HAEMATOLOGICAL AND TISSUE ENZYME CHANGES IN A SHEEP MODEL INFECTED WITH *Trypanosoma brucei rhodesiense*

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DECLARATION AND RECOMMENDATION

Declaration

This thesis is my original work and to my knowledge has not been presented, wholly or in part for the award of a degree in any Institution.

Signature……. Date……

Hawa Mavura

SD14/0157/05

Recommendation

This PhD thesis was carried out under our supervision and has our approval to be submitted for examination as per Egerton University regulations.

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DEDICATION

This work is dedicated to my children Beresha and Yusuph Mnaya Mavura

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ABSTRACT

Control and management of human African trypanosomosis faces many challenges including limited available drugs that are stage specific and also toxic, treatment failures and post treatment CNS and cardiac pathology which might be fatal. Because of ethical considerations, trypanosomosis and drug trial studies are conducted using animal models which have drawbacks. It is therefore important to develop models that can address the current drawbacks and also develop enzymatic disease staging markers to detect CNS and cardiac involvement in order to institute correct choice of trypanosome drugs and/or use of adjunct to mitigated conditions. The current study evaluated a sheep model infected with *Trypanosoma brucei rhodesiense* as a cheap and reliable model of human trypanosomosis. In addition the model was used to asses clinical signs, haematological changes and the use of total and isoenzyme CK and LDH levels in serum and CSF as markers of cardiac and staging of CNS infection, respectively. Eight sheep were infected with 1×10^4 *T.b rhodesiense* and two were uninfected controls. Blood was collected from the ear and jugular vein for parasitemia and for haematological and biochemical changes, respectively. CSF was also collected for cells and biochemical changes while total CK and LDH plus their respective isoenzymes were done by using starch gel electrophoresis and their quantification by a UN-SCAN IT densitometry. Infected sheep developed acute infection accompanied by clinical signs and haematological changes that mimicked infections in humans, including loss of body weight and fever.The infection was also characterized by an increase in MCH and MCHC. However, there was a decline in the levels of PCV, HCT, Hb, MCV and RBC counts. All parameters however recovered to control levels after treatment.This study also showed an increase in total protein, total LDH and total CK activities in both serum and CSF of infected compared to uninfected sheep. At the same time LDH and CK total enzyme and isoenzyme changes indicated cardiac involvement suggesting the diagnostic potential of the enzymes. However the infection did not develop a chronic CNS infection since following humane treatment of the infected sheep with berenil this resulted in cure of the infection and thus did not lead to late stage CNS infection. The study indicated that the *T. b.rhodesiense* sheep model can be a useful animal model of human African trypanosomosis. However, the study needs to be repeated using a less virulent or low dose of trypanosome to ensure the disease develops to the chronic stage.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATION

BBB	Blood Brain Baarrier
CNS	Central Nervous System
CK	Creatine kinase
CSF	Cerebral Spinal Fluid
CV	Coefficient of variation
dpi	day post infection
HAT	Human African trypanosomosis
KALRO	Kenya Agricultural and Livestock Organization
KARI	Kenya Agricultural Research Institute
KETRI	Kenya Trypanosomiasis Research Institute
LDH	Lactate dehydrogenase
MPM	Methyl phenazonium methosulphate
MTT	3(4, 5-diMethylthazol-2-yl)-2, 5-diphynylTetrazoliumbromide Thiozolyl blue
PCR	Polymerase Chain Reaction
PEP	Phosphoenol pyruvate
PMS	Phenazine methosulphate
SRA	Serum resistance Associate
T. b. g	*Trypanosoma brucei gambiense*
T. b. r	*Trypanosome brucei rhodesiense*
TRC	Trypanosomiasis Research Centre
WBC	White Blood Cells

CHAPTER ONE
INTRODUCTION

1.1 Background Information

Human African typanosomosis (HAT) also known as sleeping sickness is a serious tropical parasitic infection caused by parasites of the species *Trypanosoma brucei*. The parasite exits in two subspecies namely *Trypanosoma brucei rhodesiense* (*T.b rhodesiense*) and *Trypanosoma brucei gambiense* (*T. b. gambiense*) (Franco *et al*., 2014; Pepin, 1998). *T. b. gambiense* is found mainly in West and Central African countries including parts of Western Uganda, Southern Sudan and Angola (WHO, 1998, WHO, 2006) while *T.b rhodesiense* is found mainly in East and South African countries that include parts of Western Kenya, South Eastern Uganda, Tanzania, Zambia and Malawi (Franco *et al*., 2014; Hide, 1999). The trypanosomes are transmitted through bites of infected tsetse flies of the *Glossina species* (Barret *et al.*, 2003; Simarro *et al.*, 2008). Following the parasite multiplication in the skin at the site of tsetse bite, the trypanosomes enter the circulatory system and dwell free in the blood and lymphatic system. Later the trypanosomes invade various body tissues including the central nervous system (Kennedy, 2004).

Trypanosomosis is now a re-emerging disease (Kabayo, 2002; Wiser *et al*., 2003; WHO, 2006; Clerinx *et al.*, 2012) resulting from the failure in the vector control measures following civil wars in the disease endemic areas (Moore *et al.*, 1999; Moore *et al.*, 2001; Hutchinson *et al.*, 2003). Symptoms of the human disease include lack of appetite, nausea, lymphadenopathy, loss of body condition, sleepiness and ultimately death (Gelfand, 1966). *T. b.rhodesiens*e causes an acute infection with symptoms occurring only a few weeks after the infective tsetse fly bite. In the more acute course of *T.b rhodesiense* infection, pancarditis with congestive heart failure, pericardial effusion and pulmonary oedema can cause fatalities at the early stages (Barret *et al.*, 2003).

The disease progresses from the haemolymphatic stagewhen parasites appear in the blood and lymph and to the later stage when the parasites infect the central nervous system (CNS) (Atouguia *et al.*, 2000). Symptoms during the haemolymphatic stage especially in *T. b.rhodesiens*e infection are unspecific and includes fever, severe headache, joint pain, muscle aches, sweating, enlargement of lymph nodes and joint pains (Barret *et al.*, 2003; Malvy and Chappuis, 2011). However during the second phase trypanosomes invade the CNS with

symptoms including confusion, personality changes, difficulty in walking, sleep disturbance, development of cardiac problems and eventually coma (Smith *et al.*, 1997; Smith *et al.*, 1998 Kennedy, 2004; Sternberg, 2004).

However, there are few drugs available to treat *T.b rhodesiense* infection (Kennedy, 2011). The only effective drug currently available for treatment of the CNS *T.b. rhodesiense* infection is the arsenic based melarsen oxide (melarsoprol®) drug (Legros *et al.*, 2002). Melarsen oxide has severe toxic effects and causes post treatment inflammation of the brain (Pepin *et al.*, 1994) resulting in the death of 50% of treated patients (Pepin and Milrod, 1994; Iley, 2004). It is therefore important to confirm CNS involvement in patients before prescribing the toxic melarsen oxide drug. Without the possible prospect of a vaccine in the near future, drug treatment remains the main method in the control of human African trypanosomosis infection (WHO, 2013).

Several CNS trypanosome infection staging diagnostic markers have been developed (Kennedy, 2011). While all these staging biomarkers have demonstrated a potential efficacy in various studies, issues of true validity and the practical challenges of carrying out these analyses mainly under African field conditions remains a challenge (Amin *et al.*, 2010). There is therefore a need to develop novel diagnostic staging markers that are cheap, quick and easy to perform in addition to being non-invasive and easily adapted to field conditions (Kennedy, 2008; Kennedy, 2011).

Tissue enzymes have been used as sensitive diagnostic markers of CNS infection involvement during bacterial meningitis (Nussinovitch *et al.*, 1996) and also in determining the severity of CNS infection (Morang, 1991). Indeed Creatine kinase (CK) BB isoenzyme produced in the CNS during trypanosomosis infection is able to cross the blood brain barrier (BBB).Therefore the isoenzyme can be measured in blood thus enabling the avoidance in the use of CNS invasive procedures to collect cerebral spinal fluid (CSF) in the detection of brain tissue damage (Rodney *et al.*, 1977; Nussinovitch *et al.*, 1996; Sedlakov and Necas, 1999).

In addition CK and lactate dehydrogenase (LDH) have been used in other disease conditions to determine cardiac involvement (Shillingford, 1975; Nigam, 2007). Increases in body fluid levels of the tissues enzymes CK and LDH have been demonstrated in trypanosome infected in other animal models. Increases in the blood tissue enzymes have been demonstrated

during experimental animal trypanosome infections (de Souza *et al*., 2000; Ngure *et al.,* 2000) and also in infected humans (Basson *et al*., 1977). Although various animal models have been used in the past to study the pathology, therapeutic efficacy and pharmacodynamics of drugs and experimental compounds for the treatment of HAT (Kennedy, 2007; Waema *et al*., 2014), all have drawbacks. The non-human primate model for HAT has been described (Ndungu *et al.*, 1994; Ngure *et al.*, 2008) and used to generate safety and/or toxicity data on new compounds against HAT. The main drawback in the use of non-human primate is the cost of procurement and maintenance of the animals in the laboratories. HAT infection studies have been undertaken in sheep using *T. b. brucei* trypanosomes but none has been done using the human infective *T.b rhodesiense* trypanosome. Hence a need to develop a cost-effective animal model for studies on *T.b rhodesiense* CNS trypanosome infection. Animal model can also be used to evaluate the possible use of total tissue enzymes CK and LDH with their isoenzymes as reliable enzymatic markers of heart lesion and staging markers in CNS trypanosome infection.

1.2 Statement of the Problem

Pathogenesis of *T .b. rhodesiense* in animal models involving the brain is not clearly understood. The available animal models that have previously been used in trypanosomosis research have several limitations. A number of diagnostic methods like demonstration of trypanosomes, increased white cell count and protein levels in CSF exist for the CNS trypanosome disease. However, these methods have challenges and drawbacks especially the unreliability of white blood cell count and protein analysis methods. Both white blood cells and proteins only increases in later stages of CNS involvement and show wide range of variation. The collection of CSF requires a lumber puncture procedure which is a painful and patient unfriendly in addition to posing ethical considerations. Currently used markers of cardiac damage during disease are expensive and not easily adaptable to field conditions at the same time this procedure requires qualified staff to perform. Thus there is a need to identify cardiac and CNS tissue specific markers that are sensitive, specific, reliable and easy to perform at the point of trypanosoma infection care. Serum biochemical changes such as total and/or isoenzymes profiling of tissue enzymes have been shown to be more sensitive and reliable, since the levels increase with minimal tissue damage, does not need very qualified staff to assay and is reliable. The research findings will contribute to the body of knowledge on staging of

the disease and appropriate use of drugs, indirectly reduce mortality from drugs and as such improve follow up of patients following treatment.

1.3 General Objective

To determine the usefulness of sheep as a *T.b rhodesiense* model in research and the tissues enzymes as specific markers of cardiac and CNS involvement for human infective African trypanosomosis.

1.4 Specific Objectives

1. To assess clinical changes in sheep infected with human infective *T.b rhodesiense*; Parasitemia, body weight, CSF parasitosis using concentration method and serum resistance antigen PCR where trypanosomes could not be detected by concentration method.
2. To determine haematological parameters changes in sheep infected with human infective *T.b rhodesiense*.
3. To determine serum changes in total CK and LDH and their isoenzymes as markers of cardiac involvement in sheep during *T.b rhodesiense* infection.
4. To determine the CSF levels of total and isoenzyme profiles of LDH and CK as staging markers of *T.b rhodesiense* CNS infection in sheep.

1.5 Hypotheses

1. Clinical, haematological and biochemical changes in a *T.b rhodesiense* infected sheep do not mimic that of human African trypanosomosis.
2. Total CK and LDH and their isoenzymes do not change during trypanosomosis infection and hence are not of diagnostic value in determination of cardiac involvement.
3. Total and isoenzyme profiles of CK and LDH do not change during *T.b rhodesiense* CNS infection.

1.6 Justification of the Study

Rat, mice and the monkey model have been used over the years to study pathogenesis and pathology of HAT as well as drug toxicity. Access to monkeys is a challenge due to risk of other zoonosis to animal handlers, cost in the acquisition of the animalsand their

maintenance during experimental studies. In mice and rat models the collection of CSF is not possible and where possible there is a limitation on the amount of samples especially CSF that can be collected at any given time due to the size of the animals. The establishment of a robust and cost-effective *T.b rhodesiense* animal model that overcomes the limitations will enable the use of the model for pathogenesis studies as well as validation of targets for discovery of new drugs and diagnostics test. However no *Trypanosoma brucei rhodesiense* sheep model has been developed for such studies. In addition, more accurate and sensitive markers of CNS and cardiac damage during trypanosome infection are lacking and thus need to be developed. Tissue enzymes such as LDH and CK including their isoenzymes have been used in other diseases as sensitive markers of specific tissue damage including the CNS and heart during infection for diagnostic, prognostic and determining response to treatment during disease.In studying the pattern of LDH and CK isoenzyme changes in sheep during infection, the research findings will be used in evaluating the application of isoenzymes as a marker of cardiac damage and CNS involvement during trypanosome infection. The lack of vaccine, lack of safe and effective drugs and treatment failure, calls for more research in trypanosomosis treatment that can only be carried out in animal model. Sheep have shown promise of such a model.

CHAPTER TWO
LITERATURE REVIEW

2.1 Human African Trypanosomosis

Human African trypanosomosis is a major health problem in sub-Saharan Africa affecting an estimated half a million people in 36 sub-Saharan countries (WHO, 2006). The disease affects approximately 20,000 people each year and causes about 55,000 deaths annually (Barrett, 1999; Iley, 2004; WHO, 2006). Epidemics of the disease have been recorded in Angola, the Democratic Republic of Congo (DRC), Uganda, Southern Sudan and Tanzania (WHO 2006; Smith *et al.,* 1998). However, most people with the disease die before it is ever diagnosed. This under-surveillance is attributed to weaknesses in control programs, difficulty and/or inadequacies in disease diagnosis and often, inaccessibility of affected areas (Cattand *et al*., 2001) leading to very few new cases being diagnosed per year (Gibson *et al.*, 2002). There are two distinct forms of sleeping sickness namely the chronic form caused by *T. b. gambiense* occurring in West and Central Africa and the acute form, also referred to as Rhodesian sleeping sickness caused by *T.b rhodesiense* in Eastern and Southern Africa (Welburn *et al.,* 2004).

African trypanosomosis is a zoonotic disease, affecting both man and his livestock. Wild animals act as reservoirs for the disease, being infected but do not develop any apparent adverse disease (Anderson *et al.,* 2011). In addition domestic animals such as cattle and pigs also act as parasite reservoirs and these have been implicated in transmission of the parasite to humans (Wissmann *et al.,* 2011). Human African trypanosomosis is classified as a re-emerging disease resulting in increased cases in endemic areas and its appearance in non endemic areas. From its first appearance around 1900 to the early 1960s, extensive control programs managed to control the disease throughout the continent. However, with the onset of independence in many African countries, tsetse and trypanosomosis control was neglected, leading to recrudescence of the disease in the later years (Hotez, 2008). Recent resurgence of HAT has been attributed to political and civil unrest in countries such as Sudan, Angola and the Democratic Republic of Congo (DRC), which has resulted in mass migration of populations into risk situations and the breakdown of government control support systems (Ford, 2007). In addition, movement of people to tsetse infested areas such as game parks has also led to human infections (Clerinx *et al.,* 2012). Management of HAT is influenced by several factors

including the challenge posed by production of HAT drugs because pharmaceutical companies consider them to be unprofitable ventures(Etchegorry *et al.*, 2001). In addition, treatment failures with the currently available drugs due to development of drug resistance by the parasites has been reported (Legros *et al.*, 1999).

Spraying tsetse habitats by either aerial or ground-spraying has been used to control tsetse in many countries (Allsopp *et al.*, 2001; Hargrove, 2003). Indeed such efforts have been sustained in areas such as the Okavango delta in Botswana where *Glossina morsitans centralis* has been eradicated (Kgori *et al.*, 2006). In additionother control measures used includes use of traps and insect impregnated targets (Hide, 1999).

Tsetse flies of the genus *Glossina* are the most important vectors of trypanosomosis although biting flies such as tabanids and *Stomoxys* can also transmit the parasite (Desquesnes and Dia, 2004). The distribution of the tsetse fly is determined mainly by climate, but it is also influenced by altitude, vegetative cover and the presence of suitable animal hosts (Leak, 1999). Generally, tsetse flies occur in a belt across tropical Africa between 15°N and 29°S, covering about 10 million km^2 of sub-Saharan Africa and extending through 37 countries (WHO, 2006).

2.2 Life Cycle of the *Trypanosoma brucei rhodesiense* Parasite

T.b rhodesiense, the etiological agent of human African trypanosomosis found in eastern Africa, is classified as a protozoa, flagellate of the class kinetoplastida (Wiser, 2003). The trypanosomes are parasites with a two host life cycle beginning in the tsetse fly and continuing in the mammalian host. The parasite undergoes different transformation stages namely changes in morphology within their life cycle in both the mammalian host and the insect vector. The life cycle starts when the trypanosomes are ingested during a blood meal by the tsetse fly from an animal reservoir. The trypanosomes multiply over a period of two to three weeks in the fly's midgut after which the parasites then moves to the salivary glands whereby they are transformed into infective metacyclic trypomastigotes. The trypomastigotes are then transferred to the blood stream of the mammalian host as the vector feeds. Within the bloodstream, the infective form of the parasite undergoes asexual replication by longitudinal binary fission. The replicating trypanosome form is a long slender parasite. The parasites then transform to short stumpy forms which are the infective or transmission stage of the parasite

and it is the only form which survives when ingested by the intermediate hosts, the tsetse fly (Ormerod, 1970).

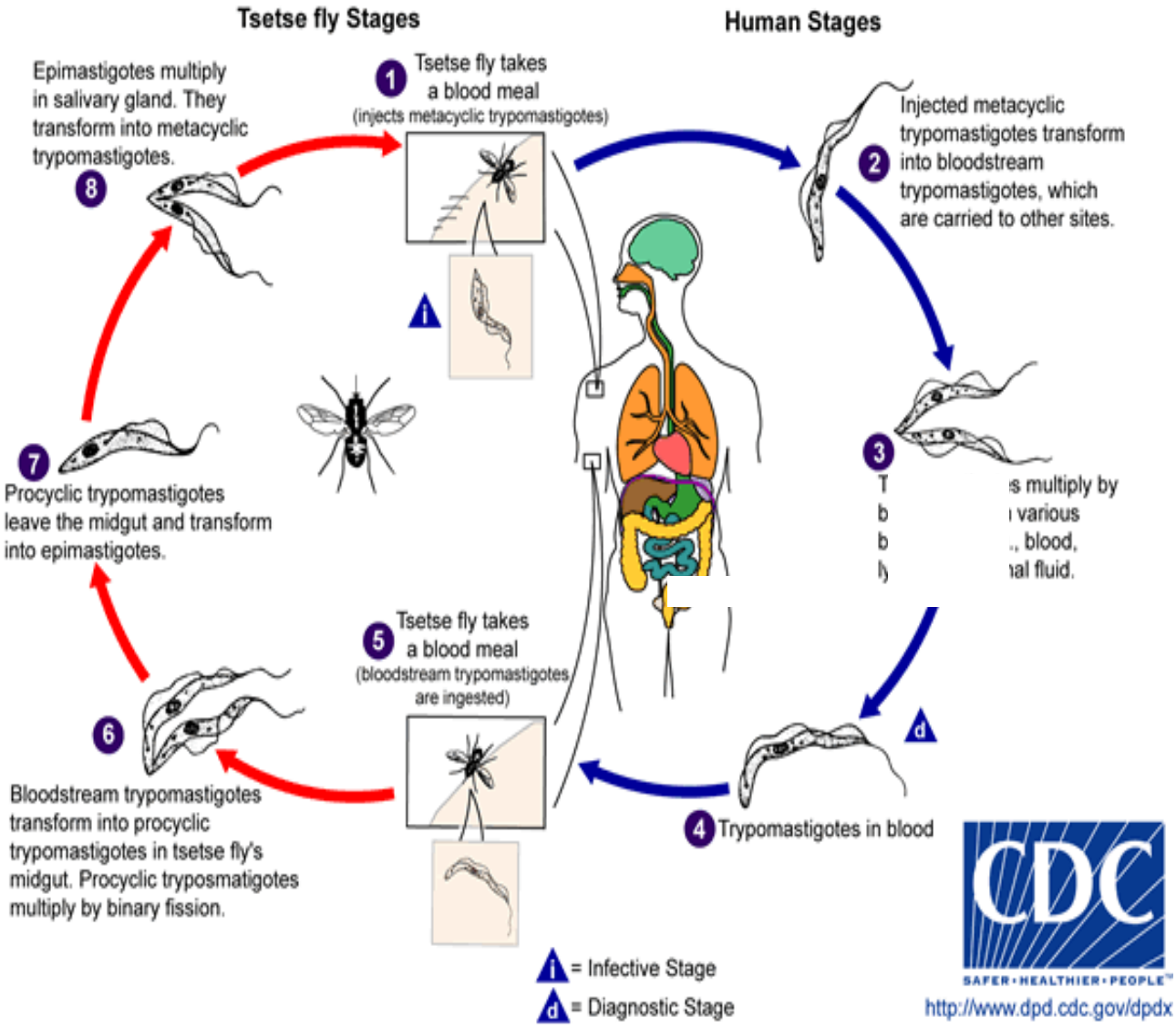


Figure 1: Life Cycle of the *Trypanosoma brucei rhodesiense* parasite (Source: CDC)

2.3 Pathophysiology and Pathogenesis

2.3.1 Hemolymphatic stage

The trypanosome infection is initiated when metacyclic trypomastigotes are inoculated into humans from the saliva of the vector via the tsetse fly bite. A primary lesion usually develops 5-15 days after the infective bite. The trypomastigotes then invade the capillary vessels and enter the general circulatory system causing episodes of fever, headache and sweating. The trypanosomes multiply and invade the lymph nodes and other tissues via blood and lymphatic system (Murilla, 2008; Barrett *et al.*, 2003; Goodwin, 1970).

Anaemia is a major and consistent component of the pathology in both blood phase of infection as well as in the late stage of infection (Mark, 1999). Indicators of anaemia include a decline in the levels of packed cell volume (PCV), red blood cell count and haemoglobin (Hb) which together gives a good indication of the disease status and also the intensity and duration of parasitemia (Ekanem *et al*., 2005; Ekanem, 2006; Katunguka *et al*., 1992).

Trypanosomes of the *brucei* subgroup inhabit the blood plasma, intercellular tissues and body cavity fluid of an infected animal precipitating anaemia and tissue damage (Nwoha and Anene, 2011a; Sharma *et al.,* 2000). The presence of trypanosomes in the blood induces increased red blood cell destruction and changes in biochemical constituents of blood (Akanji *et al.,* 2009; Ekanem and Yusuf, 2008; Igbokwe and Mohammed, 1992; Taiwo *et al.,* 2003).

Immunoglobulin M (IgM) is produced in significant amounts during trypanosome infection and this is succeeded in a few days by a rise in the immunoglobulin G (IgG). All tissues and organs are affected by the infection but of significant clinical pathological changes of importance include meningo-encephalitis, the heart, skeletal muscles and the liver. Cardiac abnormality is observed in the early stages of infection sufficient to cause electrocardiographic abnormalities (Apted, 1970; Mbala *et al.,* 1988) and indeed myocarditis is the main cause of mortality in untreated cases (Blum *et al*., 2008). Clinical signs and symptoms of cardiac involvement during trypanosomosis resulting from trypanosome infiltration into the heart leading to inflammation, fibrosis and even heart failure or death have frequently been observed (Blum *et al*., 2009; Poltera *et al.,* 1976; Poltera., 1985; Adams *et al.,* 1991).

Early diagnosis of cardiac damage can lead to treatment of infected patients for congestive failure as an adjunct in conjuction with trypanocidal drugs (Bertrand *et al.,* 1974) in order to increase success of treatment. In some instances when sleeping sickness patients are treated with trypanocidal drugs, cardiac damage increases with fatal consequences (Jones *et al.,* 1975; Junyent *et al.,* 1988). Indeed the concurrent administration of corticosteroids and trypanocidal drugs has been used with some degree of success (Bertrand *et al.,* 1974). There is therefore a need to determine if trypanosome patients have cardiac complication/involvement at time of trypanosome treatment in order to improve success of treatment.

The commonest diagnostic tool for cardiac abnormalities in animals (Ndungu *et al.,* 1991) and humans (Blum *et al.,* 2007) is the use of the electrocardiograph (ECG). The machine is expensive thus not easily available in most African Medical set-ups where trypanosomosis

occurs in addition to the fact that it cannot be easily applied in the African field condition due to lack of electricity. There is therefore need to develop a cheap and easy to apply diagnostic system/kit that can be suited for the African field conditions. Tissue enzymes have long been used in both humans clinical and laboratory animal safety testing as non-invasive, specific and sensitive biomarkers for the evaluation of cardiac injury (Walker, 2006). Blood levels of tissue enzymes are known to increase in response to early and minimal tissue damage (Rodney *et al.,* 1977) thus allowing early detection of tissue damage.

2.3.2 Central nervous system stage

The second phase of the infection occurs when the parasite invades the CNS resulting in a generalized meningoencephalitis (Kiminyo and Lucey, 2002; Kennedy, 2011; Lamers *et al.,*). The brain becomes infected when trypanosomes cross the BBB to enter the CSF. This leads to a noticeable elevation in CSF cell numbers especially the mononuclear phagocytic cells and in the later stages of the infection there is an increase in immunoglobulin levels dominated by IgM (Brun *et al*., 2010).

The CNS infection leads to the development of signs of nervous system involvement which include sleep disorders, sensory disturbances, mobility disorders and mental changes or psychiatric disorders. Finally a patient loses appetite, lapses into coma and ultimately dies if untreated (Kennedy, 2004; Kennedy, 2008).

2.4 Chemotherapy of Trypanosomosis

The treatment of trypanosomeinfection using available drugs is stage specific. There are few drugs in use including suramin, difluoromethyornithine (DFMO) and melarsen oxide (Melarsoprol®) (Mtulia, 1976; Burchmore, 2002; Brun *et al*., 2001). All the therapies have drawbacks including acute toxicity, poor efficacy, and undesirable route of drug administration and treatment failures (Bacchi, 2009; Gehrig and Efferth, 2008; Kagira *et al.,* 2007; Robays *et al*., 2007). Suramin is used for the treatment of the hemolymphatic stage as it does not cross the blood brain barrier due to its highly ionic nature (Fairlamb, 2003). DFMO on the other hand is only effective against *T. b. gambiense* parasites and is far from ideal as it is costly and high amounts have to be administered (Jose , 1999). Melasoprol®is the only effective drug used against second stage infection when the disease has progressed to the CNS. Melarsoprol® being

an arsenical compound is highly toxic and causes a serious reactive encephalopathy in 5-10% of the cases of which 50% are fatal (Orlando and Arroz 1987; Pepin and Milord, 1994). Due to the risk of encephalopathy development in response to the use of melarsoprol®, it is important that the CNS stage of the infection be established before melarsoprol® is administered. Indeed melarsoprol® should only be administered when CNS invasion by the parasites has occurred (Kennedy, 2009). Other side effects of melarsoprol are liver toxicity, severe enterocolitis, myalgia and fever, pruritus, urticaria, and gastrointestinal reactions and cardiovascular side effects particulary tachycardia (Eric *et al.*, 2008). Indeed Melarsoprol treatment during trypanosome infection when there is cardiac involvement can make the situation worse or even fatal (Jones *et al.*, 1975; Junyent *et al.*, 1988). Thus in such cases co-treatment with adjunt drugs is necessary to mitigate against the post treatment complications (Bertrand *et al.*, 1971).

2.5 Staging of Trypanosome Infection

One of the most important problems in HAT disease management is the considerable difficulty in distinguishing the early hemolymphatic from the late encephalitic stage when the parasites have crossed the BBB to enter and establish in the CNS. Treatment of the two stages is difficult with the highly toxic arsenical drug melarsoprol® being the most commonly used therapy for CNS disease (Kennedy, 2011). Since melarsoprol® kills approximately 50% of the melarsoprol® treated patients, it is vital to develop reliable diagnostic staging markers for the two stages of HAT.

Currently available staging markers for the second stage HAT disease include examination of CSF for presence of trypanosomes and elevated WBC and total protein levels (Buscher and Lejon, 2005). In addition other markers include chemokines such as CXCL10 and CXCL8 (Hainard *et al.*, 2009), lipocalin 2 leukocyte peptidase inhibitor (Amin *et al*., 2009), CSF IgM (Lejon *et al.*, 2002) anddemonstration of parasite deoxyribonucleic acid (DNA) using the polymerase chain reaction (PCR) technique (Deborggraeve *et al*., 2011). In addition Hainard *et al.*, (2011) demonstrated that intercellular adhesion molecule 1 (ICAM-1) and matrix metalloproteinase-9 (MMP-9) either alone or in combination are powerful staging markers of HAT. Although the current WHO staging criteria using CSF WBC counts of more than 5 WBC in the CSF are the most commonly used indicators of CNS involvement (Buschner and Lejon, 2005), there is still lack of consensus as to their efficacy which has necessitated the search for improved methods of HAT central nervous system diagnostic staging.

While all these staging biomarkers have demonstrated a potential efficacy in various studies, there still remain issues of true validity and the practical challenges of carrying out these sophisticated analyses widely under field conditions where the disease occurs. There is therefore great need to develop new diagnostic staging markers that are cheap, quick and easy to perform, non invasive and easily adapted to field conditions (Kennedy, 2008; Kennedy, 2011).

The key issue in HAT diagnosis and therapeutic decision making is to distinguish reliably the late encephalitic stage of HAT from early stage infection. Accurate staging of HAT is important because failure to treat a patient with CNS infection will lead inevitably to death from the disease, yet inappropriate CNS treatment in an early stage patient carries a high risk of unnecessary melarsoprol® drug toxicity (Kennedy, 2008) since it is the only effective currently availabledrug for*T.b rhodesiense* late stage infection (Legros *et al.*, 2002).

2.6 Trypanosomosis Diagnosis

There are major diagnostic challenges encountered in determination of the stage of trypanosome infection in human infections (Burri *et al*., 2002; Steverding, 2006). The diagnosis of HAT is based on a combination of clinical and investigative data. The definitive diagnosis of HAT requires the demonstration of the parasite in blood, body fluid and CSF (Blum, 2011).

Several different types of serological tests have been used to detect specific antigens or antibodies developed by the host against the infection including the indirect immunofluorescent test and enzyme linked immunosorbent assay (ELISA) (Chappuis *et al.*, 2005). However, such antigens and/or antibodies are known to persist for some time after all trypanosomes have disappeared from host organism following treatment and as such a positive result from such tests have been found to be of no proof of active infection (Cheesbrough, 1998; Kennedy, 2004).

Besides the demonstration of trypanosomes in CSF of affected patients, determination of changes in CSF total protein concentration and white blood cell (WBC) count has been used as indicators of CSF infection (Buscher and Lejon, 2005). The protein and CSF WBC changes are however not fully reliable for diagnosis or disease staging. The number of WBC in CSF of

second stage HAT patients varies from normal to around 100-300 cells/µl (Bisser *et al*., 2002) while protein concentrations in the CSF of sleeping sickness patients range from 100-200mg/l (Bisser *et al*., 2002). As a consequence, some controversy exists about the interpretation of CSF data for therapeutic decision.

2.7 Tissue enzymes in diagnosis

Due to the fact that most diagnostic methods are known to be less sensitive, less accurate and unreliable, it is important that new methods of organ specific diagnostic indicator be found. At the same time, levels of tissue enzymes are known to increase in response to early and minimal tissue damage (Rodney *et al.,* 1977) thus allowing early detection of damage to the affected organs. Several trypanosome studies have been carried out to come up with possible marker for the disease. Most of the work has been focusing on the biochemical changes taking place during the infection. A study by Ogunsamni *et al.* (1994) on the biochemical changes in sheep infected with *T. b. brucei* revealed that serum protein and mineral levels were affected. Corredor (2003) found an increase in tissue enzyme activities in *Trypanosoma cruzi* infection while Ngure *et al.,* (2008) established that total CK and LDH enzyme activity increases during *T.b rhodesiense* infection.

Increased creatine kinase and LDH levels in the CSF have been reported to indicate either tissue damage of the CNS or altered permeability of blood brain barrier (BBB) (Drent *et al*., 1996; Mayhew and Beal, 1980; Sedlakova and Necas, 1999). Valuable information concerning CSF CK activity relative to duration and severity of the disease in humans has also been reported (Safar *et al.*, 1982). Indeed, Klun (1974) reported that in humans with CNS trauma, the CSF enzyme values corresponded to the clinical severity of injury with the enzyme elevation being proportional to the extent of tissue damage. Simultaneous measurement of serum and CSF LDH activity and LDH isoenzyme profile have been found to aid in establishing status of the BBB (Heavner *et al*., 1986). In tissue enzyme determination it is important that the isomeric type be identified rather than the knowledge of total enzyme activity. Indeed, isoenzyme determination permits more precise recognition of the tissue of origin (Shillingford, 1975). CK brain isoenzyme (CK BB) is the only isoenzyme contained in the neural tissue. It is an intracellular enzyme released into CSF and diffuses into serum following various neurological conditions including meningitis (Nussinovitch *et al.*, 1996; Necas and Sedlakova, 1999).

A good number of HAT patients during the haemolymphatic and also the CNS stage have cardiac damage due to destruction of myocardial cells (Jones *et al*., 1975; Mbala *et al*., 1988) resulting in the loss of membrane integrity leading to enzymes diffusion to extra cellular spaces and drainage into blood vessels. The appearance in the serum of the various cytoplasmic enzymes has formed the basis of a variety of clinical diagnosis procedures for the detection of tissue damage of the heart and CNS using CK and LDH, respectively **(**Benjamin, 1985; Nussinovitch*et al*., 1996**).** However the data on total CK and LDH activities and protein is not adequate to confirm the site of infection, because most tissue enzymes are not tissue specific but the specificity can be enhanced by determining their isoenzymes that are relatively tissue specific.

CK and its isoenzymess is one of the most organ-specific serum enzymes in clinical use (Morag, 1991). CK reversible catalyses reaction where creatine phosphate is converted to creatine (Worthington, 1988) in cells requiring high energy. It is present in high concentration in muscles, brain and heart. The enzyme is composed of two sub units of either M or B genes, giving rise to the isomeric forms namely the muscle type MM, the brain type BB and the hybrid type MB with highest concentrations in heart muscles (Calbreath, 1992). Blood total CK measurements have frequently been used as a marker in experimental animals infected with trypanosomes. An increase in enzyme level was observed during *Trypanosoma cruzi* infection in mice (De Souza *et al.,* 2000) where the total CK levels were found to increase 4-70 times. CK (BB) also diffuses into blood from CSF and thus can be measured in blood as an indicator of brain involvement (Rodney *et al.,* 1977) without using invasive procedure on the CNS.

Lactate dehydrogenase is another enzyme whose activity level has been used as an indicator of diseasesin animals (Kaneko, 1980), belongs to non plasma specific enzymes and thus cellular damage associated with trypanosomosis will result in leakage of LDH into the plasma, causing a sharp rise in the plasma and CSF concentration of the enzyme (Kaneko, 1980).The enzyme is composed of four sub units of either heart or muscle, giving rise to five isomeric forms. It is found in different tissues and organs in various concentrations. LDH type 1 (LDH 1) consisting of HHHH (H4) form is exclusively found in the heart, LDH type 5 (LDH5) or MMMM (M4) is mostly found in muscles.

Little is known on the effect of the infection on the skeletal and heart muscles isoenzyme levels. Available information on total CK and total LDH pattern and especially their isoenzymes are therefore needed to study these enzymes as a part of the search for a better marker of lesion progression induced by *T.b rhodesiense* infection due to the fact that the two enzymes exist in a variety of isoenzymes.

CHAPTER THREE
MATERIALS AND METHODS

3.1 Experimental Animals

Ten two year old male indigenous masaai sheep breed, weighing between 15 and 27 kg body weight (bwt) were acquired from Naivasha district, a trypanosomosis free area. The choice of sheep was based on the fact that sheep have been used as models for endocrinology, reproduction and pregnancy and foetal development. Sheep was the first mammal to be cloned from an adult cell (dolly). Sheep are animals that have many similarities to humans in terms of physiology. Besides that they are easy to handle and less expensive to keep. The animals were initially quarantined in a fly proof barn for a period of 30 days for purposes of acclimatisation and then transferred to experimental wards where they were again allowed to acclimatize for two more weeks. During that time the animals were de-wormed using albendazole® (Kela, Belgium) at a dose of 25gm/kg bwt and sprayed with acaricide amitraz (triatic®) (Coopers Animals health Inc, USA). Sheep were fed twice daily on hay plus minerals and protein supplements ration (30g/day) (Vitaphosebiomin, Vetcare, Kenya) while water was provided *ad libitum.* During the experimental ward acclimatization period the animals were weighed weekly and rectal temperature recorded on a daily basis .At the same time blood was collected for biochemical analysis to provide pre-treatment values.

3.2 Trypanosomes and Preparation of Inoculum

Trypanosomes from the cryobank were propagated in donor white albino mice immunosuppresed with cyclophosphamide at a dose of 300mg/kg bwt intra-peritoneally for 3 consecutive days as described by Kagira *et al.,* (2005) before being used to infect the eight sheep. The stabilate from the Trypanosomiasis Research Centre (TRC) cryobank used in this study was KETRI 2537. The stabilate is a derivative of EATRO 1989 which was isolated from an infected human patient in Uganda and prepared by the direct inoculation of blood and lymph node aspirate into mice (Schmidt and Fink, 1980) and later cryopreserved. This stabilate is the basis for the well established monkey model of sleeping sickness (Schmidt and Sayer, 1982).

The stabilate was thawed and inoculated in three immunosuppresed donor white albino mice and the donor mice monitored daily for parasitaemia by examining tail blood under the light microscope at x 40 magnification. At the first peak of parasitaemia, the blood was

obtained by cardiac puncture from the mice and then diluted with phosphate-buffered saline (PBS) to a concentration of $1x10^4$ trypanosomes per ml. This preparation was then used to infect the eight sheep using 2ml of the stabilate via the jugular vein. Two of the remaining uninfected sheep served as un-infected controls. The choice of parasite load was based on previous studies using monkel model (Kagira *et al*., 2007b).

3.3 Experimental Design

A total of ten sheep were used. Eight animals were infected with 1×10^4 *T.b.rhodesiense* and two animals were not infected and therefore used as controls. The sheep were monitored on a daily basis for parasitemia and temperature. Body weight was monitored on weekly basis. In addition, blood for both haematological and biochemical changes during the course of infection for both groups was collected every other week up to 42 days post infection (dpi) and thereafter at every three weeks until termination of the experiment. Treatment was done when the infected sheep showed deterioration in their health status. On 42 dpi all infected sheep were treated by intra-muscular route (IM) with 5mg/kg bwt diminazene aceturate (Veriben®, Hoechst, Germany). Berenil has been used to clear parasites in the in the blood in the mouse (Jening, 1983) and in monkeys (Ndungu *et al*., 1994) thus establish CNS trypanosoma infection and thus late stage infection. The sheep were monitored for a period of 140 days when the experiment was terminated. At the end of study the sheep were euthenised using euthasol® and their carcases incinerated.

3.4 Measurement of Body Weight and Rectal Temperature

The body weight in kilograms, of each individual sheep was measured weekly using a spring weighing balance (Salter, India) while the rectal temperature in degrees centigrade was taken on a daily basis following infection using a clinical thermometer (CR.W 12 clinical thermometer, rectal, China).

3.5 Clinical Examination and Parasitaemia Estimation

Blood was examined for the presence of trypanosomes by direct microscopy at x 40 of a wet smear. In addition, blood was collected from the ear vein into heparinised capillary tubes, centrifuged in a microhaematocrit centrifuge (Hawksley, England) and the buffy coat examined for the presence of parasites (Murray *et al.*, 1977). Once parasites were seen by direct

microscopy the level of parasitaemia in blood of infected sheep was estimated using the method of Herbert and Lumsden (1976).

The values indicate the logarithm of the number of trypanosomes per milliliter as computed for *Trypanosoma brucei* infections in mouse blood examined at x 400 magnification.

Table 1: Table used for estimating lower trypanosome parasitemia.

5 fields	10 fields	20 fields
4-5 trypanosomes 6.6	2-3 trypanosomes 6.0	2-3 trypanosomes 5.7
7 trypanosomes 6.3		4 trypanosomes 5.4
		0 trypanosomes <5.4

3.6 Blood Sample Collection and Preparation for Analysis

Blood samples were collected aseptically from the jugular vein using a gauge 23 needle weekly up to 42 dpi then every two weeks up to 70 dpi but thereafter every 3 weeks. About 2ml of blood with heparin and 10mls without anticoagulant, blood volume were collected for haematological and biochemical analysis, respectively. Blood was collected from the animals weekly for the first 4 weeks and thereafter every two weeks up to the end of the experimental period of 140 dpi.

Serum for protein and enzyme assays was prepared by allowing whole blood to coagulate for thirty minutes at room temperature followed by another twelve hours at 4^0C, after which it was separated by centrifuging at 3000 revolutions per minute (rpm). Serum samples harvested were then dispensed into labelled sterile screw-capped vials and stored at -80^{o}C until required for analyses.

Every two weeks the animals were anaesthetised using a mixture of diazepam (Rotexmedica, Trittau Germany) and ketamine hydrochloride (Rotexmedica, Trittau, Germany) at a dose of 1mg/kg bwt and 10-15mg/kg bwt, respectively. A volume of 1-2ml of free flowing CSF was obtained aseptically by lumber puncture from lumber vertebral spaces 1 or 2 using a sterile 23 gauge needle and the CSF used for the total WBC and total protein determination (Ndungu *et al*., 1994). At the same time two drops of free flowing CSF was collected into a capillary tube and the content immediately transferred onto a haemocytometer chamber and presence of trypanosome noted while at the same time the number of white cells

counted. If trypanosomes were not seen in the counting chamber, approximately 1ml of CSF was collected into a pipette whose tip had been sealed by heating. The content was then centrifuged and the sealed end of the tube examined for trypanosomes using a microscope as described by Gould and Sayer (1983).

3.6.1 Determination of haematological parameters

Two sets of blood samples were collected from each sheep by jugular venipuncture at set time of the experiment as indicated in section 3.6. Blood samples of 10ml each were placed into plain vacutainer tubes for serum separation for use in the enzyme analysis. Another blood sample of 2ml wasplaced intoa sterile screw capped tube which contained ethylene diamine tetra acetic acid (EDTA) anticoagulant. The later blood samples were used for different haematological parameters analysis including packed cell volume (PCV), haemoglobin (Hb) levels, red blood cell count (RBC) and total WBC. In addition the sample with anti-coagulant was used to determine erythrocyte indexes including mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) (Kagira *et al.*, 2006). With the exception of PCV, all haematological analyses were determined using an automated haematology analyser (Beckman Coulter, Miami, USA) while the PCV was determined using micro-haematocrit method (Lorne, 1986).

3.7 Cerebrospinal Fluid PCR for Detection of Trypanosomes

Cerebrospinal Fluid (CSF) samples from all sheep that had been infected and treated with diminazine aceturate (berenil®) 42 dpi and in which trypanosomes could not be detected by the concentration method, were further analysed for the presence of trypanosome DNA using the serum resistant antigen (SRA) PCR method (Masiga *et al.*, 1992).

3.7.1 PCR analysis for the detection of *Trypanosoma brucei rhodesiense* in CSF

Trypanosome DNA was extracted using saponin lysis method as described by Mugittu *et al.*, (2001) and the extracted DNA was tested individually with *T.b rhodesiense* specific TBR1 and TBR2 primer pair to identify the *brucei* sub-group (Masiga *et al.*, 1992). The concentration of the primers used was 0.4µM. Standard PCR amplifications were carried out in 25µl reactions mixtures containing the final concentrations consisting of 10mM Tris-HCl pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, 200µm of each of the 4 deoxynucleoside triphosphates and

1 unit of *Taq* DNA polymerase (Sigma). The amplification conditions were 35 cycles at 94^0C for 1 minute then 92^0C for 30 seconds and 60^0C for 45 seconds and 72^0C for 45 seconds and a further 72^0C for 4 minutes (Masiga *et al.*, 1992). PCR products were separated by electrophoresis using 2% agarose gel containing 2.5µg/ml ethidium bromide and visualized under ultraviolet light.

3.7.2 SRA amplification from genomic DNA

Serum resistance antigen, SRA Aand SRA E primer pair were used to screen for the subspecies of *T. brucei*. The amplification was carried out using the method described by Masiga *et al.,* (1992). Typical conditions for SRA amplification included an initial denaturation done for 3 minutes at 98^0C, followed by a second denaturation at 98^0C for 30 seconds, annealing at 60^0C for 30 seconds, extension at 72^0C for 45 seconds and final extension at 72^0C for 2 minutes. PCR was carried out for a total volume of 20µl for 35 cycles and the final concentration of each component included 0.4µM of each primer, 4µl of 5 times phusion buffer containing 7.5mm $MgCl_2$, 2.5mM of dNTPs, 5µl of DNA template and 1 unit *Taq* polymerase.

The primers sequences used for PCR included:

TBR 1: 5'GAATATTAAACAATGCGCAG 3' and

TBR 2: 5' CCATTTATTAGCTTTGTTGC 3'

The SRA primers for *T.b rhodesiense* were as described by Gibson *et al.* (2002).

SRA A: 5' GACAACAAGTACCTTGGCGC 3' and

SRA E: 5' TACTGTTGTTGTACCGCCGC 3'

The PCR products were analysed in a 2% agarose gel submerged in Tris-acetate- EDTA (TAE buffer) containing ethidium bromide after which the gel was visualised under UV light.

3.8 Biochemical Analysis

3.8.1 Total CSF and serum protein levels

Total serum protein levels from individual 10 sheep on different date of sampling were determined by the biuret method (Blijenberg *et al.,* 1985). Briefly 100µl of serum was mixed with 1200µl biuret reagent and 700µl physiological saline solution (0.85% NaCl) in a micro-centrifuge tube followed by incubation of the mixture at 37^0C for 30 minutes.After the formation of a bluish purple colour coordination complex, absorbencies were measured at

540nm using anultra violet (UV) visible spectrophotometer (Cecil-CE 2021-2000 series, England). Total protein concentration in the CSF from each sheep on different date of sampling was determined by mixing 150µl of CSF, 150µl physiological saline and 450µl biuret reagent. After mixing the content, the mixture was left for 30 minutes for colour development. The absorbencies were then read at 540nm wavelength using UV visible spectrophotometer (Cecil-CE 2021-2000 series, England). Bovine serum albumin (BSA) (BIO RAD) was used as a standardto establish a standard curve by assaying serially diluted known concentrations of BSA obtained using a stock solution of 20mg/ml serially diluted in physiological saline (0.85%NaCl). The standard curve (Appendix 1) was then used to estimate the amount of protein in both serum and CSF samples.

3.8.2 Total enzyme levels

Serum and CSF samples were used for biochemical analysis. The total LDH activity of each sample was determined using the commercial lactate dehydrogenase NAC-Kinetic diagnostic kits" (Chrono Lab., Switzerland) and read on a UV-visible spectrophotometer (Cecil CE 2021-2000 Series, England).

The total LDH activity was measured by a decrease in absorbance at a wavelength of 540nm, while determination of total CK for both CSF and serum samples was undertaken in UV-visible spectrophotometer using a CK NAC-Kinetic AG diagnostic kit (Chrono Lab, Switzerland based on the method by Oliver (1955) as summarised in figure 3. Briefly, CK catalysed the reversible reaction of creatine phosphate with adenosine di-phosphate (ADP) producing adenosine tri-phosphate (ATP).The ATP was then measured indirectly by coupling to glucose using the enzyme hexokinase to produce glucose 6 phosphate which in a subsequent step is coupled to $NADP^+$ by the enzyme glucose 6 phosphate dehydrogenase to produce nicotinamide adenine dinucleotide phosphate (NADPH) as the end product. The amount of NADPH was then measured photometrically as an indicator of the amount of ATP originally present. The rate of formation of the end product NADPH, at a wavelength of 340nm was determined as it was a measure of the CK activity.

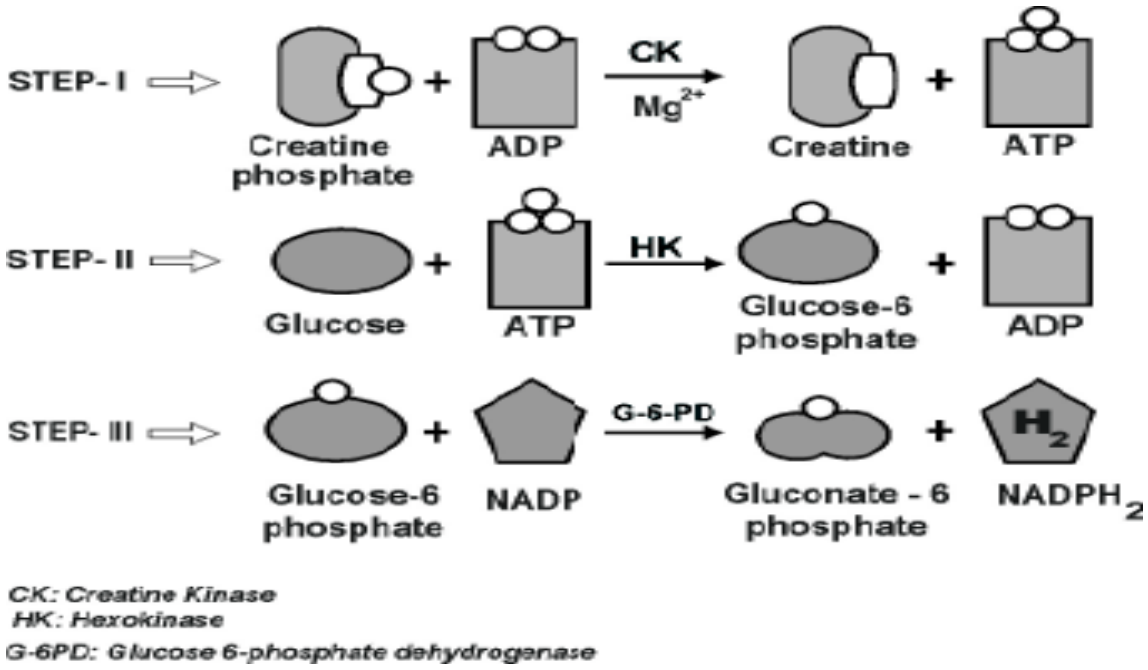


Figure 2: Reaction catalyzed by CK

3.9 Isoenzyme Analyses

The isoenzyme profiling for the enzymes CK and LDH for both serum and CSF sample aliquots at different points during infection were separated by subjecting the samples to horizontal electrophoresis on a 12% starch gel, prepared by dissolving 36g of starch in 70ml gel buffer (0.2M Tris, 0.003M citrate, 0.0003M NaOH) at pH 8.6. Electrophoresis was carried out at 10^0C for 3 hours at 350 volts, 70 milliamps (mA) using 70ml tris buffer (0.2M Tris, 0.8M citrate, 0.056M NaOH). The conditions for separating serum and CSF CK into its isoforms included passing an electric current of 70 mA at a potential gradient of 10-12V/cm, at 10^0C for 3 hours.

3.9.1 LDH staining

Lactate dehydrogenase isoenzyme bands after electrophoresis were located on the gel using the modified method described by Tanzer *et al.*, (1966). The staining solution consisted of 0.5ml of a 0.5% 2, 5, diphenyl-3-4, 5-dimethyl thiozolyl-2-tetrazolium bromide (MTT), 0.5ml of 0.5% phenazonium methyl methosulphate (PMS), 10ml of 2% agar, 2ml of 0.5M lactic acid with pH adjusted to pH 8.0 with NaOH. Substrate solution consisting of 13ml of 0.5% NAD^+ with the pH adjusted to pH 8.0 using 0.2M Tris/HCl was also prepared separately. The two solutions were then mixed to form the staining solution.The stained gels were then

incubated at 37^0C in the dark for one hour or until sufficient activity was present when the LDH isoenzyme bands stained bluish purple. Theisoenzyme bands on the gels were then scanned using a mustek1248 B scanner (Mustek Systems, Inc, China) and saved on a computer for later quantification. The proportion of the individual isoenzyme bands were determined with a UN-SCAN-IT gel software program(Silk Scientific, Inc. Utah USA) on a gel and graph digitizing software windows edition version 6.1 which works like a gel densitometer.

3.9.2 Creatine kinase staining

Creatine kinase isoenzyme bands were located by overlaying the gel with a solution containing two reagents. The first solution was a buffer/glucose consisting of Imidazol (100mM/l) pH 7.0, glucose (20mM/l), magnesium acetate (10mM/l) and EDTA (10nM/l) while the second solution is an enzyme/coenzyme/substrate consisting of ADP (2mM/l), AMP (5mM/l), $NADP^+$ (2mM/l),di-adenosine 5 pentaphosphate (10mM/L), Hexokinase (2500U/L),glucose 6 phosphate dehydrogenase (1500U/L), N-acetylcysteine (20Mm/l)and creatine phosphate (30mM/L). The gels were incubated at 37^0C in the dark for about one hour until sufficient activity was present with the bands staining bluish purple on a pinkish orange background. The gels were then scanned with Mustek 1248 B scanner (Mustek Inc, China) and the proportions of the individual isoenzymes determined with a UN-SCAN-IT gel program.

3.10 Statistical Analysis

The data for the different parameters from the individual sheep were used to calculate the mean and standard deviations. Statistical analyses was performed on haematological data, mean body weights, rectal temperature and biochemical changes data using the using Minitab version 16 data analysis tool. T-test was used for determining significant differences between the means of infected and uninfected groups of all the parameters. Significance was considered at $p< 0.05$.

CHAPTER FOUR
RESULTS

4.1 Clinical Signs and Symptoms

All the infected sheep showed clinical signs of scrotal and facial oedema commencing 5 sheep dpi. By 14 dpi, sign of severe anaemia, rough hair coat, enlarged lymph nodes and loss of body weight were observed. Two infected sheep examined were very weak and sick at 35 dpi leading to death of one sheep on the morning of 42 dpi. The death of one sheep and the deterioration in health of the remaining infected sheep necessitated the treatment with diminazene aceturate (Berenil®, Hoest, Germany) intramuscularly at a dose of 5mg/kg bwt 42 dpi on humane basis to prevent the deterioration of the animal's health and possible death .However even after treatment, one sheep died 49 dpi but the health status of the remaining infected treated sheep markedly improved and showed no effect of the disease of the sheep till 140 dpi when the experimental study was terminated. On the other hand, the uninfected sheep health status was not affected throughout the experimental period thus the two sheep were in good health.

4.2 Blood Parasitaemia Levels

The mean parasitaemia level in blood of the infected sheep is presented in figure 3. The infected sheep showed presence of trypanosomes in blood at different times post infection and pre-treatment. The first sheep was blood smear positive for trypanosomes 4 dpi while all the sheep were trypanosome positive in blood smear by the 7 dpi. This gave a mean pre-patent period of 5.12 days with a range of 4-7 days.The first peak of parasitaemia (log 2.7±1.02) occurred at 5 dpi and this was followed subsequently by several waves of parasitaemia. However, at 13 dpi the parasitaemia level rose sharply to around log 5.4±0.0 where it remained elevated until diminazene aceturate was administered. After treatment with diminazene aceturate at a dose of 5 mg/kg bwt 42 dpi, the parasite levels declined sharply and trypanosomes disappeared from peripheral blood circulation in four sheep by 53 dpi and all sheep by 56 dpi. No trypanosomes were demonstrated in blood of any of the infected sheep thereafter till termination of the study 140 days post infection.

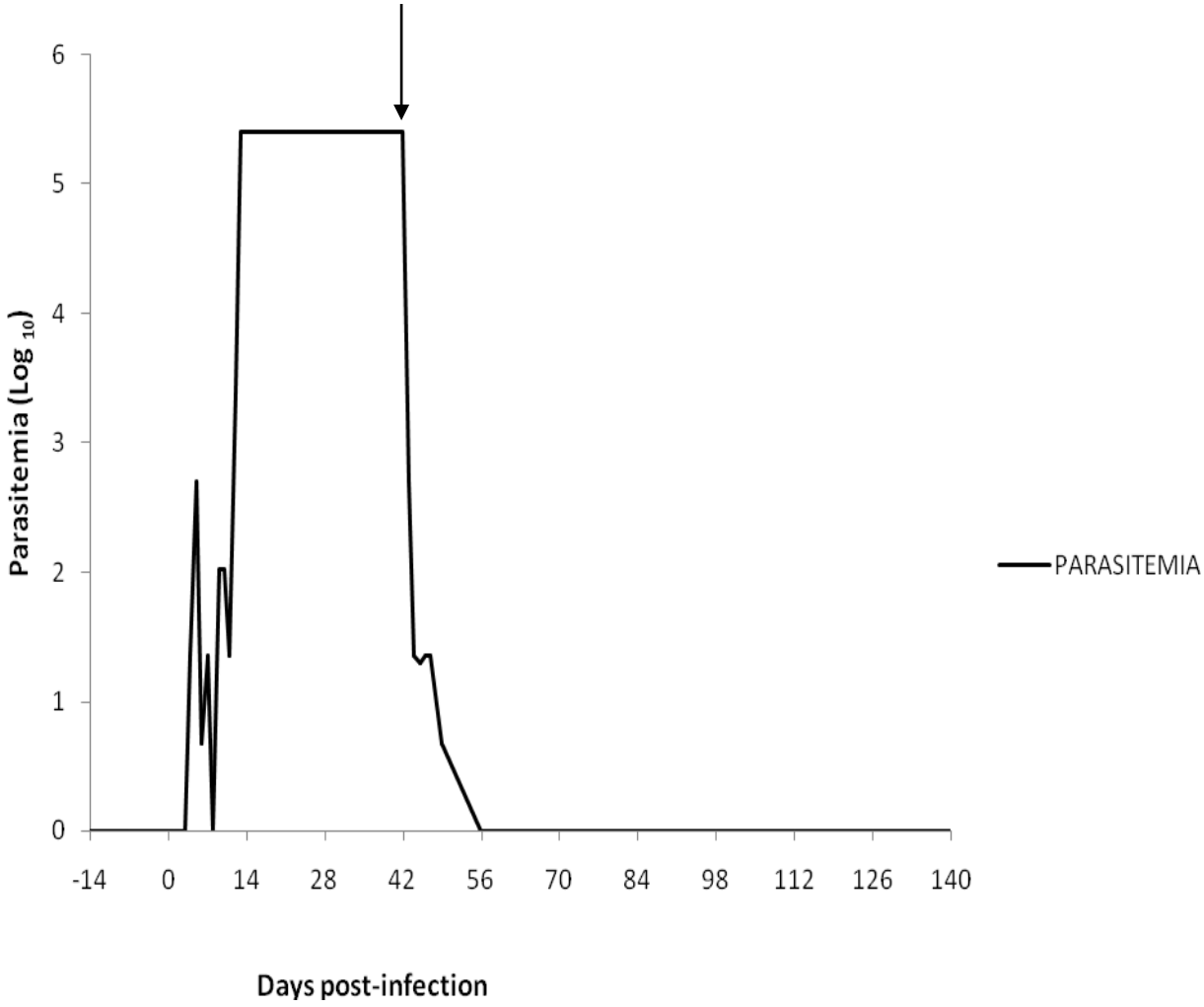


Figure 3: Mean parasitemia (Log10) levels in sheep infected with *T.b rhodesiense* (KETRI 2537) Treatment day

4.3 Rectal Temperature

The mean rectal temperature of the infected and uninfected control sheep are presented in Figure 4. The mean rectal temperature of un-infected sheep was 38.6±0.38°C and remained close to those levels throughout the experimental period. This value was similar to the mean pre-infection temperature of the infected group which was 38.4±0.32°C .However, following infection there was a rapid increase in the body temperature of the infected sheep from a mean value of 38.4 ± 0.32°C pre-infection to peak at by 28 dpi and later decreased 40.2 ± 0.25°C by 42 dpi. Following the diminazene aceturate (berenil®) treatment the rectal temperature drastically declined to reach control levels by 77 dpi at which it remained until termination of the experiment. However, between 70 and 90 dpi the values dropped to below control levels.

The mean rectal temperature of the infected sheep and un-infected control were significantly different ($p<0.05$) between 7 and 49 dpi.

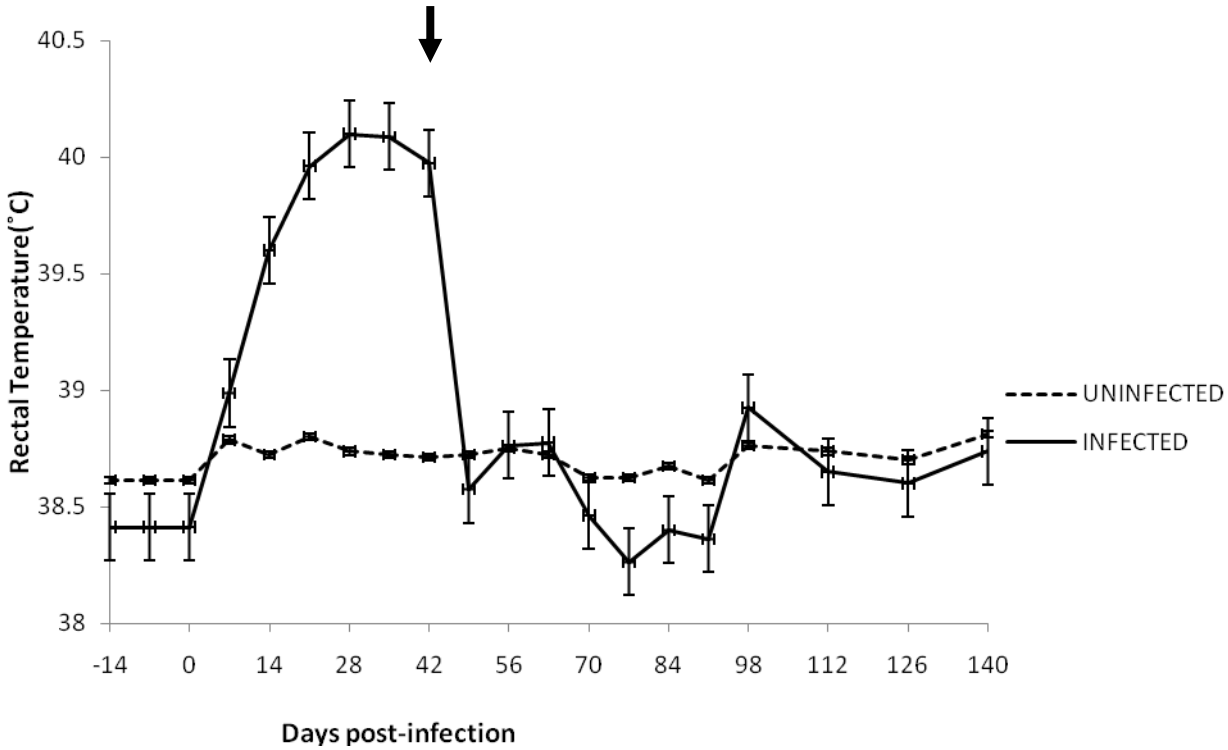


Figure 4: Mean (±SEM) body temperature of *T.b rhodesiense*-infected and uninfected sheep (↓= Treatment of infected sheep with diminazene aceturate).

Table 2: Trend in body temperature

Dpi	**-7**	**0**	**7**	**14**	**21**	**28**	**35**	**42**	**49**	**56**	**63**	**70**	**98**	**112**	**126**	**140**
Trend	**0**	**0**	**0.6**	**0.6**	**0.4**	**0.1**	**-0.01**	**-0.1**	**-1.4**	**0.1**	**0.01**	**-0.3**	**-0.2**	**-0.2**	**0.01**	**0.01**

4.4 Cerebrospinal Fluid Parasitosis

A summary of the CSF parasitosis of the trypanosome infected sheeps is presented in Table 3. Seven of the eight *T .b. rhodesiense* infected sheep became CSF parasite positive at different dpi as revealed by direct microscopic examination or following centrifugation of CSF. The first sheep become CSF positive on the 21stdpi as detected by the centrifugation method. Subsequently one more sheep became CSF positive with trypanosomes as detected by centrifugation in CSF on 28 dpi. By 35 dpi another four were CSF positive with trypanosomes in one sheep being detected by direct microscopy while in the other remaining three sheep the trypanosomes were detected following pre-centrifugation. By the 42nd dpi, four animals were

trypanosome positive in CSF, two detected by direct microscopy while the other two were detected following pre-centrifugation method.

However, of the total eight trypanosome infected sheep, only two animals were consistently parasite positive in the CSF while the other 6 sheep showed intermittent parasitosis at different time periods before diminazine aceturate treatment. The remaining 2 uninfected sheep were parasite negative by both direct microscopy and following centrifugation examination methods. Following diminazine aceturate treatment all the infected sheep became CSF negative by 56 dpi by both direct and concentration examination method and remained so throughout the experimental period on direct microscopic examination and centrifugation of the CSF.

Table 3: Summary of number of sheep that were trypanosome positive in CSF by direct microscopy and following centrifugation of CSF

Day after infection (dpi)	**21 dpi**	**28 dpi**	**35 dpi**	**42 dpi**	**>56 dpi**
Number of sheep positive for CSF trypanosomes by direct microscopy method	0 out of 8	0 out of 8	1 out of 8	2 out of 7	0 out of 6
Number of sheep positive for CSF trypanosomes by pre-centrifugation method	1 out of 8	1 out of 8	3 out of 8	2 out of 7	0 out of 6
Total positive	1 out of 8	1 out of 8	4 out of 8	4 out of 8	0 out of 6

Because fewer than 50% of the animals were CSF trypanosome negative by the time of treatment and all treated animals were negative following treatment, selected sample were analysed for trypanosome by the more sensitive PCR method in order to establish their CSF trypanosome status with results summary being presented in Table 3 with a representative gel image in Figure 5. The results indicate that more of the trypanosome negative CSF samples that were CSF negative on microscopy by both direct and after centrifugation were positive by PCR method for CSF samples collected on 21 dpi, 42 dpi and after treatment on 112 dpi. These results indicate the higher sensitivity of PCR method in detecting *T.b rhodesiense* DNA in CSF.

Table 4: Summary of number of sheep trypanosome positive by PCR method in CSF following infection and diminazine aceturate treatment

Day post infection (dpi)	**21 dpi**	**42 dpi**	**112dpi**
Total number of CSF samples analysed by PCR	7	6	6
Number of sheep positive for CSF trypanosomes by microscopy method (direct and with pre-centrifugation)	Positive 1/7	Positive 4/6	Positive 0/6
Number of sheep positivefor CSF trypanosomes by PCR method	5/7	6/6	4/6

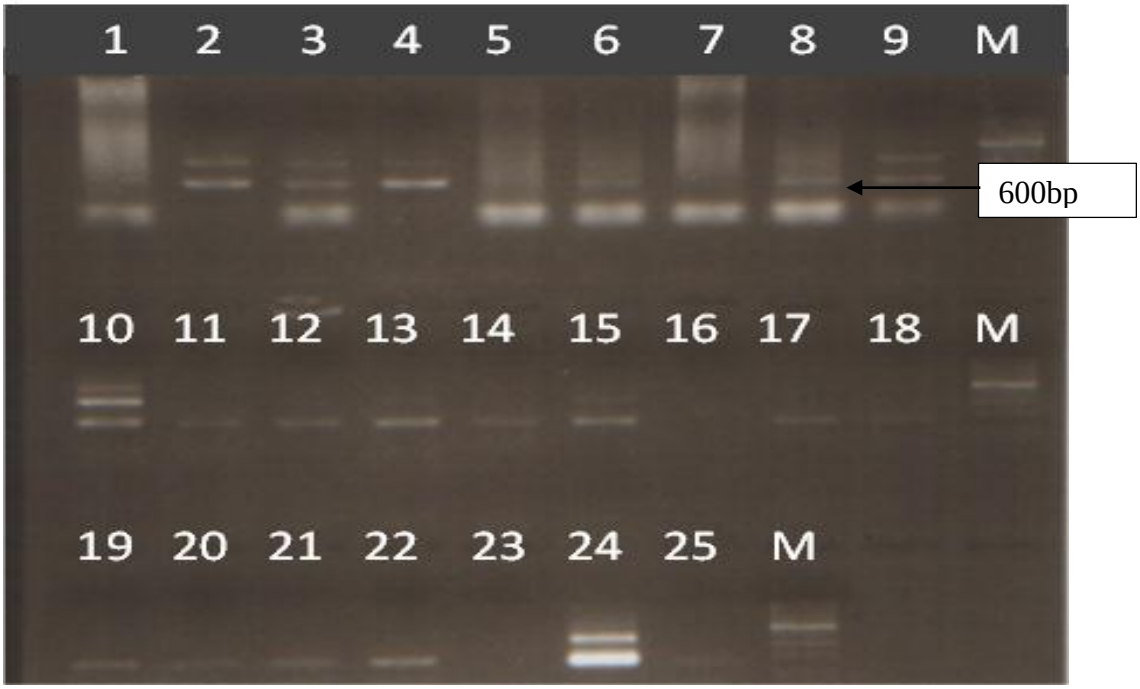


Figure 5: A 2% gel electrophoresis of trypanosome amplified *T.b rhodesiense* DNA from infected sheep's CSF. M represents molecular weight marker plus 100 bp DNA ladder

The lanes denote the following sheep CSF DNA PCR products amplified using SRA A and SRE A primers; lanes 1-7 represents samples taken 21 dpi, 8-15 represents samples taken 42 dpi, 16-23 represents samples taken 112 dpi. Lanes 24 and 25 represent positive and negative controls respectively. Lanes 11, 14, 19 and 21 represent PCR products from uninfected samples.

4.5 Body Weight

The mean weekly body weights (bwt) of un-infected control and infected sheep are presented in Figure 6. The pre-infected mean weekly live body weight was 23.50±1.06 kg while that of un-infected control sheep was 17.50±3.54 kg. The mean bwt of un-infected sheep gradually and consistently increased throughout the study period from 17.50 ±3.54 kg pre-infection to 23.50 ±1.06 kg on 140 dpi when the study was terminated. However in the infected group of sheep, the mean body weight declined steadily and gradually changed from 23.50 ± 1.06 kg to 21.94 ± 3.50 kg on day 14 post infection and further to 20.12 ± 3.40 kg on 42 dpi when the sheep were treated with diminazine aceturate.

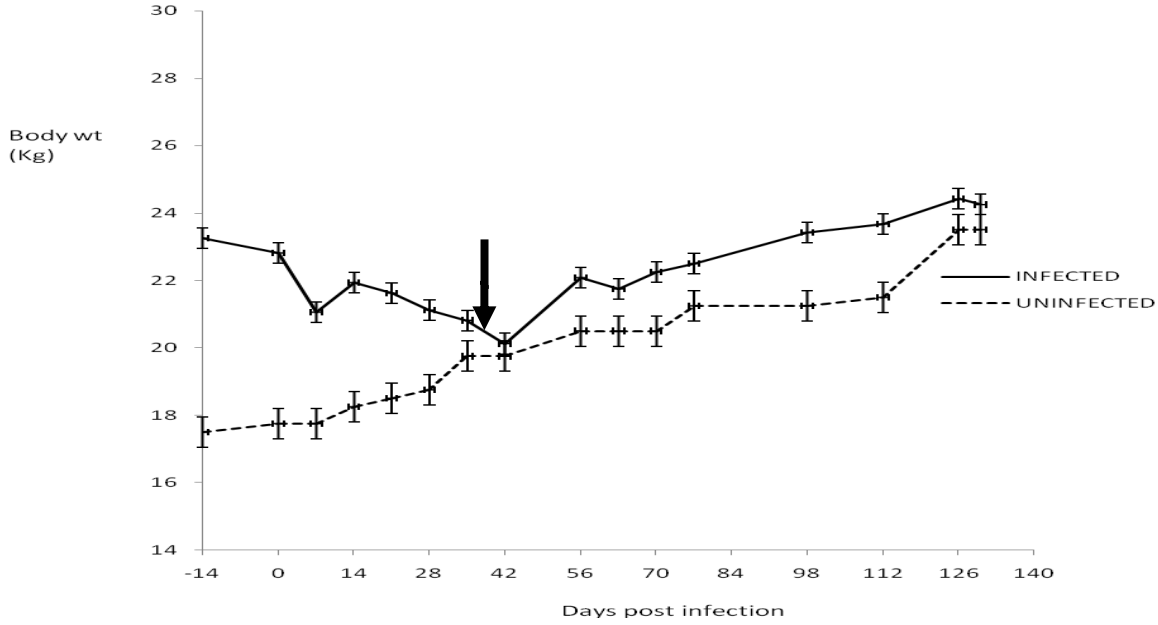


Figure 6: Mean (±SEM) body weight changes in the un-infected and *T.b rhodesiense* infected sheep ↓ = Treatment day of infected sheep with diminazene aceturate.

The changes in the mean bwt pre-treatment with diminazene aceturate was significantly different ($p<0.05$) between the infected group and the un-infected control groups. However, post diminazene aceturate treatment there was no significant differences ($p>0.05$) in the bwt change between the infected group and the un-infected control groups both showing gradual increase in body weight. After diminazine aceturate treatment of the infected sheep on 42 dpi, the mean bwt gradually increased and continued to increase throughout the experiment to pre-infection levels to reach 24.18 ± 1.9 kg by 126 dpi when the last weighing was done before the experiment was terminated.

Table 5: Trend in body weight

dpi	-7	0	7	14	28	42	56	70	84	98	126	140
Trend	-0.433	-0.667	-0.6	0.4	0.4	-0.2	0	-0.35	0	-0.05	0	-0.75

4.6 Haematological Profiles

4.6.1 Packed cell volume

The changes in PCV values is as shown in Figure 7. The mean PCV pre-infection value was 29.25 ± 2.7% and 28.25 ± 3.3% for the infected group and un-infected control group, respectively. However, following trypanosome infection of the sheep with *T.b rhodesiense* the mean PCV levels of the infected sheep showed a steady and marked decline to 25.4 ± 1.6% by 14 dpi and it reached the lowest level of 21 ± 1.1% at 35dpi. However, following diminazene aceturate treatment of infected sheep at 42 dpi (PCV = 22 ± 1.19%) the PCV levels steadily recovered to reach the pre-infection levels of 28±3.56% by 70 dpi at which it remained till termination of the study 140 dpi.

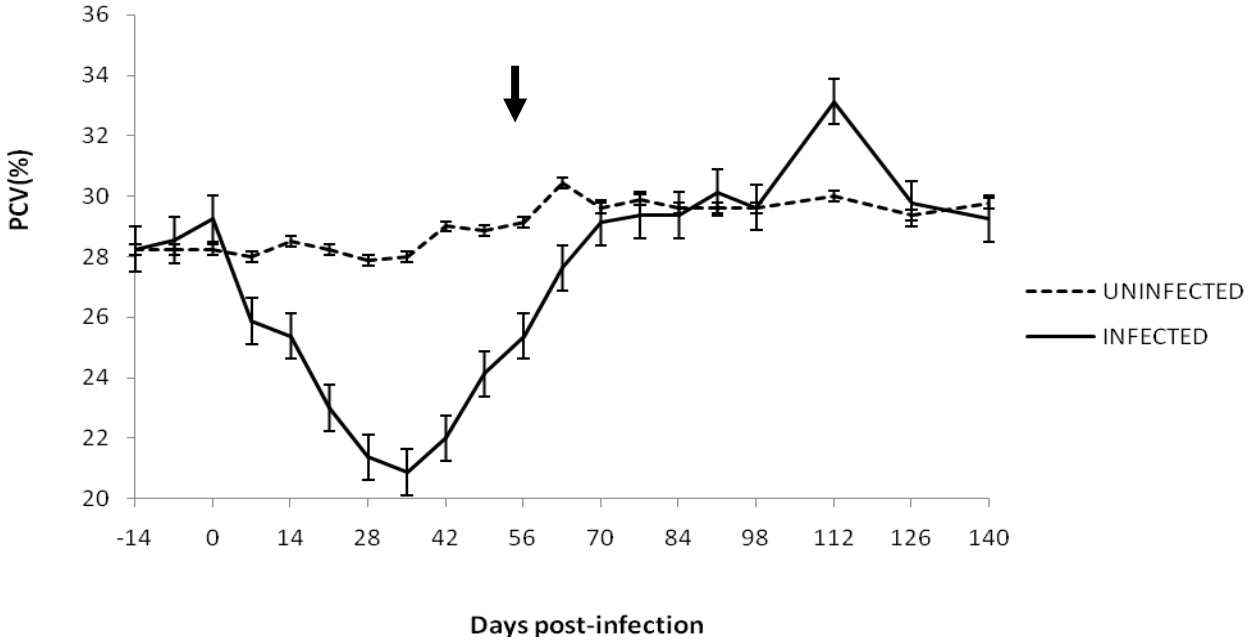


Figure 7: Mean PCV (±SEM) changes of *T.b rhodesiense* infected and un-infected sheep ↓ = Treatment day of the infected sheep with diminazene aceturate

The PCV values in the un-infected sheep remained about the pre-infection levels with minor fluctuations throughout the observation period of 140 days. The mean PCV values of infected sheep were significantly lower ($p<0.05$) than those of un-infected sheep during the period between 7 and 63 dpi but thereafter were not statistically significantly different.

Table 6: Trend in PCV

dpi	-14	-7	0	7	14	21	28	35	42	56	70	98	126	140
Trend	0.3	0.7	-3.375	-0.5	-2.375	-1.625	-0.5	1.125	2.125	1.25	2.25	1.5	0.25	0

4.6.2 Haemoglobin levels

The mean haemoglobin (Hb) levels in blood of trypanosome infected and un-infected sheep are presented in Figure 8. The mean pre-infection haemoglobin concentration values in the infected and control groups were 9.5±1.1g/dl and 9.52±1.3, respectively. Following infection, mean Hb levels in the infected sheep decreased significantly ($p<0.05$) from the mean of 9.5±1.1 g/dl at day 7 post infection to reach levels of 7.5±0.95g/dl at 28 dpi about which it remained till 42 dpi when the sheep were treated with diminazene aceturate.

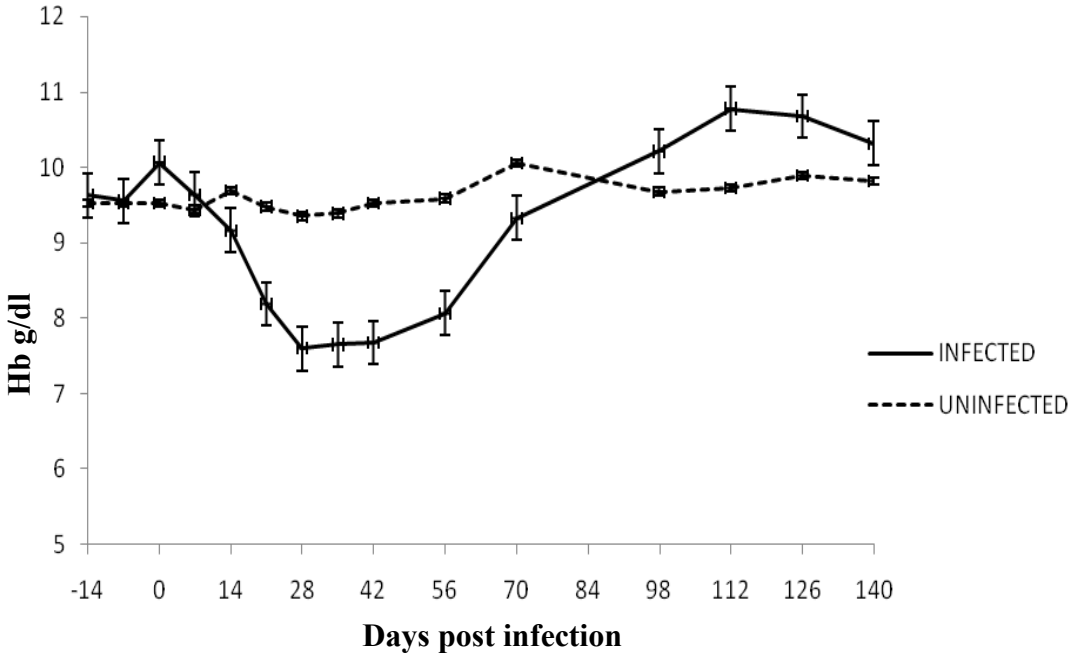


Figure 8: Mean (±SEM) haemoglobin (g/dl) profiles of *T.b rhodesiense* infected and un-infected sheep. ↓ = Treatment day of the infected sheep with diminazene aceturate

Table 7: Trend on Hb

dpi	-7	0	7	14	21	28	35	42	56	70	98	112	126	140
Trend	-0.07	0.5075	-0.42	-0.47	-0.9	-0.58	0.05	0.02	0.38	1.26	0.88	0.562	-0.1	-0.3625

However, following treatment the haemoglobin values increased gradualy to pre-infection and control levels by 82 dpi. Thereafter the Hb levels continued to increased to levels above the pre-infection and control values at 98 and 126 dpi, however, these reverted to control levels by 140 dpi when the experiment was terminated. Hb levels in control group remained similar to the pre-infection levels with some minor fluctuations. There was a significant difference ($p<0.05$) in mean haemoglobin concentrations between the infected and the uninfected groups between 14 dpi and 70 dpi.

4.6.3 Haematological index

Both pre-infection and control group RBC counts were similar at $9.6\pm0.43\times10^6$ and $9.4\pm0.35\times10^6$ per µl of blood, respectively as shown in Figure 9. However, after infection there was a marked decline in mean RBC values of infected animals from 7 dpi reaching the lowest level of $6.11\pm0.27\times10^6$ per µl of blood on day 28 post infection.

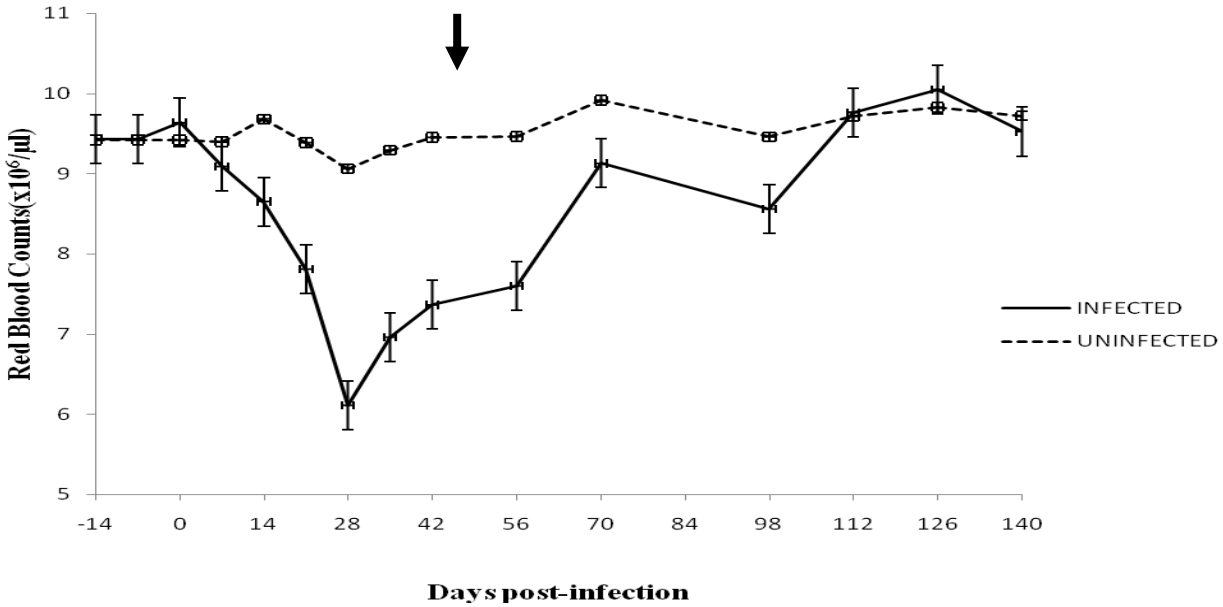


Figure 9: Mean (±SEM) red blood cell count profiles of *T.b rhodesiense* infected and un-infected sheep. ↓ = Treatment day of the infected sheep with diminazene aceturate

Table 8: Trend on Red Blood Cells analysis

dpi	-7	0	7	14	21	28	35	42	56	70	98	112	126	140
Trend	0	0.2	-0.55	-0.43	-0.84	-1.69	0.85	0.40	0.23	1.53	-0.56	1.19	0.29	-0.52

The RBC counts however increased gradualy following diminazene aceturate treatment 42 dpi to reach control levelsof 9.76±0.21 by 112 dpi at which they were maintained up to the end of the study period at 140 dpi. The mean RBC levels in the un-infected animals were maintained with no significant changes throughout the study period of 140 dpi. The decrease in the mean RBC levels of trypanosome infected sheep were significantly different ($p<0.05$) between 14 and 98 dpi.

Subsequent to this period the mean MCV values increased to reach the un-infected levels 112 dpi where it remained till termination of the experiment. The mean MCV values observed in infected sheep were found to be significantly ($p<0.05$) lower than the corresponding value observed in control group on 28 and between 77 and 105 dpi.

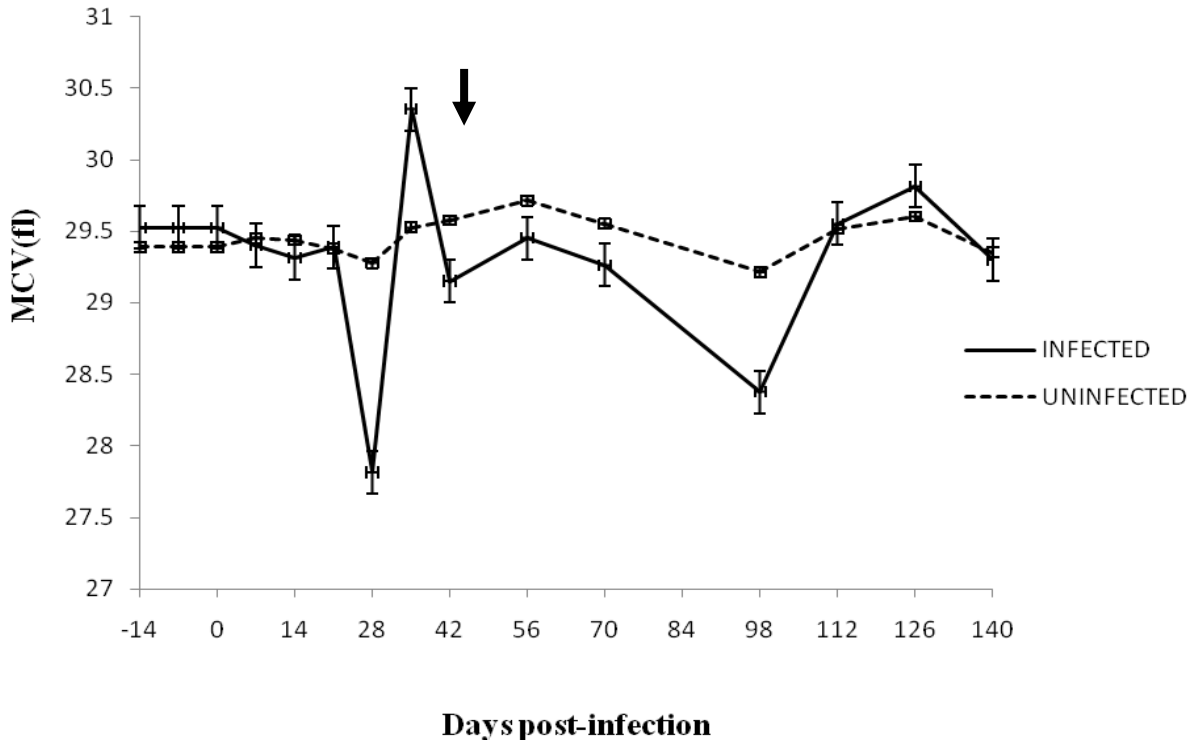


Figure 10: The mean (±SEM) mean cell volume (fl) of *T.b rhodesiense* infected and control sheep. ↓ = Treatment day of infected sheep with diminazene aceturate

Table 9: Trend on the MCV analysis

dpi	-7	0	7	14	21	28	35	42	56	70	98	112	126	140
Trend	0	0	-0.13	-0.088	0.08	-1.57	2.535714	-1.2	0.3	-0.19	-0.89	1.18	0.26	-0.51

The level of MCV first declined when the sheep was infected, it gradually increased until it attained a stable level when the fluctuations reduced. The changes in the mean corpuscular haemoglobin (MCH) which is the average amount per weight of haemoglobin in a single red blood cell are presented in Figure 11. The mean MCH values for un-infected control and the pre-infection values of the infected group were 10.45±0.12 picograms (pg) and 10.44±0.10 pg, respectively.

The mean MCH of infected sheep increased significantly from 21dpi to reach a peak of 11.76±0.19 pg on 28 dpi. Thereafter the levels declined to reach control levels 42 dpi at which they remained till 70 dpi when they increased a second time to reach a peak of 11.45±0.29 pg at 98 dpi.

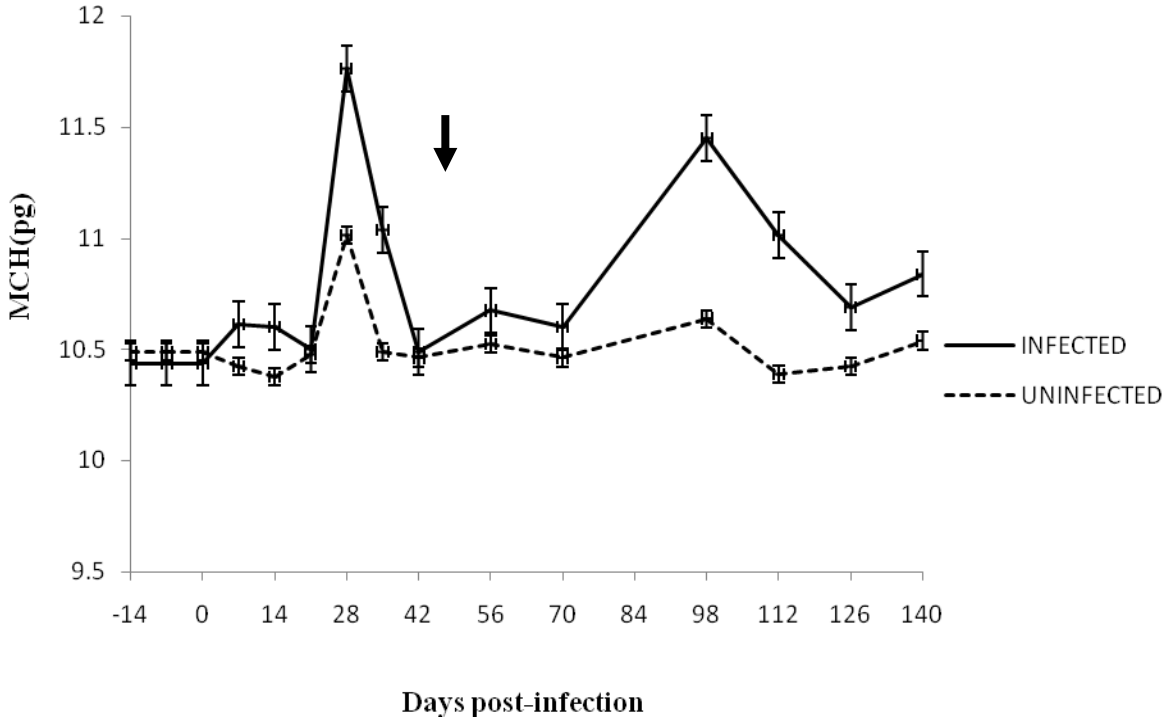


Figure 11: Mean (±SEM) mean corpuscular haemoglobin level in *T.b rhodesiense* infected and control sheep. ↓ = day of treatment of infected sheep with diminazene aceturate.

Table 10: Trend on the MCH analysis

dpi	-7	0	7	14	21	28	35	42	56	70	98	112	126	140
Trend	0	0	0.1	-0.01	-0.1	1.2	-0.7	-0.5	0.1	-0.1	0.85	-0.44	-0.33	0.15

The MCH levels thereafter declined to near control levels on 126 dpi where they remained up to the end of the experimental period. The mean MCH values of infected sheep were found to be significantly higher ($p<0.05$) than the corresponding un-infected control value observed between 21 and 35 and also between 84 and 112 dpi. The mean MCH of the infected sheep increased significantly to attain it highest, it then Started to decline. There was several fluctuations as days post infection progressed. The MCH later retained a stable level. The mean corpuscular haemoglobin concentration of infected and control sheep are presented in figure 12. The mean control and pre-infection values of MCHC were 36.11±0.71 g/dl and 35.39±0.54 g/dl, respectively.

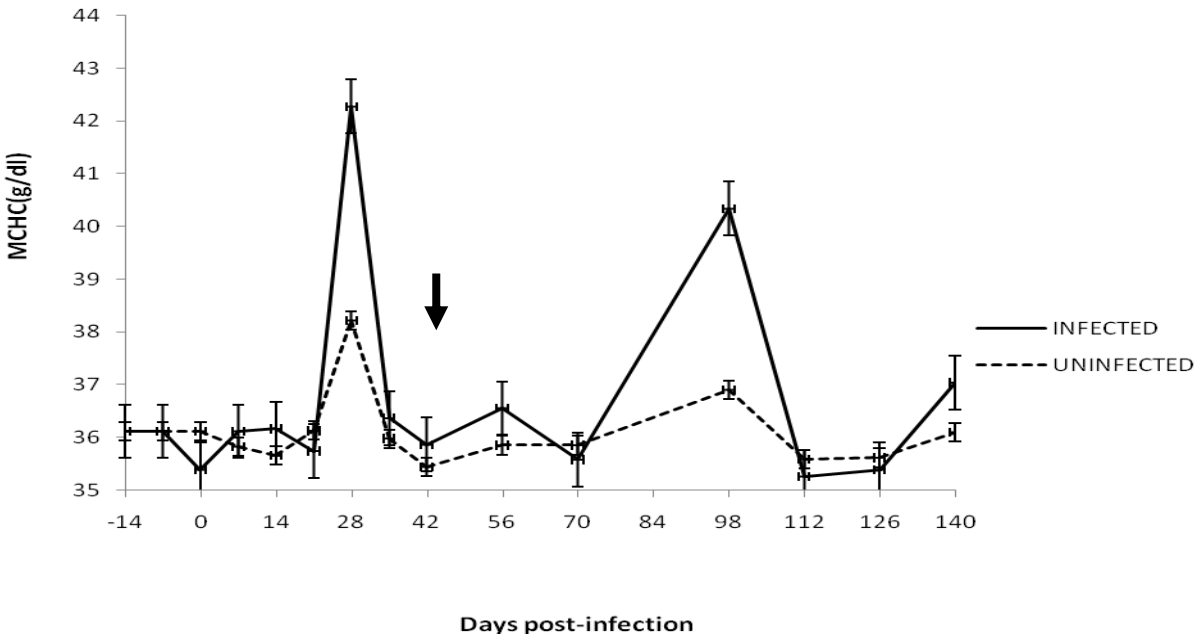


Figure 12: Mean (±SEM) corpuscular haemoglobin concentration (MCHC) in g/dl for *T.b rhodesiense* infected and uninfected control sheep. ↓ = Treatment day of the infected sheep with diminazene aceturate

During the post-infection period, the MCHC value which is the percentage of haemoglobin by weight in 100ml of packed cells value, progressively increased and reached the highest value of 42.28±0.69 g/dl by 28 dpi. Thereafter the level declined sharply at 35 dpi to reach control

levels by 42 dpi to levels of 35.86±0.4 8 g/dl when the sheep were treated with diminazine aceturate.Thereafter, the MCH levels remained at pre-treatment levels till 70 dpi when an increase occurred to give a second peak of 40.34±1.6 g/dl on 98 dpi. The values thereafter declined to 35.25±0.62 g/dl on 112 dpi at which they remained up to the end of the experiment. The MCHC mean value for the infected animals was significantly higher than the control value between 21 and 35 and also between 77 and 105 dpi.

The observed mean white blood cell count (WBC) changes in un-infected control and infected sheep are presented in Figure 13. The mean blood WBC levels for both pre-infection period and control were 16.67±3.1 $\times 10^3$ cells/ml.The WBC levels remained at control levels until day 28 dpi after which the levels increased significantly till 56 dpi even though the sheep were treated with diminazine aceturate on 42 dpi. The mean WBC level of infected sheep declined from 56 dpi to below control levels from 70 dpi to reach lowest levels (11±3.45$\times 10^3$ cells/ml), 98 dpi. The mean WBC levels thereafter increased to near control level 126 dpi at which they remained up to the end of the experimental period.

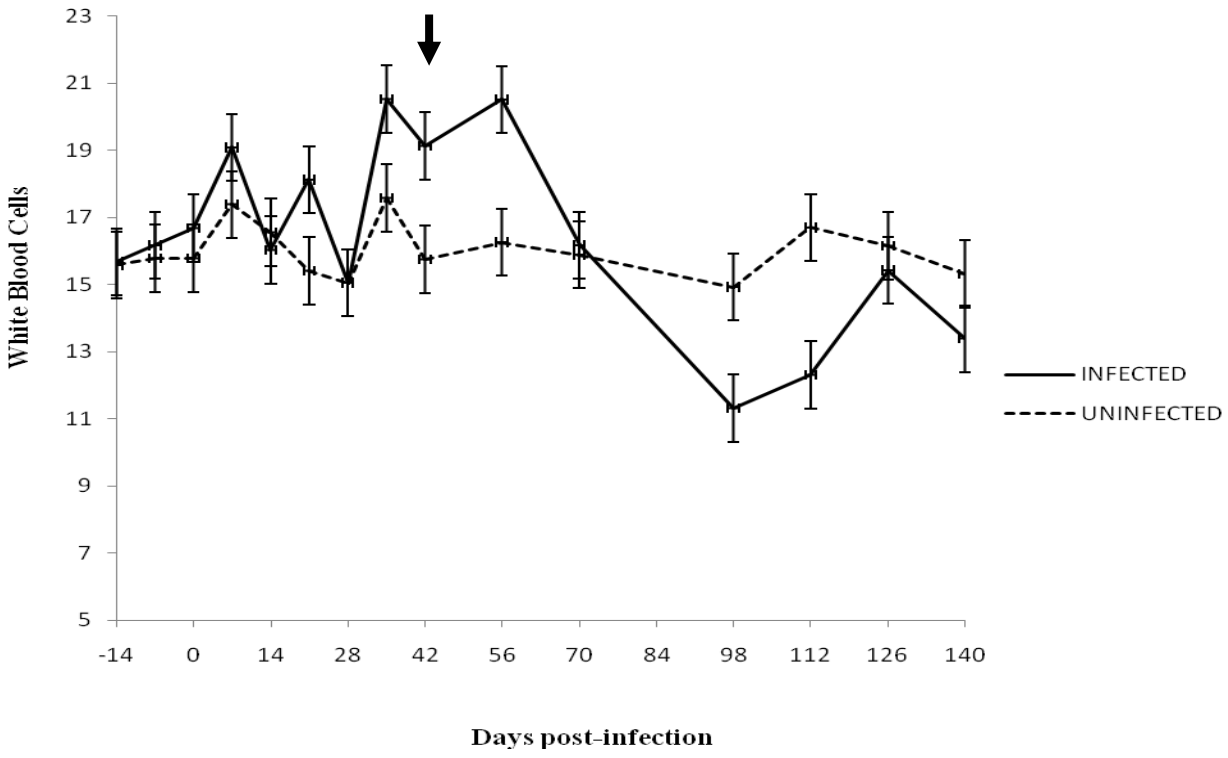


Figure 13: Mean (±SEM) white blood cell count in blood of *T.b rhodesiense* infected and un-infected control sheep. ↓ = Treatment day of the infected sheep with diminazene aceturate

Table 11: Trend on white blood cell analysis

dpi	-7	0	7	14	21	28	35	42	56	70	98	112	126	140
Trend	0.5	0.5	2.41	-3.06	2.1	-3.07	5.47	-1.38	1.37	-4.33	-4.85	0.98	3.11	-2.03

4.7 Serum Biochemical Profiles

4.7.1 Serum total protein levels

The changes in the mean serum total protein level in infected and un-infected control sheep are presented in Figure 14. The mean pre-infection total protein level was 102±2.25 mg/l and 103.57±14.55mg/l for the infected and un-infected sheep, respectively. Following infection the mean serum total protein level rose to a peak of 124.18±1.24mg/l at 7dpi. Thereafter the mean total protein level declined markedly to reach lowest value of 93.12±0.8mg/l on 28 dpi. After the low levels on 28 dpi, the levels started to increase gradually towards the un-infected values of 104.40±2.4 at 56 dpi. Thereafter, the mean total protein levels started to gradually decline from 107.11±2.77 by the 70 dpi reaching levels of 99.44±2.91mg/l at 126 dpi.

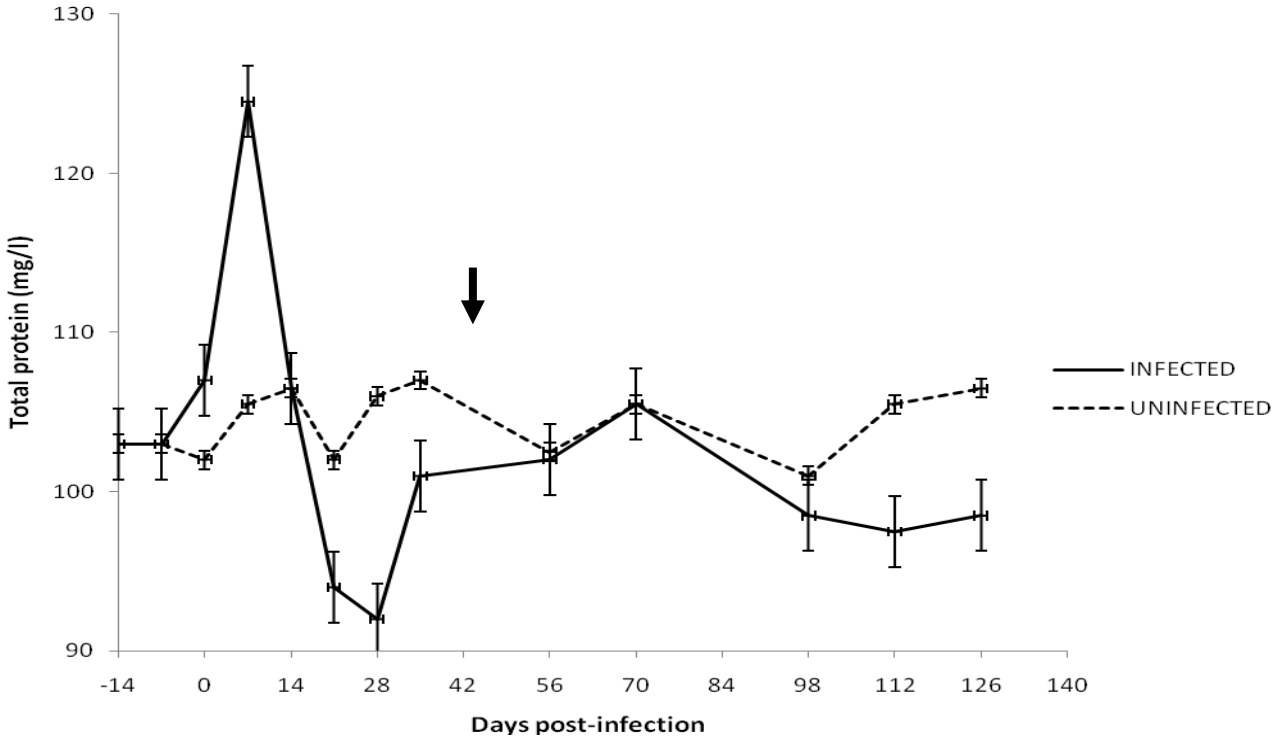


Figure 14: Mean serum total protein concentrations (±SEM) in *T.b rhodesiense* infected sheep and uninfected control. ↓ = Treatment day of the infected sheep with diminazene aceturate

Table 12: Trend on total protein levels analysis

dpi	-14	-7	0	7	14	21	28	35	42	56	70	98
Trend		0.61	-4.55	21.90	-18.956	-11.08	-2.290	9.93	0.59	-7.31	-0.38	0.29

From the total protein trend analysis, it can be observed tha the protein total started with a high level, the level droped on day zero during infection, on day 7 there was a sharp rise in the level of the protein. The level of protein dropped from day 14 to day 28 with a gradual increase within those days, on day 35 there was a high raise in the level, the protein level lowered to maintain a normal level.

4.7.2 Serum total LDH enzyme levels

The mean total LDH enzyme activity in infected and control sheep serum is presented in Figure 15. The mean LDH activity in the serum of infected sheep before trypanosome infection was 4.10±0.63U/l while that of un-infected group was 3.34±1.68 U/l. Thereafter, the mean LDH levels gradually increased for the infected group of sheep but with fluctuations to produce several peaks at 28 (8.40±1.50 U/l), 56 (7.83±2.20 U/l) and 98 (10.10±1.52 U/l) dpi. At all times the infected group from 14 dpi, had LDH levels that were significantly higher ($p<0.05$) than the un-infected groups of sheep.

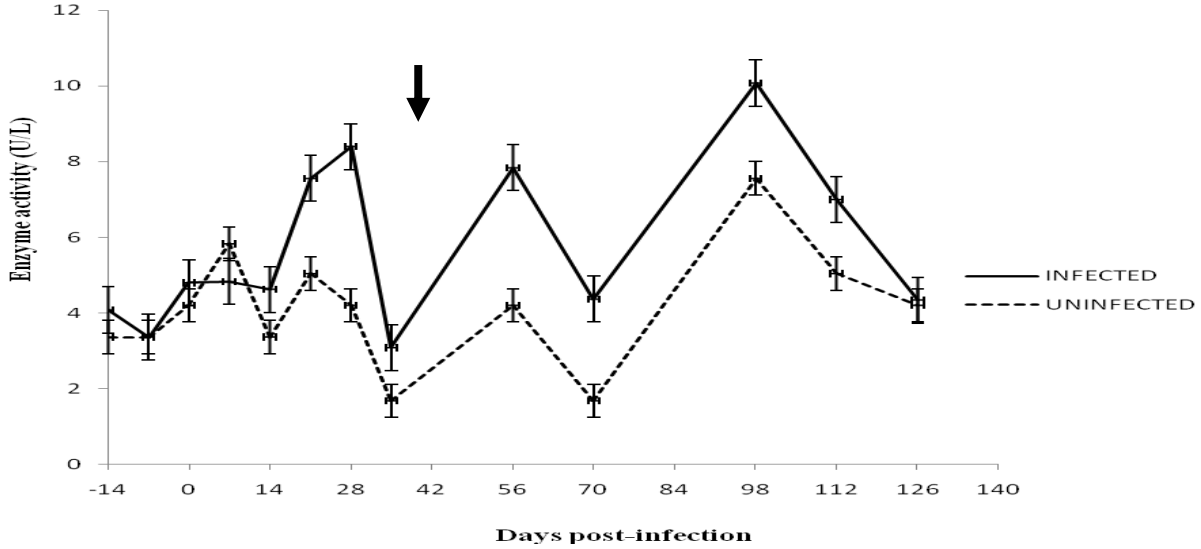


Figure 15: The mean (±SEM) serum total LDH enzyme activity in *T.b rhodesiense* infected and uninfected control sheep. ↓ = Treatment dayof the infected sheep with diminazene aceturate.

4.7.3 Serum LDH isoenzymes pattern

The serum LDH isoenzymes bands obtained from starch gel electrophoresis of infected and uninfected control sheep at various stages of infection are presented Figures 16, 17, 18, 19 and 20. Following electrophoresis of sheep serum and incubation of gels with the substrate, only four distinct bands were visible corresponding to bands of LDH1, LDH2, LDH3 and LDH4 with the fractions occurring in the order of migration farthest towards the anode (Figure 16). However, only two bands corresponding to LDH1 and LDH2 out of the four LDH isoenzymes observed were clearly visible as strong bands. The respective band intensities were directly proportion to enzyme activity in the serum samples at a given dpi.LDH2 was more pronounced in intensity at the early stages of infection (Figure 18) while LDH1 became more intense at later stages of infection (Figure 20).

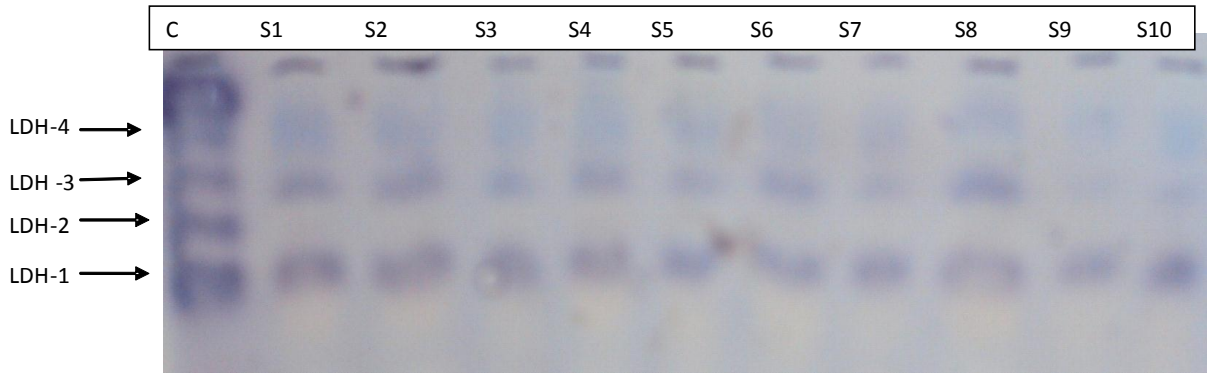


Figure 16: Serum LDH isoenzyme patterns of individual sheep 10 days after T. b. rhodesiense infection: Lane C represents LDH standard. Lanes S4 and S8 represent sera from uninfected sheep while lanes S1, S2, S3, S7, S9 and S10 represent sera from individual

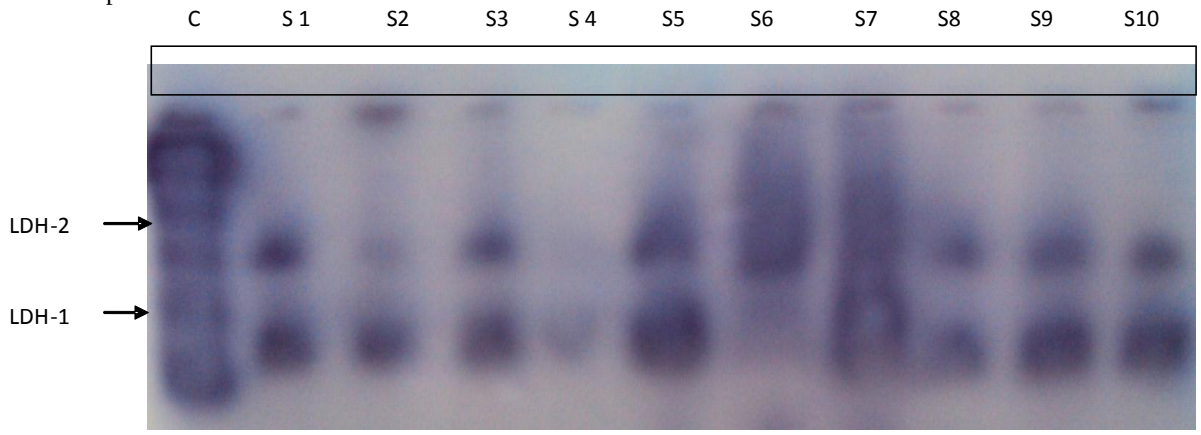


Figure 17: Serum LDH isoenzyme pattern in sheep at 21 days after *T.b rhodesiense* infection. Lane C represents standard LDH S4 and S8 represent sera from uninfected sheep, lanes S1, S2, S3, S5, S6, S7, S9, and S10 represent sera from infected sheep

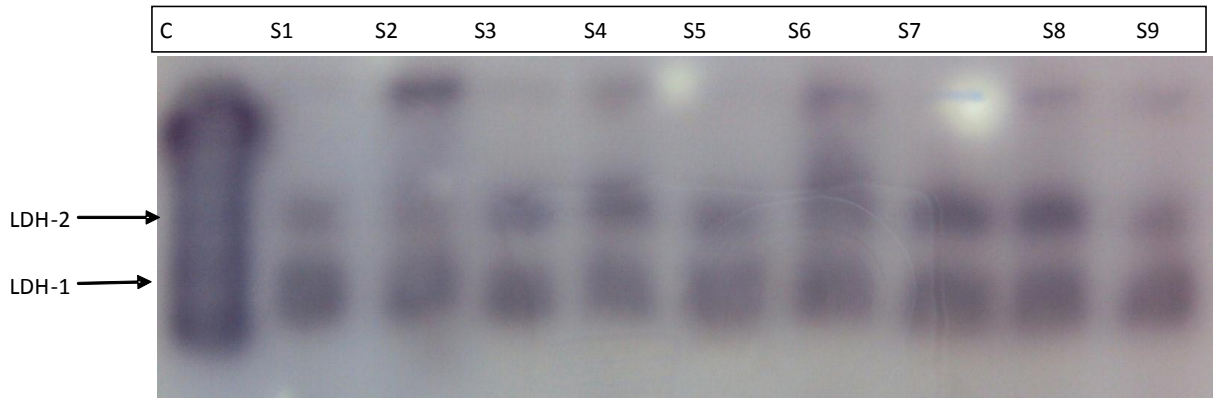


Figure 18: Serum LDH isoenzyme patterns from sheep 14 days after *T.b rhodesiense* infection. Lane C represents standard LDH. Lanes S4 and S8 represent sera from uninfected sheep while lanes S1, S2, S3, S5, S6, S7 and S9 represent sera from infected sheep

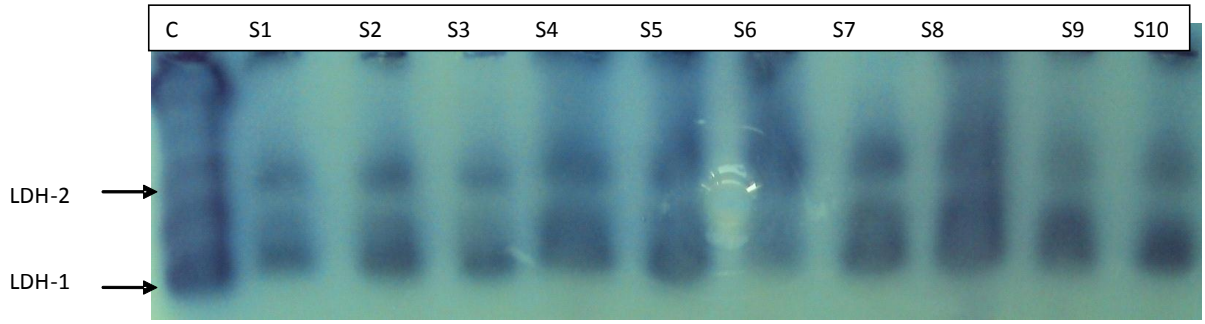


Figure 19: Serum LDH isoenzyme patterns in sheep 35 day after infection with *T.b rhodesiense*. Lane C represents standard LDH. Lanes S4 and S8 represent sera from uninfected sheep and lanes S1, S2, S3, S5, S6, S7, S9 and S10 represent sera from infected sheep

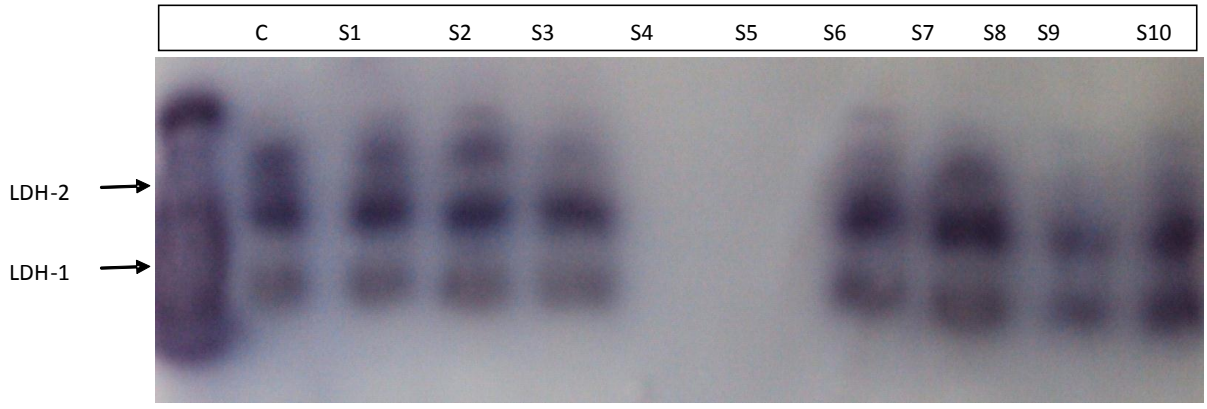


Figure 20: Serum LDH isoenzyme patterns in sheep at 98 days following *T.b rhodesiense* infection. Lane C represent LDH standard, lanes S4 and S8 represent sera from uninfected sheep, lanes S1, S2, S3, S7, S9 and S10 represent sera from infected sheep.

4.7.4 Lactate dehydrogenase isoenzyme quantification

The quantitities of LDH1 and LDH2 are presented in Figure 21. The quantities of LDH1 and LDH2 in serum of infected group of sheep were estimated in the gels in order to determine the LDH1:LDH2 ratio which can be of diagnostic importance for cardiac pathology. The percentage enzyme concentration ratio of LDH1:LDH2 was less than unity before infection. However, following infection of the sheep with trypanosome, the levels of LDH1 increased above those of LDH2 between 7 and 28 dpi. This gave an LDH1:LDH2 ratio that was above one between the said days. Thereafter, the LDH1:LDH2 ratio remained below unity till termination.

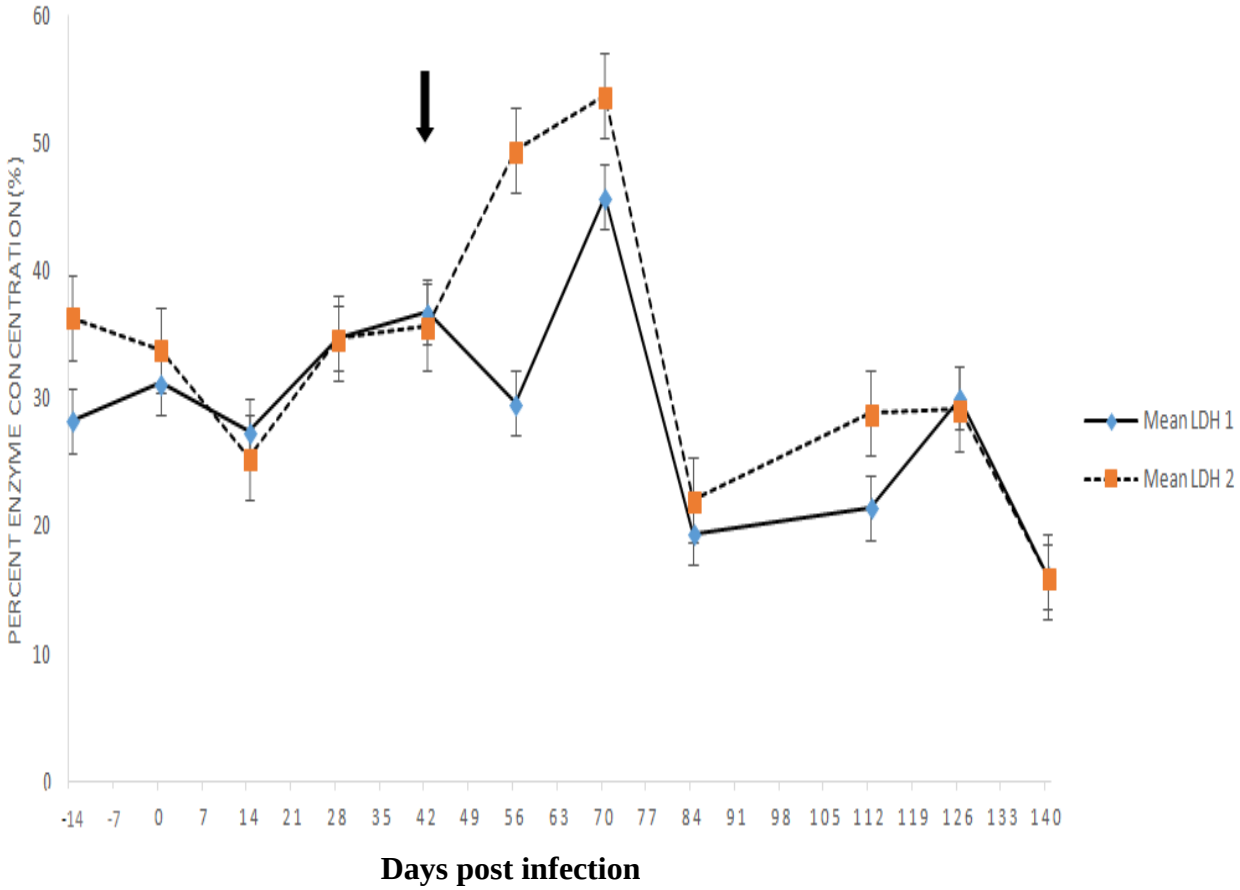


Figure 21: Percentage concentrations (%±SEM) of serum lactate dehydrogenase (LDH1 and LDH2) following infection of sheep with *T.b rhodesiense*. ↓ = Treatment day with diminazene aceturate

The summary of LDH isoenzyme ratio is presented in Table 4. The pre-infection mean LDH1 value (28%) was lower than the mean LDH2 level (36%) before trypanosome infection giving an LDH1:LDH2 ratio of less than unity. However, following infection the levels of

LDH1 increased higher than LDH2 from 14 dpi to 42 dpi resulting in a ratio higher than unity indicating a flipped over pattern. The ratio however, returned to below unity on 56 dpi and remained so throughout the remainder of the study.

Table 13: The mean percentage LDH1 and LDH2 and their ratios in serum of sheep infected with *T.b rhodesiense*

Days post infection (dpi)	**LDH1concentration (%) (±SEM)**	**LDH2concentration (%) (±SEM)**	**LDH1/LDH2 ratio**
-14	28.3±1.4	36.4±1.3	0.77
0	31.28±1.2	33.89±0.4	0.93
14	27.49±1.4	25.39±1.8	1.08
28	34.76±0.7	34.74±0.8	1.00
42	36.79±0.6	35.66±0.3	1.03
56	29.69±2.3	49.49±3.0	0.59
70	45.87±2.3	53.7±1.3	0.85
84	19.50±1.1	22.10±1.4	0.88
112	21.50±0.6	28.88±4.2	0.50
126	30.11 ±2.8	29.17±3.2	0.10
140	16.05±0.72	16.07±0.7	0.98

4.7.5 Mean serum total creatine kinase levels

The mean total serum CK enzyme activity in infected and control sheep are presented in Figure 22. The mean total serum CK levels before trypanosome infection for the infected and un-infected group of sheep were 4.57±0.93U/L and 3.48±2.09U/L, respectively. However, following trypanosome infection the total CK levels started to increase from 14 dpi to reach a significant peak of 123.19±2.1U/L by 35 dpi. These levels however decreased drastically to reach control levels by 56 dpi where it remained for the remaining part of the study. The CK

level of the infected sheep was significantly different from that of the uninfected sheep between 28 and 42 dpi. ($p \leq 0.05$).

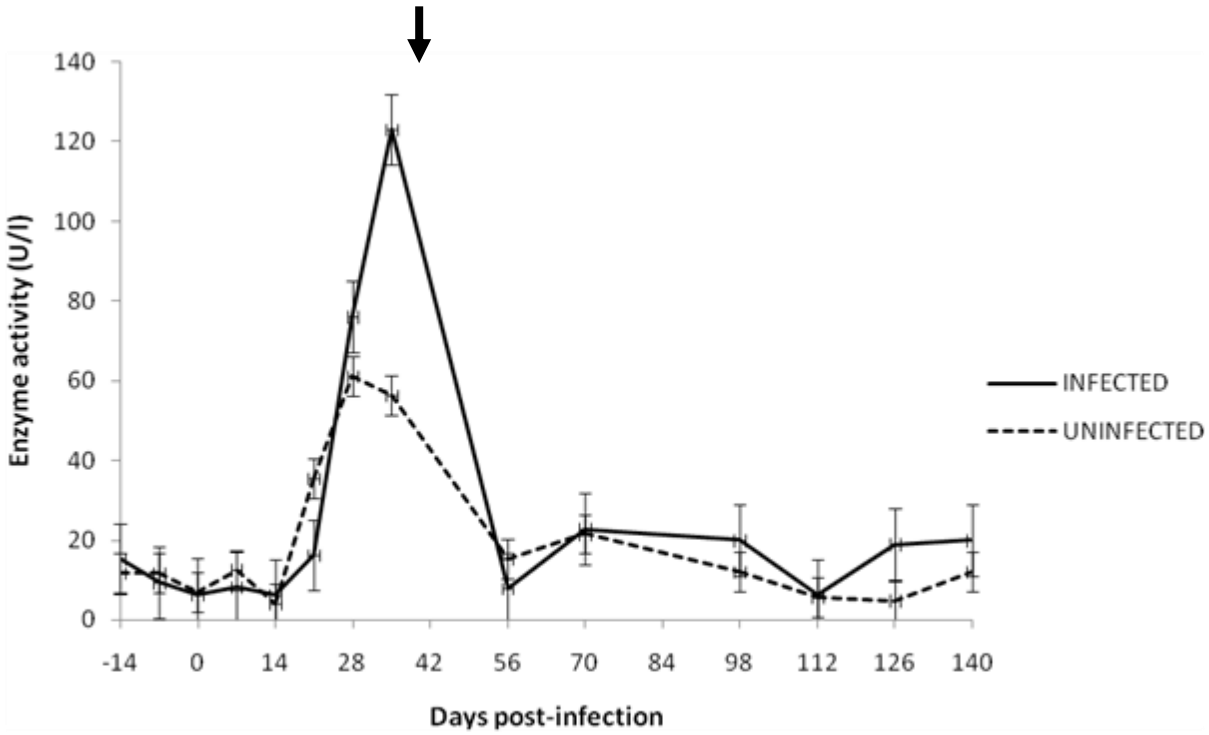


Figure 22: Mean (±SEM) total serum CK enzyme activity in *T.b rhodesiense* infected and control sheep. ↓ = Treatment day with diminazene aceturate.

Table 14: Trend on Mean LDH concentration levels analysis

dpiS	-7	0	10	14		21	28	35	56	70	98	112	126
Trend	1	-0.7	6.98	-7.79		7.07	2.03	-7.10	16.18	-23.72	-7.70	15.66	-13.96

4.7.6 Serum creatine kinase isoenzyme pattern

The CK isoenzyme starch gel electrophoresis of infected and uninfected sheep serum is presented in Figure 24. Two bands were observed following serum electrophoresis in both infected and uninfected sheep. The fractions CK (BB) and CK (MB) are shown in the order of migration farthest towards the anode. However the bands were very faint.

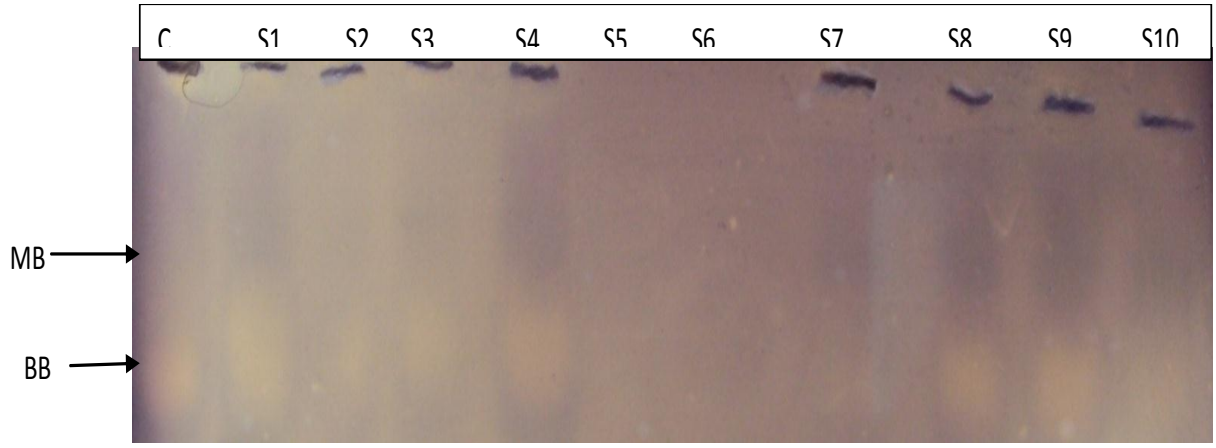


Figure 23: Serum CK isoenzyme patterns from sheep at 21 days following infection with *T. b. rhodesiense.*

Table 15: Trend on serum CK concentration analysis

dpi	-14	-7	0	7	14	21	28	35	42	56	70	98	112	126
Tend		-8.21	-25.28	-15.11	-5.92	-2.96	1.74	-1.91	9.98	59.86	47.09	-115.30	14.94	-10.8

4.8 Cerebrospinal Fluid Biochemical Profiles

4.8.1 CSF protein levels

The mean CSF protein levels of infected and uninfected control sheep is presented in Figure 24. The mean CSF protein levels of pre-infected sheep and control group at 0 dpi were 1.01±0.22 mg/l and 1.08±0.04 mg/l, respectively. Following infection, the CSF protein levels in the infected group of sheep increased from 7 dpi to a first peak of 2 gm/ml at day 21 dpi. Thereafter, the levels declined to 0.91±0.17mg/l by 42 dpi when the sheep were treated with diminazine aceturate. Following treatment the protein levels increased significantly to give a second peak of 2.5±1.42 mg/l at 56dpi. The protein levels however declined to control levels by day 70 dpi at which they remained except for the minor fluctuations up to the end of the experimental period.

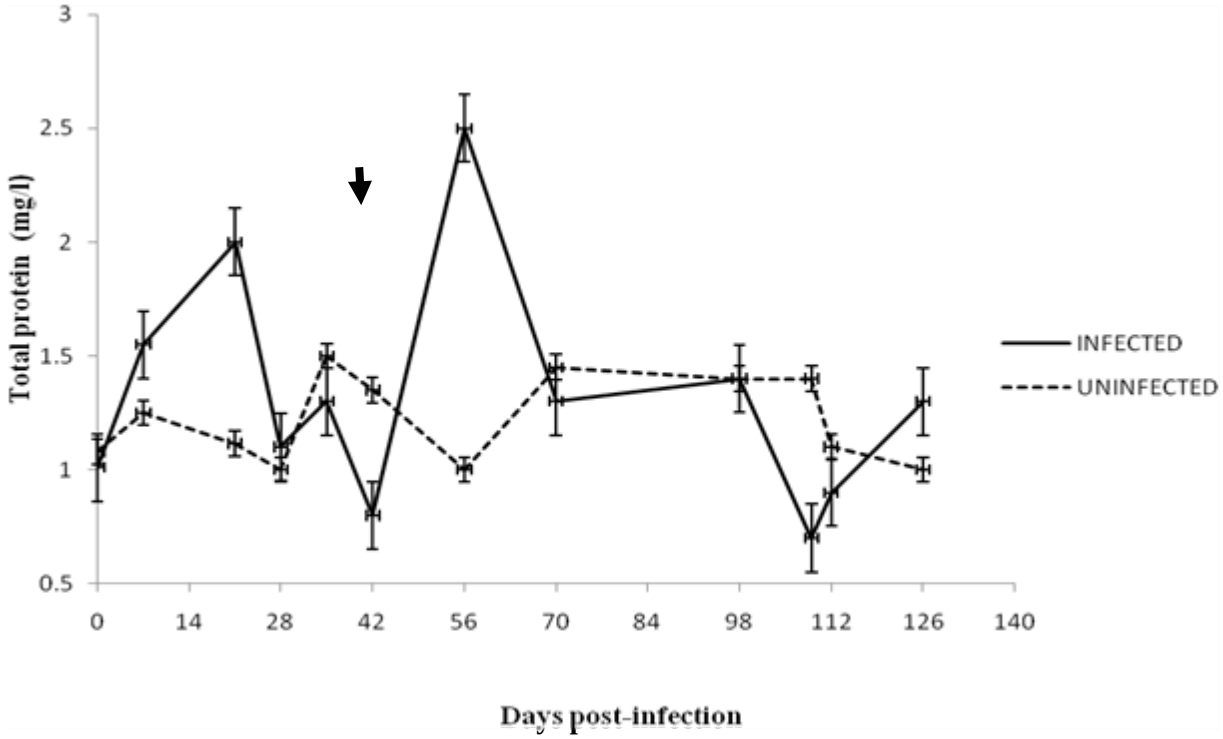


Figure 24: CSF protein (±SEM) levels in sheep infected with *T.b rhodesiense* and uninfected control. ↓ = Treatment day with diminazene aceturate\

Table 16: Trend in CSF total protein levels analysis

Days	-14	-7	0	7	14	21	28	35	42	56	70	98	112	126
Trend	0	0.60	-4.54	21.90	-18.94	-11.08	-2.28	9.92	0.59	-7.31	-0.38	0.25	-3.2	-0.2

4.9 CSF Enzymology

4.9.1 Ceresbrospinal fluid LDH activity

Changes in LDH enzyme activity in CSF of infected sheep is presented in Figure 25. The mean pre-infection and uninfected control total CSF LDH enzyme activity in sheep was 8.3±4.6 U/L and 8.39±2.37 U/L, respectively. The LDH enzyme activity for infected group of sheep increased sharply as the disease progressed starting 14 dpi to reach a peak of 74.74±1.13U/L at 21 dpi. The LDH enzyme levels declined to near control levels of 15.54±1.90 U/L by 28 dpi at which it remained except for two other minor peaks of 16.79±2.25 U/L and 33.59±0.97 U/L at 56 and 112 dpi, respectively. The mean LDH level of the infected sheep was significantly different ($p \leq 0.05$) from the controls during the peaks. Uninfected control sheep showed no significant changes in their mean CSF enzyme activity throughout to the

observation period. The CSF LDH levels were not significantly different ($p \geq 0.05$) except during the three peaks namely 21, 56 and 112 days post infection.

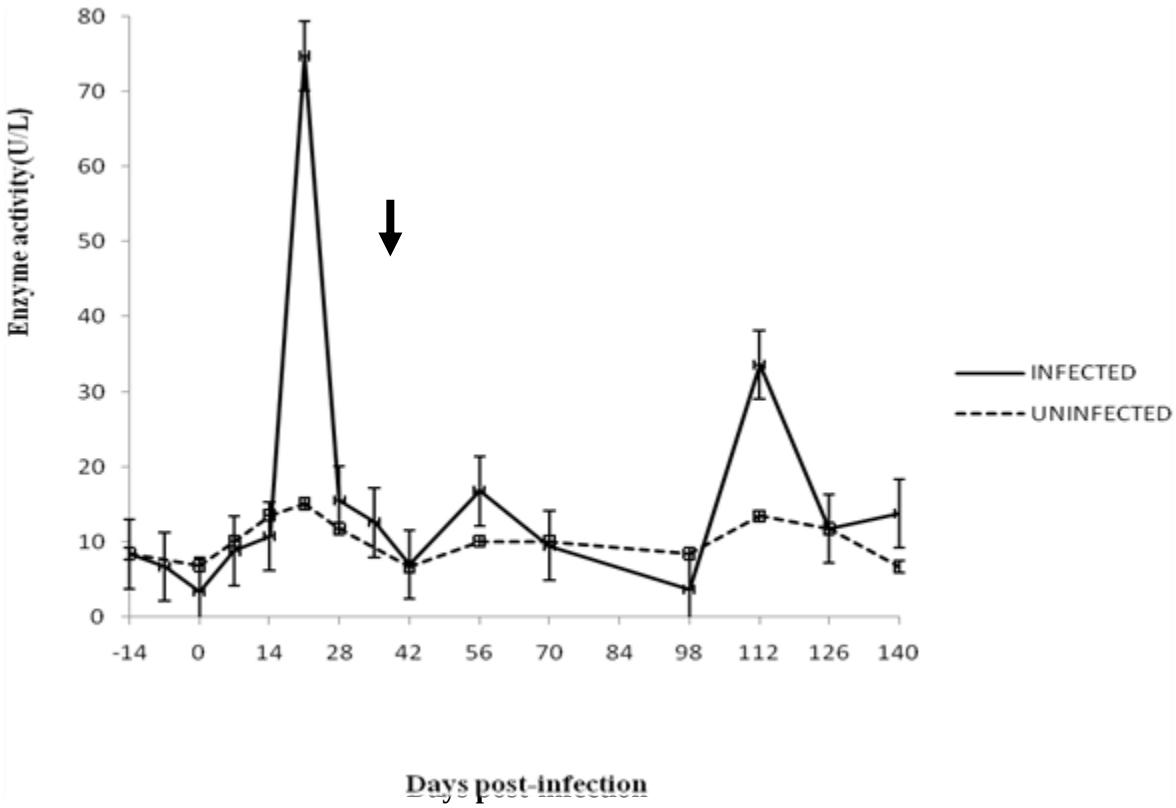


Figure 25: Total LDH (±SEM) enzyme activity changes in cerebrospinal fluid of *T.b rhodesiense* infected and control sheep. ↓ = Day of treatment with diminazene aceturate

Table 17: Trend on the Cerebrospinal fluid (CSF) levels LDH analysis

Days	-14	-7	0	7	14	21	28	35	42	56	70	98	112	126
Tend		-1.68	-3.36	5.46	1.93	63.99	-59.2	2.94	-5.59	9.79	-7.28	-5.82	-21.83	2.016

4.9.2 CK CSF enzyme activity

The mean CSF total CK enzyme activity in infected and control sheep is presented in Figure 26. The pre-infection mean CSF total CK activity in infected and control sheep were 3.8±2.6U/L and 2.65±1.78U/L, respectively. The mean CK enzyme activity for the infected sheep started to increase on 21 dpi (2.78±0.91U/L) to reach a peak of 10.61±2.06U/L at 35 dpi. Thereafter, the mean levels declined to 1.98±0.64U/L by 42dpi when the sheep were treated

with diminazine aceturate. Following treatment the mean CK activity remained at those levels throughout the remaining stages of infection. The mean cerebral spinal fluid CK enzyme activity values for the uninfected sheep were significantly lower ($p < 0.05$) than those observed in the infected group during the peak at 35dpi.

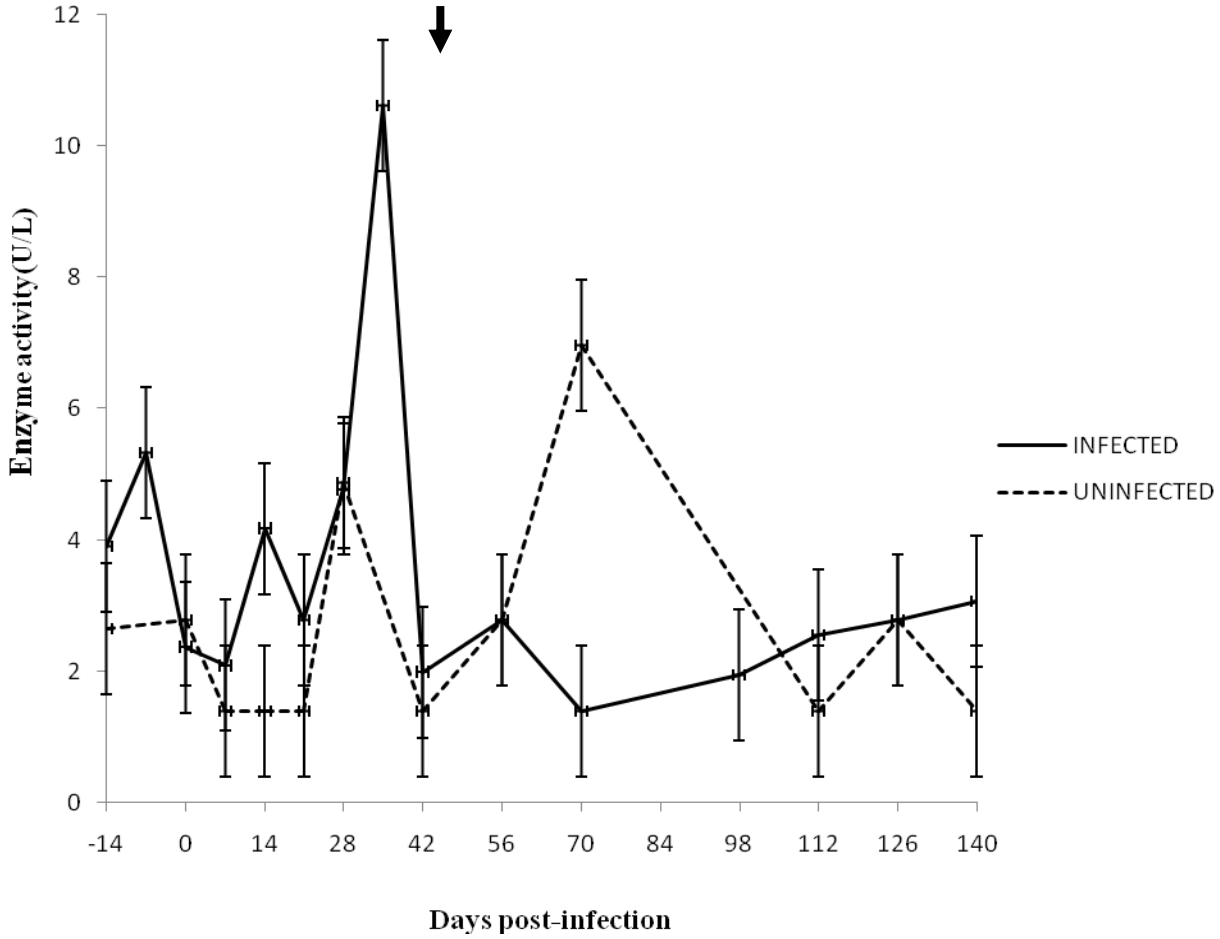


Figure 26: CSF total CK (±SEM) enzyme activity in *T.b rhodesiense* infected and control sheep. ↓ = Treatment day with diminazene aceturate

Table 18: Trend on CSF total CK concentration levels analysis

Days	-14	-7	0	7	14	21	28	35	42	56	70	98	112	126
Trend		1.43	-2.95	-0.29	2.08	-1.39	1.98	5.84	-8.62	0.79	1.16	-1.99	0.60	0.23

4.10 CSF LDH and CK Isoenzymes Pattern

Electrophoresis of the CSF for the infected and uninfected sheep to determine the isoenzyme profiles for both LDH and CK was done as previously explained for serum samples. It was however not possible to see any LDH nor CK bands in the gel following electrophoresis and stainning of the gels.

CHAPTER FIVE
DISCUSSION

5.1 Clinical Picture

Pathogenesis of *T.b rhodesiense* infections of brain tissue in humans is poorly understood. This is further complicated by the fact that very few drugs are available that are effective for CNS infection of which are also toxic (Pepin and Milrod, 1994; Maclean *et al*., 2006). These drugs have to be used sparingly and thus require the confirmation of CNS infection before administration. This necessitates for use of easy to perform, sensitive and specific markers for CNS infection. Most of the studies of both trypanosome pathogenesis and drug trials have been carried out in animal models. The available animal models that have previously been used in trypanosomosis research have several limitations. The current study was therefore aimed at establishment of a robust and cost-effective *T.b rhodesiense* sheep model and determine the usefulness of tissue enzymes as markers for cardiac and CNS involvement during trypanosome infection.

Intravenous infection of sheep produced an acute disease syndrome of an early acute stage characterized by high levels of parasitemia and the development of anaemia. In addition, the infected sheep developed intermittent pyrexia, lethargy and reduced feed intake. The infected sheep showed clinical signs of increase in rectal temperature, weight loss, oedema, enlarged lymph nodes and ultimately anaemia leading to the death of one animal before and another after berenil treatment. The rectal temperature rose with parasitemia levels to peak at 28 dpi. However, the increase in rectal temperature and clinical signs subsided with the health of the animals beings restored following treatment with diminazine aceturate.The clinical signs were consistent with observations recorded in other studies of sheep infected with *T. congolense* (Bisalla *et al*., 2007; Okaiyeto *et al.,* 2010) and *T. brucei* (Ikede and Losos, 1972). Similar signs were also observed in the monkey model (Ndungu *et al.*,1994; Ngure *et al.*, 2008; Kagira *et al.,* 2006) and human infected with *Trypanosoma b. rhodesiense* (Barret *et al.*, 2003; Brun *et al.,* 2010).

5.2 Parasitaemia in Blood

Parasites appeared in blood of infected sheep from the 4th dpi with all animals being blood positive by 7 dpi resulting in an average pre-patent period of 5 dpi and range of 4-7 dpi.

The levels of parasitaemia in blood of infected sheep developed in an exponential manner with the first peak of parasitemia occurring on the 5th dpi and coinciding with the start of clinical signs. Thereafter the parasitaemia levels showed several peaks and fluctuating waves of parasitaemia which peaked and plateued on the 13th dpi where it remained till treatment with berenil. The parasitaemia levels were characteristic of the KETRI 2537 stabilate as observed in monkey (Kagira *et al.*, 2007b). The fluctuation in parasitaemia levels are similar to those observed in humans (Franco *et al.,* 2014) and rodent animal models infected with trypanosomes (Kagira *et al.*, 2007a).

The parasitaemia waves are as a result of immune response to the trypanosome VSG/clones (Vincendeau and Bouteille, 2006). Trypanosomes have a glycoprotein coat that is encoded by genes that are antigenically distinct thus making the parasite able to engage in an immune evasion process of antigenic variation (Cross, 1990; Borst *et al.,* 1997) and thus the waves of parasitaemia (Borst and Fairlamb, 1998). When infection of host occurs the glycoprotein that are found on the surface of the trypanosome are recognised by the host's immune system, triggers the production of trypanosome specificIgG and IgM antibodies. The IgM then produce an immune destruction of trypanosome resulting to a decline in parasitemia. However, a subset of the trypanosomes change their surface coats to a new variant surface glycoprotein type that is not affected by circulating antibodies. This sequence is repeated and the immune system is therefore unable to eliminate the parasite and hence the waves of parasitaemia (Vincendeau and Bouteille, 2006).

5.3 Parasitaemia in CSF

Of the eight sheep infected with trypanosomes, only two sheep were consistently CSF positive during infection period before berenil treatment while the rest were intermittently positive in CSF by direct microscopy or following centrifugation at different point pre-treatment. This observation of intermittent appearance of trypanosome in CSF was similar to observations made by Thuita *et al.* (2008) using the same parasite in vervet monkey disease model. Indeed similar observations were noted in the same models, even when microscopic examination of CSF was done after application of concentration techniques such as double centrifugation (Gould and Sayer, 1983). Trypanosome parasites were observed more following a concentration method of CSF following prior centrifugation compared to direct microscopy

during the early stages of infection. While in the later stages parasites could be detected by direct microscopy. This further confirmed the superiority of the centrifugation methods.

Trypanosomes are infrequently seen in CSF even when a concentration technique such as double centrifugation is used to improve sensitivity detection (Barret *et al.,* 2003). Indeed improve diagnostic sensitivity of trypanosome detection in CSF has also been demonstrated in humans by concentrating trypanosomes by centrifugation using microhaematocrit centrifugation (Woo, 1970) or by anion exchange chromatography (Lumsden *et al.*, 1979). Indeed trypanosomes have been demonstrated to appear in a cyclical manner in CSF of rats infected with trypanosomes (Mogk *et al*., 2014). At the same time this phenomenon has also been observed in trypanosome infected humans where trypanosomes do not always appear in CSF at all times during infection (Dumas and Boa, 1988). Trypanosomes in CSF were detected earliest by the 21st dpi during infection. Early appearance of trypanosome in CSF has also been observed in *Trypanosome* infected rats (Mogk *et al.,* 2014), mice (Schultzberg *et al.*, 1988), monkey (Schmidt and Sayer, 1982; Ndungu *et al.*, 1994) and human (Dumas and Boa, 1988) infections.

Although trypanosomes appear in CSF early, establishment of infection in the CNS does not occur at those early times. These trypanosomes that appear in the CNS do not survive and establish CNS infection until later on. This hzzzzas lead to the conclusion that even when trypanosomes cross the blood brain barrier they do not lead to brain infection (Mogk *et al.,* 2014). Indeed others studies have shown that trypanosomes injected into the CSF cannot permanently settle in the CSF (Pentreath *et al.,* 1992; Wolburg *et al.,* 2012) and are thus easily cleared. Other studies have also shown that when trypanosomes are directly placed intrathecally into the striatum, they did not lead to a manifest of infection for until 14 day whereas those placed in the ventricle system did (Wolburg *et al.,* 2012), thus suggest that certain changes have to first occur in CNS/CSF before trypanosomes can establish a permanent infection.

Although all the infected sheep were CSF positive at different stages of infection, only four were confirmed to have been CSF positive by direct microscopy and/or microscopy following centrifugation at the time of treatment with berenil. However following treatment with berenil all the treated sheep were cleared of trypanosomes in the CSF from 56 dpi and for

the rest of the experimental period. In the current study cure of all the animals following early treatment with diminazine aceturate treatment thus shows that the infection had not fully established in the CNS by the time of treatment. Berenil is a drug that does not cross the blood brain barrier and thus does not cure established CNS trypanosome infections (Kennedy, 2004). Indeed, several authors have suggested the presence of an intermediate stage during infection where trypanosomes are in the CSF without proper infection of the CNS (Mogk *et al.,* 2014). Similar observation of cure with berenil in early trypanosomes infection though trypanosomes can be demonstrated in the CSF has also been observed in goats (Moulton, 1986). However if berenil treatment is delayed to later stages of infection, relapses due to CNS infection always occurs (Jennings *et al.,* 1979; Jennings and Gray, 1982). Similar treatment of early CNS infection where trypanosomes can be demonstrated in CSF with pentamidine, a drug that does not cross the blood brain barrier has also been observed (Doua *et al.,* 1996; Lejon *et al.,* 2003).

In order to determine if the trypanosome infected and treated animals were true negative, a PCR detection method that is more sensitive for diagnosis of trypanosome was used (Masiga *et al.,* 1992; Thumbi *et al.,* 2008). PCR method has been demonstrated to be sensitive in identifying trypanosome parasites in blood and CSF and even in a-parasitaemic cases (Deborggraeve *et al.,* 2011). Five, six and four of the six CSF samples from trypanosome infected sheep were PCR positive at 21, 42 and 112 dpi. This shows that more sample were detected positive by PCR method than by microscopy with prior centrifugation. Indeed samples that were positive by double centrifugation technique were also positive by PCR method demonstrating good concordance between the two methods (Kyambadde *et al.,* 2000). However, PCR method has been shown to be unreliable for monitoring treatment outcomes due to the persistence of the trypanosome DNA. Indeed in the current study, four of the six CSF samples that were analysed 112 dpi coinciding with 70 days post treatment were PCR positive. Similar, observation of confirmed trypanosome cured cases that continued to remain PCR positive following treatment pointing to the persistent trypanosome DNA after successful treatment have also been observed in humans (Mbati *et al.,* 1999; Deborggraeve *et al.,* 2011). It is however important to point to the fact that two of the samples were negative by PCR at 112 dpi.

5.4 Weight Changes

Decrease in the body weight of infected sheep was observed during the pre-treatment period and recovered post-treatment with berenil[@]. The decrease in the body weight has also been observed in the monkey model (Ngure *et al.*, 2008) and also humans infected with *T. brucei rhodesience* (Blum *et al.,* 2006). The decline in body weight during infection could be attributed to reduced appetite and/or defect in lipid metabolism especially lipoproteins, cholesterol and hyperlipidemia of infected host resulting in the utilization of body mass (Diehl and Risby, 1974; Ndungu *et al.,* 1994). The situation might be further aggravated by decrease in food intake due to loss of appetite. Indeed trypanosome infected animals and humans are known to have a markedly reduced appetite.

Defect in lipid metabolism is manifested by a hypertriglyceridemia observed in rabbits infected with *Trypanosoma brucei* (Rouzer and Cerami, 1980), Monkeys infected with *Trypanosoma b. rhodesiense* (Ngure *et al.,* 2008) and humans infected with *T. brucei gambiense* (Huet, 1990). This results from a marked inhibition of the adipose tissue enzyme lipoprotein lipase responsible for clearing lipids from blood circulatory system, a process that is markedly inhibited by tumour necrosis factor (Beutler and Cerami, 1988).

5.5 Haematological Changes

The findings of this study provided information on our understanding of the clinical pathology and pathogenesis of HAT in sheep. Haematological alterations indicated that sheep infected with *T.b rhodesiense* developed microcytic hypochromic anemia as the disease progressed. However, these haematological parameters recovered following the animals treatment with diminazine aceturate.

Trypanosomes of the *brucei* subgroup inhabit the blood, intercellular tissues and body cavity fluid of infected animal precipitating anaemia and tissue damage (Sharma *et al.,* 2000; Nwoha and Anene, 2011a). The presence of trypanosomes in the blood induces increased red blood cell destruction and changes in biochemical constituents of blood (Jenkins *et al*., 1985; Igbokwe and Mohammed, 1992; Taiwo *et al.,* 2003; Ekanem and Yusuf, 2008; Akanji *et al.,* 2009). Haematological and serum biochemical changes are characteristic of trypanosome infections, indeed anaemia is a consistent and significant finding in human (Sadun *et al.,* 1973) and animal (Kagira *et al.,* 2007b) trypanosomosis. Blood loss results in anaemia which till now

has been one of the cardinal signs of trypanosomosis in animals (Sadun *et al.*, 1973; Finelle, 1973; Ohaeri and Eluwa, 2011) and in humans (Woodruff *et al*., 1973; OIE, 2009). Clinically it appears as paleness of the visible mucous membrane (Nwoha and Anene, 2011b). The results of the study indicated that haematological profiles of sheep infected with *T.b rhodesiense* are in agreement to a larger extent to that observed in human suffering from human African trypanosomiasis (Sadun *et al.*, 1973).

Significant decreases in PCV and Hb signified some degree of blood loss in the infected sheep. This corroborates previous work done in trypanosomosis in other genera of animals (Eloy and Lucheis, 2009; Ezeokonkwo *et al*., 2010; Nwoha and Anene, 2011a). The severity of haematological changes is often determined by the strain of infecting trypanosome and the host (Anosa, 1988a; Anosa, 1988b). Haematological changes are influenced by the pathogenesis of the disease trypanosomosis (Anosa *et al*., 1983; Schalm *et al*., 1975) and indeed African trypanosomosis is associated with a rapid decline in red blood cell counts, haemoglobin concentration and packed cell volume (Kagira *et al.,* 2006; Thuita *et al.*, 2008). The degree of anaemia in infected animals is proportional to the severity of the disease and is normally monitored by measurement of PCV level in absence of other factors causing anaemia (Murray *et al.,* 1990; Grace *et al.*, 2007).blum

PCV levels in trypanosome infected humans, mice and other animal models have been shown to decrease with time. The observed significant decline in PCV value of parasitaemic sheep compared to those of the aparasitaemic animals were in agreement with several previous studies where Dinkaa and Abebeb (2005) reported that parasitaemic sheep and goats showed lower PCV values when compared to non-infected animals. The same has been observed in trypanosome infected monkeys with haematological parameters such as red blood cell counts, haemoglobin and haematocrit indixes decreasing. The rate of decline in the said parameter higher in acutely affected monkeys than those with chronic infection (Kagira *et al.*, 2006).

The results of the current study showed that the height and duration of parasitemia corresponded with decline of PCV, RBC, Hb, and rise in rectal temperature in all the infected sheep. Indeed it has been demonstrated that the onset and severity of anaemia in trypanosomosis is directly related to the appearance of the parasite in blood and the level of parasitaemia (Luckins and Gray, 1978; Murray, 1979; Adeiza *et al*., 2008). A decline in PCV

in sheep and goats and thus anaemia with increase in parasitaemia and its recovery with disappearance of parasite has been reported (Dargie *et al*., 1979; Saror, 1979; Adah *et al*., 1993).

Lowered levels of PCV and thus anaemia are common and critical features in the pathogenesis of African trypanosomiasis contributing to the host morbidity and mortality and thus curtailing the absence of longevity of infected hosts (Murray and Dextor, 1988; Kagira *et al.,* 2006). At the same time it has been established that the trypanotolerant animals like the N'Dama are resistant to the pathogenic effects of trypanosomes due to their ability to control the development of anaemia and the level and duration of parasitaemia (Murray *et al.,* 1977; Paling *et al.,* 1987). Indeed the ability to control parasitaemia levels and PCV using anti-trypanocidal drugs has been shown to extend the survival period of infected mice (Ngure *et al.,* 2009).

Erythrocyte indices including MCV, MCH, MCHC and red cell distribution width are used to determine the type of anaemia (Neiger *et al*., 2002; Cheesbrough, 2006; Kagira *et al.,* 2006; Thuita *et al.,* 2008). Microcytic hypochromic anaemia was observed in the early stages of the disease. This was characterized by decline in MCV and a slight decline in MCH and MCHC. The pathophysiology of anaemia in trypanosomosis is complex and multifactorial in origin (Naessens *et al*., 2005). Indeed, there is interplay of several factors acting either individually or synergistically contributing to the development of anaemia in human and animal trypanosomosis. These include erythrocyte injury caused by lashing action of trypanosome flagella, hyperplasia of bone marrow and spleen, increase haemosiderin deposits (Sharma *et al*., 2000), undulating pyrexia, platelet aggregation, toxins and metabolites from trypanosomes, lipid peroxidation and malnutrition (Murray and Morrison, 1978; Morrison *et al*., 1981; Saror, 1982; Igbokwe, 1994). It may also result from haemolysis as indicated by a shortened half life of Cr labelled RBCs (Jenning, 1976). Indeed decline in the PCV values has been shown to be due to haemolysis due to oxidative stress and/or immunological reaction (Turray, 2005).

MCH levels of infected sheep was higher than the control for most parts of the experiment especially about 28 dpi and between 84 and 112 dpi. This could due to the production of small sized RBC from the bone marrow and thus higher MCH. Indeed this is further confirmed by the small sizes of RBC as measured by the MCV an indicator of

microcytic anaemia also observed in monkeys infected with the same parasite (Kagira *et al.,* 2006).

A measure of the WBC counts indicated an increase in the acute stages of infection to 56 dpi and a decline below control post 70 dpi. The early increase in WBC levels has been shown to result from profound proliferation of T and B cells especially in the spleen and bone marrow (Mayor-Withey *et al.*, 1978). In addition leucocytosis including neutrophilia, plasmacytosis or monocytosis has been reported in humans and in experimental animals (Clayton *et a.l*, 1980; Wery *et al.,* 1982; Poltera, 1985). Indeed similar observations have also been reported in sheep infected with *Trypanosoma evansi* (Onah *et al.,* 1996). A later decline in leucocyte counts could be due to the development of a lymphopenia (Morrison *et al.,* 1978) resulting from the mitogenic like overstimulation of lymphoid tissue resulting in exhaustion of the lymphoid organ and decreased mitogenic response of the available lymphoid cells.

5.6 Biochemical Changes in Blood

5.6.1 Protein changes

Infected sheep had an initial sharp increase in the mean serum total protein levels that peaked by 14 dpi and thereafter declining to lowest levels at 28 dpi but thereafter recovered following treatment. Elevated levels of protein have been reported in other animal models of trypanosomosis including rabbits infected *T. brucei* (Orhue *et al.,* 2005), rats infected with *T. brucei* (Ekanem and Yusuf, 2008), goats infected with *Trypanosoma congolense* (Ndoutamia *et al*., 2002) and monkeys (Abega and Anosa, 2005). The same has been observed in *T.b rhodesiense* infection of man (Welde *et al.,* 1989).

The initial increase in total protein could be due to increase in the immunoglobulins/gammaglobulinaemia which is a prominent feature of trypanosomosis primarily due to increase in IgM. Indeed trypanosome antigen stimulation and antibody production especially of IgM (Anosa and Isoun, 1976; Anosa, 1988a; Ogunsanmi *etal.,* 1994) is consistent with the reports of other studies (Anosa and Isoun, 1976; Taiwo *et al.,* 2003). Another possible cause in the increase of the total protein levels could be due to increased synthesis of positive acute proteins. Indeed positive acute proteins such as haptoglobin (Ndungu *et al.,*1991), lipopolysaccharide binding protein and serum amyloid P (Ngure *et*

al.,2008; Ngure *et al.*, 2009a; Ngure *et al.*, 2009b; Ngure *et al.* ,2009c; Mungatana *et al*., 2007) are known to increase in serum of trypanosome infected animals.

The later hypoprotenemia has also been observed in trypanosome infected monkeys (Abenga and Anosa, 2005; Ngure *et al.*, 2008), mice (Karori *et al*., 2008; Eric *et al.*, 2013) and also in humans (Awobode, 2006). The hypoproteinemia could be due to the parenchyamatous disorder observed in the liver during trypanosome infection in animal models (Robertson and Jenkins, 1959a) and also in humans (Robertson and Jenkins, 1959b) resulting in reduced synthesis of various proteins since the liver is the main source in the synthesis of most plasma proteins (Limdi and Hyde, 2003). Indeed inflammation induced liver damage during trypanosomosis and other sustained inflammatory responses leads to decreased synthesis of essential biomolecules such as albumin (Limdi and Hyde, 2003).

Albumin has significant antioxidant capacity due to its free thiol group (Pupim *et al.,* 2004) thus sustained inflammatory responses such as in trypanosomosis could causes a decline in plasma albumin levels since it is used as a buffer for free radicals. Moreover, the decline in plasma albumin levels in trypanosomosis could also be due to the increased permeability of the glomerular filter (Agu and Egbuji, 2002) resulting from kidney damage thus albumin is passed in urine with the passage increasing with disease progression. Indeed gross and histological changes affecting the kidneys have been noted in trypanosome infected dogs (Murray *et al.*, 1975) and humans (Anosa *et al.*, 1988a; Anosa *et al.*, 1988b). This is further collaborated with defects observed in kidney function test in trypanosome infected monkeys (Ngure *et al*, 2008) and humans (Basson *et al*, 1977).

A decrease in albumin the major plasma protein could also be due to increased utilization by the trypanosomes as a nutrient, since they require albumin for their optimal survival (Coopens *et al.*, 1987). Thus since infection of the sheep resulted in high parasitaemia, increased utilization by the parasites probably contributed to the decrease in albumin.

5.7 Tissue Enzymes

5.7.1 Enzyme changes in blood

The current study was setup to determine the usefulness of serum total CK and LDH including their isoenzymes as markers of determining cardiac damage during *Trypanosome b. rhodesiense* infection in the sheep model. Results indicated an increase in the levels of blood

total CK and LDH levels of infected animals. In addition tissue enzyme profiles indicated the presence and elevated levels of the isoenzymes CK MB and a flipped over pattern of LDH1 and LDH2 ratio both indicative of cardiac involvement (Hidron *et al*., 2010). The changes in tissue enzyme levels and isoenzyme profiles were however reversed following treatment. The rise in concentration of total tissue enzymes observed in plasma from the trypanosome infected sheep in this study is consistent with those observed in trypanosome infected monkeys (Ngure *et al.*, 2008) and humans (Basson *et al.*, 1977; Anosa 1988a; Awobode, 2006).

Tissue enzymes are found in the cytoplasm and mitochondria of mammalian cells and are released into body fluids and circulation due to changes in cell membrane permeability or frank necrosis (De Souza *et al.,* 2000). The pathological changes in cardiac tissue in dogs (Ndungu *et al.,* 1991), monkeys (Schmidt and Sayer, 1982) and humans (Poltera *et al*, 1976) infected with trypanosomes .In addition severe damage of other organs could explain the increased enzyme concentration in plasma of infected sheep (Anosa, 1988b). Indeed *Trypanosoma brucei brucei* infection in dogs if untreated results in death due to heart failure from the severe pancarditis (Morrison *et al.,*1981; Ndungu *et al.,* 1991). At the same time a good number of HAT patients during the haemolymphatic and also the CNS stage are observed to develop cardiac damage due to destruction of myocardial cells (Jones *et al*., 1975; Mbala *et al*., 1988) resulting in the loss of membrane integrity leading to enzymes diffusion to the extra cellular spaces and drainage into blood vessels thus elevation levels of tissue enzymes in blood.

Monitoring changes in the serum tissue enzymes has been used as a diagnostic value in judging the presence and extent of tissue injury in pathological conditions in animals (Manga-Gonzalez *et al.*, 2004) and humans (Adams *et al.,* 1991; De Souza *et al.*, 2000; Rastogi, 2006). In particular, the isoenzymes of lactate dehydrogenase and creatine kinase in combination with more specific markers of cardiac injury such as the cardiac troponins, can provide information on the relative severity, extent or duration of myocardial injury (Jeremias and Gibson, 2005; Walker, 2006). At the same time results in the current study show a flipped over pattern in the ratio of LDH1:LDH2 accompanied by increase in isoenzyme CK MB, both of which has been shown to correlate with severity and duration of cardiac injury (Preus *et al.,* 1988).

5.7.2 CSF enzyme changes

The analysis of tissue enzyme CK and LDH plus their isoenzymes yielded very low levels of the total enzymes compared to that of serum. Indeed no isoenzymes bands for both tissue enzymes were observed following gel electrophoresis of CSF. This observation could be due to the low concentration of both enzymes resulting from minimal damage of the CNS by the trypanosomes. Indeed it has been observed that infection of the CNS has to be well established before any observable pathological damage/changes can be seen (Molton, 1986). In addition, the subcurative treatment with diminazine aceturate of the infected of the sheep in the current study resulted in clearance of the trypanosome from the CNS, clearly indicating that infection had not been established thus explaining the minimal increase in tissue enzymes. This is further collaborated by the results indicating the absence or the presence of low number of trypanosomes in CSF and mininmal increases in CSF protein levels. It is likely that infection of the sheep resulted in a more acute infection neccesitating their humane treatment before the establishment of the infection in the central nervous system. Indeed animals with trypanosomes in the CNS/CSF have been shown to exhibit no or minimal CNS pathology which however develops with progression of infection (Jenning and Gray, 1983).

CHAPTER SIX
CONCLUSION AND RECOMMENDATION

6.1 Conclusion

The aims of this study were to develop a *T.b rhodesiense* sheep model for human African trypanosomosis and the potential use of serum and Cerebrospinal fluid LDH and CK enzymes as diagnostic markers of cardiac involvement during infection. The infection was associated with high temperature, body weight loss and parasitemia. However, low levels of parasites were detected in CSF at various stages of infection. Therefore they are in tandem with the signs presented in human and other animal models including the non-human primate models. The infection was also characterized by an increase in MCH and MCHC. However, there was a decline in the levels of PCV, HCT, Hb, MCV and RBC counts. All parameters however recovered to control levels after treatment. This shows that the infection presented haematological changes. *T.b rhodesiense* infection resulted in increase in total protein, total LDH and total CK activities in both serum and CSF of infected compared to uninfected sheep. At the same time LDH and CK total enzyme and isoenzyme changes which indicates cardiac involvement suggesting the diagnostic potential of the enzymes. A flip over LDH1:LDH2 ratio pattern observed indicates its potential use as specific marker of cardiac development during *T.b rhodesiense* infection.

6.2 Recommendations

1. Biochemical changes in CK soenzyme were unsatisfactory compared to LDH hence the study recommends a lower than 1×10^4 dose of parasites be used to infect sheep HAT model as this might slow down disease virulence and therefore prolonging animal's life thus allowing adequate time for parasites to be well established in CNS
2. The study could not cover all aspects of *T.b rhodesiense* pathogenesis. In order to generate more information to further justify the similarities in pathogenesis between sheep and other established models of HAT, this study recommends that further work be done on other serum and CSF biochemical changes of *T.b rhodesiense* infected sheep.

REFERENCES

Abenga J N and Anosa V O (2005). Serum total proteins and creatinine levels in experimental Gambian trypanosomosis of vervet monkeys. *African Journal of Biotechnology* **4**: 187-190.

Abenga J N and Anosa V O (2006). Clinical studies on experimental Gambian trypanosomiasis in vervet monkeys. *Veterinarski Arhiv* **76**:11-18.

Adah M I, Otesile E B and Joshua R A (1993). Susceptibility of Nigerian West African Dwarf and Red Sokoto goats to a strain of *Trypanosoma congolense*. *Veterinary Parasitology* **47**: 177-188.

Adams J E, Abendschein D R and Jaffe A S (1991). Biochemical markers of myocardial injury. Is MB creatinine kinase the choice for the 1990's? *Circulation* **88**: 750-763.

Adeiza A A, Maikai V A and Lawal I (2008). Comparative haematological changes in experimentally infected Savannah brown goats with *Trypanosoma brucei* and *Trypanosoma vivax*. *African Journal of Biotechnology* **7**:2295-2298.

Agu W E and Egbuji A N (2002). Urine albumin levels in mice infected with *Trypanosoma brucei*. *Veterinary Archives* **72**: 101-108.

Akanji M A, Adeyemi O S, Oguntoye S O and Sulyman F (2009). *Psidium guajava extract* reduces trypanosomosis associated lipid peroxidation and raises glutathione concentrations in infected animals. *EXCLI Journal* **8**:148-154.

Allsopp R (2001). Options for vector control against trypanosomiasis in Africa. *Trends in Parasitology* **17:**15-19.

Amin D, Rottenberg M, Thomsen A, Mumba D, Fenger C, Krisensson K, Buscher P, Finsen B, Masocha W (2009). Expression and role of CXCL10 during the encephalitic stage of experimental and clinical African trypanosomiasis. *Journal of Infectious Diseases* **15:** 1556-1565.

Amin D, N, Ngoyi D, M, Nhkwachi G, M, Palomba M, Rottenberg M, Büscher P, Kristensson K and Masocha W (2010). Identification of stage biomarkers for human African trypanosomiasis. *American Journal of Tropical Medicine and Hygiene* **82***:* 983-990.

Anderson N E, Mubanga J Fevre E M, Picozzi K, Eisler M, C, Thomas R, and Welburn S, C (2011). Characterisation of the wildlife reservoir community for human and animal trypanosomiasis in the Luangwa Valley, Zambia. *PLoS Neglected Tropical Diseases* **5: (**e) 1211).

Anosa V and Isoun T (1976). Serum proteins, blood and plasma volumes in experimental *Trypanosoma vivax* infections of sheep and goats. *Tropical Animal Health and Production* **8**: 14-19.

Anosa V O (1988a). Haematological and biochemical changes in human and animal trypanosomiasis. Part 2. *Revue D'elevage et de Medecine Veterinaire Des trypanolytic factor of human serum Tropicaux.* **41:**151-164.

Anosa V (1988b). Haematological and biochemical changes in human and animal trypanosomiasis. Part **1**. *Revue D'elevage et de Medecine Veterinaire Des Pays Tropicaux* .**41:**65-78.

Anosa V O and Kaneko J J (1983). Pathogenesis of *Trypanosoma brucei* infection in deer mice 2(*Peromyscus maniculatus*): Hematologic, erythrocyte biochemical and iron metabolic aspects. *American Journal of Veterinary Research* **44**: 639-644.

Apted F I C (1970). Clinical Manifestations and Diagnosis of Sleeping sickness. In *The African Trypanosomiasis*. H.W Mulligan, editor. George Allen and Unwin. London. pp 661-683.

Atouguia J and Kennedy P (2000). Neurological aspects of human African trypanosomiasis. In Infectious disease of nervous system. Butterworth-Heinemann. Oxford United Kingdom pp 321-372.

Awobode H O (2006). The biochemical changes induced by natural human African trypanosome infection. *African Journal of Biotechnology* **5**: 738-742.

Bacchi C J (2009). Chemotherapy of Human African Trypanosomiasis. *Interdisciplinary Perspectives on Infectious Diseases*, 2009

Barrett M P (1999). The fall and rise of sleeping sickness**.** *The Lancet* **353**: 1113-1114.

Barrett M P, Buchmore R J S, Stich A, Lazzari J O, Frasch A C and Cazzulo J J (2003). The trypanosomiasis. *The Lancet* **362**: 1-13.

Basson W, Page M L and Myburgh D P (1977). Human trypanosomiasis in Southern Africa. *South African Medical Journal* **51**: 453-457.

Benjamin M M (1985). Outline of Veterinary Clinical Pathology. 265-267. 3rd edition. New Delhi.

Bertrand E, Loubiere R, Barabe P. and Ette M. (1974). Current aspects of cardiac symptoms in African human trypasonomiasis due *Trypanosome gambiense* (a propos of 194 cases). *Acta Cardiologica.***29**: 363-38.

Beutler B. and Cerami A. (1988). Cachectin (TNF): A macrophage hormone governing cellular metabolism and inflammatory response. *Endocrine Review* **9**: 57-65.

Bisalla M, Ibrahim N. D. G, Lawal I. A. and Esievo K. A. N. (2007). Serum total proteins, albumin and albumin globulin ratio in Yankasa sheep experimentally infected with *Trypanosoma congolense* and immunomodulated with levamisole, *Journal of Protozoology Research* **17**: 39-43.

Bisser S, Lejon V and Preux P M (2002). Blood cerebrospinal fluid barrier and intrathecal immunoglobulins compared to field diagnosis of central nervous system involvement in sleeping sickness *Journal of Neurological Sciences* **193:** 127-135.

Blijenberg B G, Roetering H A, Zwang L and Leijnse B (1985). Spinal fluid protein revisited: A reappraisal of the biuret procedure. *Clinical Chemistry and Laboratory Medicine* **23:** 225-230.

Blum A, Zellweger M, Burri C and Hatz C (2008). Cardiac involvement in African and American trypanosomiasis. *Lancet Infection Diseases* **8:** 631-641.

Blum J, Schmid C and Burri C (2006). Clinical aspects of 2541 patients with second stage human African trypanosomiasis. *Acta Tropica* **97**: 55-64.

Blum J A, Burri C, Hatz C, Kazumba L, Mangoni P and Zellweger M J (2007). Sleeping hearts: The role of the heart in sleeping sickness (human African trypanosomiasis). *Tropical Medicine and International Health* **12:** 1422-1432.

Blum J A, Schmid C, Burri C, Hatz C, Olson C, Fungula B, Kazumba L, Mangoni P, Mbo F, Deo K, Mpanya A, Dala A, Franco J R, Pohlig G and Zellweger M J (2009). Cardiac alterations in human African trypanosomiasis (*T. b. gambiense*) with respect to the disease stage and antiparasitic treatment. *PLoS Neglected Tropical Diseases* **3**: e 383.

Borst P and Fairlamb A H. (1998). Surface receptors and transporters of *Trypanosoma brucei*. *Annual Reviews in Microbiology* **52:** 745-778.

Borst P, Bitter W, Blundell P, Cross M, McCulloch R, Rudenko G and Coombs G H (1997). The expression sites for variant surface glycoproteins of *Trypanosoma brucei*. *Trypanosomiasis and Leishmaniasis: Biology and Control* eds Hide G.,Mottram J., Coombs G.,Holmes P., (British Society for Parasitology/CAB International Oxford .109-131.

Brun R, Blum J, Chappus F and Burri C (2010). Human African trypanosomiasis. *Lancet* **375**: 148-159.

Brun R, Schumacher R, Schmid C, Kunz C and Burri C (2001). The phenomenon of treatment failures in human African trypanosomiasis. *Tropical Medicine and International Health* **6**: 906-914.

Burchmore R J (2002). Chemotherapy of human African trypanosomiasis. *Current Pharmaceutical Design* **8:** 256-267.

Burri C and Brun R (2002). African trypanosomiasis. In: Manson's Tropical Diseases ed.Cook G C, Zumla A). 21. London. pp 1303-1323.

Buscher P and Lejon V (2004). Diagnosis of human African trypanosomiasis: In *Trypanosomiasis*. Mauldine I., Holmes P. and Miles M. A. editors. CABI Publishing, Oxfordshire. pp. 203-218.

Buscher P and Lejon V (2005). CSF in human African trypanosomiasis: A key to diagnosis, therapeutic decision and post treatment follow up. *Tropical Medicine and International Health* **5:** pp 395-403.

Calbreath D (1992). Clinical enzymologyII: Use of enzymes in diagnosis. In: Clinical Chemistry: *A fundamental textbook.* W. B. Saunders Philadephia, USA.pp 179-223.

Cattand P, Jannin J and Lucas P (2001). Sleeping sickness surveillance: An essential step toward elimination. *Tropical Medicine and International Health* **6:** pp 348-361.

Chappuis F, Loutan L, Simarro P and Buscher P (2005). Options for field diagnosis of human African trypanosomiasis. *Clinical Microbiology Review* **18:** pp 133-146.

Cheesbrough M (2006). District laboratory practice in tropical countries. Part 2, 2nd ed. Cambridge University Press, pp 299-319.

Cheesebrough M (1998). Examination of blood, lymph fluid and CSF for trypanosomes causing African trypanosomiasis. District Laboratory Practice in Tropical countries. Cambridge University Press, Cambridge. pp 259-266.

Clayton C E, Selkirk M E, Corsini C A, Ogilvie B M and Askonas B A (1980). Murine trypanosomiasis: Cellular proliferation and functional depletion in the blood, peritoneum and spleen related to changes in bone marrow stem cells. *Infection and Immunity* **28**: 824-831.

Clerinx J, Vlieghe E, Asselman V, Van de Casteele S, Maes M B and Lejon V (2012). Human African trypanosomiasis in a Belgian traveller returning from the Masai Mara area, Kenya. February 2012. *Eurosurveillance* **17**: pp 1334-1335

Coopens I., Opperdoes F. R., Courtoy P. J. and Baubhuin P. (1987). Receptor mediated endocytosis in the bloodstream form of *Trypanosoma brucei*. *Journal of Protozoology* **34**: pp 465-473.

Corredor O. M. (2003). Serum enzyme pattern and local enzyme gradients inchronic chaggasic patients. *Acta Cientifica Venezola* **53**: pp 1-15.

Cross G. M. (1990). Cellular and genetic aspects of antigenic variation in trypanosomes. *Annual Review in Immunology* **8**: pp 83-110.

Dargie J. D., Murray P. K., Murray M. and McIntyre W. M. (1979). The blood volume and erythrokinetic of Ndama and Zebu cattle experimentally infected with *Trypanosoma brucei.Research in Veterinary Sciences* **26**: pp 245-247.

De Souza A P (2000). Enzymatic markers of heart lesion in mice infected with *Trypanosoma cruzi* and submitted to benzinidazole chemotherapy *Parasitology Research* **86**: pp 800-808.

Deborggraeve S, Claes F and Laurent T (2006). Molecular dipstick test for diagnosis of sleeping sickness. *Journal of Clinical Microbiology* **44**: pp 2884-2889.

Deborggraeve S, Lejon V, Ekangu R A, Mumba Ngoyi D, Pati Pyana P, Llunga M, Mulunda J P and Buscher P (2011). Diagnostic accuracy of PCR in gambiense sleeping sickness diagnosis, staging and post-treatment follow-up: A 2 year longitudinal study. *PLoS Neglected Tropical Diseases* **5**: e972.

Desquesnes M and Dia M L. (2004). Mechanical transmission of *Trypanosoma vivax* in cattle by the African tabanid *Atylotus fuscipes*. *Veterinary Parasitology* **119:** pp 9-19.

Diehl E J and Risby E L (1974). Serum changes in rabbits experimentally infected with *Trypanosoma gambiense. The American Journal of Tropical Medicine and Hygiene* **23**: pp 1019-1022.

Dinkaa H and Abebeb G (2005). Small ruminants trypanosomosis in the southwest of Ethiopia. *Small Ruminant Research* **57**: 239-243.

Doua F, Miezan T W, Sanon S R., Boa Y F and Baltz T (1996). The efficacy of pentamidine in the treatment of early-late stage *Trypanosoma brucei gambiense* trypanosomiasis. *American Journal of Tropical Medicine and Hygiene* **55** 586-588.

Drent E F M, Cobben N M, Henderson R F, Woulter E F and van Dieijen-Visser M (1996). Usefulness of lactate dehydrogenase and its isoenzymes as indicators of lung damage or inflammation. *European Respiratory Journal* **9**: 1736-1742.

Dumas M. and Boa F. Y. (1988). Human African trypanosomiasis. In Handbook of Clinical Neurology (Harris AA Ed) Elsevier Science Publishers Essex pp 339-344.

Ekanem J T and Yusuf O K (2008). Some biochemical and haematological effects of black seed (*Nigella sativa*) oil on *T. brucei*-infected rats. *African Journal of Biomedical Research* **11**: 79-85

Ekanem J T, Majolagbe O R, Sulaiman F A and Muhammad N (2006). Effects of honey supplemented diet on the parasitaemia and some enzymes of *Trypanosoma brucei*-infected rats. *African Journal of Biotechnology* **5**: 1557-1561

Ekanem J T, Sulyman F A and Adeyemi O S (2005). Therapeutic properties and serum iron in *T. brucei* infected rats treated with amodiaquine and mefloquine. *Biokemistri* **17**: 115-121.

Eloy I J and Lucheis S B (2009). Canine trypanosomiasis: Etiology of infection and implications for public health. *Venomous Animals and Toxins including Tropical Diseases.* **15**: 589-611.

Eric S K, Ngure R M, Chome J, Korir S, Mdachi R E and Mburu J N (2013). Effects of long term administration of *Solanum nigrum* extracts in female swiss white mice infected with *Trypanosoma brucei rhodesiense*. *Science Journal of Medicine and Clinical Trials* 2276-7487, doi:0.7237/sjmct/290.

Eric M F, Wissmann B V, Welburn S.C., Lumburn P (2008). The burden of human African trypasonomiasis, PLoS Neglected diseases.**2**:e333

Etchegorry G, Helenport J, Pecoul B,Jannin J and Legros D (2001). Availability and affordability of treatment for human African Trypanosomiasis.*Tropical Medicine and International Health.***l6:** 957-959.

Ezeokonkwo R C, Ezeh I O, Onunkwo J I, Obi P O, Onyenwe I W and Agu W E (2010). Comparative haematological study of single and mixed infections of mongrel dogs with *Trypanosoma congolense* and *Trypanosoma brucei brucei*. *Veterinary Parasitology,* **173**: 48-54.

Fairlamb A H (2003). Chemotherapy of human African trypanosomiasis: Current and future prospects. *Trends in Parasitology* **19**:488-494.

Fevre1 E M, Wissmann B V, Welburn S C and Lutumba P (2008). The burden of human African trypanosomiasis, *PLoS Neglected Diseases*.**2** e 333

Finelle P (1973). African animal trypanosomiasis. *World Animal Review* **8**: 24-27.

Ford L B (2007). Civic conflict and sleeping sickness in Africa in general and Uganda in particular. *Conflict and Health* **1**: 6

Franco J R, Simaro P P, Diarra A, Ruiz J.A and Jannin J (2014). The Journey towards Elimination of Gambiense Human African Trypanosomiasis: not far, nor easy. *Parasitology.* **141**:748-760.

Gehrig S and Efferth T (2008). Development of drug resistance in *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense*. Treatment of human African trypanosomiasis with natural products (Review).*International Journal of Molecular Medicine* **22**: 411-419.

Gelfand, M (1966). The early clinical features of Rhodesian tryponasomiasis with special reference to the "chancre" (local reaction). *Transactions of Royal Society of Tropical Medicine and Hygiene.* **60**:376-379.

Gibson W, Backhouse T and Griffiththns A (2002). The human serum resistanceassociated gene is ubiquitous and conservrved in *Trypanosoma brucei brucei rhodesiense* throughout East Africa. Infection, Genetics and Evolution. *Journal of Molecular Epidemiology and Evolutionary Geneticsin infectious Diseases.*1: 207-214.

Goodwin L G (1970). The pathology of African trypanosomiasis. *Transaction of the Royal Society of Tropical Medicine and Hygiene* **64**: 797-812.

Gould S S and Sayer P D (1983). A simple concentration technique for the direct detection of trypanosomes in the cerebrospinal fluid of vervet monkeys. In the proceedings of the International Scientific Council for the Trypanosomiasis Research and Control (OAU). Seventh Meeting Arusha, Tanzania pp 259-260.

Grace D, Himstedt H, Sidibe I, Randolph T and Clausen P H (2007). Comparing FAMACHA (c) eye color chart and hemoglobin color scale tests for detecting anemia and improving treatment of bovine trypanosomosis in West Africa. *Veterinary Parasitology* **147**: 26-39.

Hainard A, Tiberti N, Robin X, Lejon V, Matovu E, Ngoyi D, Enyaru J Muller M, Turck N, Ndung'u M, Lejon V, and Sanchez C. (2011). Amatrix metalloproteinase-9- and intercellular adhesion molecule I are powerful staging markers for human African trypanosomiasis. *Tropical Medicine and International Health* **16**: 119-126.

Hargrove J W (2003). Tsetse eradication; Sufficiency, Necessity and Durability, DFID Animal Health Programme, editor, Edinburg: Centre for Tropical Veterinary Medicine. pp 134

Heavner J E, Colaianne J and Roper M (1986). Lactate dehydrogenase isoenzymes in blood and cerebrospinal fluid from healthy beagles. *American Journal of Veterinary Research* **47:** 1772-1775.

Herbert W and Lumsden W (1976). *Trypanosome brucei*: A 'rapid matching method' for estimation of host's parasitemia. *Experimental Parasitology* **40**: 427-431.

Herbert W J and Parrat D (1979). Virulence of trypanosomes in the vertebrate host. In Lumsden WHR Evans DA edition Biology of kinetoplastida New York Academic Press pp 482-521.

Hide G (1999). History of sleeping sickness in East Africa. *Clinical Microbiology Review* **12**: 112-125.

Hidron A, Vogenthaler N, Santos-Preciado J I, Rodriguez-Morales A J, Franco-Paredes C and Rassi A (2010). Cardiac involvement with parasitic infections. *Clinical Microbiology Reviews* **23**: 324-349.

Hoffman R, Benz Jr. E J, Silberstein L E, Heslop H, Weitz J and Anastasi J (2013). Hematology: Basic Principles and Practice. Elsevier Health Sciences, London, UK. pp. 2343.

Hotez P J (2008). Forgotten diseases, forgotten people: *The Neglected Tropical Diseases and their Impact on Global Health and Development*. ASM Press. pp. 218

Huet G, Lemmesre J L, Grand G, Noerie F, Boutignn F, Dieu M C Jannin J and Degand P (1990). Serum lipid and lipoprotein abnormalities in human African trypanosomiasis. *Transaction of the Royal Society of Tropical Medicine and Hygiene* **84**: 792-794.

Hutchinson O C, Fèvre E M, Carrington M and Welburn S C (2003). Lessons learned from the emergence of a new *Trypanosoma brucei rhodesiense* sleeping sickness focus in Uganda. *Lancet Infectious Diseases* **3**: 42-45.

Igbokwe I O (1994). Mechanisms of cellular injury in African trypanosomiasis. *Veterinary Bulletin* **64**: 611-620.

Igbokwe I O and Mohammed A (1992). Some plasma biochemical changes in experimental *Trypanosoma brucei* infection of Sokoto red goats. *Revue d'elevage et de Medecine. Veterinaire des Pays Tropicaux* **45**: 287-290.

Ikede B O and Losos G J (1972). Pathology of disease in sheep produced experimentally by *T. brucei.Veterinary Pathology* **9**: 278-289.

Iley K (2004). Sleeping sickness spreading in Angola.Reuters AlertNet. http://www.alertnet.org.

Iqbal J and Chaudhary Z (2000). Incidence, biochemical and haematological alterations induced by natural trypanosomiasis in racing dromedary camels. *Acta Tropica* **77:** 209-213.

Jenkins R and Robertson HH (1959a). Hepatic dysfunction in human trypanosomiasis ii: Serum proteins in *Trpanosoma rhodesiense* infections and observations on the alterations found after treatment and during convalescence. *Transactions of Royal Society of Tropical Medicine and Hygiene* **53**: 524-533.

Jenkins R and Robertson H (1959b). Hepatic dysfunction in human trypanosomiasis i: Abnormalities of excretory function with observations on the alterations of these tests during treatment and convalescence. *Transactions of Royal Society of Tropical Medicine and Hygiene* **53**: 511-523.

Jenkins G and Facer C (1985). Hematology of African trypanosomiasis, in: TIZARD, I**.** Immunology and Pathogenesis of Trypanosomiasis. Boca Raton, Florida: C.R.C. Press, pp 13-44.

Jennings F W (1976). The Anaemia of Parasitic Infections. In: *Pathophysiology of Parasitic Infection* Soulsby, E.J.L. (Ed.). Academic Press, New York, USA. pp: 41-67.

Jennings F W and Gray G D (1983). Relapse parasitemia following chemotherapy of chronic *T. brucei* infections in mice and its relation to cerebral trypanosomes. *Contribution to Microbiology and Immunology* **7:** 147-154

Jennings F W, Gray G D and Urquhart G M (1982). The use of Erlangen diamidine 98/202 in relasing *Trypanosoma brucei* infection in mice. *Transaction of the Royal Society of Tropical Medicine and Hygiene* **76**: 204-207.

Jennings F W, Whitelaw D D, Holmes P H, Chizyuka H G B and Urguhart G M (1979). The brain as a source of relapsing *Trypanosoma brucei* infection in mice after chemotherapy. *International Journal of Parasitology* **9**: 381-384.

Jeremias A and Gibson CM (2005). Narrative review: Alternative causes for elevated cardiac troponin levels when acute coronary syndrome are excluded. *Annal of Internal Medicine* **142**: 786-791.

Jones I G, Lowenthal M N and Buyts H (1975). Electrocardiographic changes in African trypanosomiasis caused by *Trypanosoma brucei rhodesiense*. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **69**: 388-395.

Jose C (1999). Therapy of human African trypanosomiasis: Current situation. *Memour of the Institute of Osvaldo Cruz, Rio de Janeiro* **94:** 221-224.

Junyent J M G, Rozman M, Corachan M, Estruch R and Urbano-Marquez A (1988). An unusual course of West African trypanosomiasis in a Caucasian man. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **81:** 931-932.

Kabayo J (2002). Aiming to eliminate tsetse from Africa. *Trends in Parasitology* **18**: 473-474.

Kagira J, Thuita J, Ngotho M, Mdachi R, Mwangangi D and Ndung'u J (2006). Haematology of experimental *Trypanosoma brucei rhodesiense* infection in vervet monkeys. *African Journal of Health Science* **13:** 59-65.

Kagira J M and Maina N W (2007). Occurrence of multiple drug resistance in *Trypanosoma brucei rhodesiense* isolated from sleeping sickness patients. *Onderstepoort Journal of Veterinary Research* **74**: 17-22

Kagira J M, Maina N W, Thuita J K, Ngotho M and Han J (2005). Influence of cyclophosphamide on the haematological profile of laboratory bred African soft furred rats. *Scandinavian Journal Laboratory Animal Science* **32**: 153-158.

Kagira J M, Ngotho M and Thuita J (2007a). Development of a rodent model for late stage rhodesian sleeping sickness. *Journal of Protozoological Research* **17**: 48-56.

Kagira J M, Ngotho M, Thuita J K, Maina N W and Hau J (2007b). Hematological changes in vervet monkeys (*Chlorocebus aethiops*) during eight months' adaptation to captivity. *American Journal of Primatology* **69:** 485-490.

Kaneko J J (1980).Serum Enzymes of Clinical Diagnostic Importance. Clinical Biochemistry of Domestic Animals. 3rd edn. Academic Press.Califonia. pp 183-188.

Karori S, Ngure R M, Wachira F N, Wanyoko J K and Mwangi J N (2008). Different types of tea products attenuate inflammation induced in *Trypanosoma brucei brucei* infected mice. *Journal of Parasitology International* **57**: 325-333.

Katunguka-Rwakishaya E, Murray M and Holmes P H (1992). The pathophysiology of ovine trypanosomiasis: Haematological and blood biochemical changes. *Veterinary Parasitology* **45:** 17-32.

Kennedy P (2011). Difficulties in diagnostic staging of human African trypanosomiasis. *Journal of Neuroparasitology* **l2:** 1-3.

Kennedy P G (2004). Human African trypanosomiasis of the CNS: Current issues and challenges**.** *Journal of Clinical Investigation* **113**: 496-504.

Kennedy PG (2009). Diagnosing central system trypanosomiasis: Two stage or not to stage? *Transactions of the Royal Society of Tropical Medicine and Hygiene* **102**: 306-307.

Kennedy P G E (2007). Animal models of human African trypanosomiasis-very useful or too far removed? *Transactions of the Royal Society of Tropical Medicine and Hygiene* 101: 1061-1062.

Kennedy P G E (2008). The continuing problem of human African trypanosomiasis (sleeping sickness). *Annals of Neurology* **64**: 116-126.

Kgori P, Modo S and Torr S (2006). Spraying to eliminate tsetse from the Okavanga Delta of Botswana. *Acta Tropica* **99**: 184-199.

Kiminyo K.K and Lucey D.R (2002). African Trypanosomiasis (Sleeping Sickness). *eMedicine Journal*: 101

Klun B (1974). Spinal fluid and blood serum activity in brain injuries. *Journal of Neurosurgery* 41: 224-228.

Koten J W and De Raadt P (1969). Myocaditis in *Trypanosoma rhodesiense*. *Transcation of the Royal Society of Tropical Medicine and Hygiene* **63:** 485-489.

Kyambadde J W, Enyaru J C K, Matovu E, Odiit M and Cerasco J F (2000). Detection of trypanosomes in suspected sleeping sickness patients in Uganda using polymerace chain reaction. *Bulletin of the World Health Organisation* **78**: 119-124.

Lamers K J B and Wevers R A (1995). Cerebrospinal fluid diagnostic: Biochemical and clinical aspects. *Klin Biochemical Metabolism* **3**: 63-75.

Leak S (1999). Tsetse Biology and ecology: Their role in the epidemiology and control of Trypanosomiasis. Book review.CAB International,Wallingford and New York, pp 568.

Legros D, Evans S, Maiso F, Enyaru J and Mburamberi D (1999). Risk factors for treatment failure after melarsoprol for *Trypanosoma brucei gambiense* trypanosomiasis in Uganda. *Tropical Medicine and Parasitology* **93**: 439-442.

Legros D, Ollivier G, Gastellu-Etchegorry M, Paquet C, Burri C, Jannin J and Buscher P (2002). Treatment of human African trypanosomiasis--present situation and needs for research and development. *Lancet Infectious Diseases* **2**: 437-440.

Lejon V, Legros D, Richer M, Ruiz J A, Jamonneau V, Truc P and Magnus E (2002). IgM quantification in the cerebrospinal fluid of sleeping sickness patients by a latex card agglutination test. *Tropical Medicine and International Health* **7:** 685-692.

Lejon V, Legros D, Savignoni A, Gastellu Etchegrorry M, Mbulamberi D and Buscher P (2003). Neuro-inflammatory risk factors for treatment failure in early second stage sleeping sickness patients treated with pentamidine. *Journal of Neuroimmunology* **144**: 132-138.

Limdi J K and Hyde G M (2003). Evaluation of abnormal liver function tests. *Postgraduate Medical Journal* **79**: 307-312.

Ljubuncic P, Mujic F, Winterhalter M, Hukovic N, Vidovic Z and Duricic S (1992). Isoproterrenol toxicity in the myocardium in an experiment.*Croatian* **43**: 11-20.

Lorne S (1986). *Trypanosomiasis.* A Veterinary Perspective. New York: Pergamon Press, pp 351-420.

Luckins A G and Gray A R (1978). An extravascular site of development of *T. congolense.Nature* **272**: 613-614.

Lumsden W H R, Kimber C D, Evans D A and Doig S J (1979). *Trypanosoma brucei*: Miniature anion-exchange centrifugation technique for detection of low parasitaemias: Adaptation for field use. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **73:** 312-317.

MacLean L, Odiit M, Sternberg M (2006). Intrathecal cytokineresponses in *Trypanosoma brucei rhodesiense* sleeping sickness patients.Transaction of the Royal Society of Tropical Medicine and Hygiene .100 **(3).** 270-275.

Malvy D and Chappuis F (2011). Sleeping sickness. *Clinical Microbiology and Infection: the official publication of the European Society of Clinical Microbiology Clinical* **17:** 986-995.

Manga-Gonzalez M Y, Ferreras M C, Ocampo R, Gonzalez-Lanza C, Perez V and Garcia-Merin J F (2004). Hepatic marker enzymes, biochemical parameters and pathological effects in lambs experimentally infected with *Dicrocilium dentriticum* (Degenea). *Parasitology Research* **93**: 344-355.

Mark A M, Boken D, Ruma T A, Vuchetich P J and Smith P W (1999**).** Rhodesian trypanosomiasis in a splenectomized patient. *American Journal of Tropical Medicine and Hygiene* **61*:*** 428-430.

Masiga D K, Smyth A J, Hayes P, Bromidge T J and Gibson W C (1992). Sensitive detection of trypanosomes in tsetse flies by DNA amplification. *International Journal for Parasitology* **22**: 909-918.

Mayhew I G and Beal C R (1980). Techniques of analysis of cerebrospinal fluid. *Veterinary Clinics of North America Small Animal Practice* **10**: 155-176.

Mayor-Withey K S, Clayton C E, Roelants G E and Askonas B A (1978). Trypanosomiasis leads to extensive proliferation of B and T cells and null cells in spleen and bone marrow. *Clinical and Experimental Immunology* **34**: 359-363.

Mbala P T, Blackett K, Mbonifor CL, Leke R and Etoundi R (1988). Funtional and immunoathological disturbances in *T. gambiense* human African trypanosomiasis. *Bulletin de la Societe de Pathologie Exotique et des ses Filiales* **81:** 490-501.

Mbati P A, Hirumi K, Inoue N, Situakibanza N H and Hirumi H (1999). Towards developing a diagnostic regimen for the treatment follow-up of *Trypanosoma brucei rhodesiense*. *Korean Journal of Parasitology* **37:** 289-292.

Mogk S, Meiwes A, Shtopel S, Schraermeyer U, Lazarus M, Kubata B, Wolburg H and Duszenko M (2014). Cyclical appearance of African trypanosomes in the cerebrospinal fluid: New insights in how trypanosomes enter the CNS. *PLoS one* **9**:e91372.

Moore A and Richer M (2001). Re-emergence of epidemic sleeping sickness in southern Sudan. *Tropical Medicine and International Health* **6:** 342-347.

Moore A, Richer M, Enrile M, Losio E, Roberts J and Levy D (1999). Resurgence of sleeping sickness in Tambura County, Sudan. *American Journal of Tropical Medicine and Hygiene* **61**: 315-318.

Morag G K (1991). Clinical Enzymology-Plasma enzymes in diagnosis. Veterinary laboratory medicine: Clinical Biochemistry and Haematology. Blackwell Scientific Publication. London. pp 109-115.

Morrison W I, Murray M, Sayer P D and Preston J M (1981). The pathogenesis of experimental induced *Trypanosoma brucei* infection in the dog: 1 tissue and organ damage *American Journal of Pathology* **102:** 168-181.

Morrison W I, Roelants G E, Mayor K S and Murray M (1978). Susceptibility of inbred strain of mice to *Trypanosoma congolense*: Correlation with changes in spleen lymphocyte populations. *Clinical and Experimental Immunology* **32**:25-40.

Moulton J E (1986). Relapse infection after chemotherapy in goats experimentally infected with *Trypanosoma brucei*: Pathological changes in central nervous system. *Veterinary Pathology* **23**: 21-28.

Mtulia A T (1976). Drugs used in treatment of trypanosomiasis. Pharmacology and Therapeutics. AMREF. Nairobi, Kenya. pp 38-40.

Mugittu K N, Silayo R S, Majiwa P A O, Kimbita E K, Mutayoba B M and Maselle R (2001). Application of PCR and DNA probes in the characterisation of trypanosomes in the blood of cattle in farms in Morogoro, Tanzania. *Veterinary Parasitology* **94:** 177-189.

Mungatana N, Ngure R and Yole D (2007). Acute phase response of albumin and haptoglobin in experimental infection of the olive baboon, *Papio Anubis*, with *Schisostoma mansoni*. The *Scandinavian Journal of Laboratory animal Science* **34**: 119-126.

Murilla G (2008). Pathogenicity of bloodstream and cerebrospinal fluid forms of *Trypanosoma brucei rhodesiense* in swiss white mice. *African Journal Health Sciences* **15**: 34-41.

Murray M and Morrison W I (1978). Parasitaemia and host susceptibility in African trypanosomiasis, Proceedings of a workshop Nairobi, Kenya, Nov.20-23, pp.71-81.

Murray M, Lambert P H and Morrison W I (1975). Renal lesions in experimental trypanosomiasis. *Medical Malady of Infection* **5**: 638-641.

Murray, M and dextor, T (1988). Anaemia in bovine African trypanosomiasis. A review. *Acta tropica.* **45**: 389-432.

Murray M, Murray P K and McIntyre W I M (1977). An improved parasitological technique for the diagnosis of African tNdoutrypanosomiasis. *Transaction of the Royal Society of Tropical Medicine and Hygiene* **71**: 325-326.

Murray M, Trail J C M and d'Ieteren G D M (1990). Trypanotolerance in cattle and prospects for the control of trypanosomiasis by selective breeding. *Revue Scientifique et Technique* (International office of Epizootics) **9**:369-386.

Murray M. (1979). Anaemia of bovine African trypanosomiasis: An overview, in: Losos G, and Chouinard A. (Eds.). Pathogenecity of Trypanosomiasis, IDRC, Ottawa.

Myburgh E, Coles J A, Ritchie R, Kennedy P G, McLatchie A P, Rogers J, Taylor M, Barrett M P, Brewer J M and Mottram J C (2013). *In vivo* imaging of trypanosome-brain interactions and development of a rapid screening test for drugs against CNS stage trypanosomiasis. *PLoS Neglected Tropical Diseases* **7**: e2384.

Naessens J, Kitani H, Yagi Y, Sekikawa K and Iraqqi F (2005). TNF-a mediates the development of anaemia in a murine *Trypanosoma brucei rhodesiense* infection, but not the anaemia associated with a murine *T. congolense* infection. *Clinical and ExperimentalImmunology* **139**: 403-410.

Ndoutamia G, Mbakasse R N, Brahim A and Khadija A (2002). Influence of *Trypanosoma congolense* infection on haematological parameters, minerals and protein- energy in Sahelian goats of Chad. *Journal of Veterinary Medicine* **153:** 395-400.

Ndungu J, Ngure R M, Ngotho J M, Sayer P D and Omuse J K (1994). Total protein and white cell changes in the cerebrospinal fluid of vervet monkeys infected with *Trypanosoma rhodesiense* and the post treatment reaction. *Journal of Protozoology Research* **4**: 124-135.

Ndungu J M, Eckersall P D and Jennings F W (1991). Elevation of the concentration of acute phase proteins in dogs with *Trypanosoma bruceiActa Tropica* **49**: 77-85.

Ndungu J M, McEwan N A, Jennings F W and Murray M (1991). Cardiac damage in dogs infected with *Trypanosoma brucei*: Clinical and electrocardiographic feature. *Journal of Small Animal Practice* **32:** 579-584.

Sedlakova D and Necas A (1999). Changes in the creatine kinase and lactate dehydrogenase activities in cerebrospinal fluid of dogs with thoracolumbar disc disease. *Acta Veterinaria Brno* **68**: 111–120.

Neiger R, Hadley J and Pfeiffer D U (2002). Differentiation of dogs with regenerative and non-regenerative anaemia on the basis of their red cell distribution width and mean copurscular volume *Veterinary Records* **150**: 431-434.

Ngure R, Eckersall P, Mungatana N K Mburu J, Jennings F W Burke J and Murray M (2009). Lipopolysacharridebinding protein in the acute phase response of experimental murine *Trypanosoma brucei brucei* infection. *Research in Veterinary Science* **86**: 394-398.

Ngure R M, Burk J, Eckersall P D, Jennings F W, Mbai F and Murray M (2009b). Secondary bacterial infection plasma endotoxin levels and acute phase response of mice infected with *Trypanosoma brucei brucei.Parasite Immunology* **31**: 357-365.

Ngure R M, Eckersall P, Burke J, Karori S M, Mwangi W W, Wachira F N, Maathai R and Murray M (2009a). Endotoxin-like effects in acute-phase response to *Trypanosomabrucei brucei* infection are not due to gastrointestinal leakage. *Parasitology International* **58**: 325-329.

Ngure R M, Eckersall P D, Jennings F W, Mburu J, Burke J, Mungatan N and Murray M (2008). Acute phase response in mice experimentally infected with *Trypanosoma congolense*: A molecular gauge of parasite host interaction. *Veterinary Parasitology* **151**: 14-20.

Ngure R M, Eckersall D, Jennings W, Mburu J, Burke J, Mungatana N and Murray M. (2008). Acute phase response in mice experimentally infected with *Trypanosoma congolense*: A molecular gauge of parasite-host interaction. *Veterinary Parasitology* **151**: 14-20.

Ngure R M**,** Eckersall P D, Mungatana N K, Mburu J N, Jennings F W, Burke J and Murray M (2009c). Lipopolysacharide binding protein in the acute phase response of experimental murine T*rypanosoma brucei brucei* infection. *Research in Veterinary Sciences* **86**: 394-398.

Ngure R M, Ndungu J M, Gatere L M and Ngotho J M (2000). Application of the VetTest 8008 system in biochemical analysis of vervet monkeys plasma. *Veterinary Record* **146**: 612-613.

Ngure R M, Ndungu J M, Ngotho J M, Mungatana N K, Maathai R G and Gateri L M (2008). Biochemical changes in plasma of vervet monkeys (*Chlorocebus aethiops*) experimentally infected with *Trypanosoma brucei rhodesiense*. *Journal of Cell and Animal Biology* **2**: 150-157.

Ngure R M, Ndungu J M, Ngotho J M, Sayer P D and Omuse J (1994). Total protein and white cell changes in cerebrospinal fluid of vervet monkey infectedwith *Trypanosoma rhodesiense* and the post-treatment reaction**.** *Journal of Protozoology* **4:** 124-135.

Ngure R M, Ngotho J M, Nancy M K, Maathai R G and Gateri L M (2008). Biochemical changes in the plasma of vervet monkeys (*Chlorocebus aethiops*) experimentally infected with *Trypanosoma brucei rhodesiense*. *Journal of Cell and Animal Biology* **2**: 150-157.

Ngure R M, Ongeri B, Karori S M, Wachira W, Maathai R G, Kibugi J K and Wachira F N (2009) Anti-trypanosomal effects of *Azadiracta indica* (neem) extract on *Trypanosoma brucei rhodesiense* infected mice. *Eastern Journal of Medicine* **14:** 2-9.

Nigam P K (2007). Biochemical markers of myocardial injury**.***Indian Journal of Clinical Biochemistry* **22**: 10-17.

Nussinovitch M, Klinger G, Soen G, Magazanik A, Volovitz B and Varsano I (1996). Increased creatine kinase brain isoenzyme concentration in cerebrospinal fluid with meningitis. *Clinical Paediatrics* **35:** 349-351.

Nwoha R I O and Anene B M (2011a). Changes in packed cell volume and hemoglobin concentration in dogs with single and conjunct experimental infections of *Trypanosoma brucei* and *Ancylostoma caninum*. *Philippian Journal of Veterinary and Animal Science* **37**: 151-158.

Nwoha R I O and Anene B M (2011b). Clinical signs and pathological changes in dogs with single and conjunct experimental infections of *Trypanosoma brucei brucei* and *Ancylostoma caninum*. *Journal of Veterinary Parasitology* **25**: 97-102

pOgunsamni A O, Anosa V O and Akpavie S O (1994). Serum biochemical changes in West African dwarf sheep experimentally infectedwith *Trypanosoma brucei*. *Revue D'elevage et de Medecine Veterinaire Des Pays Tropicaux* **47:**195-200.

Ohaeri C C and Eluwa M C (2011). Abnormal biochemical and haematological indices in trypanosomiasis as a threat to herd production. *Veterinary Parasitology* **177:** 199-202.

OIE (2009). Standard operating Procedures (SOP) for IOA Validation and Certification of Diagnostic AssaysVersion 2.1 World Organization for Animal Health

Okaiyeto S O, Ajanusi O J, Sackey A K and Tekdek L B (2010). Changes in some haematological values associated with mixed *Trypanosoma congolense* and *Haemonchus contortus* infection in Yankassa Sheep, *Veterinary Research* **3**: 9-13.

Oliver I T (1955). A spectrophotometric method for the determination of creative phosphokinase and myokinase. *Biochemical Journal* **61:** 116.

Onah D N, Hopkins J and Luckins A G (1996). Haematological changes in sheep experimentally infected with *Trypanosoma evansi*. *Parasitology Research* **82**: 659-663.

Orhue N, Nwanze E and Okafor A (2005). Serum total protein, albumin and globulin levels in *Trypanosoma brucei*-infected rabbits: Effect of orally administered *Scoparia dulcis*. *African Journal of Biotechnology* **4**: 1152-1155.

Orlando J and Arroz L (1987). Melarsoprol and reactive encephalopathy in *Trypasoma brucei rhodesiense. Transaction of the Royal Society of Tropical Medicine and Hygiene* **81:** 192

Ormerod W E (1970). Pathogenesis and Pathology of Trypanosomiasis in Man. In *The African Trypanosomiasis*. H.W Mulligan, editor. George Allen and Unwin.London. pp 587-599.

Paling R W, Moloo S K and Scot J R (1987). The relationship between parasitaemia and anaemia in N'Dama and Zebu cattle following four sequential challenges with *Glossina morsitan centralis* infected with *Trypanosoma congolense. The International Scientific Council for Trypanosomiasis Research and Control. Togo, AOU/STRC publication* **114**: 256-264.

Pays E, Vanhollebeke B, Vanhamme L, Paturiaux-Hanocq F, Nolan D P and Perez-Morga D (2006). The trypanolytic factor of human serum. *Nature Reviews in Microbiology*.**4**:477-486.

Pentreath V W, Owolabi A O and Doua F (1992). Survival of *Trypanosoma brucei brucei* in cerebrospinal fluid. *Annals of Tropical Medicine and Parasitology* **86:** 29-34.

Pepin J (1998). African trypanosomiasis.Hunter's Tropical Medicine. WB Saunders; 7th edition Philadelphia, USA pp 643-653.

Pepin J and Milord F (1994). The treatment of human African trypanosomiasis. *Advances in Parasitology* **33:** 1-47.

Poltera A A (1985). Pathology of African trypanosomiasis with reference to experimental African trypanosomiasis and infections of the central nervous system. *British Medical Bulletin* **41**: 169-174.

Poltera A A, Cox J N and Owor R (1976). Pancarditis involving the conduction system and all valves in humans. *British Heart Journal* **38**: 827-837.

Preus M, Bhargava A S, Kharter A E and Gunzel P (1988). Diagnostic value of serum creatine kinase and lactate dehydrogenase isoenzyme determinations for monitoring early cardiac damage in rats *Toxicology Letters* **42**: 225-233.

Pupim L B, Flakoll P J and Ikizler T A (2004). Nutritional supplementation acutely increase albumin fractional synthetic rate in chronic hemodialysis patients. *Journal of the American Sociology of Nephrology* **15**: 1920-1926.

Rastogi S C (2006). Isoenzyme. In Biochemistry. Tata McGraw-Hill Publishing Company, New Delhi, India. pp 148-150.

Robays J, Lefevre P, Lutumba P, Lubanza S, Kande Betu-Ku-Mesu V, Van der Stuyft P and Boelaert M (2007). Drug toxicity and cost as barrier to community participation in HAT

control in Democratic Republic of Congo. *Tropical Medicine and International Health* **12**: 290-298.

Robertson D H and Jenkins R (1959). Hepatic dysfunction in human trypanosomiasis. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **53**: 511-523.

Rodney D B, Roger N, Rosenberg, Ruby T and Mukherjee A (1977).Creatine kinase BB isoenzyme levels by radioimmunoassay in patients with neurological disease. *Annals of Neurology* **3**: 52-59.

Rouzer C A and Cerami A (1980). Hypertriglyceridemia associated with *Trypanosoma brucei brucei* infection in rabbits: Role of defective triglyceride removal. *Molecular Biochemical Parasitology* **2**: 31-38.

Sadun E H, Johnson A J, Nagle R B and Duxbury R E (1973). Experimental infections with African trypanosomes. *American Journal of Tropical Medicine and Hygiene* **22**: 323-330.

Safar J, Kulenda Z and Horakova E (1982). Creatine kinase activity of the cerebrospinal fluid and serum in focal cerebral ischemia. Dynamics of changes in the fluid composition. *Casopís lékařů českých* **121**:1379.

Saror D I (1979). Observations on the course and pathology of *Trypanosoma vivax* in Red Sokoto goats. *Research in Veterinary Science* **28**: 36-38.

Saror D I (1982). Aspects of the anaemia of acute bovine trypanosomiasis, Proceedings of the first National Conference on Tsetse and Trypanosomiasis Research, pp. 12-14, August 10-12, Kaduna, Nigeria, 1981.

Schalm O W, Jain N C and Carrol EJ (1975). Veterinary Haematology 3rd Edition Lea and Febiger, Philadelphia pp 15-81.

Schmidt H (1983). The pathogenesis of trypanosomiasis of the CNS. *Virchow Archives (Pathology Anatomy* **399**: 333-343.

Schmidt H and Fink E (1980). Preclinical testing of potential trypanocidal drugs inprimates: Preliminary investigation of an experimental diamidine in vervets. In: Recent Development in Medical Research in East Africa (Njogu, A.R *et al*) Nairobi. pp 173-182.

Schmidt H and Sayer P D (1982). *Trypanosoma brucei rhodesiense* infection in vervet monkeys. I Parasitologic, haematologic, immunologic and histologic results. *Tropen Medicine Parasitologic* **33**: 249-254.

Schultzberg M, Ambatsis M, Samuelson E B, Kristensson K and van Meirvenne N (1988). Spread of *Trypanosoma brucei* to the nervous system: Early attack on the circumventricular organs and sensory ganglia. *Journal of Neuroscience Research* **21**: 56-61.

Eric S K, Ngure R M, Chome J, Korir S, Mdachi R and Mburu J (2013). Effects of long term administration of *Solanum nigrum* extracts in female swiss white mice infected with *Trypanosoma brucei rhodesiense*. *Science Journal of Medicine and Clinical Trials*. ISSN 2276-7487. 2013. doi:0.7237/sjmct/290.

Sharma D K, Chauhan P S, Saxena V K and Agrawal R D (2000). Haematological changes in experimental trypanosomiasis in Barbari goats. *Small Ruminant Research* **38**: 145-149.

Shillingford J P (1975).Creatine kinase isoenzymes: Their separation, quantitative determination and use in the assessment of acute myocardial infarction.*Biochemical Society Transactions* **3:** 423-429.

Simarro P, Jannin J and Cattand P (2008). Eliminating human African trypanosomiasis: Where do we stand and what comes next? *PLoS Medicine* **5**: e55.

Smith D H (1998). HAT: An emerging public health crisis. Tropical medicine achievement and prospects. *British Medical Bulletin* **54**: 341-355

Smith D H and Bailey J W (1997). Human African trypanosomiasis in South Eastern Uganda: Clinical diversity and isoenzyme profiles. *Annals of Tropical Medicine and Parasitology* **91**: 851-856.

Sternberg J M (2004). Human African trypanosomiasis: Clinical presentation and immune response. *Parasite Immunology* **26**: 469-476.

Steverding D (2006). A new initiative for the development of newdiagnostic tests for human African trypanosomiasis.*Kinetoplastid Biology* **5**: 1475-2070**.**

Taiwo V O, Olaniyi M O and Ogunsanmi A O (2003). Comparative plasma biochemical changes and susceptibility of erythrocytes to *in vitro* peroxidation during experimental *Trypanosoma congolense* and *Trypanosoma brucei* infections of sheep. *Israel Journal of Veterinary Medicine* **58**: 112-117.

Tanzer M L and Gilvard C (1966) Creatine and creatine kinase measurement. *Journal of Biological Chemistry* **234**: 3201-3204**.**

Thuita J K, Kagira J M, Mwangangi D, Matovu E, Turner C M R and Masiga D (2008). *Trypanosoma brucei rhodesience* transmitted by a single tsetse fly bite in vervet

monkeys as a model of human African trypanosomiasis *PLoS Neglected Tropical Diseases*: **2**: e238

Thumbi S M, McOdimba F A, Mosi R O and Junga J O (2008). Comparative evaluation of three PCR based diagnostic assays for the detection of pathogenic trypanosomes in cattle blood. *Parasites and Vector* **1**:46.

Turner C M R, Aslam N and Dye C (1995). Replication, differentiation, growth and virulence of *Trypanosoma brucei* infection. *Parasitology* **111**: 289-300.

Turray A A, Nwobu G O, Okogun G R A, Igwe C U, Adeyeye K, Aghsti K E, Okpala H O and Tatfeng Y M (2005). A comparative study on the suceptibility of male and female albino mice to *Trypanosoma brucei brucei*. *Journal of Vector Borne Diseases* **42**: 15-20.

Vincendeau P and Bouteille B (2006). Immunology and immunopathology of African trypanosomiasis. *Anais da Academia Brasileira de Ciencias* 78: 645-665.

Waema M, Maina N, Karanja S, Gachie B, Ngotho M and Kagira J (2014). Development of a safer laboratory vervet monkey model for the study of human African trypanosomiasis. *African Journal of Laboratory Medicine* **1**: 1-6

Walker D B (2006). Serum chemical biomarkers of cardiac injury for nonclinical safety testing. *Toxicology Pathology* **34**: 94-104.

Welburn S, Fevre E, Coleman P and Maudlin I (2004). Epidemiology of Human African Trypanosomiasis. In: The Trypanosomiasis. Maudlin, I., Holmes, P.H. & Miles, M.A. (Eds), CABI Publishing, Wallingford, Oxfordshire, UK. pp219

Welde B T, Readon M J, Mwangi J and Asenti A (1989). Presenting features of Rhodesian sleeping sickness in the Lambwe valley, Kenya. *Annal of Tropical Medicine and Parasitology* **83**: 73-89.

Wery M, Mulumba P M, Lambert PH and Kazyumba I (1982). Hematologic manifestation, diagnosis and immunopathology of African trypanosomiasis. In: *Seminars in Hematology* **2**: 83-92.

WHO (2013). Epidemiology and Control of African trypanosomiasis. Report of a WHO Expert Committee.World Health Organization Technical Report series No. 984. 1-237, Geneva, Switzerland.

WHO (1998). Control and Surveillance of African trypanosomiasis. World Health Organization Technical Report series No. 881.WHO, Geneva, Switzerland.

World Health Organization (2006). Human African trypanosomiasis (sleeping sickness) Epidemiological update, Geneva, Switzerland. *Weakly Epidemiological Records* **81:** 71-80.

Wiser M F (2003). Kinetoplastid Biology and Human Disease. http://www.tulane.edu

Wissmann B, Machila N, Picozzi K, Fèvre E M, deC. Bronsvoort B M, Handel I G and Welburn S C (2011) Factors associated with acquisition of human infective and animl infective trypanosome infections in domestic livestock in Western Kenya. *PLoS Neglected Tropical Diseases* **5:** e941.

Witola W H and Lovelace C E (1997). Serum proteins in indigenous Zambian goats with trypanosomiasis. In Meeting abstract*FASEB Journal* **9:** 1257-1257.

Wolburg H, Mogk S, Acker S, Frey C, Meinert M, Schonfeld C and Duszenko M (2012). Late stage infection in sleeping sickness. *PLoS One* **7**: e34304.

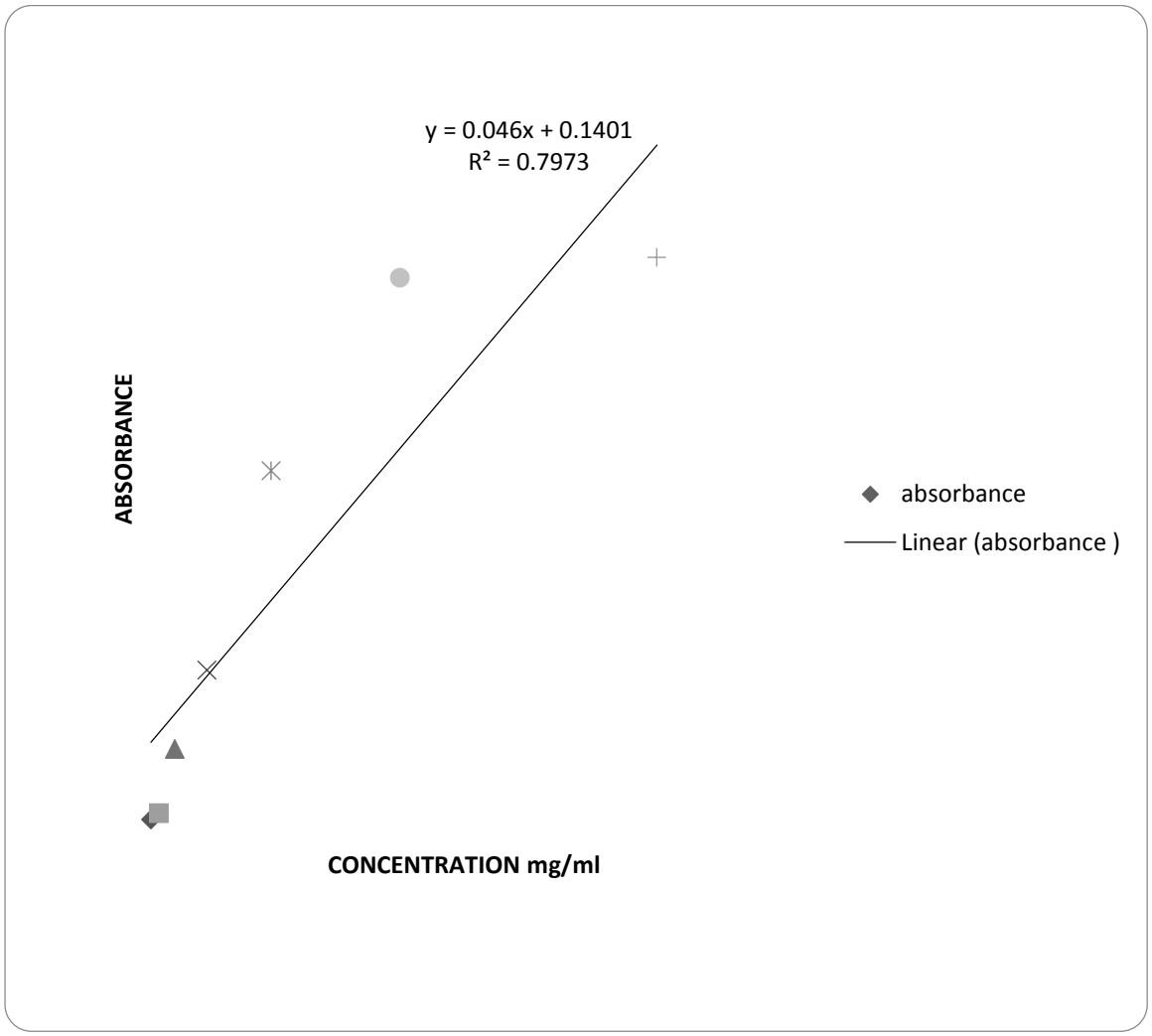
Woo P T K (1970). The haematocrit centrifugation technique for the diagnosis of African trypanosomiasis. *Acta Tropica* **27**: 384-386.

Woodruff A W (1973). Mechanisms involved in anaemia associated with infection and splenomegaly in the tropics. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **67:**313-325.

Worthington C C (1988). Enzymes and Related Biochemicals Worthington Biochemical Corporation. Freehold, NJ, USA. pp 106-205.

APPENDICES

Appendix 1: Standard curve for protein analysis by buiret method



Appendix 2: Statistical analysis

Rectal temperature

Two-Sample T-Test and CI: INFECTED, CONTROL

```
Two-sample T for INFECTED vs CONTROL

Difference = mu temperature (INFECTED) - mu temperature
(CONTROL)
Estimate for difference:  0.5469
95% CI for difference:  (0.3591, 0.7347)
T-Test of difference = 0 (vs not =): T-Value = 5.84  P-Value =
0.000  DF = 52
```

Two-Sample T-Test and CI: INFECTED, CONTROL

```
Two-sample T for INFECTED vs CONTROL

           N   Mean  StDev  SE Mean
INFECTED  51  25.93   3.02     0.42
CONTROL   51  27.71   4.99     0.70


Difference = mu PVC %( INFECTED) - mu PVC %( CONTROL)
Estimate for difference:  -1.788
95% CI for difference:  (-3.413, -0.162)
T-Test of difference = 0 (vs not =): T-Value = -2.19 P-Value =
0.032 DF = 82
```

Two-Sample T-Test and CI: INFECTED, CONTROL

```
Two-sample T for INFECTED vs CONTROL


Difference = mu RCB (INFECTED) - mu RCB (CONTROL)
Estimate for difference:  -1.043
95% CI for difference:  (-1.792, -0.294)
T-Test of difference = 0 (vs not =): T-Value = -3.04 P-Value =
0.010 DF = 12
```

Two-Sample T-Test and CI: INFECTED, CONTROL

```
Two-sample T for INFECTED vs CONTROL

           N    Mean StDev SE Mean
INFECTED 13 10.823 0.399     0.11
CONTROL 13 10.515 0.164    0.046
```

```
Difference = mu (INFECTED) - mu (CONTROL)
Estimate for difference:  0.308
95% CI for difference:  (0.053, 0.563)
T-Test of difference = 0 (vs not =): T-Value = 2.57 P-Value =
0.021 DF = 15
```

MCHC

Two-Sample T-Test and CI: INFECTED, CONTROL

```
Two-sample T for INFECTED vs CONTROL

           N    Mean StDev SE Mean
INFECTED 13   36.77   2.11     0.59
CONTROL   13 36.097 0.733     0.20


Difference = mu (INFECTED) - mu (CONTROL)
Estimate for difference:  0.676
95% CI for difference:  (-0.655, 2.006)
T-Test of difference = 0 (vs not =): T-Value = 1.09 P-Value =
0.294 DF = 14
```

White blood cells

Two-Sample T-Test and CI: INFECTED, CONTROL

```
Two-sample T for INFECTED vs CONTROL

           N    Mean StDev SE Mean
INFECTED 13   16.44   2.97     0.82
CONTROL   13 16.060 0.830     0.23


Difference = mu (INFECTED) - mu (CONTROL)
Estimate for difference:  0.384
95% CI for difference:  (-1.467, 2.234)
T-Test of difference = 0 (vs not =): T-Value = 0.45 P-Value =
0.662 DF = 13
```

Appendix 3: IACUC Approval

KENYA AGRICULTURAL RESEARCH INSTITUTE

Trypanosomiasis Research Centre, MUGUGA

P.O. BOX 362, KIKUYU, 00902, TEL: 020-2700604, 2700654, FAX: 066-32397

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Email:Centre@ Kari-trc.org, trccd@kari-trc.org

Ref: C/TR/2/14/VI/3 25th October 2008

KARI-TRC Animal Care and Use Committee (IACUC)

Mrs Hawa Mavura (Principal Investigator, PI)

Dear Hawa

RE: IACUC Approval

The Institutional Animal Care and Use Committee (IACUC) of the Kenya Agricultural Research Institute-Trypanosomiasis Research Centre (KARI-TRC) has reviewed your proposal entitled **"Use of Isoenzyme profiles in the development of a rapid diagnostic method in staging of *Trypanosoma brucei rhodesiense* infection using sheep model"**. The committee notes that current diagnostic and staging methods for human African trypanosomiasis (HAT) suffer from inadequate sensitivity and therefore the proposed study addresses an important topic with potential public health benefits. The methods described for the in vivo component of your study are appropriate and are consistent with KARI-TRC IACUC recommendations as well as National (KVA) standards of animal welfare. The committee has therefore resolved to support your project. The committee will hold you (the PI) personally responsible to ensure that high standards of animal welfare are observed at all times of the project. The committee may make impromptu visits to ensure compliance with its regulations.

Yours Sincerely

Dr John Thuita

IACUC Chairman, KARI-TRC

Appendix 4: Publications

International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Index Copernicus Value (2015): 78.96 | Impact Factor (2015): 6.391

Tissue Enzymes as Markers of Cardiac Damage during *Trypanosoma brucei rhodesiense* Infection of Sheep

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[4]KARLO, Biotechnology Research Centre, Muguga, Kenya

Abstract: ***This study was done to determine markers of cardiac and CNS involvement during HAT using sheep where eight sheep were intravenously inoculated with 1 × 10[4] T. b. rhodesiense. Two were uninfected. Jugular blood vein samples were used for parasitological analyses and determination of serum concentrations of total protein, LDH and CK and their isoenzymes. There was an increase in total protein, total LDH and total CK activities in both serum and CSF. A flip over pattern LDH1:LDH2 ratio was observed. It was concluded that serum LDH1/LDH2 ratio, has a potential diagnostic value in determination of cardiac damage.***

Keywords: Trypanosomiasis, LDH, CK, CSF, Serum

1. Introduction

Tissue enzymes have been used as sensitive diagnostic markers of CNS infection involvement during bacterial meningitis [1] and also in determining the severity of CNS infection [2]. Indeed CK BB isoenzyme produced in the CNS during disease is able to cross the BBB and therefore can be measured in blood thus enabling the avoidance in the used of CNS invasive procedures to collect cerebral spinal fluid (CSF) in the detection of brain tissue damage [3; 1]. In addition CK and LDH have been used in other disease conditions to determine cardiac involvement [4;5]).

2. Literature Review

Increases in body fluid levels of the tissues enzymes creatine kinase (CK) and lactate dehydrogenase (LDH) have been demonstrated in trypanosome infected animal models. Indeed increases have been demonstrated during experimental animal trypanosome infections [6;7] and infected humans [8]. There is therefore a need to develop a cost-effective model for studies on *T. b. rhodesiense* CNS trypanosome infection and also evaluate the possible use of tissue enzymes CK and LDH as reliable enzymatic markers of heart lesion and staging markers in CNS trypanosome infection.

A good number of HAT patients during the haemolymphatic and also the CNS stage have cardiac damage due to destruction of myocardial cells [9;10] resulting in the loss of membrane integrity leading to enzymes diffusion to extra cellular spaces and drainage into blood vessels. The appearance in the serum of the various cytoplasmic enzymes has formed the basis of a variety of clinical diagnosis procedures for the detection of tissue damage of the heart and CNS using CK and LDH, respectively [11;1]. In *T. b. rhodesiense* infection, its more acute course, pancarditis with congestive heart failure pericardial effusion and pulmonary oedema can cause fatalities at the early stages [12]

3. Statement of the Problem

Currently used markers of cardiac damage during disease are expensive and not easily adaptable to field conditions at the same time this procedure requires qualified staff to perform. Thus there is a need to identify cardiac and CNS tissue specific markers that are sensitive, specific, reliable and easy to perform at the point of trypanosoma infection care. Serum biochemical changes such as total and/or isoenzymes profiling of tissue enzymes have been shown to be more sensitive and reliable, since the levels increase with minimal tissue damage, does not need very qualified staff to assay and is reliable. A number of diagnostic methods like demonstration of trypanosomes, increased white cell count and protein levels in CSF exist for the CNS trypanosome disease. However, these methods have challenges and drawbacks especially the unreliability of white blood cell count and protein analysis methods. Both white blood cells and proteins only increases in later stages of CNS involvement and show wide range of variation. The collection of CSF requires a lumber puncture procedure which is a painful and patient unfriendly in addition to posing ethical considerations.

4. Materials and Methods

Experimental Animals: Ten male indigenous Maasai breed sheep aged two years and weighing between 15 and 27kg were acquired from Naivasha district-Kenya a trypanosomosis free area were used for the study. The sheep were initially housed in a fly proof house as part of quarantine for 30 days for purposes of their acclimatization. During that time the animals were de-wormed using albendazole® at a dose of 25gm/kg bwt and sprayed with acaricide amitraz (triatic®) to ensure that they were free from endo-parasites and ecto-parasites. The sheep were fed twice daily on ratio consisting of hay plus minerals and protein supplements. Water was provided *ad libitum*. Each of the sheep was ear-tagged for identification and transferred to experimental wards to acclimatize for a further two

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HAEMATOLOGICAL CHANGES IN SHEEP (*Ovis aries*) INFECTED WITH HUMAN INFECTIVE *Trypanosoma brucei rhodesiense*

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Abstract

The haematological parameters of sheep (Ovis aries) an experimental model of Human African Trypanosomiasis (HAT) were determined. Ten sheep were used; eight animals were infected with 1× 104 Trypanosoma brucei rhodesiense while two animals were not infected and hence used as control. Clinical evaluation was done daily while weekly animals of both groups were bled from the jugular vein and blood samples examined for Packed Cell Volume (PCV), Haemoglobin (Hb), Red Blood Cell count (RBC), platelet counts, total white blood cell counts and erythrocyte indexes of Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH) and Mean Corpuscular Haemoglobin Concentration (MCHC).

Clinical evaluation of the disease in the sheep corresponded closely to that described in human patients. Infected sheep showed an increase in body temperature, MCH and MCHC. However the levels of PCV, Hb, MCV and RBC counts declined drastically on infection. All parameters however recovered to control after treatment. Analysis of data revealed significant changes in all these parameters and erythrocyte indexes in infected sheep when compared with controls. From the changes observed in this study, we can conclude that microcytic anaemia was the major feature in the initial stages of the disease and while macrocytic anaemia was observed in the later stages of disease in the infected sheep.

Keywords: *Sheep hematology, Trypanosomosis, Haematology, Anaemia, Serum, HAT.*