Use of Loop-Mediated Isothermal Amplification (LAMP) of DNA in Diagnosis and Monitoring Treatment of *Trypanosoma brucei rhodesiense* Infections in Vervet Monkeys (*Chlorocebus aethiops*)

Lenny Mwagandi Chimbevo

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DECLARATION

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

Signature…… Date…….

Lenny Mwagandi Chimbevo

This thesis has been submitted for examination with our approval as university supervisors.

Signature…… Date……

Dr. Naomi W.N. Maina

JKUAT, Kenya

Signature…….. Date……

Dr. Johnson O. Ouma

TRC, Kenya

Signature…… Date……

Dr. Simon M. Karanja

JKUAT, Kenya

DEDICATION

To my loving parents; the late Chimbevo Mwagambo and Mbodze Nyale and my uncle Joseph Mwangala.

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ABBREVIATIONS AND ACRONYMS

DNA	Deoxyribonucleic acid
kDNA	Kinetoplast DNA
EDTA	Ethylene-diamine tetra acetic acid
TAE	Tris acetic acid buffer
PCR	Polymerase chain reaction
dNTPs	Deoxynucleoside tri-phosphate
Taq	*Thermus aquaticus*
LAMP	Loop-Mediated Isothermal Amplification
SRA	Serum resistant antigen
Bst	*Bacillus stearothermophilus*
mL	Millilitres
mM	Millimolar
ESR	Erythrocytes sedimentation rate
WBC	White blood cells
^{o}C	Degrees centigrade
µL	Microlitres
pM	PicoMolar

WHO World health Organization

OC Oligochromatography

CSF Cerebrospinal fluid

HAT-PCR-OC Human African trypanosomiasis PCR oligochromatography

ELISA Enzyme-linked immunosorbent assay

VSG Variant specific Glycoproteins

HCT Haematocrit centrifugation techniques

QBC Quantitative buffy coat

m-AECT mini-Anionic exchange centrifugation technique

TE Tetra-ethanolamine

RIME Repetitive insertion mobile element

Bwt Body weight

PSG Phosphate saline glucose

Dpi Days of post infection

Dpt Days of post treatment

HCl hydrochloric acid

KCl Potassium chloride

$MgSO_4$ Magnesium sulphate

ABSTRACT

The recently developed loop-mediated isothermal (LAMP) based on *RIME* gene was used in diagnosis, staging and post-treatment follow-up of HAT in infected vervet monkeys (*Chlorocebus aethiops*) and compared with microscopic methods. The monkeys (A and B) were infected with *Trypanosoma brucei rhodesiense* 2537 and sub-curatively treated with diminazene aceturate (Berenil®) at 35 day of post infection (dpi) and later with Melarsoprol (Mel B®) at 113 dpi (78 day of post treatment (dpt) with Berenil®) and 171 dpi (136 dpt with Berenil®) respectively.

Pure and crude DNA from the samples for LAMP was extracted using Saponin method and heat treatment respectively. Amplification was conducted in a thermocycler and a water bath set between 60^0C to 65^0C. The test results were assessed visually by addition of SYBR green I dye and by ultra violet (UV) illumination of DNA bands in 1% ethidium bromide stained electrophoresed agarose gel. Parasitaemia, cerebrospinal fluid (CSF) parasitosis, packed cell volume (PCV), white blood cell (WBC) counts and total CSF protein concentration were also determined.

In the blood and CSF, microscopy detected 28.21% and 21.18% positive cases in the collected samples respectively. In the blood, serum and CSF, LAMP detected 60.26%, 55.13% and 79.49% positive cases in the collected samples respectively. The Chi-square (X^2)-Statistics of 16.734 (p=0.000 and 38.023 (p=0.000) was obtained between LAMP and microscopy in the blood and CSF, respectively.

The percentage trypanosome DNA detection on different sample preparation and amplification methods on LAMP was also assessed. Pure DNA on a thermocycler had 60.27%, 55.13% and 78.12% in the whole blood, serum and CSF respectively. For pure DNA on a water bath, the percentage detection was 46.15%, 48.72% and 75.64% in the whole blood, serum and CSF, respectively. For crude DNA on a thermocycler, the percentage detection was 56.41%, 56.41% and 76.92% while crude DNA in a water bath had 48.72%, 44.87% and 64.10% detection rate was in the whole blood, serum and CSF, respectively.

Trypanosome DNA was detected at 7 dpi in the blood and serum and starting at 21 dpi in the CSF. After subcurative Berenil® treatment, trypanosome DNA cleared at 56 dpi (21 dpt with Berenil®) in the blood and serum of both monkeys, and re-appeared at 77 dpi (42 dpt with Berenil®) and 129 dpi (84 dpt with Berenil®) in the blood and serum of monkey A and B respectively. After Mel B® treatment, trypanosome DNA cleared after 40 and 90 days and 90 and 150 days in the blood and serum and CSF of vervet monkey A and B respectively.

The comparison between LAMP and microscopy for crude DNA on thermocycler had k values of; 0.397 and 0.602 and X^2-value of 13.141 (p=0.000) and 35.247 (p=0000) in the blood and CSF respectively. Percentage agreement (k) and X^2) between LAMP and microscopy in detection of trypanosomes in the late stages of the disease was 0.600 and 15.000 (p=0.000) respectively in the CSF.

For post-treatment follow-up the k and X^2 values were 0.472 and 19.429 (p=0.000) and 0.527 and 21.346 (p=0.000) in the blood and CSF respectively. Therefore, heat treatment and amplification on a water bath may be use as sample preparation and amplification methods respectively. Blood and CSF may are the preferred sample for early and late stage of the disease.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Rhodesian form of human African trypanosomiasis (HAT) or sleeping sickness was an emerging public health problem (Kabayo, 2002; Waiswa *et al*., 2003; WHO, 2005) and currently it's under control. Unfortunately it has been neglected in terms of drug development and research in understanding the pathogenesis, immunological and pathological changes (Truc, 2003). This acute, complex, lethal and debilitating disease is caused by infections with *T. b. rhodesiense* transmitted by a bite of tsetse flies of *Glossina spp*. It occurs in eastern, central and southern parts of Africa (Molyneux *et.al.,* 1996; WHO, 1998) with an estimated 66 million people at risk of being infected (WHO, 2006).

Wild game animals and cattle were the reservoir of *T. b. rhodesiense* (Onyango *et al*., 1966). However, humans also harbor the parasite (Apted, 1970). Horse-flies, *Tabanidae* and *Stomoxydinae* possibly could play a role in mechanical transmission in special situation (Cherenet *et al*., 2004). Other *Trypanozoon* are *T. b. gambiense, T. b. brucei* and *T. evansi*. *Trypanosoma b. gambiense* causes a devastating, chronic form of the disease in western and central Africa (Scott, 1970). *Trypanosoma b. brucei* is lysed by human serum thus not infective in humans but cause disease in livestock along with *T. evansi*. However, research indicate that a human case of *T. evansi* infections has previously been reported in India (WHO, 2005; Joshi *et al*., 2005; Truc *et al*., 2006),

highlighting the need to develop definitive diagnostic tests for trypanosomes in human infections.

Devastating epidemics of *T. b. rhodesiense* have in the past occurred in Uganda and western Kenya (Hide, 1999; de Raadt, 2005). In kenya, human African trypanosomiasis (HAT) has been a major threat to human and public health specifically in Lambwe valley of south Nyanza district since 1960s (Mikoh *et al*., 1989). Despite the efforts to control tsetse flies, cases of HAT in western Kenya were on the rise upto 2002. Since then, cases have declined due to success of efforts to control tsetse flies.

Currently, endemic areas are located only in a few districts (south Nyanza, Bungoma, Busia and Teso) close to the Ugandan border (WHO, 2008). Despite a solid case detection by the Trypanosomiasis Research Centre (TRC) from 2002 to 20010, one case was diagnosed in Bungoma by January 2006 (WHO, 2008) and another bone in Busia in 2008.

The search for a vaccine proves more arduous than anticipated. The number of drugs available for therapy and prophylaxis are limited due to toxicity (WHO, 1998; Pepin & Milord, 1994; Stich *et al*., 2002). Control and monitoring treatment outcome of HAT relies on detecting trypanosomes. Diagnosis of the disease relies heavily on direct microscopic visualization of the trypanosomes in clinical samples (Van Meirvenne, 1999). This is limited due to low sensitivity as result of low fluctuating parasitaemic waves (Chappuis *et al*., 2005).

Currently diagnosis of Rhodesian HAT involves a combination of parameters such as origin of the patient, symptoms, detection of trypanosomes by microscopy. There are no pathogenic signs of the disease. Clinical symptoms are not specific, variable and inconstant, thus limited for the diagnosis and monitoring treatment (WHO, 1998). Early and accurate diagnosis is thus important in interpreting transmission cycle of the parasite and progress of the disease to the late stage (Kennedy, 2004).

Treatment of early stage HAT is easier and safer though side effects have been reported upon the use of suramin (Lejon *et al*., 2003a). Definitive diagnostic tests are thus crucial for the early detection cases as transition of early to late stages is not always distinct (Atouguia and Kennedy, 2000). Such tests should minimize the false positive and hence reduce exposure of patients to toxic drugs with unguaranteed efficacy (Inojosa *et al*., 2006).

Treatment of patients with late-stage disease after central nervous system (CNS) invasion by the parasite is difficult. It involves long treatment schedule, requiring hospitalization and considerable care in the use of toxic drugs (WHO, 1998). Melarsoprol, the only drug effective for the late stage *T. b. rhodesiense* form of the disease is toxic (WHO, 1998; Pepin & Milord, 1994). Modifications of treatment regime through introduction of new drugs and in combination have not been able to reduce mortality (Schmidt *et al*., 2004). Treatment failure has been reported in the field upon the use of these treatment regimes (Legros *et al*., 1999; Matovu *et al*., 2001).

Despite improved laboratory diagnostic methods such as mini-Anionic exchange centrifugation technique (m-AECT); Lumsden *et al*., 1979), polymerase chain reaction (PCR); (Welburn *et al*., 2001; Gibson *et al*., 2002; Radwanska *et al*., 2002 Jamonneau *et al*., 2003) and PCR-Oligochromatography molecular dipstick test for trypanosomes (HAT-PCR-OC); (Deborggraeve *et al*., 2006), diagnosis still remain unsatisfactory. Undiagnosed patient will remain untreated and will die soon or later. In the mean time, the HAT patient becomes a burden to the family and acts as a reservoir of the disease. The molecular technique; Loop-mediated isothermal amplification (LAMP) of DNA (Notomi *et al*., 2000) may provide answers to some of the difficulties in diagnosis and monitoring treatment outcome of HAT in endemic areas.

1.2 Problem statement

Diagnosis of HAT is based on a combination of investigative and clinical data (Connor, 1993). However, the non-specific nature of clinical features found in *T. b. rhodesiense* infections makes it imperative to exclude other tropical fevers such as malaria, leishmaniasis, toxoplasmosis, typhoid, viral encephalitis, tuberculosis and HIV which mimic or even coexist with HAT (Atouguia and Kennedy, 2000). Microscopic methods available to confirm the clinical suspicion are cumbersome and less sensitive to ascertain absence and monitoring success of treatment (Lejon and Buscher, 2005).

In the absence of reliable blood tests able to detect CNS invasion by the parasite, hunan African trypanosomiasis (HAT) staging relies on microscopic examination of CSF obtained by lumbar puncture, an invasive procedure (Cattand *et al*., 1988; Lejon *et al*., 2001). Therefore a quick, specific, easy to perform and reliable diagnostic methods for

the disease are needed. Since LAMP is a molecular method that is specific, sensitive, quick and easy to perform, it may be used for diagnostic purposes. Nonetheless, there is need to assess LAMP, its potential in diagnosis and monitoring treatment outcome of *T. b. rhodesiense* infections in non-human primate model of the disease.

1.3 Justification of the study

Human African trypanosomiasis (HAT) cause mortality and morbidity during outbreak with 17000 cases of infections reported (WHO, 2005). Control of HAT mainly relies on chemotherapy. Treatment requires admission to hospital and is potentially dangerous (WHO, 1998; Pepin & Milord, 1994). There is need for proper methods for diagnosis, staging and monitoring treatment outcome.

Parasitological diagnostic methods have limitations while dipstick tests and PCR are costly and still under development (Chappuis *et al*., 2005). Amplification using LAMP (Notomi *et al*., 2000), a rapid, simple and sensitive molecular technique has been used in detecting trypanosome DNA (Kuboki *et al*., 2003; Thekisoe *et al*., 2005; Njiru *et al*., 2007; Njiru *et al*., 2008) and has been promising in the field. Investments in clinical and patho-physiological research to asses LAMP potential in diagnosis and monitoring treatment of *T. b. rhodesiense* infections are thus needed. Therefore, LAMP on vervet monkey model of HAT may help in addressing the problem of diagnosis, staging and post treatment follow-up of HAT.

1.4 Hypothesis

The LAMP test cannot be used in diagnosis and monitoring of treatment outcome of *T. b. rhodesiense* infections in vervet monkey model.

1.5 Objectives of the study

1.5.1 General Objective of the study

To evaluate the performance of LAMP in diagnosis, staging and monitoring treatment outcome in vervet monkeys infected with *T. b. rhodesiense*.

1.5.2 Specific objectives

1. To determine the performance of LAMP in detecting *T. b. rhodesiense* on various biological samples, different sample preparation and amplification methods;
2. To determine the performance of LAMP in diagnosis of HAT infections in infected vervet monkeys;
3. To determine the performance of LAMP in monitoring treatment outcome of HAT in infected vervet monkeys.

CHAPTER TWO

LITERATURE REVIEW

2.1 Biology of trypanosomes

Trypanosomes are members of the sub-kingdom protozoa and the phylum sarcomastigophora. They are attributed to the family *Trypanosomatidae*, order kinetoplastida due to the flagellum arising from a flagella pocket and a single mitochondrion extending the length of the body containing a DNA-rich nucleoid called kinetoplast DNA (kDNA) (Molyneux and Ashford, 1983). The kDNA is usually responsible for the polymorphism of trypanonosomes in different hosts (Vickerman, 1966). All trypanomastids are parasites in vertebrates, invertebrates or plants (Michel's *et al*., 1990). The 20-50µM long spindle shaped *T. b. gambiense*, *T. b. rhodesiense* and *T. b. bruce*i are morphologically similar and are classified within the sub-genus *Trypanozoon* (WHO, 1986). *Trypanosoma b. gambiense* and *T. b. rhodesiense* are the causative agents of HAT whereas *T. b. brucei* is lysed by human serum and therefore is not infective in man. However, it causes cause Nangana in livestock along with *T. evansi*.

Typically *T. brucei* spp trypanosomes live in the blood and intercellular spaces of the host's tissues (Vickerman, 1985). Trypanosomes are protozoa thus have a number of cellular structures which are common to other eukaryotes. However they exhibit some unique features of sub-cellular organisms (Hajduk *et al*., 1992). Among several peculiarities are a discontinuous transcription and transplicing, an unusual network of mitochondrial DNA (mDNA), editing of mitochondrial RNA, glycosomes and an

unusual glutathione (trypanothione) metabolism (Michel's *et al*., 1991). In different hosts where they develop, trypanosomes are transmitted cyclically, involving at least six distinct morphological stages (Vickerman, 1985). After ingestion by a blood sucking tsetse fly, they differentiate, multiply and undergo a series of morphological and biochemical changes (Vickerman, 1985). Subsequently, they migrate from the gut to the salivary glands from where they emerge as infective metacyclic trypanosomes (epimastigote) to be inoculated into another host, multiplication is generally by longitudinal binary fission.

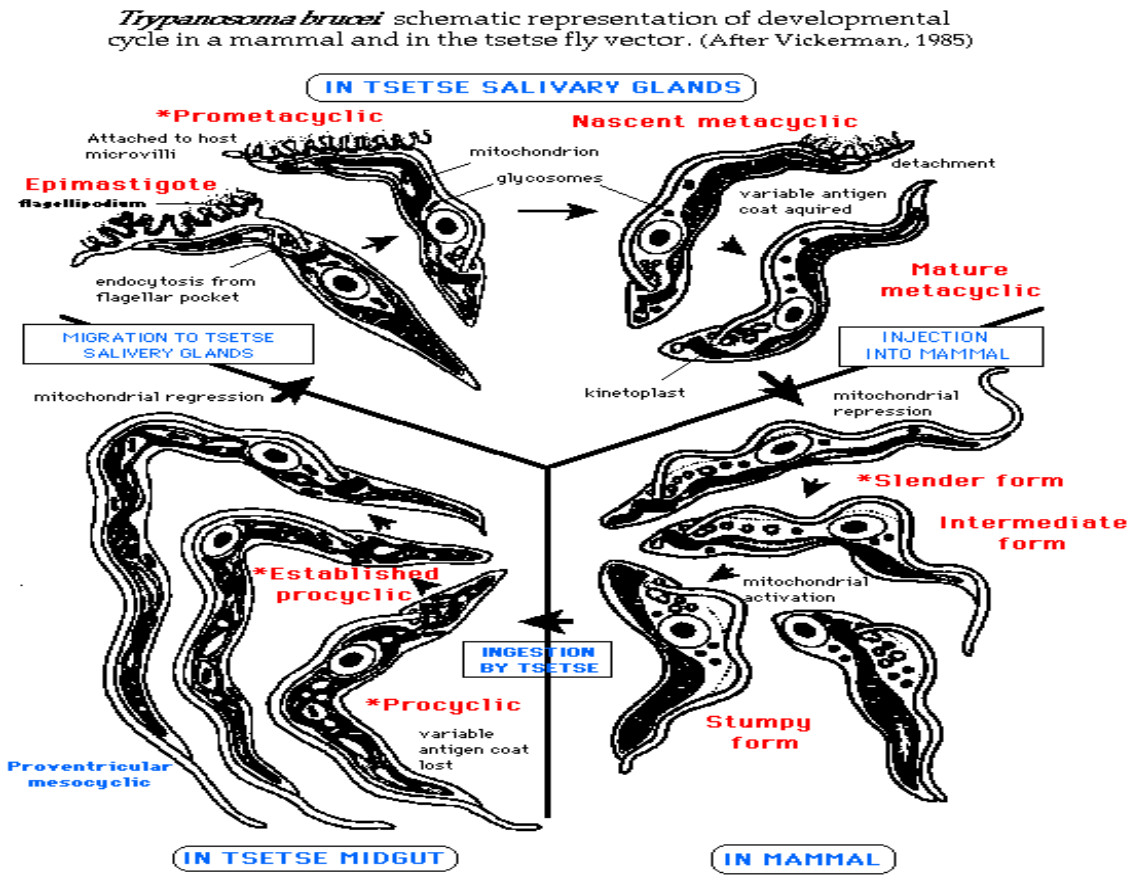

Figure 2.1: Schematic presentation of Trypanosoma brucei developmental cycle in mammal and in the tsetse fly (Vickerman, 1985).

After inoculation of the metacyclic (epimastigote) forms into the dermal connective tissues, an inflammation occur at the site of injection called a chancre. The trypanosomes proliferate and transform to trypanomastigote, the blood stream forms in the skin and subsequently evade the lymphatic and blood systems. The trypanomastigote are able to cross the walls of the blood and lymph capillaries into the connective tissues and even across the choroids plexus to the CSF, which marks the beginning of second (late) or encephalitic stage of the disease.

Two distinct forms of trypanomastigote, the blood stream form are seen in the blood stream of the infected host. Early after infections, rapid diving, long slender forms predominates, while short stumpy forms can be observed at peak and declining parasitaemia (Vickerman, 1985). During the trypanomastigote stage, energy production is exclusively through glycolysis. The cells lack detectable cytochrome and the Kreb cycle is not functional (Hajduk *et al*., 1992).

Trypanomastigote transforms to procyclic forms once ingested by the tsetse fly in the mid gut. Procyclics development is associated with switch from the utilization of glucose to proline as a principal energy source, abundant in the tsetse fly midgut. Activation of mitochondrion in the procyclics is also associated with a switch to cytochrome mediated terminal respiration. The parasites pass down the gut to the posterior end of the peritrophic membrane where they escape into the ectoperitrophic space. From there, they move to the proventiculus and by a long journey via the

oesophagus, mouth parts and salivary gland ducts to the salivary gland (Vickerman, 1985).

There, the procyclics transform to the metacyclic (epimastigote) form and massive multiplication occurs. They attach to microvilli which border the epithelial cells of the glands by means of a junction complex (hemidesmosome) between the flagellum and microvilli. Around 15-30 days after infections the tsetse fly becomes infective to a vertebrate host. Unattached metacyclic trypanosomes develop which carry a surface coat and in which the Kreb cycle and the cytochrome systems are depressed.

The alteration of trypanosomes during the live cycles is very complex. The most important changes occur in the mitochondrion system (Molyneux and Ashford, 1983) and the surface glycoprotein coat of the flagellates (Rudenko *et al*., 1998). There are major antigenic types of parasites present in the infections with different variant-specific glycoproteins (VSGs) (Borst *et al*., 1998; Van hamme *et al*., 2001; Pays, 2006). A fraction of the parasite population escapes the initial mammalian host humoral immune response by extensive antigenic variation of the parasite VSGs (Rudenko *et al*., 1998).

Proliferation of the parasites usually continues until the new VSGs are recognized by a new generation of specific antibodies, mainly immunoglobulin M (IgM) type (Overath *et al*., 1994a). This phenomenon of antigenic variation contributes to the parasite virulence in the host. The minor heterotypes continue to multiply until an immune response can be mounted against these parasites (Hajduk *et al*., 1992). Thus the infections become chronic because the parasites population always contains variants

covered with a new VSG against which the host has not yet produced specific antibodies (Overath *et al*., 1994a).

This phenomenon explain the fluctuating number of circulating trypanosomes in the patient's blood (Ross and Thomson, 1910), contributes to the limited sensitivity of parasitological detection methods in clinical practice (Chappuis *et al*., 2005). Variant-specific glycoproteins, besides evasion of the host immune system, shield other proteins on the surface from immune attack and inhibite phagocytosis by the host phagocytes (Overath *et al*., 1994a; Rudenko *et al*., 1998).

After uptake in the midgut of the tsetse fly, the trypanomastigote, the blood stream form VSG-coat is replaced by a glycoprotein, procyclin or procyclic protein (PARP) (Hajduk *et al*., 1992). Morphological transformation includes increase in body length with noticeable elongation of post-kinetoplast portion of the parasites as the simple mitochondrion expands into a net work of discoid rather than tubular cristea (Vickerman, 1985). The relative mitochondrial volume increases from 5% to 25% (Hucker, 1980).

The surface of the blood stream forms is coated by a single type of glycoprotein molecule, the VSG (Borst *et al*., 1998) that protect the parasite against lysis by complement (Rudenko *et al*., 1998). Only when specific antibodies are present against the available VSG are the parasites destroyed. This surface coat is linked to the surface of the plasma membrane by a glycosyl-phosphotidyl-inosital (GPI) anchor and is essential for African trypanosomes' survival (Overath *et al*., 1994b). It protects the

parasite in two ways; firstly the dense packing of the coat acts as a macromolecular diffusion barrier; secondly, the surface coats undergo antigenic variation (Masterson, 1990).

Only one out of a thousand VSG genes present is expressed at a time (Rudenko *et al*., 1998). Hence, switching from expressing one VSG to another, a completely different coat is produced. In this way the parasite can evade the host immune response (Borst *et al*., 1998). Approximately one in every 10^{-2} to 10^{-7} trypanosomes undergoes antigenic variation during a cell doubling time (Overath *et al*., 1994a). This change of VSG composition occurs spontaneously and is independent.

2.2 Pathology and pathogenesis of sleeping sickness

Understanding of the pathogenesis of sleeping sickness is still inadequate. However, most theories implicate immunopathological processes (Bales, 1988). The development of the disease is ultimately linked to the inability of the immune system to eliminate the parasites (Pentreath, 1991). The late stage manifestations results from the increased and altered levels of cytokines (Maina *et al*., 2004), nitric oxide production (Sternberg *et al*., 1998), from influencing macrophages, lymphocytes and astrocytes (WHO, 1986). The reason for the more virulent nature of *T. b. rhodesiense* remains unclear (Pentreath, 1991).

Histological vasculitis and perivascular agglumerulation of lymphocytes, macrophages and plasma cells and occasionally morular cells (Mott cells) in the brain are observed (Mott, 1906; Green-Wood & Whittle, 1976b). The pia-arachnoid is thickened due to

infiltration and parasites may enter the CNS via the choroids plexus and other regions where the blood-brain barrier is impaired or destroyed (Pentreath, 1989). The strongly immunogenic VSGs of the trypanosomes elicit high levels of IgM (Green-Wood and Whittle, 1973) leading to an increase in the value of erythrocytes sedimentation rate (ESR) and formation of immune complexes (Green-Wood and Whittle, 1976a). The immune complexes induce the release of active substances of the kallikrein-kinin system leading to the increased vascular permeability causing oedema, hypertension and inflammation (Poltera, 1985). Further, a wider variety of auto-antibodies directed against DNA, erythrocytes and brain myelin is reported (Asonganyi *et al*., 1989; Hunter *et al*,. 1992). Strong immunosupression is a prominent feature of trypanosomiasis where the humoral and T-cell mediated immune response are both affected (Askonas, 1985).

Trypanosoma brucei releases a protein factor, *T. brucei*-derived-lymphocyte-triggering factor (TLTF) (Bakheit *et al*., 1993) which is reported to have specific effect on $CD4^+$ and $CD8^+$ T-cells sensitizing them to produce interferon-γ (IFN-γ) a potent activator of macrophages and also stimulates the growth of the parasites (Olsson *et al*., 1991). The production of IFN and localization of trypanosomes in sensory ganglia has been associated with the rise in temperature (fever) produced in HAT (Kristerson *et al*., 1994).

Activated macrophages on their part play a key role in immunosupression during infections (Olsson *et al*., 1992). On the other hand released prostaglandins (PGs) impair the production of interleukin-2 (IL-2). Additionally, the expression of IL-2 receptor (IL-

2R) on both $CD4^+$ and $CD8^+$ cells is suppressed by co-operation of IL-2 and other soluble factors released from the activated macrophages (Pentreath, 1991). Inhibition of IL-2 secretion and down regulation of IL-2R expression inhibits proliferation of T-cells and culminates in a profound state of T-cells unresponsiveness (Lucas *et al*., 1993).

Therefore, the control of the disease is ultimately restricted to T-cells independent B-cells (Pentreath, 1991). On the other hand macrophages produce large amount of tumor necrosis factor-α (TNF-α) involved in the genesis of anaemia and inflammation. Additionally, TNF-α was reported to be a negative regulator of trypanosome growth. An interplay of TNF-α and IFN-γ may thus determine the course of parasitaemia (Lucas *et al*., 1993).

Infections with trypanosomes markedly leads to elevated IgM in the brain, mainly produced in the brain tissues by morular cells rather than entering via damaged blood-brain barrier (Pentreath, 1989). Astrocytes appear to play a key role in the co-ordination of the immune responses for causireathng and limiting inflammation of the CNS to the perivascular areas. The cells function as antigen presenting cells after stimulation by activated T-cells (Pentreath *et al*., 1990). They produce immune responses modifying substances like IL-2, PGD2, PGE2 and TNF-α which can induce inflammation and may cause extravasations of lymphocytes and plasma cells into the CNS (Hunter *et al*., 1992).

Several immune responses modifiers have been shown to be important in the control of normal sleep and its increase during infection and fever. The IL-1α and IL-1β are

somnogenic and are thought to be linked in regulatory pathways to produce further somnogenic agents, notably PGD2, and sleep inhibiting substances via negative feedback like PGE2 (Hunter *et al*., 1992). The PGD2, one of the major sleep inducing substances was found to be elevated in Gambian sleeping sickness (Pentreath *et al*., 1990).

2.3 Clinical manifestations of T. b. rhodesiense sleeping sickness

The acute, sometimes fullminate febrile illness in Rhodesian HAT starts 1 to 3 weeks after the infective bite (Stich *et al*., 2002). A chancre at the site of inoculation of the trypanosomes by a bite of the tsetse fly is frequently observed. The first symptoms like headache, general malaise and fever lasting for 1 to 7 days are non-specific and thus often misinterpreted and overlooked (WHO, 1986, 1998). Intermittent fever attacks, persistent headache, joint pains, weight loss and pruritus are common symptoms of the first (early) or haemolymphatic stage of the disease. A week or months after the infection, general spleenomegarly and lymphadenopathy may develops (Apted, 1970). However the enlargement of posterior cervical lymph nodes ("winter bottom signs") is often not distinct in Rhodesian HAT. Generalized endocrine disorders like reduced libido, amenorrhea, abnormal thirsty or appetite, oedema and anaemia are frequent. However, due to the fodrouyant course of the Rhodesian disease, the typical symptoms like somnolence may not occur (Gentillin *et al*., 1986).

Rhodesian sleeping sickness is characterized by an acute progressive course with CNS invasion which may lasts for weeks or months before death (Stich *et al*., 2002; Kennedy, 2004). It cannot be distinguished clinically from other tropical fevers (Atouguia and

Kennedy, 2000). There is less demarcation between first and second-stages of the illness. The CNS involvement can be clinically limited to drowsiness and tremor (Burri and Brun, 2003).

The onset of clinically different late stage is defined by the detection of trypanosome in the CNS. It results into a chronic meningoencephalitis. The meninges are infiltrated with lymphocytes, plasma cells and occasionally morular cells that are filled with IgM containing vacuoles (Mott, 1906; Green-Wood and Whittle, 1973; Green-Wood and Whittle, 1980). The inflammatory cells infiltration extends along the Virchow-robin spaces into the substances of the brain producing the characteristic picture of perivascular cuffing (Greenwood and Whittle, 1980). Early changes of the personality and behaviors may be subtle. Speech may become indistinct, low and frequently extra-pyramidal occur, with tremors of the tongue and fingers.

The most common sign is day time somnolence, often alternating with insomnia at night. Epileptic form of seizureness and euphoria are observed and the patients become indifferent to the environment. The final phase is characterized by progressive mental deterioration and general wasting. Death results from the sleeping sickness itself, immunosupression, concurrent or opportunistic infections, often pneumonia or malnutrition (Molyneux and Ashford, 1983; Atouguia and Kennedy, 2000). Cardiac involvement is prominent (Manson-Bahr and Charters, 1963) and pancrearditis may be common (WHO, 1986; Bales, 1988). Death may occur even before major CNS damage has developed.

Laboratory investigation reveals anaemia and thrombocytopenia (Green-Wood and Whittle, 1980; Robins-brown *et al*., 1975). The liver and the spleen may be slightly enlarged; localized oedema may be observed in the eyelids, perineum and the skin of the back. Auto-agglutination of the red blood cells (RBC) and high erythrocytes sedimentation rate (ESR) as well as markedly increased levels of unspecific IgM in the serum is observed (Lejon *et al*., 2003b). The number of white blood cells (WBC) and protein content are elevated in the CNS after its invasion by trypanosomes (WHO, 1986, 1998; Lejon *et al*., 2001).

2.4 Diagnosis of Human African Trypanosomiasis

2.4.1 Clinical diagnosis

The diagnosis of HAT is difficult and a major challenge. Not only are there no specific clinical signs, but the intermittent and usually fluctuating parasitaemia makes detection of the trypanosomes difficult in the blood (Connor, 1993; Kennedy, 2004; Chappuis *et al*., 2005). Furthermore, the infections are not specific for the disease; many sub-clinically affected individuals live in delicate balance with potentially pathogenic trypanosomes (Molyneux *et al*., 1996). The element of clinical judgment in diagnosis of the disease become less important as the disease occurs at the same time as other infections that may be more readily detected by these parameters (Atouguia and Kennedy, 2000). This makes the underlying problem and so the extent of the disease commonly not understood.

Rhodesian HAT is usually presented as an acute febrile illness with non-specific signs unless the initial characteristic chancre is observed. Biological blood parameters such as

low anaemia manifesting as low haematocrit or packed cell volume (PCV) (Woodruff *et al* 1973), increased ESR (Green-Wood and Whittle, 1973), coagulation and thrombocytopenia (Barret-Connor *et al*., 1973; Green-Wood and Whittle, 1976b; Stephen, 1986; Robins-Browne *et al*., 1975), hypoalbuminemia accompanied with hypergammaglobulinemia (Lejon *et al*., 2003b) and low serum complement III (C3) levels and split C3 products (Green-Wood and Whittle, 1976a) accompanied by other features (Gear and Muller, 1975) can be observed. However, these findings are of little use in field settings where the tests can be done and are limited in endemic areas.

2.4.2 Diagnosis of HAT in the field

To confirm diagnosis of HAT, it's necessary to find and identify trypanosomes. Rhodesian form of HAT causes a high parasitaemia in the blood of host (Kennedy, 2004), thus detection parasites may be therefore successful. However, parasitological diagnostic methods for field purposes pose problems due to fluctuation of parasitaemic waves (Connor, 1993; Kennedy, 2004; Chappuis *et al*., 2005).

2.4.3 Diagnostic tests used in the laboratory

2.4. 3.1 Parasitological diagnostic methods

The simplest parasitological techniques commonly used in HAT endemic areas are direct microscopic examination techniques. They include wet preparation and fixed (thin and thick) smear. Parasites concentration techniques such as microhaematocrit centrifugation techniques (HCT) (Woo, 1970), Quantitative buffy coat (QBC) which has been extended in the diagnosis of haemoparasites including trypanosomes (Levine *et al*., 1989; Bailey and Smith, 1992) and m-AECT (Lumsden *et al*., 1979) are also used in

detection of trypanosomes. These techniques are time consuming and have less sensitivity. They cannot detect trypanosomes in low pasitaemia (Chappuis *et al*., 2005). Moreover, parasite detection is labor-intensive thus failure to detect the parasites therefore, does not necessarily exclude infections in individuals.

2.4. 3.2 Serological diagnostic methods

There is no equivalent to the Card agglutination test for trypanosomiasis (CATT; Magnus *et al*., 1978) for Rhodesian HAT (Chappuis *et al*., 2005). However, existing serological tests such as immunofluoresence and Enzyme-linked immunosorbent assay (ELISA) (Nantulya, 1989) have varied specificity and sensitivity. This is because of cross-reactivity with non-human trypanosomes and is performed in reference centres by highly trained technicians (Lejon & Buscher, 2004). They cannot be used for clinical decisions. In addition, serum-conversion occurs after clinical symptoms in *T. b. rhodesiense* infections hence the limited use of serological tests. Furthermore, serological tests lack specificity and currently detect antibodies after 3 to 4 weeks of infection (Van Hamme *et al*., 2001) and antibodies can persist up to 3 years after cure or successive treatment (Paquet *et al*, 1992).

2.4.3.3 Diagnostic methods based on propagation of trypanosomes

In vitro cultivation and animal inoculation are also methods that can be used in the diagnosis of HAT. Procedures for *in vitro* cultivation of *T. brucei* have been described, but their success has been irregular over the years (OIE, 2008). Moreover, the method needs sophisticated equipments, produce results after a considerable delay and not suitable for large-scale use. In the field, the major challenges of *in vitro* cultivation are

contaminations, high cost and the long delay before obtaining the results. The sub-inoculation of suspected blood and other body fluids into susceptible animals such as mice, rats and rabbits is particularly useful in revealing sub-patent infections (OIE, 2008). Animal inoculation may be more sensitive than direct examination techniques. However, the method is not practical; it is expensive and results are not immediate. Multiplication of trypanosomes upon inoculation on a suitable medium takes 3 to 4 weeks to reach detectable level. Sub-inoculation of suspected blood and other fluids into susceptible animals is also limited by long period of time to get the results. Trypanosomes may fail to multiply (Poltera, 1985) since some of the animals are able to control the parasitaemia levels (Ndung'u *et al*., 2009). These facts limit the use of *in vitro* cultivation and animal inoculation in field routine diagnosis.

2.4.3.4 Other diagnostic methods

Proteomic signature analysis (Papadopoulos *et al*., 2004) and neuroimaging techniques like computer topomography and magnetic resonance imaging (Gill *et al*., 2003) have recently been used in HAT patients traveling from Africa in Canada. These tests are impracticable in the field and are not available in areas of endemics in the developing countries.

2.5 Screening of the disease

Screening of Rhodesian form of HAT relies on clinical signs which are non-specific. There is no equivalent of CATT for screening the Rhodesian form of HAT (Chappuis *et al*., 2005). Therefore, clinical symptoms are implicated in areas where *T. b. rhodesiense* HAT is endemic. Diagnostic confirmation which relies on the finding of trypanosomes

in the chancre fluid aspirates, blood, lymph nodes aspirates, bone marrow, CSF and any other body fluid is thus recommended during screening of Rhodesian HAT.

2.6 Staging of the disease

2.6.1 Detection of trypanosomes in the CSF

The examination of CSF plays a key role in staging (van Meirvenne *et al*., 1999; Buscher & Lejon 2004; Kennedy, 2004). The collection of CSF by lumbar puncture is difficult and introduces risk to the patient (Apted, 1970; Woo, 1970; Cattand *et al*., 1988). Staging of *T. b. rhodesiense* infection relies on WHO recommendations that is increased cell count (>5cell/µl) or total protein concentration (>37mg/100ml) are used (WHO, 1986, 1998). However, these criteria are not satisfying (Doua *et al*., 1996; Lejon & Buscher, 2005; Chappuis *et al*., 2005). A definitive stage diagnosis of *T. b. rhodesiense* infections requires the actual detection of trypanosomes in the CSF after single or double centrifugation (Cattand *et al*., 1998; WHO, 1998; Miezan *et al*., 2000). Microscopic examination of the parasites in CSF (van Meirvenne *et al*., 1999) is of low sensitivity and trypanosomes are not easily detectable in the CSF (Lejon *et al*., 1995) since CSF is not a good medium for trypanosome growth (Pentreath *et al*., 1992)

2.6.2 Elevated levels of WBC in the CSF

The cut off value of WBC count was set at 5 cells/mm^3 in the CSF (WHO, 1986, 1998). Patient with WBC count >5 cell/mm^3 are considered in the second stage. The WBC in the CSF of meningo-encephalitic stage HAT varies from normal to around 100-300 cell/mm^3 and but not exceeding 1200 cell/mm^3 (Lejon *et al*., 2003b).

Increase in the WBC count (cytorachia) in the CSF may be considered pathogenic but may also occur in neuro-infectious diseases of other origins (Kristerson and Bentivoglia, 1999; Lejon and Buscher, 2001). A persistently elevated WBC count in the CSF can be observed in recovering patients or immediately after treatment (Dumas and Girard, 1978). Although a change in WBC count is more helpful diagnostically, this can be misleading (Lejon and Buscher, 2001; Lejon and Buscher, 2005). Elevation of WBC in CSF during late stage sleeping sickness has low sensitivity due to cell lysis (Green-Wood and Whittle, 1976b) and is not specific to HAT (WHO, 1986; Lejon and Buscher, 2001). The accurate enumeration of cells in CSF is further hampered by the detection limit of the haemocytometer that is about equal to the upper limit of normal cytorachia (Burechailo and Cunningham, 1974).

Almost half of the patients with WBC counts between 6-20 cells/mm^3 without trypanosomes in the CSF that are classified in the late stage may show no signs of neuro-inflammation (Lejon *et al*., 2003b). Thus, several authors proposed an increased cut-off value of WBC count between 10-20 cells/mm^3 (Bisser *et al*., 2002; Lejon *et al*., 2003b). Therefore, this suggestion has been questioned (Lejon and Buscher, 2005). Trypanosome DNA was detected in the CSF patients suspected to be in the haemolymphatic stage (Truc *et al*., 1999; Jamonneau *et al*., 2003).

2.6.3 Total CSF protein concentration

Protein concentration (proteinorachia) in the CSF of sleeping sickness patients ranges from 10-30mg/dl (Bisser *et al*., 2002; Lejon *et al*., 2003b). The cut off value of proteinorachia in the CSF of sleeping sickness patients differ depending on the protein

quantification method applied (Lejon *et al*., 1995). These include 25mg/dl in trichloroacetic and precipitation, 37mg/dl in colorimetric methods, 45mg/dl in sulfosalicylic and precipitation (WHO, 1983), 30mg/dl in Biorad method and 23-30 mg/dl in Fortress method. Protein quantification in CSF is omitted because the higher limits are sometimes accepted as normal (Lejon *et al*., 1995).

In practice proteinorachia is rarely performed for stage determination and post-treatment follow-up due to shortage of adequate instruments, the need of reagents that are not often available in rural health centres and the belief that proteinorachia does not provide additional information (Miezan *et al*., 1998). Associated increase in protein content above 25 mg/dl in the Method of Siccard and Cantaloube may occur in the late stage (WHO, 1983). However, this still remain limited to few individuals exhibiting the feature thus cannot used for diagnosis, staging and post-treatment follow-up (Lejon and Buscher, 2005).

2.6.4 Biomolecular tests under development

Currently it is accepted that IgM and IgG are present in high concentrations in the serum as a results of polyclonal B-cell activation (Green-Wood and Whittle, 1973). The second stage trypanosomiasis lead to blood brain barrier damage. The inflammation of the CNS is accompanied by intrathecal synthesis of IgA, IgM and IgG and intrathecal trypanosome specific antibodies (Bisser *et al*., 2002; Lejon *et al*., 2003b Ngotho *et al*., 2008). Due to diffusion of these antibodies present in high concentration in the serum into CSF especially intrathecal IgM, elevated levels of these bio-markers can occur in the first stage of HAT (Lejon *et al*., 2007). Pro-inflammatory cytokines (γ-Interferon,

TNF-ά and soluble TNF receptor 1) profiles indicate that they are up-regulated in the haemolymphatic stage (Maina *et al*., 2004). Nitric oxide and IL-10 are prominently elevated during late and early stage of the disease, respectively (Sternberg *et al*., 1998; Ngotho *et al*., 2006) and may present reliable and useful biomarkers of the disease staging.

2.4.5 Treatment follow-up

In the past, treatment of infected humans has less effect on incidences of infections in humans as diagnosis is done in medical centres with no surveillance practiced except during epidemics (Apted, 1970). Currently, surveillance and screening programmes have been in place to avoid transmission and spread of the disease. For follow-up after treatment, the blood and CSF of the patient need to be re-examined on several occasions usually 3, 6, 12, 18, 24 and 36 months after the end of treatment (Lejon and Buscher, 2001). A patient is considered cured of the disease when during the two to three years follow-up period; no trypanosomes are detected in the blood, lymph or CSF (WHO, 1998; Lejon and Buscher, 2005).

There are no guidelines that have been described for total CSF protein concentration during treatment follow-up. Therefore, examination of CSF plays a key role in the disease stage diagnosis selection of treatment and post-treatment follow-up or monitoring of sleeping sickness patients (van Meirvenne, 1999; Kennedy, 2004; Buscher and Lejon, 2004). However, these criteria are not entirely satisfying for the purpose of post-treatment follow-up.

2.5 Molecular diagnostic methods

2.5.1 Polymerase Chain Reaction (PCR)

Different PCR assays now exist; however, none of them has been validated for HAT diagnostic purposes (Kuboki *et al*., 2003). The sensitivity of PCR depends on target sequence to be amplified, with repetitive sequences being more sensitive than low-copy or single-copy sequences (Gibson *et al*., 2002). In principle, PCR can be applied to any patient sample that may contain trypanosome DNA. However the problem of reproducibility of PCR in diagnosis of HAT especially on samples from seropositive but apparent negative cases have been reported (Solano *et al*., 2002). Samples should be stabilized in special buffers or filter papers for easy handling and protection of DNA from degradation. However the amount of sample that can be applied on a filter paper is small limiting the chances for it to contain enough DNA for detection (Chappuis *et al*., 2005).

Polymerase chain reaction is not used as a routine diagnostic tool for HAT due to the cost involved (Gibson *et al*., 2002). The method is also inadequate to amplify targets reliably in clinical samples without parasite multiplication in suitable and susceptible animal such as mice due to usage of low-copy number genes, besides the elaborate precision instruments required (Gibson *et al*., 2002). Moreover, *Thermus aquaticus* (*Taq*) DNA polymerase is easily inactivated by tissue- and blood-derived inhibitors such as myoglobin, haeme-blood protein complex, IgG and other immune complexes (Akane *et al*., 1994; Al-Soud *et al*., 2000; Belec *et al*., 1998; Johnson *et al*., 1995; Al-Soud *et al*., 2001). There is difficulty in optimizing PCR reaction conditions, thus its use in

diagnosis in field conditions in areas where HAT is endemic is unfeasible. Currently PCR is restricted only for research purposes (Chappuis *et al*., 2005).

2.5.2 Molecular dipstick test

The molecular dipstick format for vector bone diseases developed by Renuart *et al*., (2004), uses Oligochromatography (OC) that provides a simple dipstick test for detection of amplified PCR products. The human African trypanosomiasis-PCR-Oligochromatography (HAT-PCR-OC) or molecular dipstick test (Deborggraeve *et al*., 2006) based on OC, combines both sensitivity and specificity of PCR with simple speed of membrane chromatography, visible after 5 minutes by naked eyes. In contrast to convectional amplicon detection techniques, OC does not require post amplification preparations neither does it require sophisticated equipments for the detection of the PCR amplicon. The major drawbacks of HAT-PCR-OC are cost due to the instrumentation used in the initial stages of amplifying the DNA to get PCR amplicon and contamination with PCR products leading to false positive results.

2.5.3 Loop-Mediated Isothermal Amplification (LAMP) of DNA

Loop-mediated isothermal amplification (LAMP) of DNA developed by Notomi *et al*., (2000) is a novel strategy for gene amplification. It relies on the auto-cycling strand displacement synthesis of DNA by *Bacillus stearothermophilus* (*Bst*) DNA polymerase large fragment under isothermal conditions (60-65^{o}C) (Notomi *et al*., 2000). Since LAMP is carried out under isothermal conditions, simple incubators such as water bath or heat block are sufficient for DNA amplification (Njiru *et al*., 2007). The reaction also

shows high tolerance to biological products meaning that DNA extraction is not necessary (Enowoto *et al.*, 2005; Yamada *et al*., 2006; Kaneko *et al*., 2006).

In LAMP, the amplification is done without thermal cycling, instead a chemical; betaine is used to denature the DNA (Notomi *et al*., 2000). The technique uses a set of 4-6 primers recognizing 6-8 regions of the targeted DNA respectively (Notomi *et al*., 2000; Nagamine *et al.*, 2002). Simultaneously initiation of DNA synthesis in LAMP by multiple primers makes the technique highly sensitive and increases the specificity, efficiency and rapidity. Moreover, LAMP can synthesize 10 to 20µg of targeted DNA three-fold every half cycle within 30 to 60 minutes and the reaction is only limited by the amount of deoxyribonucleoside triphosphate (dNTPs) and primers (Notomi *et al*., 2000; Hafner *et al*. 2001). The product can be inspected visually by the addition of fluorescent dyes SYBR Green I (Iwamoto *et al*., 2003; Mori *et al*., 2006; Monis *et al*., 2007) or calcien (Boehem *et al*., 2007) and measurement of turbidity derived from the white precipitate of magnesium pyrophosphate in the mixture of LAMP amplicon (Mori *et al*., 2001).

Loop-mediated isothermal amplification (LAMP) of DNA has been used successfully in detection of human infectious agent in periodontis (Yoshida *et al*., 2005), peptic ulcers (Minami *et al*., 2006), malaria (Poon *et al*., 2006; Paris *et al*., 2007) and in tuberculosis (Boehem *et al*., 2007). Through this novel technology, genetic analysis of infectious diseases has be developed and detailed genetic information obtained on virulence and

drug resistance without a significant influence of the co-presence of non-target DNA (Notomi *et al*., 2000).

The potential of LAMP in diagnosis of trypanosomiasis was demonstrated by Kuboki *et al*., (2003); based on single copy target Para-flagella protein (*PFRA*) gene for detection of *T. brucei sp*. Recently Njiru *et al*., (2007) reported LAMP for *Trypanozoon* based on the *RIME* gene. On the other hand Thekisoe *et al*., (2007) reported LAMP for *T. evansi*, *T. vivax*, *T. congolese* and *T. b. gambiense*. The usefulness of LAMP in diagnosis of *T. b. rhodesiense* infection was demonstrated by Njiru *et al*., (2008) based on human serum resistant antigen (SRA) gene.

The evaluation of LAMP in the diagnosis of malaria has been documented (Boehm *et al*., 2006, Paris *et al*., 2007). However, the evaluation of LAMP with other diagnostic methods in diagnosis of trypanosomiasis was demonstrated by Thekisoe *et al*., (2005). More recently, Thekisoe *et al*., (2008) evaluated the stability of LAMP reagents and its amplification efficacy on crude trypanosome DNA and was found to be suitable in detecting trypanosome DNA. Therefore LAMP may be a useful tool in diagnosis and monitoring treatment outcome of HAT.

Indeed, availability of sequenced genomes of several species of trypanosomes has provided information about genes that could be targeted as diagnostic markers for the development of molecular diagnostic methods. An example is the repetitive insertion mobile elements (RIME) gene (Hasan *et al*., 1984). As such, LAMP can offer an attractive strategy for diagnosis of HAT in sub-Saharan Africa where facilities are

minimal. Its speed, independence of specialized heating systems and the fact that the results can be visually inspected may make it a well established molecular method for diagnosis and monitoring treatment. It is therefore important to evaluate its potential in diagnosis, staging and monitoring treatment of *T. b. rhodesiense* infection in appropriate experimental animal models.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Animals

Two adult vervet monkeys (*Chlorocebus aethiops*), labeled A an adult female and B an adult male weighing 3.3 and 3.25 kg respectively were obtained from the Institute of Primate Research (IPR), Nairobi, Kenya. The animals were housed in quarantine for 90 days, screened for evidence of any diseases including zoonotics such as hepatitis, tuberculosis, schistosomiasis and helmenthic infections. They were dewormed and treated for any ectoparasite infections. During this quarantine period, the monkeys became accustomed to staying in individual squeeze-back stainless steel cages and human handling.

The animals were maintained on green maize, fresh vegetables (bananas, tomatoes and carrots) and commercial monkey cubes (Monkey pellets®, Unga Feeds Ltd, Kenya), fed twice daily (9.00–9.30 am and 3.00–3.30 pm). Drinking water was provided *ad libitum.* Before experimental infection, the animals were transferred to experimental wards and allowed to settle for another three weeks. Pre-infection (baseline) data was collected before the commencement of the experiments. The international animal care and use committee (IACUC) and Institution's scientific ethical review committee (ISERC) of TRC contacted to approve the protocol of this study

3.2 Trypanosomes

The stabilate *T. b. rhodesiense* KETRI 2537 a derivative of EATRO 1989 was used. This stabilate was isolated in Uganda in 1972 from a human patient by direct inoculation

of the patient's blood and lymph node fluid into a monkey and later cryopreserved (Fink and Schmidt, 1980).

3.3 Drugs

Melarsoprol (Mel B®; Aventis, Germany) and diminazene aceturate (Berenil®, Bayer, Germany) were used in the treatment of the vervet monkeys. Berenil® was weighed and reconstituted in distilled water to give a volume of 1mL for injection in a dosage of 5mg/Kg body weight (bwt) intra muscularly (IM). Mel B® was administered in a dosage of 3.6mg/Kg bwt intra venous (IV) solution after reconstitution with 50% water and 50% propylene glycol injection.

3.4 Experimental study design

The two vervet monkeys were used due to economic and ethical considerations. The cryopreserved trypanosomes were injected into gamma-irradiated mice where trypanosomes were harvested from these mice at the peak parasitaemia then diluted to 10^4. The vervet monkeys were infected by intravenous injection with 10^4 *T. b. rhodesiense* using femoral vein in 1mL of phosphate saline glucose (PSG). Before and during the course of the disease, presence of trypanosomes in ear vein-pricked-blood was determined on daily basis. The parasitaemia was estimated using the rapid matching method (Herbert and Lumsden, 1976). On weekly basis, the animals were anaesthetized with diazepam (May and Baker, U.K) at a dosage of 10mg/kg bwt and ketamine hydrochloride (Rotexmedica, Trittau, German) at a dosage of 15mg/kg bwt for detailed clinical examination and sample collection.

Two milliliters of CSF was obtained by lumbar puncture. 5mL of Ethylene-diamine-tetra-acetic acid (EDTA) blood was collected by femoral vein puncture for LAMP test while non-EDTA blood collected was processed into serum. The animals were sub-curatively treated with Berenil® at 5mg/kg btw intra muscularly (IM) at 35 day of post infection (dpi) for three days. The animals were not cured but developed clinical meningo-encephalitis and were treated with Mel B® at a dosage of 3.6mg/Kg bwt intravenously at 78 and 136 dpt with berenil on monkey A and B for three days respectively. The animals were then monitored during a follow-up for a period of 250 days.

3.5 Parasitaemia determination

Trypanosomes numbers in the blood were counted using wet blood film on blood obtain from an ear vein-prick. The parasitaemia was estimated using the rapid matching method (Herbert and Lumsden, 1976). A drop of blood obtained from ear-pricked vein was placed on a glass slide overlaid with a cover slip and parasitaemia read under a microscope at X400 total magnification. For low parasitaemia <antilog 5.4, haematocrit centrifugation technique (HCT) (Woo, 1970) was used. About 70µL of EDTA ear-prick-blood was transferred into a capillary tube about three quarter full. One end of the capillary tube was sealed with cristaseal then centrifuged in a micro-haematocrit centrifuge for 5 minutes at 10,000 rpm. The spun capillary tube was placed on a microscopic slide and a drop of water dropped between the plasma and the buffy coat. The slide placed on a microscope stage was examined at X400 magnification for the presence of trypanosomes, at the buffy coat region.

3.6 Packed cell volume

The packed cell volume (PCV) was determined using the standard micro-haematocrit method (Stephen, 1986). About 70µL of infected EDTA ear-prick-blood was transferred into a capillary tube (75X1.5mm) to about three quarter full. One end of the capillary tube was sealed with cristaseal. The capillary tube was centrifuged in a micro-haematocrit centrifuge for 5 minutes at 10,000 rpm. The spun capillary tube was placed on the PCV reader and percentage PCV recorded, as previously described by Stephen, (1986).

3.7 Cerebrospinal fluid trypanosome and white blood cells counting

At least 2ml of CSF was collected by lumbar puncture into cryovials. The CSF was also collected by a capillary and was used to charge the haemocytometer. A drop of fresh CSF (about 2µL) was immediately transferred into a haemocytometer and the number of WBC and trypanosomes counted as described by Gould and Sayer, (1983).

3.8 Analysis of the samples at sampling points

3.8.1 Estimation of proteins in the Cerebrospinal fluid

Fortress protein assay kit (Serolab, UK) was used to estimate the total proteins in the CSF. For the sample, 25µL of CSF was mixed with 1.5mL of pyrogyllol red reagent. For the standard, 25µL of standard was mixed with 1.5mL pyrogyllol red reagent. For the reagent blank, 25µL of ultra pure water (PCR grade) (Fishher Biotec) was mixed with 1.5mL pyrogyllol red reagent in test tubes. The test tubes were incubated at 37^{o}C for 5 minutes. The absorbance of the samples and the standard were measured against

the blank at 600 nm in a spectrophotometer (Jenway®, Genova, Switzerland) and the protein concentration calculated using the formula below.

$$\text{Protein concentration (mg/dl)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \{\text{Standard concentration}\}$$

3.8.2 Total DNA extraction

3.8.2.1 Crude total DNA extraction

Crude total DNA was extracted as described by Njiru *et al*., (2007). 15µL of the sample (blood, serum) was mixed with 40µL of ultra pure water (PCR grade, Fishher Biotec, Japan), boiled for 5 minutes in a water bath and centrifuged at 20,800g for 10 minutes. The CSF was boiled 5 minutes and centrifuged at 20,800g without dilution. About 10-15µL of supernatant of both blood and CSF was collected for LAMP test.

3.8.2.1 Pure total DNA extraction

Total DNA was extracted using Saponin method (Plowe *et al*., 1995). 500µL of the sample (blood, serum, CSF) was mixed with 500µL of Saponin lysis buffer (0.15%w/v Saponin, 0.2%w/v NaCl and 1mM EDTA) and vortexed. The mixture was then centrifuged at 11,000g for 10 minutes in a microcentrifuge followed by four washes Saponin lysis buffer. The resulting pallet was then re-suspended in 100µL of PCR buffer (50mM KCl, 1.5mM MgCl2, 10mM of Tris-HCl, pH 8.3) and incubated at 95^{o}C for 20 min, cooled and stored at -20^{o}C until further analysis.

3.8.3 Oligonucleotide primers

A total of six LAMP primers designed from the *RIME* gene (Njiru *et al*., 2007) shown in table 3.1 were used in the study.

Table 3.1: Oligonucleotide primers used in LAMP and their sequences

Primer type	Sequence
Forward inner primer (FIP)	5'-GGAATACAGCAGATGGGGCGAGGCCAATTGGCATCTTTGGGA-3'
Backward inner primer (BIP)	5'–AAGGGAGACTCTGCCACAGTCGTCAGCCATCACCGTAGAGC-3'
Forward outer primer (F3)	5'-CTGTCCGGTGATGTGGAAC-3'
outer backward primer (B3)	5'-CGTGCCTTCGTGAGAGTTTC-3'
loop forward primer; (LF)	5'–GCCTCCCACCCTGGACTC-3'
Loop backward primer (LB)	5'-AGACCGATAGCATCTCAG-3'.

3.8.4 LAMP reaction

Amplification of DNA was carried out as described by Njiru *et al*., (2007) using Loop DNA amplification kit (Eiken Chemical Co. Ltd., Japan). The reaction mixture (25µL) contained template, 40pmol each of FIP and BIP, 5pmol each of F3 and B3, 5pmol of each LF and LB, 8 U of *Bst* DNA polymerase large fragment (New England Biolabs, MA, USA), 1.4mM dNTPs, 0.8M betaine, 20mM Tris-HCl (pH 8.8), 10mM KCl, 10mM $(NH_4)_2SO_4$, 8mM $MgSO_4$, and 0.1%Tween 20. 1µL of pure total extracted DNA and 2-4µL of crude total extracted DNA was used as the template for LAMP reaction. The test was carried out for 45 minutes at 65°C in a Rotor-Gene 3000 thermocycler (Corbett

Research, Sydney, Australia) and in water bath. In both cases, termination of the reaction was done by increasing the temperature to 80°C for 4 minutes.

3.8.5 Detection of LAMP product

Amplification of DNA in the LAMP reaction was monitored as describe by Njiru *et al*., (2007) through direct visual inspection after addition of 1 µL/mL of 1/10 dilution of SYBR Green I (Invitrogen, Australia) and electrophoresis in 1% ethidium bromide stained agarose gel and the bands visualized after illumination with UV light. To confirm that LAMP amplified the correct target, the product was digested with specific restriction enzyme *Ndei*I (New England Biolabs, Ma, USA) at 37°C for 3h, followed by electrophoresis in 1% agarose stained with ethidium bromide solution (1µg/mL). The bands were visualized after illumination using ultra-violet (UV) light.

3.9 Data Analysis

Data generated was stored in hard and soft copies in Ms Access as data base. Descriptive statistics were used to describe PCV, body weight, temperature, WBC count, total CSF protein and parasitaemia data with their trends expressed in figures. Student t-test was used to check whether there was a significant difference of the individual monkeys and significant changes during the course of infections for the above parameters at 95 % confidence level. Chi-Square (X^2) test was used test the significant difference between LAMP and microscopy in diagnosis and also the significant difference between different samples, sample preparation and amplification methods in diagnosis using LAMP. A $p<0.05$ indicate a significant difference between any two methods, preparation and amplification methods and types of samples.

The Kappa (k) statistics was used to measure level of agreement between LAMP and microscopy in diagnosis. The Kappa statistic measures the observed percentage of agreement between tests: (No. positive by both tests + No. negative by both tests)/*N* (Total number of positive and negative by both test), against what might be expected by chance. The efficiency of different sample preparation and amplification methods was also assed using Kappa (k) statistics. Kappa (k) values of 0 to 0.4 indicated marginal agreement, 0.41 to 0.75 indicated good agreements and above 0.76 indicated excellent agreement between the different diagnostic methods and also sample preparation and amplification methods.

CHAPTER FOUR

RESULTS

4.1 Clinical parameter

The animals developed clinical signs of HAT starting 5 dpi. These were characterized by mild fever, spleenomegarly and lymphadenopathy. As the infections progressed, clinical signs intensified but disappeared 7 days after Berenil® treatment at 35 dpi. Clinical signs re-appeared 21 days later indicating relapse of infections and disappeared after Mel B® treatment at 113 and 171 dpi in monkey A and B respectively.

4.2 Body weight

The baseline body weight ranged from 3.3 to 3.6 kg and 3.2 to 3.8 Kg in vervet monkey A and B respectively. A significant decrease in body weight was observe in the early stages of the infections ($p<0.05$). After sub-curative Berenil® treatment, there was an increase in body but reversed few days later in both monkeys (Figure 4.1). Significant gain in body weights were recorded after curative Mel B® treatment. The changes in body weight between the monkeys differed significantly ($p<0.05$), and was more severe in monkey B (Figure 4.1).

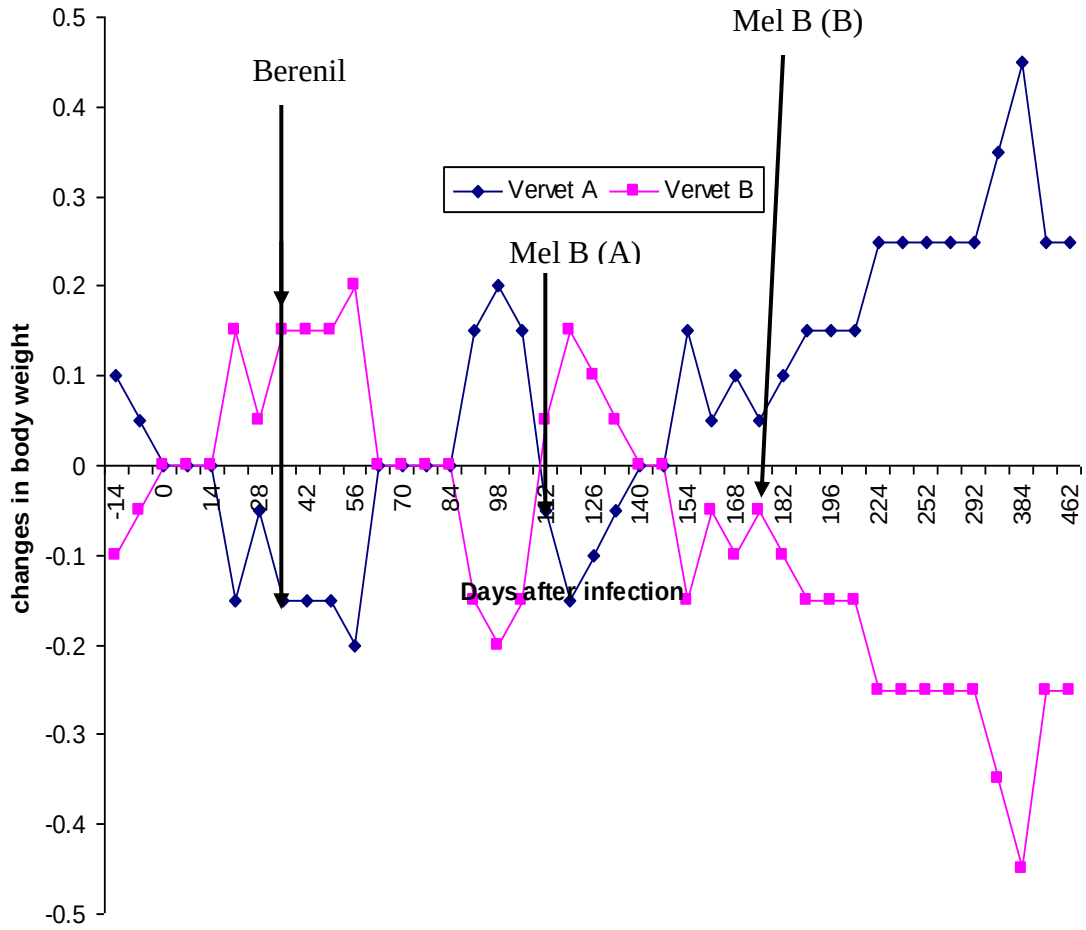

Figure 4.1: Body weight changes in *T. b. rhodesiense* infected vervet monkeys treated with Berenil® and Mel B®.

4.3 Temperature

The baseline body temperature ranged from 36 to 38^{0}C. During the initial stages of infections, a significantly increase in body temperature ($p<0.05$) of as high as 40^{0}C was observed at 14 dpi (Figure 4.2). After sub-curative and curative Berenil® and Mel B® treatment respectively, there was a decrease in body temperature returning to pre-infection levels. Temperature changes between the two monkeys did not differ

significantly ($p>0.05$). A number of temperature variation was observed between 196 to 384 dpi

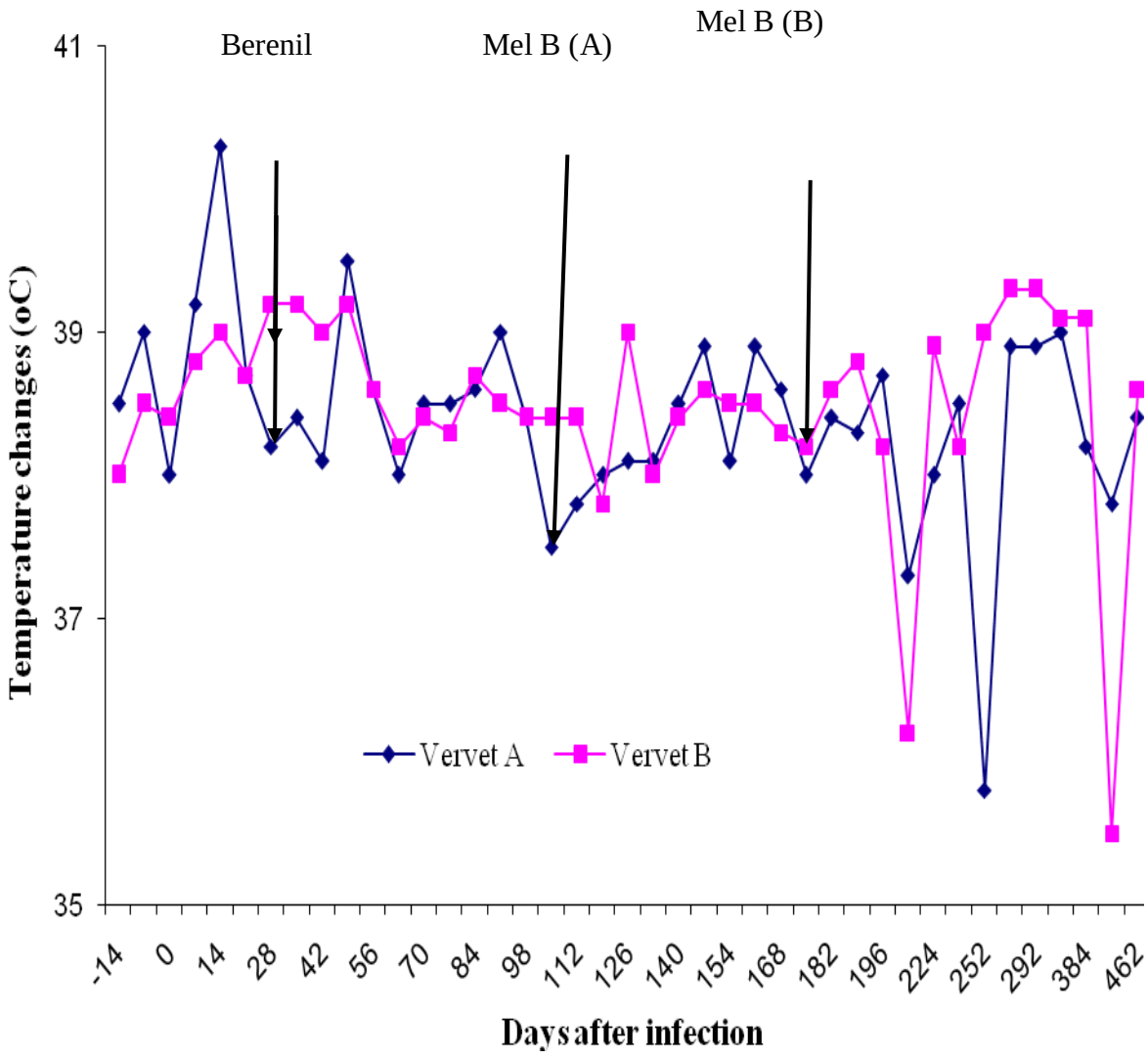

Figure 4.2: Temperature changes in *T. b. rhodesiense* infected vervet monkeys treated with Berenil® and Mel B®.

4.4 Packed cell volume

Before infection, the PCV of both vervet monkeys ranged from 50% to 61%. After infections, a significant decrease in PCV ($p<0.05$) was observed in both monkeys. There was an increase in PCV after Berenil® treatment. As the diseases progressed, PCV decreased and returned to normal after curative Mel B® treatment (Figure 4.3). Significant increase in PCV was observed after Mel B treatment at 113 dpi (78 dpt with Berenil®) and 171 dpi (136 dpt with Berenil®) for monkey A and B respectively (Figure 4.3).

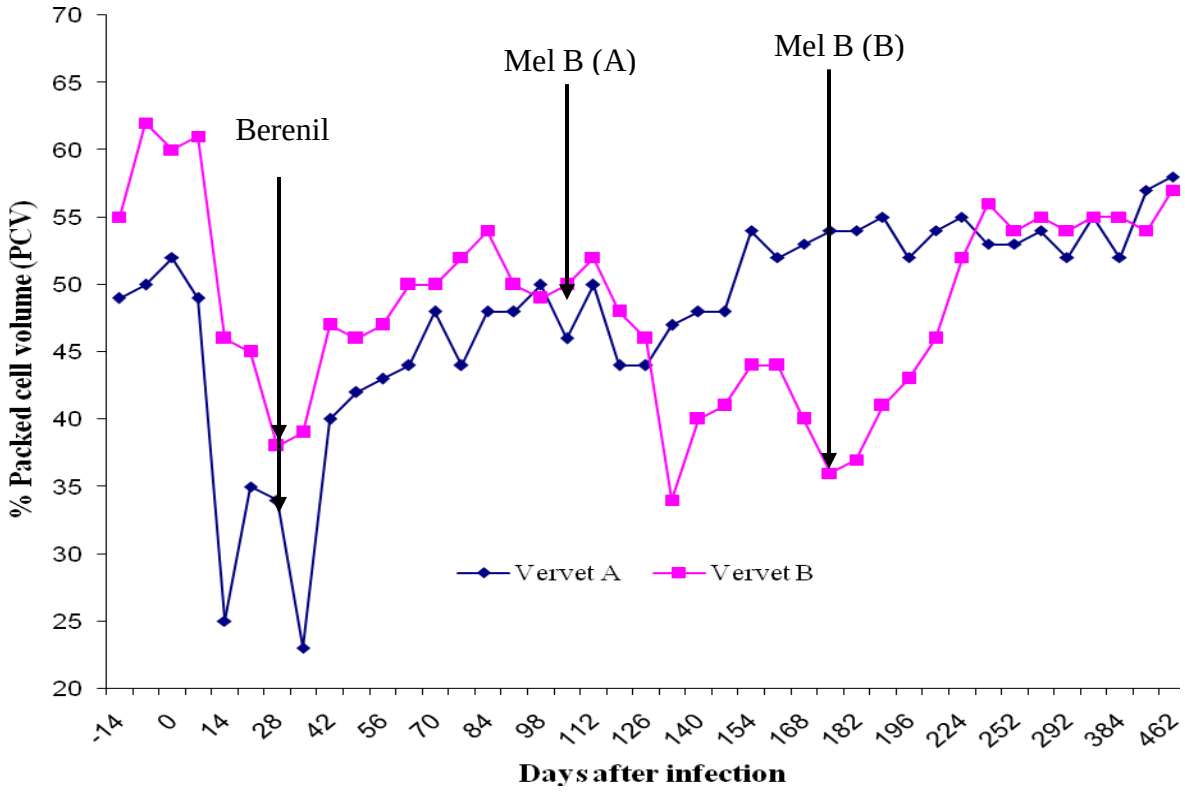

Figure 4.3: PCV changes in *T. b. rhodesiense* infected vervet monkeys infected and treated with Berenil® and Mel B®.

4.5 Parasitaemia and CSF parasitosis

Following infection, trypanosomes were detected in the blood at 4 and 5 dpi in vervet monkeys A and B respectively. Parasitaemia remained high (antilog ≈ 7) characterized by minor fluctuations (Figure 4.4). Following sub-curative Berenil® treatment, no parasites could be detected in both monkeys 3 days after treatment. Trypanosomes relapsed in the blood of monkey B between 154 and 168 dpi (129 and 133 dpt with Berenil®) and cleared 3 days after curative Mel B® treatment at 171 dpi (136 dpt with Berenil®). After subcurative Berenil® treatment, trypanosomes were not detected in the blood of vervet monkey A (Figure 4.4).

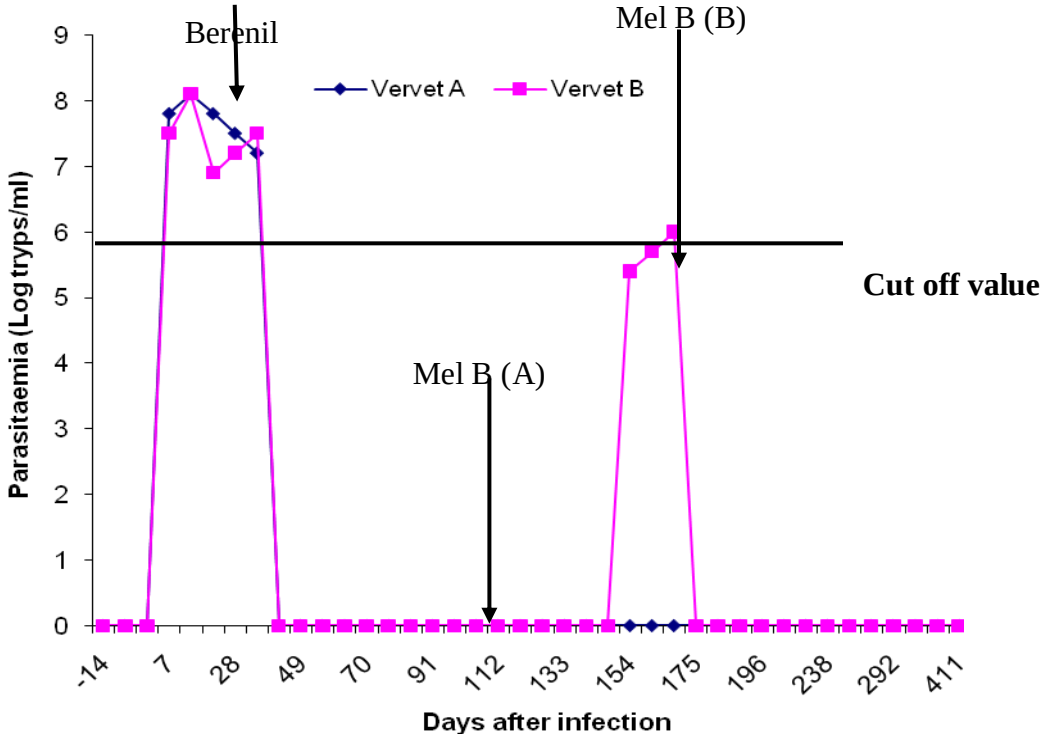

Figure 4.4: Parasitaemia levels in *T. b. rhodesiense* infected monkeys treated with Berenil® and Mel B®.

Trypanosomes were first detected between 21-28 dpi in the CSF of both monkeys. Monkey B had trypanosomes relapse in the CSF at 127 dpi (92 dpt with Berenil®) and Mel B® treatment was done at 17i dpi (136 dpt with Berenil®). Vervet monkey A had no relapse of trypanosomes in the CSF but developed signs of late stage of the disease of biting its finger at 97 dpi (62 dpt with Berenil®). Mel B® treatment was done at 113 dpi (78 dpt with berenil). Trypanosomes were cleared off in the CSF of both monkeys 3 days after Mel B® treatment. During the post-treatment follow-up period of up to 462 from the day of infection, the animals were aparasitaemic with no parasites detected in the CSF.

4.6 CSF white blood cell count

In both vervet monkeys, the WBC counts in the CSF during pre-infection was <5 cell/mm^3. After infection, the WBC counts increased to >5 cell/mm^3 by 14 dpi and 21 dpi (figure 4.5). Subcurative Berenil® treatment at 35 dpi had no effect on WBC count in the CSF thus remained >5cell/mm^3 (Figure 4.5). The WBC count increased<5 cell/mm^3 in monkey A and B respectively with a lot of fluctuations. After curative Mel B® treatment at 113 dpi (78 dpt wit Berenil®) and 171 dpi (136 dpt with Berenil®) in monkey A and monkey respectively, WBC started to decrease. The WBC counts in the CSF dropped to levels < 5cell/mm^3 at 224 dpi in both monkeys. It took 120 and 53 days after curative Mel B® for WBC count to drop to levels < 5cell/mm in monkey A and monkey B respectively.

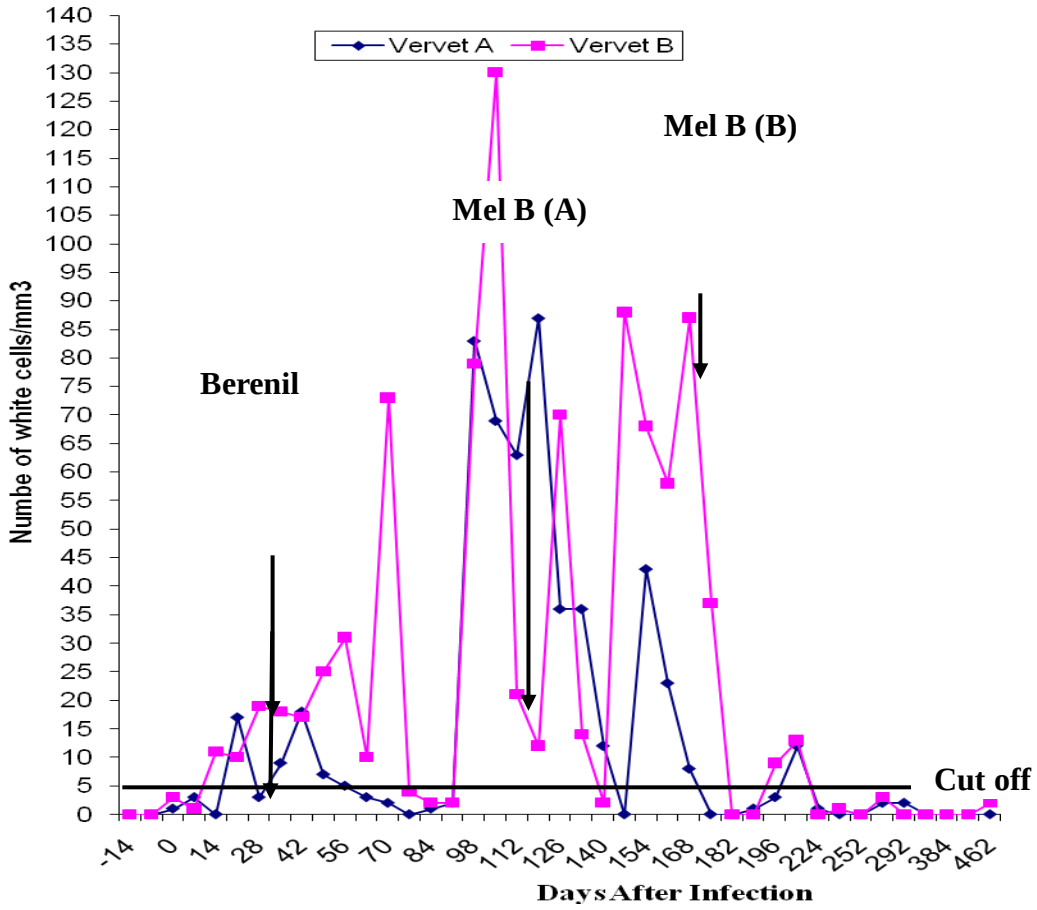

Figure 4.5: The number of White blood cells (No/mm^3) in the CSF of *T. b. rhodesiense* infected vervet monkeys treated with Berenil® and Mel B®.

4.7 Total cerebrospinal fluid protein concentration

In both vervet monkeys, the baseline level of total protein concentration ranged from 23-25 mg/dl. There was a significant increase in total protein concentration ($p<0.05$) which was >100mg/dl at 49 dpi. Total protein concentration increased above 25 mg/dl between

14 and 21 dpi. There was no significant difference in total protein concentration between the monkeys ($p>0.05$). After subcurative Berenil® treatment at 35 dpi, there was a rapid increase of total protein concentration in the CSF at 49 dpi (14 dpt with berenil). Curative Mel B® treatment at 113 dpi (78 dpt with Berenil®) and 171 dpi (136 dpt with Berenil®) in monkey A and B respectively, lead to decrease in total CSF protein concentration. However the total protein concentration in the CSF was above the pre-infection level. Protein concentration in the CSF did not return to the pre-infection level through out the study period.

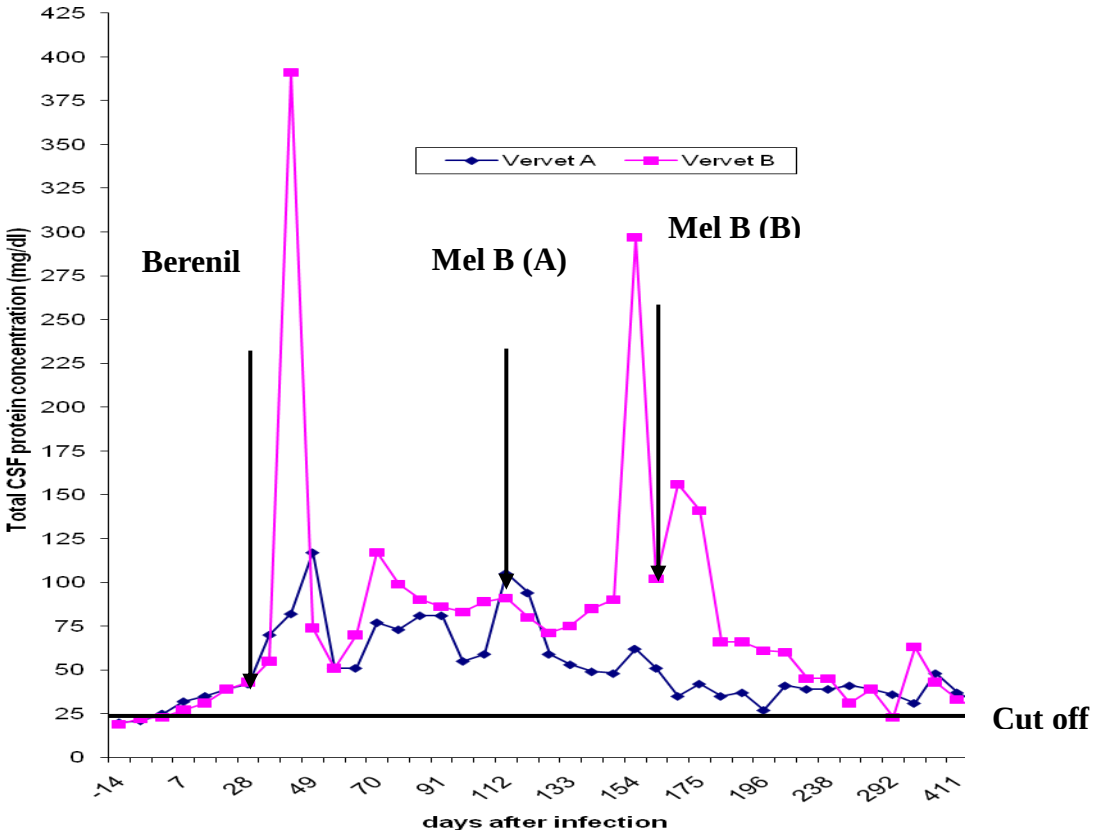

Figure 4.6: Total protein concentration in the CSF of *T. b. rhodesiense* infected vervet monkeys treated with Berenil® and Mel B®.

4.8 LAMP test

4.8.1 Detection of trypanosome DNA in the LAMP amplicon

All positive LAMP reactions turned green on addition of SYBR green 1, while negative ones remained orange (Figure 4.7). All positive LAMP reactions produced a characteristic ladder of multiple bands after electrophoresis on ethidium bromide (1µg/ml) stained agarose gel (Figure 4.8). *Ndei*I restriction enzyme digestion and electrophoresis gave the predicted sizes of 89 bp and 134 bp (Njiru *et al*., 2007) (Figure 4.9).

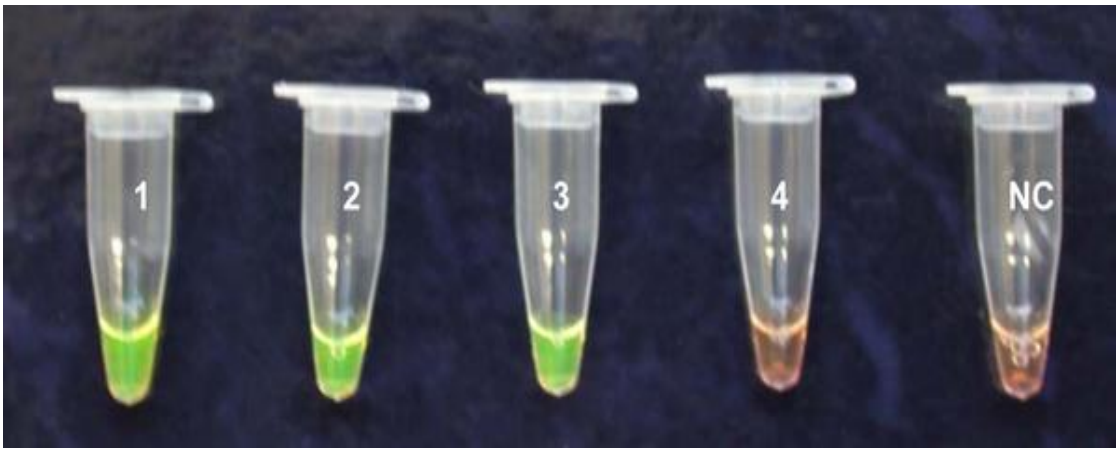

Figure 4.7: Visual appearance of LAMP reactions from samples after the addition of SYBR green I. The positive samples produce a green colour (tubes 1, 2 and 3) while negative samples remain orange (tube 4) and negative control (NC)

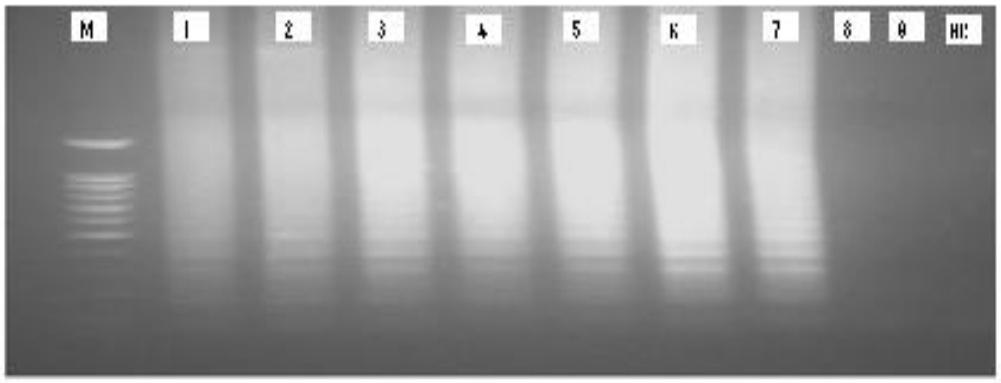

Figure 4.8: Electrophoresis analysis of LAMP amplified products after crude DNA extraction. M, 100 bp marker, lane 1-7 positive samples, lane 8 and 9 negative samples, NC, negative control.

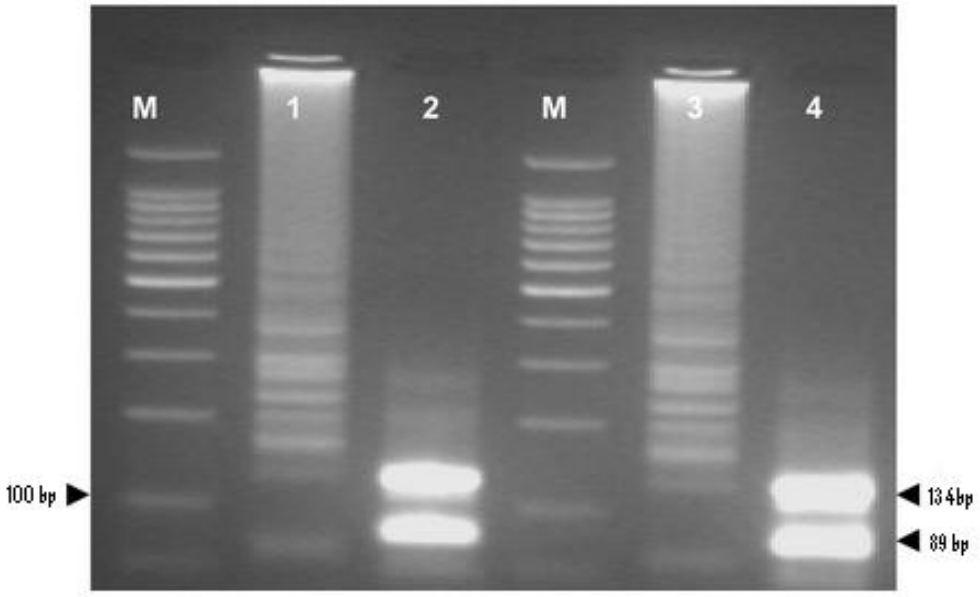

Figure 4.9: Electrophoresis analysis of LAMP amplified product results for positive samples lane 1 and 3 and their *Ndei*I restriction enzyme digests (lane 2 and 4). M, 100 bp marker

4.8.2 LAMP in different DNA extraction and amplification methods

The percentage trypanosome DNA detection ({No. of positive cases/total sample collected} x 100) in different DNA extraction and amplification methods in the blood, serum and CSF is presented in table 4.1.

Table 4.1: percentage trypanosome DNA detection by lamp for different DNAextraction and amplification methods in blood, serum and CSF

Sample	Method of DNA extraction and amplification and percentage (%) detection			
	Pure DNA; thermocycler (%)	Pure DNA; water bath (%)	Crude DNA; thermocycler (%)	Crude DNA; water bath (%)
Blood	60.27	46.15`	56.41	48.72
Serum	55.13	48.72	56.41	44.87
CSF	78.21	75.64	76.92	64.10

Amplification on a thermocycler resulted into higher detection rate than amplification on a water bath. The use of serum had low detection rate when compared to blood and CSF, while CSF had high detection rate regardless of DNA extraction and amplification method. The X^2-Statistics comparing the trypanosome DNA detection in different DNA extraction and amplification methods in blood serum and CSF is presented in Table 4.2.

Table 4.2: Chi-Square values (X2) indicating the differences between DNA extraction and amplification method in detection of trypanosome in blood, serum and CSF by LAMP

DNA extraction and amplification method	Chi-Square value (X^2); p-values		
	Blood	Serum	CSF
Pure DNA Vs crude DNA; thermocycler (N=78)	0.026 (p=0.873)	0.646 (0.421)	1.53 (p=0.216)
Pure DNA Vs Crude DNA; water bath (N=78)	0.273 (p=0.626)	0.026 (p=0.872)	0.037 (p=0.848)
Pure DNA; thermocycler Vs Pure DNA water bath	3.153 (p=0.078)	0.642 (p=0.423)	0.001 (p=0.975)
Crude DNA; Thermocycler Vs Crude DNA; Water bath	1.258 (p=0.262)	3.108 (p=0.068)	2.026 (p=0.155)

There were no significant differences between the performance of LAMP in detection of trypanosome on a thermocycler and a water bath method in the blood, serum and CSF. Similarly, there were no significant differences between pure and crude DNA in detection of trypanosome in the blood, serum and CSF. Therefore, there were no significant differences of LAMP in the detection of trypanosome for the different DNA extraction and amplification methods in the blood, serum and CSF.

The agreement of LAMP between different DNA extraction and amplification methods in detection of trypanosome DNA was also assessed and is presented in Table 4.3.

Table 4.3: Agreement of LAMP between different DNA extraction and amplification method in detection of trypanosome DNA in different samples

sample preparation/amplification method for LAMP	Percent agreement (k)		
	Blood	Serum	CSF
Pure DNA Vs crude DNA; thermocycler	0.039	0.013	0.116
Pure DNA Vs Crude DNA; water bath	0.038	0.052	0.038
Thermocycler Vs water bath; Pure DNA	0.141	0.064	0.025
Thermocycler Vs Water bath; Crude DNA	0.088	0.012	0.435

There were marginal agreements between pure and crude DNA on a thermocycler and a water bath in detection of trypanosome in the blood, serum and CSF. Amplification on a thermocycler and a water bath had marginal agreements for pure DNA in the blood, serum and CSF. However, for crude DNA amplified on a thermocycler and a water bath, the agreement was marginal in the blood and serum and good in the CSF. Therefore, the performance of LAMP on the different DNA extraction and amplification methods was similar in the blood, serum and CSF.

4.8.3 LAMP in different Samples

LAMP showed no significant difference and marginal agreements between different DNA extraction and amplification methods (Table 4.3 and Table 4.4). Therefore, crude DNA on a thermocycler was used in evaluating the performance of LAMP different samples. The difference of between serum and CSF in detection of trypanosome using LAMP with respect to blood was assessed. The agreement and X^2-Statistics comparing

the difference between different samples in detection of trypanosome using LAMP for crude DNA on a thermocycler is presented in Table 4.

Table 4.4: Chi-Square values (X2) of LAMP in detection of trypanosome DNA in serum and CSF versus blood

Sample	Difference or agreement		
	Chi-Square values (X^2); p-values	Percent agreement (k)	Remarks
Serum	14.501 (p=0.000)	0.017	Marginal agreement
CSF	9.510 (p=0.002)	0.205	Marginal agreement

There was a significant difference between blood and serum in detection of trypanosome DNA using LAMP. A significant difference between blood and CSF in detection of trypanosome DNA using LAMP was also obtained. There were marginal agreements between blood and serum and between blood and CSF in detection of trypanosome using LAMP.

4.8.4 Comparison of LAMP with microscopy in detection of trypanosomes

Microscopy and LAMP detected live trypanosomes and trypanosome DNA respectively. Parasitological detection is used as a criterion in diagnosis of the disease. The percentage trypanosome detection, agreement (k) and X^2-Statistics comparing LAMP and microscopy in detection of trypanosomes for crude DNA on a thermocycler in the blood and CSF is presented in Table 4.5.

Table 4.5: Percentage trypanosome detection, agreement (k) and Chi-Square (X2) values indicating the agreement and difference between LAMP and microscopy in detection of trypanosome in blood and CSF.

Sample	Detection method			
	Microscopy (% detection)	LAMP (% detection)	% Agreement (k)	Difference (X^2; p-values)
Blood	28.21	60.26	0.397	13.141 (p=0.000)
CSF	21.18	79.49	0.602	35.247 (p=0.000)

Of the total 78 sample points for the two vervet monkeys in the blood, serum and CSF, LAMP had higher detection rate than microscopy in the blood and CSF. There were significant differences ($p<0.05$) between LAMP and microscopy in detection of trypanosomes both in the blood and CSF (Table 5)**.** The agreement between LAMP and microscopy in detection of trypanosomes was marginal in the blood. However, the agreement of LAMP and microscopy in detection of trypanosome in the CSF was good (Table 4.5).

4.8.5 LAMP and microscopy at acute and late stage disease and after treatment

Trypanosome DNA was detected at 7 dpi in the blood and serum and starting at 21 dpi in the CSF. After subcurative berenil treatment, trypanosome DNA cleared by 56 dpi (21 dpt with Berenil®) in the blood and serum of both monkeys. However, trypanosome DNA was later detected at 77 dpi (42 dpt with Berenil®) and 129 dpi (84 dpt with Berenil®) in the blood and serum of monkey A and B respectively. Following **s**ubcurative Berenil® treatment, CSF remained positive of trypanosome DNA. After Mel B® treatment at 113 and 171 dpi, in monkey A and B respectively, trypanosome

DNA cleared after 40 and 90 days and 90 and 150 days in the blood and serum and CSF of vervet monkey A and B respectively. Trypanosome DNA cleared earlier in the serum than in the blood.

In staging of the disease, trypanosome DNA detection using LAMP was compared with the gold standard microscopy examination in detection of trypanosomes at different stages of the disease in blood and CSF. The percentage agreement (k) and X^2-Statistics between LAMP and microscopy in detection of trypanosomes in the blood and CSF at different stages of the disease is presented in Table 4.6.

Table 4.6: Percentage agreement (k) and Chi-Square values (X2) between LAMP and microscopy in detection of trypanosomes at different stages of the disease

Sample	Percent agreement (k) and Chi-Square value (X^2); p-values		
	Percent agreement (k)	Remarks	(X^2); p-values
	Early stage (0-35 dpi)		
Blood	0.143	Marginal agreement	0.000 (p=1.000)
CSF	0.088	Marginal agreement	0.168 (p=0.042)
	Late stage (35 dpi - until treatment with Mel B)		
Blood	0.233	Marginal agreement	3.270 (p=0.071)
CSF	0.600	Good agreement	15.000 (p=0.000)
	After treatment with Mel B® (post-treatment follow-up period)		
Blood	0.472	Good agreement	19.249 (p=0.000)
CSF	0.527	Good agreement	21.346 (p=0.000)

In the early stages of the disease, there was no significant difference ($p>0.05$) between LAMP and microscopy in detection of trypanosomes both in the blood and CSF. As the disease progressed to the late stage, there was no significant difference ($p>0.05$) between LAMP and microscopy in diagnosis in the blood. However, a significant difference ($p<0.05$) between LAMP and microscopy was obtained in the CSF. After treatment, LAMP and microscopy had significant differences ($p<0.05$) in detection of trypanosomes both in the blood and CSF.

During early stage, there were marginal agreements between LAMP and microscopy in detection of trypanosomes both in the blood and CSF. In the late stage, the agreement between LAMP and microscopy was marginal in the blood. However, in the CSF, the agreement between LAMP and microscopy was good. After treatment, LAMP and microscopy had good agreements both in the blood and CSF.

CHAPTER FIVE

DISCUSSION

5.1 Diagnosis of HAT using clinical parameters parasitological methods

Diagnosis of HAT relies on clinical signs and microscopy. These parameters may not be sufficient for diagnosis. In the study, fever, loss of body weight, spleenomegarly and lymphadenopathy were observed. However, in earlier studies, the development of clinical signs in different vervet monkeys infected with the same stabilate differed (Thuita *et al*., 2008). This was replicated in the study when the two vervet monkeys infected with the same stabilate *T. b. rhodesiense* KETRI 2537 and produced different course of the disease in term of development of clinical signs. This, therefore further suggests that clinical parameters are not sufficient enough in diagnosis of the disease.

Parasitaemia detected by microscopy progressed differently in the two vervet monkeys. At the same time parasites in the CSF were undetectable. These observations confirmed the insufficient nature of clinical signs and microscopy in diagnosis of the disease. Therefore for proper diagnosis, clinical parameters and microscopy should be supplemented with other diagnostic methods.

5.2 White blood cell counts and total CSF proteins in staging the disease

Staging and post-treatment follow-up is done by detecting trypanosomes, elevated total protein concentration and WBC counts in the CSF. Increased WBC counts >5cell/mm^3 and total protein concentration >25mg/dl starting from 14 dpi was observed in the CSF indicating the initiation of late stage. Trypanosomes were detected in the CSF at 21 dpi. One limitation of the study was that CSF sampling done on weekly basis because of

ethical issues. However, the study shows that the vervet monkey model can be used in staging of the disease (Schimdt and Sayer, 1982)

After Berenil® and Mel B® treatment, parasites were undetectable in the blood and CSF respectively, after 3 days. However, the DNA was detected. The WBC counts decreased to normal level between 50-150 days post Mel B® treatment. Increased WBC counts >5cells/mm^3 is regarded as a reliable test for staging the disease (WHO, 1983). However, the effectiveness of this parameter as a staging and a diagnostic measure has been doubtful (Kagira *et al*., 2006). Another disadvantage of WBC count in CSF is that the accuracy of the haemocytometer is about equal to the upper limit of normal count (Burechailo and Canningham, 1974) and limited by cell lysis during counting (Green-wood and Whittle, 1976b).

The total protein concentration in the CSF decreased after Mel B® treatment but was still >25mg/dl at end of monitoring period. Earlier studies have noted that total protein concentration vary with age and method used (WHO, 1983; Lejon and Buscher, 2001). Increase in total protein concentration and WBC count for staging HAT are not specific (Ndung'u *et al*., 1994) while detection of trypanosomes in the CSF was difficult. Therefore the two methods cannot be separately used for staging of HAT.

5.3 LAMP in different DNA extraction and amplification methods

Although the trypanosome detection rate using both thermocycler and a water bath on pure and crude DNA varied, these differences were not significant ($p>0.05$) and the agreements were marginal. Therefore, the performance of LAMP on the different DNA

extraction and amplification methods was similar. This means that crude DNA on a water bath may be adopted in LAMP. The invention of a heat block has resolved the challenges of controlling the temperature. This further extends the application of LAMP in areas where thermocyclers cannot be used besides reducing the operation cost of the test

Assessing the results of crude and pure DNA on LAMP, marginal agreements were obtained and there was no significant difference ($p>0.05$). This implies that the pure DNA extraction step which is very expensive and require expertise can be omitted further shortening the time for the test and reducing the cost. Similar findings using *RIME* gene were reported by Njiru *et al*., (2007).

5.4 Detection of trypanosome using LAMP and microscopy

Detection of trypanosome DNA in LAMP amplicon by electrophoresis in ethidium stained agarose gel is a post-amplification processing. This increased the time for LAMP test to give results and may also introduce risks for contaminations (Njiru *et al*., 2007). Therefore, visualization of colour changes after addition of fluorescent dye, SYBR Green I dye gave results immediately. There is no post-amplification processing thus reducing the time to give results and also reduces the cost of the test.

Both microscopy and LAMP detected trypanosomes at different times with higher detection rate in LAMP. The significant difference ($p<0.05$) between the two methods in detection of trypanosomes suggests the sensitive of LAMP compared to microscopy.

These results are in agreement with those of Njiru *et al*., (2007) and Matovu *et al*., (2011)

5.5 LAMP in detection of trypanosomes in different samples

The high detection rate of LAMP in the CSF had regardless of DNA extraction and amplification method may be attributed to its purity. Indeed a marginal agreement and a significant difference ($p<0.05$) was noted between blood and CSF. Blood and serum may contain myoglobin, haeme-blood protein complex, IgG and other immune complexes which inhibit PCR (Akane *et al*., 1994; Al-Soud *et al*., 2000; Belec *et al*., 1998; Johnson *et al*., 1995; Al-Soud *et al*., 2001). These compounds may also inhibit LAMP but are absent in CSF, thus the good performance of LAMP in CSF than blood and serum. Althouth marginal agreement and significant difference ($p<0.05$) was noted between blood and serum in detection of trypanosome DNA in LAMP test, blood can be the preferred sample owing to tiresome and time consuming procedure of processing blood for serum.

5.6 LAMP at different stages of the disease

5.6.1 Early stage

Although trypanosome DNA was detected at 7 dpi in the blood and serum, it was expected to be detected much earlier (Jamonneau *et al*., 2003). However, samples were collected on weekly basis starting 7 dpi. Sample collection on daily basis was not possible due to ethical considerations when dealing with non-human primate experiments. The marginal agreements and insignificant difference ($p>0.05$) noted between LAMP and microscopy in detection of trypanosomes during early stages of the

infections needs to be investigated. Therefore, for proper evaluation of LAMP during early stages in such studies, it's recommended that sample be collected on daily basis.

5.6.2 Late stage

Trypanosomes were detected in the CSF at 21 dpi in vervet monkey A and 28 dpi in vervet monkey B. However, the DNA was detected at 21 dpi in both vervet monkeys confirming the sensitivity of LAMP. As the disease progressed to the late stage, good agreement and a significant difference ($p<0.05$) was noted between LAMP and microscopy in detection of trypanosomes in the CSF. In the blood marginal agreement and insignificant difference ($p>0.05$) was note. Due to the low sensitivity of CSF parasitosis, LAMP test in CSF can be a good test for staging the disease. Another advantage of LAMP is that it is a specific test as compare to WBC count and protein concentration.

5.6.3 Post-treatment

After curative Mel B® treatment, good agreements and significant differences ($p<0.005$) were noted between LAMP and microscopy in detection of trypanosomes. Microscopy, could not detect trypanosomes both in the blood and CSF 3 days after treatment. However, trypanosome DNA was still present and could be detected by LAMP in the CSF 120 to 150 dpt. Different PCR assays have been developed in detection of trypanosome DNA (Welburn *et al*., 2001; Gibson *et al*., 2002; Radwanska *et al*., 2002 Jamonneau *et al*., 2003), none of them has been evaluated. However, LAMP has been evaluated in diagnosis of HAT (Thekisoe *et al*., 2005; Thekisoe *et al*., 2008, Matovu *et al*., 2010)

These observations of all the studies that were done therefore, suggest that LAMP can be used as an alternative method in monitoring treatment outcome of the disease. This study shows that for post-treatment follow-up, at least 5 months may be adequate for LAMP. The current methods used (microscopy, total protein concentration and WBC count), follow-up is done for a period of 24 to 36 months. In this context therefore, it's important to note that LAMP could be useful in diagnosis, staging and monitoring treatment outcome.

5.7 Practicability of LAMP in diagnosis of HAT

The trypanosomes-infected vervet monkey model of HAT (Ngotho *et al*., 2008; Maina *et al*., 2004; Ngure *et al*., 2000; Gichuki and Brun, 1999; Ndung'u *et al*., 1994) has been shown to develop both clinical and pathological changes mimicking the human disease. However, no comparable studies have been conducted in vervet monkey model for the purpose of diagnosis and monitoring treatment outcome using LAMP. The present study investigated the use of LAMP in diagnosis, staging and monitoring treatment of *T. b. rhodesiense* form of HAT in vervet monkey model.

LAMP has been used in diagnosis of HAT using archived clinical samples (Kuboki *et al*., 2003; Thekisoe *et al*., 2005; Njiru *et al*., 2007; Njiru *et al*., 2008). In this study, LAMP based on *RIME* gene (Njiru *et al*., 2007) was used. It has been suggested that the amplification method may be suitable for both diagnosis and post-treatment follow-up. This is because, LAMP utilizes *Bst* DNA polymerase large fragment with optimal temperature ranging from 60^{o}C to 65^{o}C inactivated at 80^{0}C after the LAMP reaction

(Notomi *et al*., 2000). This reduces the prospects of non-specific priming limiting false positive results.

A set of two specially designed inner and outer primers; forward inner primer (FIP) and backward inner primer (BIP) and outer primers; forward outer primer (F3) and backward outer primer (B3) are used (Notomi *et al*., 2000). Two more primers; loop forward primer (LF) and loop backward primer (LB) may be included. This increases sensitivity and acceleration of the reaction, reducing the time by a half (Nagamine *et al*., 2002). The use 4-6 primer pairs recognizing 6-8 region of targeted DNA increases specificity and sensitivity of the test (Notomi *et al*., 2000; Nagamine *et al*., 2002)

LAMP results can be visualized by addition of fluorescent dyes such as SYBR Green I under UV light (Iwamoto *et al*., 2003; Mori *et al*., 2006) or by turbidity that can be seen by the naked eyes (Mori *et al*., 2001). The dye, SYBR Green I was used and similar results noted in the study. This eliminates the need for electrophoresis in ethidium bromide stained agarose gel, a post- amplification processing and reduce the exposure to ethidium bromide, a potential mutagen.

The use of crude and pure DNA in LAMP was also assessed. The results of crude and pure DNA on LAMP had marginal agreements and no significant difference. This implies that the pure DNA extraction step can be omitted further shortening the time for the test. Similar findings using RIME gene were reported by Njiru *et al*., (2007). Blood and serum had similar results. Therefore, blood can be the preferred sample owing to tiresome and time consuming procedure of processing blood for serum.

Thermocycler and simple incubators such as heat block and water bath were previously noted to allow low concentration of DNA to be amplified within 35 minutes in a laboratory (Njiru *et al*., 2007). This makes LAMP cost friendly as compared to other molecular methods. In previous studies, LAMP showed superior tolerance to biological substances (Enomoto *et al*., 2005; Yamada *et al*., 2006; Kaneko *et al*., 2006). This was also noted in the study. The use of various heat-treated templates; blood, serum, CSF, native sera and buffy coat (Poon *et al*., 2006; Njiru *et al*., 2007) eliminate the need of pure DNA extraction, shortening further the time for LAMP reaction.

Previous studies have also shown that the use of reagents stored at varied temperature between 25^{o}C and 37^{o}C which is above the recommended storage temperature for LAMP reagents had no effects on the detection sensitivity (Thekisoe *et al*., 2008). This further suggests the cost friendliness of LAMP and promisibility for use and storage in Africa where high temperature are common. The use of different DNA template preparation and amplification methods (Njiru *et al*., 2007; Thekisoe *et al*., 2008) further promise the practicability of LAMP in diagnosis, staging and monitoring treatment of HAT.

Therefore, developing LAMP for detection of animal and human trypanosomes is of economic importance (Kuboki *et al*., 2003; Thekisoe *et al*., 2005; Thekisoe *et al*., 2007). However, further improvements on rapidity and simplicity of DNA preparation methods are required to enhance the practicability of LAMP for diagnosis, staging and monitoring treatment of HAT.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Diagnosis, staging and monitoring treatment follow-up of HAT has been a problem. Most of the methods that have been validated for this purpose are not satisfactory. This initiated the search for other methods to be used in diagnosis, staging and post-treatment follow-up. Because of closer phylogenetic relationship of between human and other non-human primates, the current study was investigating LAMP as an alternative method in vervet monkey model of the disease.

This study is aimed at solving the problems associated with diagnosis, staging and monitoring treatment of HAT. The study indicates that LAMP may be used to exclude false negative results caused by the less sensitive Gold standard microscopic methods in diagnosis and monitoring treatment. Although LAMP has been promising, further evaluation has to be done to evaluate its use and potential for it to be applied in the field. The study has shown that LAMP can be used as a treatment monitoring test, thereby reducing the current monitoring period from 24-36 months to 5 months.

6.2 Recommendations

From the results obtained in this vervet monkey model of HAT, LAMP can be a strong candidate in diagnosis, staging and monitoring treatment. Both blood and serum can be used as samples for LAMP in early stage. However, blood may be preferred due to the time consuming and tiresome procedure of processing blood for serum. Crude DNA

extraction through heat treatment may be adopted as sample preparation method. Amplification in a water bath can also be adopted.

Visualization of LAMP amplicon by addition of SYBR Green I dye immediately after amplification is cheap and thus recommended. It also reduces the time to get results of agarose gel electrophoresis and avoids the cost involved in the use ethidium bromide a potential mutagen. LAMP can be performed for both early and late diagnosis of the disease and during post-treatment follow-up. Further evaluation of LAMP on the same model, a large number of vervet monkeys and at the same time many sample obtained from many HAT patients is recommended.

Due to the zoonotic nature of African trypanosomiasis, development of LAMP based on a universal gene for all trypanosomes will be a breakthrough in solving the problem of diagnosis and monitoring treatment. Therefore development of LAMP based in all groups of trypanosomes should be a research priority. This will eliminate the problems in diagnosis and post-treatment follow-up of the disease.

Other tropical infections sometimes act as HAT-co-infections as they may co-exist or mimic HAT. Therefore, evaluation of LAMP test on other tropical primates or non-human primates model of HAT need to be undertaken. In diagnosis and monitoring treatment of HAT, a sensitive, faster, cheap, and easy to perform test is essential. Therefore, comparison of the cost of LAMP and other molecular tests, PCR and molecular dipstick test need to be done. Finally, development of LAMP kits that are user friendly needs to done probably by lyophilization of the LAMP master mix.

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APPENDICES

Appendix 1: Components of DNA extraction and other buffers; Saponin lysis buffer

0.15%w/v Saponin, 0.2%w/v NaCl and 1mM EDTA

Appendix 2: PCR buffer

50mM KCl, 1.5mM MgCl2 and 10mM of Tris-HCl, pH 8.3)

Appendix 3: TE Buffer

10mM Tris-HCL pH 8.0 and 0.25mM EDTA

Appendix 4: 1XTAE Buffer

Prepare 50X: 242g Tris base, 57.1ml glacial acetic acid and 100ml 0.5M EDTA (pH 8.0) and then dilute to 1XTAE working solution with distilled H_20