIFE CYCLE OF AFRICAN TRYPANOSOMES

The life cycle of the African trypanosomes involves a stage in the insect vector and another stage in the mammalian host (Figure 2.2). Transmission of African trypanosomes from one animal to the other is initiated when infected blood feeding tsetse flies inject metacyclic trypomastigotes into the host during feeding. The parasite multiplies at the site of the bite before invading the blood and lymphatic tissues. Metacyclic trypomastigotes differentiate into trypomastigotes (long slender and short stumpy forms) which maintain bloodstream infection. Parasites eventually reach other tissues and organs including the Central Nervous System. When a tsetse fly takes a blood meal from an infected human or animal, it ingests the bloodstream forms of the parasite (trypomastigotes). Upon uptake, the bloodstream short stumpy trypomastigotes, which are well adapted for survival in the vector transform into procyclic trypomastigotes in the tsetse fly’s midgut (Chappuis *et al.*, 2005). At this stage the dense VSG coat of the bloodstream trypomastigotes is replaced by a relatively less dense EP/GPEET procyclin coat (Matthews, 2009). The trypanosomes also develop an elaborate mitochondrion in the insect vector. The procyclic trypomastigotes proliferates in the midgut and then migrate to the salivary gland where they are transformed into epimastigotes. The epimastigotes attach to epithelial cells via the flagella. They undergo further replication in the salivary gland and then transform into the metacyclic trypomastigotes, which are the infective forms of the parasite. At this stage the dense
VSG coat reforms and the parasite detaches from the epithelial cells, free in the salivary gland lumen and well adapted to survive in a mammalian host upon infection (Langousis & Hill, 2014).

![Life cycle of Trypanosoma brucei. Adapted from Stuart et al. (2008).](image)

**Figure 2.2:** Life cycle of *Trypanosoma brucei*. Adapted from Stuart *et al.* (2008).

### 2.6. PATHOGENESIS AND CLINICAL MANIFESTATION

Trypanosomes begin replicating in interstitial spaces at the site of infection. A chancre develops as the parasite replicates due to a buildup of metabolic waste and cell debris (Baral, 2010). The trypanosomes then migrate to the lymphatic tissues and blood. In the blood, the parasite continues to replicate and this result in high persistent parasitaemia. Some trypanosome species
such as *T. congolense* and *T. simiae* localize in endothelial cells of small blood vessels and capillaries whilst *T. brucei brucei* and *T. vivax* invade body tissues (Zwart, 1989; Maré, 2004). Severe acute anaemia, thrombocytopenia, lymphadenopathy, coagulopathy and damage to blood vessels and other body organs occur at this stage due to the presence of the parasite in blood, lymph, tissues and organs such as the lymphatic tissue, heart, kidneys, liver and spleen (Zwart, 1989). There is a vigorous immune response elicited by the host against the parasite. Immune complexes trigger inflammation, which contributes to fever and other clinical manifestations of the disease. Phagocytosis of blood cells such as erythrocytes and platelets by macrophages occur (Osório *et al.*, 2008). The host immune system also recognizes expressed VSG and produce antibodies that mediate clearance of particular antigenic type of parasites (Baral, 2010). Due to antigenic variation and other immune evasion strategies, the parasite is able to evade the immune system and persist in the host. This can lead to chronic infection and high mortality as observed in cattle and other animals. Chronically infected animals that recover from acute symptoms remain clinically normal with low parasitaemia for a long time (Batista *et al.*, 2009). Other parasitic infections, physical stress or poor nutrition can, however, reactivate the clinical manifestations.

The severity of disease varies with species and age of animals infected and the infecting species of trypanosomes (Peterson & Grinnage-Pulley, 2015). Incubation period usually takes about a week to a month. The major clinical manifestations include; fever, anaemia, lymphadenopathy, thrombocytopenia, apathy, enlarged lymph nodes, pale mucous membranes, weakness, infertility, abortion and progressive weight loss (Itard, 1989; Batista *et al.*, 2009; Silva *et al.*, 2013). During chronic infection, however, swollen lymph nodes, serous atrophy of fat, and anaemia are seen (Peterson & Grinnage-Pulley, 2015).
2.7. TRYPANOTOOLERANCE

Trypanotolerance is the ability of some livestock breeds to tolerate trypanosome infection by controlling levels of parasitaemia and showing no severe clinical manifestation (most prominently anaemia) and production loss that are characteristic of infections in susceptible animals (Kemp & Teale, 1998). Studies have suggested that natural resistance to trypanosomes involves at least two mechanisms, an innate mechanism that checks parasite growth, and the haemopoietic system, that is able to limit anaemia (Naessens et al., 2002). In cattle, trypanotolerance has been well documented with the N’Dama and West African shorthorn (WASH) breeds recognized as the predominant trypanotolerant breeds (d’Ieteren et al., 1998). Variable degrees of resistance have also been observed in trypanosusceptible Zebu cattle breeds. The Sanga breed of cattle has also been claimed to have some degree of trypanotolerance but this breed is relatively susceptible to trypanosomiasis compared to the N’Dama and WASH breeds (Stein, 2011).

2.8. DIAGNOSIS OF TRYPANOSOMIASIS

Accurate diagnosis and identification of causative trypanosome species provides data which is vital for control of AAT. Diagnosis at point of treatment is currently dependent on detection of parasites in body fluids and clinical signs (Pillay et al., 2013). Diagnosis using clinical signs is, however, unreliable since the clinical features of infection are exhibited in other blood parasite infections and thus not sufficiently specific (CDC, 2012). Currently methods used in diagnosis of AAT include parasitological, serological and molecular techniques.
2.8.1. Parasitological Diagnosis

Parasitological diagnosis relies on detection of trypanosomes in various body fluids. Currently, the gold standard for diagnosis of AAT is microscopic examination of blood for presence of parasites (Pillay et al., 2013). Microscopic examination of wet-mount blood slides and thick or thin blood films stained with Giemsa are some techniques used for detection. In cases of low parasitaemia, the sensitivity of microscopy is increased using concentration methods such as the microhematocrit centrifugation (Maré, 2004). The usefulness of parasitological diagnosis is, however, limited since in chronic infection parasitaemia is low and intermittent, making it difficult to detect parasites (Pillay et al., 2013). Microscopy also requires expertise in order to detect and differentiate different species of parasites.

2.8.2. Serological Diagnosis

Serological tests have been used for some time now in the diagnosis of African trypanosomiasis. Tests such as the Indirect Fluorescent Antibody Test (IFAT), Antibody Enzyme linked Immunosorbent Assay (ELISA) and Card Agglutination Test for Trypanosomiasis (CATT) have been used in diagnosis of trypanosomiasis (Uilenberg & Boyt, 1998; Chappuis et al., 2005). These tests are based on detection of antibodies produced against trypanosomes in infected animals. Although these tests are sensitive and genus specific, they are not species specific (Pillay et al., 2013). Antigen detection techniques have also shown low sensitivity and species specificity. Although antibody detection techniques are very sensitive, they are not able to distinguish between past and present infections.
2.8.3. Molecular Diagnosis

The introduction of molecular diagnostic techniques has overcome the limitations of sensitivity and specificity of microscopy and serology (Morlais et al., 1998). Molecular techniques such as synthesis and sequencing of DNA, DNA probing and polymerase chain reaction (PCR) have been applied in diagnostic methods for detection/diagnosis of various infectious diseases (Desquesnes & Dávila, 2002). Some molecular tools that have been used in diagnosis of African trypanosomiasis include; PCR based tests, Loop-Mediated Isothermal Amplification method (LAMP) and Nucleic Acid sequence based amplification assay (NASBA).

Most PCR diagnostics aim to characterize the parasites at the species level using various targets in the genome of trypanosomatids with high copy numbers (Desquesnes & Dávila, 2002). Several PCR based diagnostic methods such as use of species-specific primers, single ITS-based PCR and Nested ITS-based PCR have been developed to enhance detection of pathogenic trypanosomes (Thumbi et al., 2008). Species-specific primers may require 5-6 different PCR reactions since up to six different pathogenic trypanosomes occur in cattle in sub-Saharan Africa. Single ITS-based PCR may not identify trypanosomes under conditions of low parasitaemia. However, nested ITS based PCR has been shown to be very sensitive and specific (Cox et al., 2005; Thumbi et al., 2008).

Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification method that amplifies DNA rapidly with high specificity and efficiency at a constant temperature (Nagamine et al., 2002; Mori et al., 2004). This method requires a simple heating device and amplification is detected by visual turbidity or colour change (Wastling et al., 2010). It is, therefore, more practicable for use in the field compared to traditional PCR which requires
expensive automated thermocyclers for amplification, and electrophoresis for detection. *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense* have been successfully detected using LAMP based on amplification of the serum resistance-associated (SRA) gene and the *T. b. gambiense*-specific glycoprotein (TbgsGP) gene respectively (Ngotho *et al*., 2015; Njiru *et al*., 2008).

Nucleic Acid Sequence Based Amplification Assay (NASBA) is a sensitive, isothermal, transcription based amplification system for the specific replication of nucleic acids *in vitro* (Deiman *et al*., 2002). It involves the use of three enzymes and is specifically designed to detect RNA targets. This method has been established for detection of trypanosome RNA in blood for diagnosis of Human African Trypanosomiasis (Mugasa *et al*., 2009).

### 2.9. IMMUNE RESPONSE DURING INFECTION WITH AFRICAN TRYPANOSOMES

Trypanosomes as extracellular parasites encounter both innate and adaptive immune response in the mammalian host. The parasites first encounter the innate immune system as first line of defense and later adaptive immune response (Baral, 2010). There has been conflicting evidence on the immunological factors that influence the extent of resistance to trypanosomiasis in various animal models (Namangala *et al*., 2001a). However, different host cells have been shown to be activated by trypanosomal factors leading to activation of acute inflammatory response (Baral, 2010). Macrophage activation is a major characteristic of trypanosome infection. Studies show increased quantity and activity of macrophages in tissues of animals infected with trypanosomes (ILRAD, 1984; Paulnock & Coller, 2001; Mansfield & Paulnock, 2005). Macrophages are among the first host immune cells that encounter trypanosome antigens and they become classically activated to secrete inflammatory mediators like TNF, IL-12 and NO (Namangala *et al*., 2001a).
Receptor-mediated macrophage activation leads to initial production of pro-inflammatory cytokines which initiates inflammatory response and affect downstream maturation of activated T cells (Mansfield & Paulnock, 2005). Macrophages play a double role as initiators of acquired immunity and as major components of innate immunity to trypanosomes. With the acquired/adaptive immune responses, host Th-cell and B-cell responses are produced to alternate determinants of the VSG coat (Mansfield & Paulnock, 2005). Due to antigenic variation non-specific polyclonal B-cell activation occurs during infection producing IgM in large quantities (Krishna et al., 2015). VSG-specific B cell activation is however obstructed by the intrinsic molecular structure of switch alternate surface coats (Mansfield & Paulnock, 2005). T cells are equally important to the control of trypanosomes. Although they do not act on trypanosomes in a similar manner as the cytotoxic T cells in other infectious diseases such as viral infections, they secrete various cytokines that modify the functions of B cells and Macrophages (Mansfield & Paulnock, 2005; Vincendeau & Bouteille, 2006).

### 2.10. CYTOKINES

Cytokines are low molecular weight proteic or glycoprotein mediators that play a major role in the communication between immune cells (Bienvenu et al., 2000). They can be divided into different functional classes, some are mainly lymphocyte growth factors, others play a role as pro-inflammatory or anti-inflammatory molecules whereas other cytokines polarize the immune response to antigens (Dinarello, 2007). The production and release of cytokines from innate immune cells are crucial responses to inflammation and infection in the body (Lacy & Stow, 2013). Pro-inflammatory cytokines (IL-12, TNF-α and IFN-γ) play crucial and synergistic roles in inflammation whilst anti-inflammatory cytokines (IL-10 and IL-4) inhibit inflammation and
T-cell proliferation (Elenkov & Chrousos, 2002). The cytokine milieu and changes in the Th1/Th2 cytokine balance can affect progression of trypanosomiasis in infected animals (O’Gorman et al., 2006).

2.10.1. Interleukin 12 (IL-12)

Interleukin 12 (IL-12) is a pro-inflammatory cytokine produced mostly by phagocytic cells in response to intracellular parasites, bacteria, bacterial products and to some extent by B cells (Trinchieri, 1995). IL-12 induces interferon-γ production and triggers CD4+ T cells to differentiate into type 1 T helper (Th1) cells (Hamza et al., 2010). Interleukin 12 has been shown to play vital roles in defense against trypanosomes and other parasitic infections.

2.10.2. Interleukin 10 (IL-10)

IL-10 is an anti-inflammatory cytokine that has become known as a key regulator of immunity during infection with viruses, bacteria, fungi, protozoan and helminths (Couper et al., 2008). Several studies have shown that IL-10 inhibits production of pro-inflammatory cytokines, such as TNF-a, IL-1 and IL-6, leading to down regulation of pro-inflammatory and cell-mediated immune responses (Schneider et al., 2004).
2.10.3. Interleukin 4 (IL-4)

Interleukin 4 is a Th2 anti-inflammatory cytokine secreted by activated Th2 and NKT cells. It has pleiotropic effect on many cell types (Choi & Reiser, 1998). IL-4 regulates numerous immune functions including Ig isotype switching, expression of class II MHC by B cells, and the differentiation outcome of some T cell subsets (Brown, 2008). The down regulation of Th1 cells by IL-4 plays a role in the prevention of autoimmunity, but its exact role in prevention of autoimmunity is not clear (Choi & Reiser, 1998).

2.10.4. Tumour Necrosis Factor Alpha (TNF-α)

Tumour Necrosis Factor alpha (TNF-α), is a strong immunomediator and pro-inflammatory cytokine produced by macrophages/monocytes in the course of acute inflammation and accounts for varied signaling events within cells, leading to necrosis or apoptosis (Idriss & Naismith, 2000; Maddahi et al., 2011). This cytokine is known to exert both homeostatic and pathophysiological functions in the central nervous system (Olmos & Lladó, 2014).

2.10.5. Interferon- gamma (IFN-γ)

IFN-γ is a pleiotropic cytokine which acts in heightening of both innate and acquired immune responses. It is a unique cytokine that is involved in regulation of all phases of inflammatory response including, activation and differentiation of T cells, B cells, NK cells, macrophages, and others (Gattoni et al., 2006). Interferon gamma inhibits viral replication, controls intracellular bacterial infections and protects against chronic parasite infection (Bao et al., 2014).
2.11. SERUM BIOCHEMISTRY

Serum biochemistry provides vital information that serve as basis for the diagnosis, treatment, and prognosis of diseases (Yokus & Cakir, 2006). The levels of the serum biochemical indices give an indication of the metabolic profile of the body. Several factors categorized into genetic (breed and genotype) and non-genetic (age, sex, management system, medication, nutrition and climate) influence the serum biochemistry of various livestock animals (Onasanya et al., 2015). Infections, tissue damage and other factors that result in decrease or increase in the amount of a nutrient or metabolite in the blood impacts on serum metabolite levels (Thurnham & Mccabe, 2012). Interpretation of the physiological status of bovines through blood biochemical methods provide information regarding animal health, growth, nutrition and maintenance technologies (Prisacaru, 2014). Some of the serum haematological/biochemical parameters measured in livestock include; packed cell volume (PCV), mean corpuscular volume (MCV), total blood glucose (TBG), total protein (TP), urea, creatinine, uric acid, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) (Onasanya et al., 2015). Others include; Lactate dehydrogenase (LDH), creatinine kinase (CK), albumin (Alb), Gamma-glutamyl transpeptidase (GGT), amylase, globulin, cholesterol, very low density lipoprotein (VLDL), triglyceride, folate, vitamin A and E, triiodothyronine (T3), thyroxine (T4), free triiodothyronine (fT3) and free thyroxine (fT4) concentrations, serum retinol and α-tocopherol concentration (Onasanya et al., 2015). Changes in the levels of some of these serum biochemical parameters have been reported to occur during infection with various pathogens including trypanosomes. These changes are indicators of pathophysiological responses, hence providing baseline report on the epidemiology of livestock animals towards assessing nutrition, improving health management practices and reducing economic loss (Onasanya et al., 2015).
2.11.1 Blood Glucose

Glucose is the most important monosaccharide involved in energy production in the body. In the fed state, blood glucose levels are high but during fasting, gluconeogenesis and glycogenolysis maintain glucose levels. The measurement of serum glucose is therefore a measure of an animal’s nutrition but blood glucose level is usually used to monitor metabolism and physiology. Serum glucose determination is an important marker for most disease conditions (Kosiborod & Mcguire, 2010; Upadhyay, 2015). High levels of glucose are linked to diseases such as renal failure and pancreatitis while low glucose levels are associated with insulin-induced hypoglycemia and neoplasms (Nakano et al., 1996; Fernandez et al., 2009).

2.11.2 Creatinine

Creatinine is a waste product formed from the metabolism of creatine in muscles but may also originate from cooked meat. It is removed from circulation through the kidneys. Serum creatinine is therefore commonly used to screen for renal disease or to investigate urinary sediment abnormalities, hypertension or non-specific symptoms such as tiredness (Nankivell, 2001).

2.11.3 Cholesterol

Cholesterol is a prominent lipid that circulates in the blood and is an important structural component of all mammalian cells. It is a chemical precursor to bile acids, steroids, and vitamin D and also plays important roles in cell signaling (Berg et al., 2002). The liver produces about 75% of the body’s cholesterol whiles food accounts for 25%. Cholesterol is important in the
pathogenesis of cardiac and brain vascular diseases but it has also been implicated in dementias, diabetes, cancer as well as several rare monogenic diseases (Ikonen, 2006; Ikonen 2008).

2.11.4. Serum Bilirubin

Bilirubin is an end-product of hemoglobin catabolism within the reticuloendothelial system, which is released in unconjugated form that enters into the liver, converted to conjugated forms bilirubin mono- and diglucuronides by the enzyme UDP-glucuronyltransferase (Mauro et al., 2006). Serum bilirubin determination is an important indicator for the diagnosis of several diseases. Elevated levels are associated with hemolysis, genetic defect such as the Gilbert's syndrome, Crigler-Najjar syndrome and reabsorption of large hematomas and ineffective erythropoiesis (Fevery, 2008). Viral hepatitis, hepatocellular damage, toxic or ischemic liver injury are also associated with elevated levels of serum conjugated bilirubin (Gowda et al., 2009).

2.11.5. Alkaline phosphatase (ALP)

ALP is a hydrolase enzyme that removes phosphate groups from molecules facilitating lipid transportation in the intestines and calcification in bones. Bone and liver diseases are the commonest origins of pathological increase in ALP levels, although ALP may come from other tissues, such as the placenta, kidneys or intestines, or from leukocytes (Giannini et al., 2005). Serum ALP is, therefore, used as an important marker of liver and bone diseases.
2.11.6. Alanine amino transferase (ALT)

ALT is a metabolic enzyme expressed primarily in the liver and is involved in cytoplasmic catalyses of transamination reactions (Gowda et al., 2009). Increased levels of ALT are observed most often in animals and persons with diseases such as viral hepatitis, ischemic liver injury (shock liver) and toxin-induced damage to liver cells (Gowda et al., 2009). ALT is, therefore, an important marker of liver disease.

2.11.7. Total Serum Protein

Serum proteins are major macromolecules in blood and important components of cells and tissues. Changes in blood protein levels occur during various disease manifestations. Clinicians therefore obtain vital information regarding disease states in different organs by measuring protein concentrations in blood. Elevated total protein levels may be observed in metabolic and nutritional disorders as well as kidney and bone marrow diseases. Decreased total protein on the other hand can be seen in malnutrition and chronic liver disease (McClatchey, 2002; Thapa & Walia, 2007).
CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Chemicals and Reagents

S-monovette blood collection tubes (Sarstedt AG & Co, Nümbrecht, Germany); 5U Mango Taq polymerase (BIOLINE, USA); QIAamp DNA Blood Maxi Kit and QIAquick purification kit (QIAGEN, Hilden, Germany); Primers (Invitrogen Life Technologies); Ethidium bromide (Sigma-Aldrich, Inc. Missouri, USA); Nuclease free water (Sigma-Aldrich, Inc. Missouri, USA); Ultrapure agarose (Invitrogen Life Technologies); Direct load Wide Range DNA Marker 50bp – 10,000bp (Sigma-Aldrich, Inc. Missouri, USA); IL-10, IL-4, IFN-γ, TNF-α and IL-12 sandwiched bovine cytokine ELISA kits (Cusabio Biotech Co., Ltd, Wuhan, China); Blood Glucose (BG), Total Serum Protein (TSP), Bilirubin (BIL), Creatinine (CRT), Total Cholesterol (CHOL), Alkaline Phosphatase (ALP) and Alanine Aminotransferase (ALT) reagent kits (ELITech Group, Puteaux, France).

3.1.2 Study Area and Study Population

The study was conducted at two separate study sites, the University of Ghana Livestock and Poultry Research Farm at Accra in the Greater Accra region and a private cattle ranch at Adidome in the Volta region (Figure 3.1). Accra lies in the coastal savanna vegetation zone and has a low tsetse fly density and low prevalence of animal trypanosomiasis. Adidome (located at
about 130km from Accra) lies in the transitional forest vegetation zone and has a high tsetse fly density and a high prevalence of animal trypanosomiasis.

The study population included cattle at the study sites aged between 6 months to 1 year irrespective of the sex and breed. A total of 40 cattle were randomly selected for the study; twenty from the Accra study site (University of Ghana Livestock and Poultry Research Farm) and twenty from the Adidome cattle ranch. The breeds of cattle selected at the Accra study site were Sanga, Sanga Cross and West African Short Horn (WASH). At Adidome study site, all selected cattle were of the Sanga breed.
Figure 3.1: Geographical location of study sites. Adapted from Mahama et al. (2003). Red (University of Ghana Livestock and Poultry Research Farm). Green (Cattle Ranch, Adidome).
3.2 METHODS

3.2.1 Sample Collection

Peripheral blood samples were collected from cattle at the two study sites at approximately 5 weeks intervals over six different time points. Two blood samples (5 ml each) were collected by jugular venipuncture from each animal into S-monovette blood collection tubes, one coated with EDTA and the other without additive. Samples in EDTA tubes were placed on ice and all samples transported to the Molecular Biology Laboratory, Department of Biochemistry, Cell and Molecular Biology, University of Ghana for processing and analysis. Ethical clearance was obtained from the Council for Scientific and Industrial Research (CSIR)-Ghana.

3.2.2 Isolation of DNA from Whole Blood Samples

DNA was extracted from uncoagulated whole blood samples (blood in EDTA tubes) using the QIAamp DNA Blood Maxi Kit (Spin Protocol) (QIAGEN, Hilden, Germany) following the manufacturer’s protocol. Five milliliters of each blood sample was transferred into 50 ml centrifuge tubes. A volume of 500 μl QIAGEN protease was then added and the contents of the tubes mixed briefly. Lysis buffer AL (6 ml) was added to each sample and the tubes inverted 15 times followed by vigorous shaking for about 1 min. Tubes were incubated at 70°C for 10 min. A 5 ml volume of ethanol was added to the samples and then mixed by inverting the tubes 10 times, followed by additional vigorous shaking. The solutions were transferred onto QIAamp Maxi spin columns placed in 50 ml centrifuge tubes. The samples were then centrifuged at 4000 rpm for 3 min. The QIAamp Maxi columns were removed, the filtrates discarded and the columns placed back into the tubes. Wash buffer AW1 (5 ml) was added to the QIAamp columns
and then spun at 5000 rpm for 1 min. The flow through was discarded and the columns placed back into the tubes. Wash buffer (AW2) was then added to the QIAamp columns and the solution centrifuged at 5000 rpm for 15 min. The QIAamp Maxi columns were then transferred into clean 50 ml centrifuge tubes and the collection tubes containing the filtrate discarded. Elution buffer AE (1 ml) equilibrated to room temperature was added directly onto the membrane of the QIAamp Maxi spin columns and incubated for 5 min. The solution was then spun at 5000 rpm for 2 min. The flow through was reloaded onto the QIAamp Maxi columns, incubated at room temperature for 5 min and then spun at 5000 rpm for 5 min in other to obtain highly concentrated DNA. Aliquots of 200 μl were pipetted from each tube into four separate Eppendorf tubes and stored at -20ºC. DNA was later purified and used for PCR.

3.2.3 Purification of Extracted DNA

The extracted DNA was purified using the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) following the manufacturer’s protocol. After the DNA extraction, crude DNA was purified using the QIAquick PCR purification kit. The QIAquick column was placed in a 2 ml collection tube provided. Crude DNA sample (200 μl) was applied to the QIAquick column and centrifuged for 30-60 s. The flow through was discarded and the QIAquick column placed back in the same tube. The QIAquick column was then washed by adding 750 μl Buffer PE to the column and centrifuging for 30-60 s. The flow through was discarded and the QIAquick column placed back into the tube. The column was centrifuged once more in the 2 ml collection tube for 1 min to remove residual washing buffer. It was then placed in a clean 1.5 ml microcentrifuge tube. DNA was eluted by adding 100 μl Buffer EB (10 mM Tris HCl, pH 8.5) to the center of the QIAquick membrane and the tube allowed to stand for 1 min, followed by centrifugation for 1
min. Eluent from the column is the purified DNA. This procedure was performed on each of the extracted (crude) DNA samples and the purified DNA stored at -20°C.

### 3.2.4 Serum Separation

Upon arrival at the laboratory, blood samples in S-monovette tubes without additive were allowed to clot and serum separated by centrifugation at 3000 rpm for 10 min. The separated serum was then aliquoted into four 1.5 ml eppendorf tubes. The serum samples were stored at -80°C until use. The processing of the serum samples was carried out 3-4 h after sample collection.

### 3.2.5 Nested ITS Based PCR

Trypanosome DNA was amplified by nested ITS-based PCR technique as described by Cox *et al.* (2005) with slight modifications. For the first round of PCR, 1 µl of the purified DNA sample was added to a PCR reaction mixture of 29 µl. The reaction mixture contained 1X Taq buffer with 1.5 mM MgCl₂, 0.3 µM outer primers TM1 (GAT TAC GTC CCT GCC ATT TG) and TM2 (TTG TTC GCT ATC GGT CTT CC), 0.3 mM dNTP’s and 2.5 U Taq polymerase. The thermal cycling was carried out in an Analytik Jena thermocycler (Biometra, Gottingen, Germany) under the following reaction conditions; 1 cycle of 95°C for 5 min followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 1 min, and then 1 cycle of 72°C for 5 min followed by cooling at 4°C. For the second round of PCR reaction (nested step), 1 µl of the PCR product from the first round reaction was placed in a fresh tube and 29 µl of the reaction master mix added. The reaction mixture was composed of 1X Taq buffer with 1.5 mM MgCl₂, 0.3 µM Outer primers TM3 (GGA AGC AAA AGT CGT AAC AAG G) and TM4 (TGT TTT CTT TTC
CTC CGC TG), 0.3 mM dNTP’s and 2.5 U Taq polymerase. The cycling conditions used were the same as that for the first PCR reaction. After the amplification, 10 µl of the PCR products from each sample were mixed with 2 µl of 6X loading dye (glycerol, bromophenol blue) and run on a 1.2 % agarose gel (containing 2µl of 10 mg/ml ethidium bromide) at 100 V for 1 h. The gel was then visualized under ultraviolet (UV) light using a UV transilluminator.

3.2.6 Measurement of Serum Biochemical Parameters

The levels of blood glucose (BG), total serum protein (TSP), total bilirubin (BIL), creatinine (CRT), total cholesterol (CHOL), alkaline phosphatase (ALP) and alanine aminotransferase (ALT) were determined using commercial reagent kits (ELITech Group, Puteaux, France) and Flexor E automated clinical chemistry analyser (Vital Scientific NV, Dieren, The Netherlands). CHOL, BG and CRT were measured by enzymatic colorimetric methods. ALP and ALT were measured by the Kinetic Colorimetric DGKC method and Kinetic UV IFCC method (Schumann & Klauke, 2003), respectively. BIL was measured by End Point Modified Malloy Evelyn method and TSP measured by Biuret method (Gornall et al., 1948). After calibration of the automated analyser using standards and blanking using appropriate blanks, serum samples (500 µl each) were placed in appropriate sample wells in the sample rotor. Reagents were also placed in their corresponding wells in the reagent rotor. Different volumes of the samples are aspirated for the measurement of the different parameters. Samples aspirated were mixed with the appropriate reagents and incubated in the cuvette. Absorbance was then measured at appropriate wavelengths for each parameter and concentration calculated and reported on the screen of the computer.


3.2.7 Quantification of Serum Cytokines in Cattle

Serum concentrations of IL-10, IL-4, IFN-γ, TNF-α and IL-12 were measured using commercially available sandwiched bovine cytokine ELISA kits (Cusabio Biotech Co., Ltd, Wuhan, China) following the manufacturers protocol. Serum separated and stored at -80˚C was thawed at room temperature.

For IL-4, IL-12 and IFN-γ, 100 µl of assay standards and serum samples (diluted two fold) were added in duplicate to appropriate wells of the pre-coated assay plates and incubated for 2 h at 37˚C. This was followed by removal of the liquid from the wells and subsequent addition of 100 µl of 1X biotin-labeled cytokine antibodies to each well. The assay plates were then covered with adhesive strip and incubated at 37˚C for 1 h. The wells were aspirated and washed three times using 200 µl wash buffer, inverting and blotting the plates against clean paper towels after the last wash. Afterwards, 100 µl of 1X HRP-avidin conjugate were added to each well and incubated at 37˚C for 1 h. The wells were again aspirated and washed three times as before. TMB substrate (90 µl) was then added to each well and plates incubated for 20 min in the dark at 37˚C. Finally, 50 µl stop solution were added to each well to terminate the enzyme-substrate reaction. Optical densities (ODs) of the wells were read within 5 min at 450 nm using the microplate reader.

For TNF-α and IL-10, 50 µl each of standards and serum samples were added to appropriate wells, leaving a set of wells as blanks. HRP-conjugate (50 µl) and antibody (50 µl) were added to each of the wells except the blanks. Plates were then incubated at 37˚C for 1 h. The wells were aspirated and washed three times using 200 µl wash buffer, inverting and blotting the plates against clean paper towels after the last wash. After washing, 50 µl substrate A and substrate B
were added to each well. Plates were then incubated at 37°C for 15 min in the dark. Finally, 50 µl stop solution were added to each well to terminate the enzyme-substrate reaction. Optical densities of the wells were read within 10 min at 450 nm using the microplate reader. Concentrations of all the cytokines were extrapolated from calibration curves constructed for the bovine cytokine standards using the measured ODs.

3.2.8 Data Analysis

Data obtained from the study was organized in Microsoft Excel and analysed using STATA 13.0 statistical software (StataCorp LP, Texas, USA) and GraphPad Prism (version 6, San Diego, CA, USA). For investigation of the effect of gender, breed and geographical location on the levels of serum biochemical parameters, values generated for the different groups were represented as mean ± standard error of the mean. As the data for the other comparisons made in the study were largely non-parametric, Mann-Whitney U test was used for comparison of medians between these groups at each time point. Optical densities obtained after ELISAs were converted to concentrations using an Auditable Data Analysis and Management System for ELISA (ADAMSEL) and further analysis carried out using GraphPad Prism.
CHAPTER FOUR

4.0 RESULTS

4.1 IDENTIFICATION OF TRYPANOSOMES IN CATTLE

For each time point at both study sites, PCR was carried out on DNA samples extracted from all 40 cattle. Figures 4.1 and 4.2 show representative gels obtained after running of PCR products on a 1.2 % agarose gel. As observed in Figure 4.1, eight out of the twenty cattle were infected at time point 2 at the Accra study site. Amplified products of approximately 980bp were obtained. The PCR products, when compared to the expected amplicon sizes in the NCBI database are identified as Trypanosoma theileri. In Figure 4.2, twelve out of the 20 cattle were infected at time point 4 at the Adidome study area. Two types of amplified DNA of approximately 610bp and 980bp were obtained. These when compared to the expected amplicon sizes in the NCBI database were identified as Trypanosoma vivax and Trypanosoma theileri, respectively. Table 4.1 shows results obtained on the identification of trypanosome species across all six time points. For the 20 cattle at the Accra study site, at the first time point, 12 cattle were infected and the infecting species were T. theileri, T. vivax and T. simiae. Three, eight and six cattle were infected at time points 3, 4 and 5, respectively. The infecting species at all three time points were T. theileri. At time point 6, however, 8 cattle were infected with either T. vivax or T. theileri. For the other 20 cattle at the Adidome study site, 8, 6, 9, 4 and 8 cattle were infected at time points 1, 2, 3, 5 and 6, respectively. The infecting species identified from time point 1 to 5 were T. theileri and T. vivax. At time point 6, however, all the cattle were infected with T. vivax. The species of trypanosomes identified by the PCR product length were confirmed by sequencing 11 random
positive samples from both study sites (Table 4.1). Trypanosome species identified after sequencing were same as those identified using the PCR product length (Table 4.1).

Figure 4.1: Nested ITS-based PCR products for Accra study site, time point 2. N: Negative Control; P: Positive Control (*T. brucei brucei*); M: DNA marker from 50 - 10,000 bp.

Figure 4.2: Nested ITS-based PCR products for Adidome study site, time point 4. N: Negative Control; P: Positive Control (*T. brucei brucei*); M: DNA marker from 50 - 10,000 bp.
Table 4.1: Trypanosome species identified at Accra and Adidome

<table>
<thead>
<tr>
<th>Time point</th>
<th>Adidome</th>
<th>Accra</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of cattle infected</td>
<td>Trypanosome species detected</td>
</tr>
<tr>
<td>1</td>
<td>8</td>
<td>5 <em>Trypanosoma vivax</em> &lt;br&gt; 3 <em>Trypanosoma theileri</em></td>
</tr>
<tr>
<td>2</td>
<td>6 (1)</td>
<td>6 <em>Trypanosoma vivax</em> &lt;br&gt; 1 <em>Trypanosoma theileri</em></td>
</tr>
<tr>
<td>3</td>
<td>9</td>
<td>5 <em>Trypanosoma vivax</em> &lt;br&gt; 4 <em>Trypanosoma theileri</em></td>
</tr>
<tr>
<td>4</td>
<td>12 (1)</td>
<td>5 <em>Trypanosoma vivax</em> &lt;br&gt; 8 <em>Trypanosoma theileri</em></td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>1 <em>Trypanosoma vivax</em> &lt;br&gt; 3 <em>Trypanosoma theileri</em></td>
</tr>
<tr>
<td>6</td>
<td>8</td>
<td><em>Trypanosoma vivax</em></td>
</tr>
</tbody>
</table>

Values in parenthesis represent number of mixed infections.

4.2 SPECIES OF TRYPANOSOMES IDENTIFIED IN CATTLE AT ADIDOME AND ACCRA

The species of trypanosomes identified in the infected cattle at both study sites using PCR product length and confirming by sequencing are presented in Table 4.1. Overall, three species of trypanosomes, namely *Trypanosoma vivax*, *T. theileri* and *T. simiae*, were identified from the study. *Trypanosoma vivax* was the most predominant trypanosome species at the Adidome study site. At the Accra study site on the other hand, *T. theileri* was the most predominant trypanosome species detected. Both trypanosome species were, however, detected at the two study sites. *Trypanosoma simiae* was the least common species as it was detected only at time point 1 at the Accra study site. Mixed infections with two different trypanosome species were detected at time point 1 (4 *T. theileri* & *T.vivax* and 1 *T. theileri* & *T. simiae*) in Accra and at time points 2 (1 *T. theileri* & *T.vivax*) and 4 (1 *T. theileri* & *T.vivax*) in Adidome.
4.3 SERUM BIOCHEMICAL PARAMETERS IN CATTLE AT ACCRA AND ADIDOME DURING NATURAL TRYPANOSOME INFECTION

In order to establish the levels of serum biochemical parameters during trypanosome infection, serum concentrations of glucose, creatinine, cholesterol, alkaline phosphatase (ALP), alanine aminotransferase (ALT), total bilirubin and total protein were compared between infected and uninfected cattle at six time points. Due to variation in the dynamics of infection from one time point to the next (Table 4.1), comparisons were made at each time point.

For cattle at Adidome, it was observed that there was no significant difference in the levels of glucose and ALT across all the 6 time points (Figure 4.3A, E). ALP was, however, significantly elevated in trypanosome infected cattle at time point 1 (infected = 613.15 U/L, uninfected = 342.45 U/L, p = 0.02) and time point 5 (infected = 462.10 U/L, uninfected = 271.10 U/L, p = 0.02) (Figure 4.3D). Cholesterol levels, on the other hand, were not statistically different between infected and uninfected cattle from time point 1 to time point 5. However, at time point 6, infected cattle had significantly lower levels of cholesterol (infected = 2.45 mmol/L, uninfected = 3.15 mmol/L, p = 0.04) (Figure 4.3C). Total bilirubin was also not significantly different from time point 1 to time point 5 but at time point 6, infected cattle had significantly higher (p=0.02) levels of bilirubin compared to uninfected cattle (Figure 4.3G). Median values of total bilirubin were 2.45 µmol/L and 1.96 µmol/L for infected and uninfected cattle, respectively. Creatinine was significantly elevated in infected cattle only at time point 5 (infected = 101.90 µmol/L, uninfected = 77.75 µmol/L, p = 0.01). Total protein was also significantly higher in infected cattle at time point 5 only (infected = 77.14 µmol/L, uninfected = 71.26 µmol/L, p = 0.01).
Figure 4.3: Differences in serum biochemical parameters between trypanosome infected cattle and uninfected controls in Adidome. Levels of (A) Glucose (B) Creatinine (C) Cholesterol (D) ALP (E) ALT (F) Total protein (G) Total bilirubin were measured. The Mann-Whitney-U-Test was used for testing statistical significance between the two groups at each time point, * p < 0.05, ** p < 0.01. Time points are at 4 to 5 week intervals.
For cattle at Accra, the levels of glucose, ALP, total bilirubin and total protein were not significantly different between infected and uninfected cattle across all six time points (Figure 4.4). However, creatinine was significantly elevated in infected cattle at time point 5 (infected = 150.2 µmol/L, uninfected = 111.65 µmol/L, p = 0.01). Cholesterol and ALT were not significantly different at time points 1, 2, 3, 4 and 6. At time point 5, however, both biochemical parameters were significantly lower in infected cattle. Median values of cholesterol were 3.55 mmol/L (infected) and 4.0 mmol/L (uninfected) with P value = 0.03. Median values of ALT were 25.25 U/L (infected) and 35.0 U/L (uninfected) with P value = 0.01.

Results also show generally higher biochemical parameters in cattle at Adidome compared to cattle in Accra for both infected cattle and uninfected cattle (Figures 4.3 and 4.4).
Figure 4.4: Differences in serum biochemical parameters between trypanosome infected cattle and uninfected controls in Accra. Levels of (A) Glucose (B) Creatinine (C) Cholesterol (D) ALP (E) ALT (F) Total protein (G) Total bilirubin were measured. The Mann-Whitney-U-Test was used for testing statistical significance between the two groups at each time point. * $p < 0.05$, ** $p < 0.01$. Time points are at 4 to 5 week intervals.
4.4 EFFECT OF GENDER ON THE LEVELS OF SERUM BIOCHEMICAL PARAMETERS

In order to determine whether the gender of cattle had an effect on the levels of serum biochemical parameters, the mean levels of biochemical parameters between bulls and heifers were compared over the six time points. There was no significant difference (P > 0.05) in all measured parameters between bulls and heifers at the Accra study site over the six time points (Figure 4.5). With biochemical parameters measured in cattle at the Adidome study site, however, creatinine levels were significantly higher in the heifers compared to bulls at time points 1 (p=0.04), 2 (p=0.003) and 3 (p=0.03) (Figure 4.6B). ALP on the other hand was significantly higher for heifers at time point 2 (p=0.04) and higher for bulls at time point 6 (P=0.04) (Figure 4.6D). The other biochemical parameters measured were not different between the heifers and bulls. Cattle at both Accra and Adidome could not be separated by infection status before comparison due to low numbers of cattle in each group.
Figure 4.5: Comparison of serum biochemical parameters in cattle at Accra according to gender. (A) Glucose (B) Creatinine (C) Cholesterol (D) ALP (E) ALT. (F) Total bilirubin (G) Total protein. Data presented are mean ± SE. The Mann-Whitney-U-Test was used for testing statistical significance between the two groups at each time point. Time points are at approximately 5 weeks intervals.
Figure 4.6: Comparison of serum biochemical parameters in cattle at Adidome according to gender. (A) Glucose (B) Creatinine (C) Cholesterol (D) ALP (E) ALT. (F) Total bilirubin (G) Total protein. Data presented are mean ± SE. The Mann-Whitney-U-Test was used for testing statistical significance between the two groups at each time point, * p < 0.05. Time points are at approximately 5 weeks intervals.
4.5 EFFECT OF THE BREED OF CATTLE ON SERUM BIOCHEMICAL PARAMETERS

Serum biochemical parameters were compared between the Sanga Cross (n=8) and West African Shorthorn (WASH) (n=7) breeds of cattle at Accra. The Sanga breeds were only two animals and were therefore left out of the statistical comparison. The levels of glucose, ALP, ALT, total bilirubin and total protein were not significantly different between the Sanga Cross breed and the WASH across all the time points (Figure 4.7 (A, D-G)). Creatinine and cholesterol however were significantly different only at time point 1 (Figure 4.7 (B, C)). Cattle could not be separated by infection status (infected and uninfected) before comparison due to low numbers of cattle in each group.
Figure 4.7: Comparison of serum biochemical parameters between WASH and Sanga Cross cattle breeds at Accra (A) Glucose (B) Creatinine (C) Cholesterol (D) ALP (E) ALT. (F) Total bilirubin (G) Total protein. Data presented are mean ± SE. The Mann-Whitney-U-Test was used for testing statistical significance between the two groups at each time point, * p < 0.05. Time points are at approximately 5 weeks intervals.
4.6 SERUM BIOCHEMICAL PARAMETERS IN PATHOGENIC AND NON-PATHOGENIC TRYPANOSOME INFECTIONS

The levels of serum biochemical parameters were compared between cattle infected with pathogenic trypanosome parasites (*T. vivax*) and cattle infected with non-pathogenic trypanosome parasites (*T. theileri*) at timepoint 4 at Adidome. Figure 4.8 shows the individual values of serum biochemical parameters measured in *T. vivax* infected cattle and *T. theileri* infected cattle. Glucose, cholesterol, ALT, total protein, creatinine, total bilirubin and ALP were similar between *T. vivax* and *T. theileri* infected cattle (p>0.05).

Figure 4.8: Serum biochemical parameters in pathogenic and non-pathogenic trypanosome infections. (Levels of (A) Glucose (B) Creatinine (C) Cholesterol (D) ALP (E) ALT (F) Total protein (G) Total bilirubin were measured. The Mann-Whitney-U-Test was used for testing statistical significance between the two groups. Data from time point 4 at Adidome were used in the analyses.)
4.7 SERUM BIOCHEMICAL PARAMETERS IN MIXED AND SINGLE TRYpanosome INFECTIONS

Comparing the serum levels of glucose, creatinine, cholesterol, ALP, ALT, total bilirubin and total protein between cattle with mixed trypanosome infection and cattle with single infection at time point 1 at Accra, there was no significant difference (p>0.05) for all 7 biochemical parameters measured. The serum biochemical parameters in cattle with mixed infection were within a similar range as cattle with single trypanosome infection (Figure 4.9).

Figure 4.9: Serum biochemical parameters in mixed and single trypanosome infections. Levels of (A) Glucose (B) Creatinine (C) Cholesterol (D) ALP (E) ALT (F) Total protein (G) Total bilirubin. The Mann-Whitney-U-Test was used for testing statistical significance between the two groups, Data from time point 1 at Accra were used in the analyses.
4.8 SERUM LEVELS OF PRO-INFLAMMATORY (IL-12, IFN-γ AND TNF-α) AND ANTI-INFLAMMATORY (IL-10 AND IL-4) CYTOKINES IN TRYPANOSOME INFECTED AND UNINFECTED CATTLE

To determine the kind of immune response that is mounted during natural infection with trypanosomes, serum levels of pro-inflammatory and anti-inflammatory cytokines were compared. At the Accra study site at time point 1, the serum levels of IL-10, an anti-inflammatory cytokine and TNF-α, a pro-inflammatory cytokine were significantly elevated in trypanosome infected cattle compared to the uninfected cattle (p<0.05) (Figure 4.10). The median concentration of IL-10 was 273.65 pg/ml (Range = 147.6 pg/ml to 393.4 pg/ml) in trypanosome infected cattle and 155.05 pg/ml (Range = 63.4 pg/ml to 268.4 pg/ml) in the uninfected cattle. The median concentration of TNF-α on the other hand was 5.30 pg/ml (Range = 2.6 pg/ml to 7.0 pg/ml) in trypanosome infected cattle and 2.95 pg/ml (1.2 pg/ml to 6.3 pg/ml) in uninfected cattle. The median concentration of IL-10 was 51.6 pg/ml (18.9 %) higher than TNF-α in infected cattle indicating higher levels of the anti-inflammatory cytokine. With the other three cytokines (IL-4, IL-12 and IFN-γ), their serum levels were not significantly different in the trypanosome infected cattle compared to the uninfected cattle.

At the Adidome, study site at time point 4, on the other hand, the levels of IL-10, IL-4, IL-12, IFN-γ and TNF-α were all not significantly different between trypanosome infected cattle and uninfected cattle (Figure 4.11).
Figure 4.10: Comparison of serum cytokines between infected and uninfected cattle in Accra. Levels of (A) IL-10 (B) IL-4 (C) IL-12 (D) IFN-γ (E) TNF-α. Dots represent individual cytokine values in cattle. Horizontal bar represents the median. Statistical significance was tested using the Mann-Whitney U test, * p < 0.05, ** p < 0.01. Data from time point 1 at Accra were used in the analyses.
Figure 4.11: Comparison of serum cytokines between infected and uninfected cattle in Adidome. Levels of (A) IL-10 (B) IL-4 (C) IL-12 (D) IFN-γ (E) TNF-α. Dots represent individual cytokine values in cattle. Dots represent individual cytokine values in cattle. Horizontal bar represents the median. Statistical significance was tested using the Mann-Whitney U test. Data from time point 1 at Accra were used in the analyses.
The profiles of pro-inflammatory and anti-inflammatory cytokines in infected cattle were compared between the two study areas (Figure 4.12). Levels of IL-4, IL-12 and INF-γ were not different between the infected cattle at Adidome and Accra. IL-10 and TNF-α were, however, significantly elevated in trypanosome-infected cattle in Accra compared to infected cattle in Adidome. The median levels of IL-10 were 126.05 pg/ml in cattle at Adidome and 273.65 pg/ml in cattle at Accra (p = 0.001, Mann-Whitney U test). The median levels of TNF-α on the other hand were 2.3 pg/ml in cattle at Adidome and 5.3 pg/ml in cattle at Accra (p = 0.002, Mann-Whitney U test).
Figure 4.12: Comparison of serum cytokines between infected cattle in Accra and Adidome. Levels of (A) IL-10 (B) IL-4 (C) IL-12 (D) IFN-γ (E) TNF-α. Dots represent individual cytokine values in cattle. Horizontal bar represents the median. Statistical significance was tested using the Mann-Whitney U test, **p < 0.01. Data from time point 1 at Accra and time point 4 at Adidome were used in the analyses.
4.10 CYTOKINE LEVELS IN PATHOGENIC AND NON-PATHOGENIC TRYPANOSOME INFECTION

The serum concentrations of all the measured pro-inflammatory and two anti-inflammatory cytokines were not significantly different in pathogenic (T. vivax) trypanosome infection (n=7) compared to non-pathogenic (T. theileri) infection (n=5) (Figure 4.13). *Trypanosoma vivax* infected cattle, however, had slightly elevated levels of IL-10, IL-4 and TNF-α (29%, 92% and 42%, respectively) than the *T. theileri* infected cattle.

![Graph showing cytokine levels](image)

**Figure 4.13:** Comparison of serum cytokines between Pathogenic and non-pathogenic trypanosome infections. Levels of (A) IL-10 (B) IL-4 (C) IL-12 (D) IFN-γ (E) TNF-α. Horizontal bar represents the median. Statistical significance was tested using the Mann-Whitney U test. Data from time point 4 at Adidome were used in the analyses.
4.11 CYTOKINE LEVELS IN MIXED AND SINGLE TRYPANOSOME INFECTION

Comparing the levels of serum cytokines between cattle with mixed trypanosome infection and single infection, there was no significant difference in pro-inflammatory and anti-inflammatory cytokines levels in the two groups ($P > 0.05$) (Figure 4.14).

![Graphs showing cytokine levels](image)

**Figure 4.14:** Comparison of serum cytokines between cattle with single and mixed trypanosome infections. Levels of (A) IL-10 (B) IL-4 (C) IL-12 (D) IFN-$\gamma$ (E) TNF-$\alpha$. Horizontal bar represents the median. Statistical significance was tested using the Mann-Whitney U test. Data from time point 1 at Accra were used in the analyses.
4.12 TH1/TH2 CYTOKINE BALANCE IN TRYPANOSOME-ENDEMIC (ADIDOME) AND NON-ENDEMIC (ACCRA) AREAS.

In order to establish immune profiles in cattle without trypanosome infections at the two study areas, baseline serum levels of pro-inflammatory to anti-inflammatory (Th1/Th2) cytokine ratios were compared between uninfected cattle in Accra and uninfected cattle in Adidome (Figure 4.15). Cattle in Adidome had significantly higher INF-γ/IL-10 cytokine ratios than cattle in Accra (Adidome=12.40, Accra=0.91, P= 0.01). Also the IFN-γ/IL-4 cytokine ratios were significantly higher in cattle in Adidome compared to cattle in Accra (Adidome=19.70, Accra=1.78, P= 0.04). The levels of IL-12/IL-10, IL-12/IL-4, TNF-α/IL-10 and TNF-α/IL-4 were all not significantly different between cattle in the two locations.

Comparing the pro-inflammatory to anti-inflammatory (Th1/Th2) cytokine ratios between infected cattle in Accra and infected cattle in Adidome, IL-12/IL-4, INF-γ/IL-10 and IFN-γ/IL-4 were all not significantly different (Figure 4.16). IL-12/IL-10 was however significantly higher in infected cattle at Adidome (Adidome=9.85, Accra=3.99, P= 0.02). TNF/IL-4 cytokine ratio was significantly lower in cattle at Adidome compared to Accra (Adidome = 0.01, Accra=0.13, P = 0.04).
Figure 4.15: Baseline Th1/Th2 cytokine concentration in uninfected cattle at Adidome and Accra. Levels of (A) IL-12/IL-10 (B) IL-12/IL-4 (C) INF-γ/IL-10 (D) IFN-γ/IL-4 (E) TNF-α/IL-10 (E) TNF-α/IL-4. Horizontal bar represents the median. Statistical significance was tested using the Mann-Whitney U test, * p < 0.05.
Figure 4.16: Th1/Th2 cytokine concentration in infected cattle at Adidome and Accra. (A) IL-12/IL-10 (B) IL-12/IL-4 (C) INF-γ/IL-10 (D) INF-γ/IL-4 (E) TNF-α/IL-10 (F) TNF-α/IL-4. Horizontal bar represents the median. Statistical significance was tested using the Mann-Whitney U test, * p < 0.05.
Variation in pro- to anti-inflammatory cytokine response was assessed in individual cattle that had the same state of infection (either infected or uninfected) throughout all the six time points. In cattle at Adidome (AD-3780 and AD-7759) infected throughout the six time points, increasing trends in IL-12/IL-10 and IL-12/IL-4 cytokine ratios were observed (Figure 4.17 A, B). Although the cytokine ratios fluctuated at some time points, comparing the ratios at time point 1 to time point 6, there was generally an increase in the ratios of IL-12/IL-10 (For AD-3780, Time point 1= 0.62, Time point 6=3.39; For AD-7759, Time point 1=0.20 , Time point 6=6.62 ) and IL-12/IL-4 (For AD-3780, Time point 1=5.61, Time point 6=18.03; For AD-7759, Time point 1=1.22 , Time point 6=18.73). In cow AD-7743, which was uninfected throughout all six time points however, the IL-12/IL-10 cytokine ratio was not very different comparing the value at time point 1 (3.09) to time point 6 (3.60). IL-12/IL-4 was also not very different comparing the value at time point 1 (15.25) to time point 6 (20.34).

In cattle at Accra (UG-164 and UG-166) infected throughout the six time points, decreasing trends in IL-12/IL-10 and IL-12/IL-4 cytokine ratios were observed (Figure 4.17 C, D). Although fluctuations in the cytokine ratios were observed at some time points, comparing the ratios at time point 1 to time point 6, a general decrease in the ratios of IL-12/IL-10 (For UG-164, Time point 1=12.90, Time point 6=3.59; For UG-166, Time point 1=4.03 , Time point 6=3.39 ) and IL-12/IL-4 (For UG-164, Time point 1= 51.92, Time point 6=1.91; For UG-166, Time point 1=33.72 , Time point 6=13.96) were observed.
Figure 4.17: Cytokine ratios in individual cattle at Adidome (AD) and Accra (UG) over six time points. (A) IL-12/IL-10 levels in two cows (AD-7780 & AD-7759) infected throughout all six time points and a cow (AD-7743) uninfected throughout all the time point at Adidome. (B) IL-12/IL-4 levels in two cows (AD-7780 & AD-7759) infected throughout all six time points and a cow (AD-7743) uninfected throughout all the time point at Adidome. (C) IL-12/IL-10 levels in two cows (UG-164 & UG-166) infected throughout all six time points in Accra. (D) IL-12/IL-4 levels in two cows (UG-164 & UG-166) infected throughout all six time points in Accra. Time points are at 4 to 5 week intervals.
CHAPTER FIVE

5.0 DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 DISCUSSION

To enhance current understanding of the pathophysiology and immune response during trypanosome infection, this longitudinal study characterized trypanosome infections and determined the associated levels of serum biochemical parameters and cytokines in naturally infected cattle at two areas of different endemicity. Using PCR product length, three different species of trypanosomes were identified. Sequencing data confirmed the species of trypanosomes that were identified using the PCR product length. *T. theileri, T. vivax* and *T. simiae* were identified in Accra with *T. theileri* being the most predominant species. In Adidome, *T. theileri* and *T. vivax* were identified with *T. vivax* being the most predominant. These identified species have been previously characterized in livestock in Ghana (Nakayima et al., 2012). The two study areas differ in endemicity, tsetse fly density and vegetation covers. These factors may have influenced the predominant infecting species in the localities. Mixed infections previously reported in literature (Mekata et al., 2008; Nakayima et al., 2012) were also observed in some cattle at both Accra and Adidome. The dynamics of infection varied from one time point to the next in individual cattle. However, some cattle remained infected throughout all the six time points suggesting possible chronic infections.

Previous studies assessing the levels of some biochemical parameters in animals have shown the influence of multiple factors such as disease, breed, age, gender, season, muscle activity,
nutrition, gestation, heat, and stress on the levels of the parameters (Xie et al., 2013; Bhat et al., 2014; Durak et al., 2015).

In this study, the influences/effects of breed and gender on the levels of serum biochemical parameters in cattle were investigated over six time points. The mean values of the biochemical parameters measured in cattle at the two study locations fell within previously reported reference ranges (Jackson & Cockcroft, 2002) except for total protein levels which were higher. The higher total protein levels observed could be due to several genetic and non-genetic factors such as climatic conditions, age and genotype of animals, since these have been reported to cause variation in total protein levels in ruminants. Cozzi et al. (2011) and Alberghina et al. (2011) have however reported total protein levels (82 ± 6.1 g/L and 67.5 ± 11.5 g/L respectively) which are comparable to mean values (77.8 ± 8.4 g/L to 69.5 ± 5.2 g/L) obtained in this study.

Serum biochemical parameters between bulls and heifers were similar at Accra and Adidome except for creatinine and ALP which differed significantly at some time points in Adidome. These differences could however be due to factors other than gender since the differences did not run through all the time points or differ at the Accra study area. The data from the study therefore suggests that gender of cattle has no effect on the levels of serum biochemical parameters. This agrees with observation reported in previous studies by Bhat et al., (2014) and Kiran et al., (2012) in small ruminants. Prisacaru (2014), however, found significantly higher glucose and ALP levels in bulls. The study was however cross-sectional and therefore the observation made could be due to some other factors.

For the effect of breed on serum biochemical parameters, there were similar levels of the measured parameters in the Sanga cross and WASH except creatinine and cholesterol which
differed significantly at time point 1. These significant differences in only cholesterol and creatinine could also be due to factors other than the breed since this was observed only at one time point out of the six time points. The data therefore suggests that cattle breed has no effect on the levels of serum biochemical parameters. This agrees with findings reported in a study in Romanian cattle (Prisacaru, 2014).

In Accra, trypanosomiasis is less endemic and *Trypanosoma theileri* was identified as the predominant infecting trypanosome species. *T. theileri* as a non-pathogenic parasite is well tolerated by cattle and, therefore, may not cause any significant pathology associated with trypanosome infection. This could, therefore, be the possible reason for the similar levels of serum biochemical parameters observed in infected and uninfected cattle at most time points in this area. Although *T. theileri* is considered a non-pathogenic parasite, its presence in animals exhibiting clinical symptoms such as peritonitis has led to suggestions of the parasite’s involvement in tissue inflammation (Sood *et al*., 2011). Significant differences in creatinine, ALT and cholesterol between infected and uninfected cattle at time point 5 could therefore be a result of the presence of the parasite in body fluid and tissues. Also trypanosome infection in animals often progresses to chronic states and during early chronic infections most serum biochemical parameters drop to normal or near normal values (Whitelaw *et al*., 1980; Dufour *et al*., 2000; Peterson & Grinnage-Pulley, 2015). The non-significant difference observed in cattle at Accra across all six time points could be due to chronicity of the parasite infection. As chronic infections are usually characterized by very low parasitaemia (Whitelaw *et al*., 1980), the deduction that cattle were chronically infected can be supported by the absence of parasites when selected samples were screened microscopically but these same samples tested positive for trypanosome infection when trypanosome DNA was amplified using PCR. *T. theileri* infections
are known to manifest chronically throughout the life of the host (Mott et al., 2011). Hence the high prevalence of *T. theileri* infections in this study area also implies that most cattle in this study area are possibly chronically infected with *T. theileri*.

At Adidome which is an endemic area for trypanosome infection (with predominant *T. vivax* species), the similarities in some biochemical parameters in infected and uninfected cattle at some time points could be due to animals being chronically infected or having sub-microscopic infections as no positive infections were detected by microscopy. Also, periodic treatment of cattle with anti-trypanosomal drugs at Adidome could account for this observation. Since transmission is high in endemic regions, the significant differences observed at some specific time points could either be due to infection with new parasites or a rise in parasitaemia at these time points as a result of new homotypes of parasites unrecognizable by the host immune system. The significantly elevated levels of ALP in infected cattle at time point 1 and 5 could, therefore, be attributed to a number of factors. ALP is a leakage enzyme usually found in the bone, the intestines and predominantly in the liver (Girling et al., 2015). The elevated levels of ALP in the infected cattle therefore suggest a probable invasion of the vital body organs and inflammation particularly of the intestines and liver (Awobode, 2006; Girling et al., 2015). The high levels of ALP in the infected group may also be due to cellular damage caused by lyses of trypanosomes by the host immune system (Enwezor & Sackey, 2005). Several studies have reported significantly elevated ALP levels in both clinical and sub-clinical trypanosome infections (Ngure et al., 2008; Takeet & Fagbemi, 2009; Oluyomi & Sulaiman, 2012).

Creatinine, total protein and total bilirubin were also significantly elevated in trypanosome infected cattle whereas cholesterol was significantly decreased. These observed changes are
consistent with reports from studies in cattle and other animals. Increased levels of serum creatinine during trypanosome infections have been linked to damage to host tissues or renal malfunction (Abenga & Anosa, 2005). Elevation in total bilirubin levels in infected cattle as observed in this study is suggestive of hemolytic anemia due to trypanosomes in blood or jaundice as previously reported in some trypanosome infected animals (Takeet & Fagbemi, 2009). For total protein levels, some studies have demonstrated decreased levels in trypanosome infected animals, however these reports have all been linked to acute infections where feed intake is reduced in animals due to illness (Sow et al., 2014). Chronically infected animals, however, tend to have increased total protein levels due to increase in gamma globulins produced as part of host immune response (Sow et al., 2014; Sivajothi et al., 2015). The decreased cholesterol levels observed in infected cattle in this study is consistent with findings in cattle (Traore-Leroux et al., 1987), pigs (Adamu et al., 2009) and sheep (Katunguka-Rwikishaya et al., 1992). High energy demand of trypanosomes in infected animals and impaired synthesis or release of cholesterol from the liver, due to pathologic changes induced by trypanosome infection could be responsible for the decreased serum cholesterol levels (Faye et al., 2005; Adamu et al., 2009).

The similarity observed in serum biochemical parameters in pathogenically infected and non-pathogenically infected cattle as well as in mixed and single trypanosome infections implies that regardless of the pathogenicity of the infecting trypanosome species or the type of trypanosome infection (mixed or single), levels of the serum biochemical parameters do not differ.

Several studies have demonstrated the role of cytokines in the regulation of immune response during trypanosome infections (Uzonna et al., 1999; Namangala et al., 2001; de Sousa et al.,
Some of these studies have suggested the influence of cytokine responses in disease outcomes (de Sousa et al., 2011). However, most of these studies have been carried out in experimentally infected mice. A few studies carried out in cattle have also been based on experimental infections. This particular study investigated cytokine profiles in naturally infected cattle at two areas of different endemicity.

IL-10 and TNF-α were elevated in trypanosome infections at Accra (non-endemic). Both IL-10 and TNF-α have been shown to play essential roles in immune response to trypanosome infections. TNF-α is involved in both parasite control and infection mediated pathology (Magez et al., 1999). Mice deficient in TNF-α showed increased parasitaemia in T. brucei infections (Magez et al., 1999). Also TNF-α has been shown to be involved in reduced T-cell responses in acute infections (Taylor & Mertens, 1999). IL-10 on the other hand is an anti-inflammatory cytokine that is known to play a crucial role in resistance to African trypanosomes by down regulating excessive production of pro-inflammatory cytokines (Namangala et al., 2001b; Okwor, et al., 2009). This effect results in reduced pathology associated with immune response. Up-regulation IL-10 in the infected cattle, therefore, suggests a possible down-regulation of Th1 immune response in the cattle to mediate immunopathology. Since IL-10 a Th2 cytokine is associated with chronic infections when elevated (Taylor & Mertens, 1999; Ouyang, et al., 2011), the infected cattle at Accra are most likely experiencing chronic infections. The predominance of T. theileri, which is known to cause chronic infections and the low transmission intensity of trypanosomes in Accra, could be responsible for this high anti-inflammatory cytokine response observed. Comparing cytokines between cattle infected with different species and types of infections (single or mixed), it was observed that immune response was not
dependent on the infecting species or the type of infection. A similar finding was reported in naturally infected cattle in Uganda (Katiti, 2014).

At the Adidome study area, there was no difference in individual cytokines compared between infected and uninfected cattle. Investigating the pro- to anti-inflammatory cytokine ratios however, at basal levels and during infection, significantly higher pro-inflammatory/anti-inflammatory (Th1/Th2) cytokine ratios were observed in cattle at Adidome compared to Accra. This indicates a higher Th1 immune response in Adidome. As cattle in this area are occasionally treated with anti-trypanosomal drugs, clearance of parasites may occur. However, due to the high transmission intensity in this area, the cattle may be re-infected with new parasites of different species or parasites of the same species expressing different antigenic coats. This could probably be the reason for the high pro-inflammatory cytokine response as cattle are repeatedly exposed to new/different antigens. This is supported by observations in cattle that remained infected throughout all six time points. In Adidome where the infecting species differed at some time points, an increasing trend in pro-inflammatory (Th1) immune response was observed from time point 1 to time point 6. However, cattle in Accra infected with the same species at all the time points, had an increasing anti-inflammatory immune response from time point 1 to time point 6. These findings collectively suggest that, cattle in an endemic area repeatedly infected with parasites of different species and possible different antigenic types demonstrate high pro-inflammatory (Th1) immune response whereas cattle in a non-endemic area with chronic infections demonstrate high anti-inflammatory (Th2) immune response.

Some confounding factors such as haemoparasitic diseases and worm infections may have affected the measured serum biochemical parameters and cytokine profiles in the cattle. The
differences in the parameters measured may, therefore, not be solely due to presence or absence of trypanosome infections. However, comparing uninfected and infected cattle exposed to same environmental conditions, control for some of these confounders to some extent.

5.2 CONCLUSIONS

From this study, *Trypanosoma theileri* was identified as the predominant infecting trypanosome species in Accra whilst *T. vivax* was identified as the predominant species in Adidome. The breed and gender of cattle were found not to affect the levels of the parameters measured. However, geographical distribution was found to influence levels of the serum biochemical parameters. Infected cattle in Accra generally had similar serum biochemical parameters as uninfected controls. In Adidome however, creatinine, ALP, total protein and total bilirubin were significantly elevated in trypanosome infected cattle whereas cholesterol was significantly decreased at specific time points. With regards to the cytokine profiles, a high pro-inflammatory response was observed in cattle at Adidome and a high anti-inflammatory response in infected cattle at Accra. Levels of biochemical parameters and cytokines were also found not to be dependent on the infecting species or the type of infection (single or mixed).

This study demonstrates a complicated and much more dynamic host parasite interaction during natural infections. Findings generally demonstrate that cattle in the non-endemic area, which were predominantly infected with non-pathogenic *T. theileri*, have different biochemical profiles and immune responses compared to cattle in the endemic area with predominantly pathogenic trypanosome (*T. vivax*) infections. This suggests higher biochemical aberrations and pro-inflammatory response in areas with high *T. vivax* infections. Exploring the biomarker potential of biochemical parameters and cytokines will therefore require careful consideration of the stage
of infection (acute or chronic), endemicity/transmission intensity of the area, geographical
distribution and treatment practices by livestock farmers. From the study, baseline serum
biochemical parameters for cattle across two different populations were also established.

5.3 RECOMMENDATIONS

1. To get a better picture of what happens in the field, cattle should be followed from the time of
birth and the biochemical parameters and cytokines measured prior to first exposure.

2. Parasite load should also be measured to correlate this with the changes in biochemical
parameters.

3. The Variant Surface Glycoprotein (VSG) repertoires during natural trypanosome infection
should be studied to establish whether repeated infection with new and different antigenic types
leads to high pro-inflammatory immune response.
REFERENCES


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Figure A1: Agarose gels for nested ITS based PCR for cattle at Adidome across six time points. (A) Time point 1, (B) Time point 2, (C) Time point 3, (D) Time point 4, (E) Time point 5, (F) Time point 6. Some amplified DNA appeared very faint on gels and were not visible when pictures were taken.
Figure A2: Agarose gels for nested ITS based PCR for cattle at Accra across six time points. (A) Time point 1, (B) Time point 2, (C) Time point 3, (D) Time point 4, (E) Time point 5, (F) Time point 6. Some amplified DNA appeared very faint on gels and were not visible when pictures were taken.
Table A1: Median concentrations of serum biochemical parameters in infected and uninfected cattle at Accra with p-values

<table>
<thead>
<tr>
<th>TIME POINT</th>
<th>GLUCOSE(mg/dL)</th>
<th>CREATININE(µmol/L)</th>
<th>CHOLESTEROL(mmol/L)</th>
<th>ALP(U/L)</th>
<th>ALT(U/L)</th>
<th>TOTAL PROTEIN(g/L)</th>
<th>TOTAL BILIRUBIN(µmol/L)</th>
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</thead>
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Table A2: Median concentrations of serum biochemical parameters in infected and uninfected cattle at Adidome with p-values

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<th>TIME POINT</th>
<th>GLUCOSE(mg/dL)</th>
<th>CREATININE(µmol/L)</th>
<th>CHOLESTEROL(mmol/L)</th>
<th>ALP(U/L)</th>
<th>ALT (U/L)</th>
<th>TOTAL PROTEIN (g/L)</th>
<th>TOTAL BILIRUBIN(µmol/L)</th>
<th>P-value</th>
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Table A3: Serum concentrations of IL-10, IL-4, IL-12, INF-γ and TNF-α in cattle at Adidome

<table>
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<tr>
<th>SAMPLE ID</th>
<th>INFECTION</th>
<th>IL10(pg/ml)</th>
<th>IL4(pg/ml)</th>
<th>IL12(pg/ml)</th>
<th>INF-γ(pg/ml)</th>
<th>TNF-α(pg/ml)</th>
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<td>911.5</td>
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<td>2124.4</td>
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Table A4: Serum concentrations of IL-10, IL-4, IL-12, INF-γ and TNF-α in cattle at Accra

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<th>IL12(pg/ml)</th>
<th>INF-γ(pg/ml)</th>
<th>TNF-α(pg/ml)</th>
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Table A5: Serum concentrations of IL-10, IL-4, IL-12, INF-\(\gamma\) and TNF-\(\alpha\) in cattle with the same state of infection over six time points

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