MOLECULAR CHARACTERIZATION AND PATHOGENICITY OF MELARSOPROL RESISTANT AND SENSITIVE *Trypanosoma brucei rhodesiense* ISOLATES FROM UGANDA

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DECLARATION AND RECOMMENDATION

I hereby declare that this thesis is my original work and has not been submitted wholly or in part for any award in any other institution of learning to the best of my knowledge.

Kavunga Membo Hugo

Signature:…… Date:……..

APPROVAL

We wish to confirm that this work was done under our supervision and has our approval to be presented for examination as per the Egerton University regulations

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DEDICATION

To my wife, Yanette Kavunga Akem for her immeasurable love, support and for her understanding when I had to be away from home to undertake the study.

To my parents, Joachim Kavunga Kiamfu and Helene Nsoki Kizinga for the sacrifices they made towards my education.

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LIST OF ABBREVIATIONS

BecANet	Biosciences eastern and central Africa network
CNS	Central nervous system
CSF	Cerebrospinal fluid
DFMO	Difluoromethylornithine
Dpi	Days post-infection
EATRO	East Africa Trypanosomiasis Research Organization
EDTA	Ethylene diamine tetra-acetic acid
GPI	Glycosyl phosphate inositol
HAPT	High affinity pentamidine transporter
HAT	Human African trypanosomosis
HAD	Helicase dependant amplification
LAPT	Lower affinity pentamidine transporter
MSF	Medecins sans frontiers
NEPAD	New partnership for African's development
ODC	Ornithine decarboxylase
PCV	Packed cell volume
PGS	Phosphate glucose sacharose
PSG	Phosphate saline glucose
RLFP- PCR	Restriction length fragment polymorphism-polymerase chain reaction
SRA	Serum resistance associated
TCA	Tricarboxylic acid

ABSTRACT

Molecular characterization and pathogenicity of two isolates of *Trypanosoma brucei rhodesiense*, one resistant to melarsoprol and the other sensitive to melarsoprol were investigated in Swiss white mice. The two trypanosome populations had been isolated from sleeping sickness patients in Uganda. The study aimed at investigating whether there are any molecular and pathogenesis differences between the two isolates. Each form of parasite was inoculated into eighteen mice at 10^4 trypanosomes/ml and treated with melarsoprol at various dosages while six mice were used as un-infected controls. Parasitaemia progression was monitored every two days for sixty days to confirm the status of cure. Molecular characterization was undertaken by extracting and amplifying specific parasite DNA for the *brucei* subgroup first using TBR1 and TBR2 primers then for determining the presence of SRA gene in the strains. Pre-patent period, parasitaemia progression, packed cell volume, body weight and survival time were monitored for sixty days in infected Swiss white mice as markers of pathogenicity. The sensitivity test revealed that all mice infected with melarsoprol sensitive isolate and treated were cured. However, only one mouse infected with melarsoprol resistant isolate and treated was aparasitaemic for sixty days post infection. No molecular differences were observed since the fragment of DNA was amplified at the same size in both strains using all the primers. Mice infected with melarsoprol sensitive isolate had the shortest pre-patent period and survival time, the parasitaemia levels of this group of mice increased faster than in the group infected with resistant isolate. The rate of decline of packed cell volume and body weight was faster in the drug sensitive group than the drug resistant group of mice, however the differences were not significant ($P>0.05$). Results from this study demonstrated that the sensitive isolate appeared relatively more pathogenic than the resistant isolate. It is recommended that similar studies using a higher number of isolates from different regions and using also more advanced molecular techniques such as DNA sequencing, RFLP-PCR be carried out, this would shed some light on the genetic basis of pathogenesis.

CHAPTER ONE
INTRODUCTION

1.1 Background Information

Human African trypanosomosis (HAT) constitutes a serious public health threat in Africa, particularly in East and Central Africa, where approximately 60 million people are at risk of contracting the disease (WHO, 1998). The 45,000 new cases reported by the World Health Organization do not accurately reflect the real situation. It is however estimated that 300,000-500,000 persons are infected with Human African trypanosomosis (WHO, 2000). Since 1970 the incidence of the disease has been increasing with the control of African trypanosomosis being complicated by poverty, political instability and civil wars often occurring in areas endemic for the parasite (Gibson, 2002). The parasite responsible for causing African sleeping sickness belongs to a group of closely related trypanosomes in the *Trypanosoma brucei (T.b)* species complex (Vickerman, 1985). Three morphologically indistinguishable species are recognized, namely *Trypanosoma brucei brucei*, *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense*.

T. b. brucei is a natural parasite of wild game in Africa and is non-infective to humans (Anene *et al*, 2001; Barret *et al*, 2003). The inability to infect humans is due to a 'trypanosome lytic factor' found in human sera (Vanhammel *et al*, 2003). As the names imply, *T. b. gambiense* and *T. b. rhodesiense* are distinguished by their geographical distributions. *T. b. rhodesiense* is found in East and southern Africa and *T. b. gambiense* is found in West and Central Africa.

The restricted distributions of the African trypanosomes are determined by the vectors subspecies, commonly known as the tsetse. In addition to being transmitted by different vectors species, *T. b. gambiense* and *T. b. rhodesiense* are distinguished by animal reservoirs, epidemiology and disease virulence (Baker, 1974). The tsetse vector of *T. b. rhodesiense* is found in woodland or dry bush environments. Its natural vertebrate host is the antelope and other wild ungulates. Disease transmission depends upon contact with infected vectors and is often associated with hunters or safaris (Lean *et al*, 2004). *T. b. rhodesiense* is primarily a zoonosis and little human-fly-human transmission occurs.

The disease typically progressess rapidly and is often fatal without treatment (Molyneux *et al*, 1982). Although variant of *T. b. rhodesiense* which cause slowly progressing chronic disease have been identified, molecular data suggest that *T. b. rhodesiense* may be a host range variant of *T. b. brucei* (Jennings & Urquhart, 1985).

T. b. gambiense is transmitted by vectors found along rivers and lakes which are often in close proximity to human habitation. Consequently, the disease tends to be endemic and domestic animals may serve as reservoirs (Mehlitz *et al*, 1982). The disease is much less virulent and is characterized by a slow progression from an acute disease to a chronic disease. It is believed that *T. b. gambiense* has been associated with humans for much longer than *T. b. rhodesiense* and thus possibly accounts for the lower virulence of *T. b. gambiense* (Gibson, 2002). African trypanosomes are not only pathogenic parasites but also highly fascinating protozoa suitable for molecular analyses. *Trypanosoma brucei* spp. can be cultivated axenically and cloned *in vitro* (Brun & Schonenberger, 1979; Baltz *et al*, 1985; Carruthers & Cross, 1992), and are amenable to genetic manipulations such as gene knock-out by homologous recombination (Carruthers *et al*, 1993 ; Carruthers *et al*, 1996) or gene silencing by RNAi (Ngo *et al*, 1998). African trypanosomes already served as model parasites a hundred years ago in early pharmacological research, owing to their good visibility under a microscope and the reproducible course of infection in rodents (Hereld *et al*, 1986).

1.2 Statement of the Problem

Human African trypanosomosis is found in 36 African countries (WHO, 2000). Today, there are only a handful of active drugs available for treatment of human African trypanosomosis. No significant drug development has been made over the last 2 decades. Studies carried out show that some trypanosomes have developed resistance to both early and late stage of treatment. At the same time variation in pathogenicity of *T. b. gambiense* and *T. b. rhodesiense* have also been shown. There is therefore need to understand the genetic basis of drug resistance in *T. b. rhodesiense* and its implications in pathogenesis to circumvent existing resistance problem and avoid the emergence of resistance to the next generation of drugs.

1.3 Main Objective

To determine the molecular differences between melarsoprol resistant and melarsoprol sensitive *T. b. rhodesiense* isolates from Uganda and the effect of the difference in parasite pathogenesis.

1.3.1 Specific objectives

1. To establish the drug response profiles of melarsoprol sensitive and melarsoprol resistant isolates of *T. b. rhodesiense* from Uganda following cryopreservation.
2. To determine molecular identity between melarsoprol sensitive and melarsoprol resistant *T. b. rhodesiense* isolates from Uganda.
3. To evaluate differences in pathogenesis of melarsoprol sensitive and melarsoprol resistant *T. b. rhodesiense* isolates from Uganda in Swiss white mice.

1.4 Hypotheses

1. Profiles of melarsoprol sensitive and melarsoprol resistant isolates from Uganda do not change with cryopreservation.

2. There are no molecular identity differences between melarsoprol sensitive and melarsoprol resistant *T. b. rhodesiense* isolates from Uganda.

3. There is no correlation between *T. b. rhodesiense* pathogenicity and melarsoprol sensitivity/resistance.

1.5 Justification

The problem of drug resistance in *Trypanosoma brucei* appears to be increasing in the field. Sleeping sickness has recently become resurgent in sub–saharan Africa and the emergence of drug resistance is hindering efforts to control the disease. In particular melarsoprol treatment failures have reached alarming levels in several HAT endemic foci. Some *T. b. rhodesiense* are known to cause chronic infection while others cause acute infections. The trypanosomal gene *Trypanosoma brucei* adenosine transporter1 (*Tb*AT1) encodes purine permease P2, which mediates influx of melarsoprol and diamine. The disruption of *Trypanosoma brucei* adenosine transporter1gene reduced sensitivity to these

trypanocides. *Trypanosoma brucei* multidrugs resistance proteins associated (*Tb*MRPA) encodes a putative trypanothione-conjugate efflux pump and over expression of *Tb*MRPA in *Trypanosoma brucei* causes melarsoprol resistance. The severity of the disease and response to treatment might be dependent on pathogenic and genetic aspects of the parasite. Thus, better understanding of genetic and/or pathogenic basis of drug resistance may lead to early detection of drug resistant trypanosome in the field and thus improve drug treatment for sleeping sickness.

1.6 Expected Output

1. Profiles of melarsoprol sensitive and melarsoprol resistant cryopreserved isolates determined.

2. The molecular diversity between melarsoprol sensitive and melarsoprol resistant isolates established.

3. Better understanding of the relationship between the disease severity and melarsoprol resistant/sensitivity.

4. Thesis for a Master of Science degree in Biochemistry and publications in peer review journal.

CHAPTER TWO

LITERATURE REVIEW

2.1 Human African Trypanosomosis

There are two species of trypanosomes that affect humans namely *T. b. rhodesiense* and *T. b. gambiense* which are morphologically undistinguishable from the closely related *T. b. brucei* that cause disease in animals (Dumas *et al*, 2000). Human African trypanosomosis is found in sub-saharan Africa between latitudes 14^0N and 29^0S within the geographical location of tsetse fly where, according to WHO (1998), about 200 endemic foci exist in 36 countries. An estimated 60 million people are at risk, with about 50,000 cases being reported annually (Kinung'hi *et al*, 2006). It is believed that the actual number of infected people could be between 300,000 and 500,000 (WHO, 2000). Human African trypanosomosis occurs in two forms (chronic and acute) which differ in clinical manifestation and geographical distribution. *T. b. gambiense* is found in West and Central Africa and is transmitted principally by *Glossina palpalis* and *Glossina tachinoides*. The disease is known as Gambian sleeping sickness and is usually more chronic. On the other hand *T. b. rhodesiense* occurs in East and Southern Africa and is transmitted by *Glossina morsistant, Glossina pallidipes, Glossina fuscipes, Glossina tachinoides*. This form of the disease, also known as Rhodesian sleeping sickness is more acute and could cause death within few weeks or months., WHO statistics indicate that *gambiense* trypanosomosis has a wider geographical spread than Rhodesian disease with nearly 80% of Human African Trypanosomosis endemic countries infected with *T. b. gambiense* (Ripamonto *et al*, 2002).

2.1.1 Gambian sleeping sickness

The epidemiology of *T. b. gambiense* sleeping sickness is not fully understood. *T. b. gambiense* has a long evolutionary history with humans and has become successfully adapted to establishing infections in human hosts without manifesting severe symptoms. The disease is typically chronic, with low levels of trypanosomes in circulating blood, which makes it difficult to detect the parasites in blood smears (Jordan, 1985).

Several different reservoirs for *T. b. gambiense* have been identified, suggesting that animals such as the African domestic pigs may act as reservoir host and thus maintain the persistence

of sleeping sickness in human populations (Gibson, 2002). Pigs have been identified as reservoir host for *T. b. gambiense* and are associated with the persistence and epidemics of sleeping sickness in Uganda, Equatorial Guinea, Cameroon and Nigeria (Mehllitz *et al*, 1982). Other mammalian host include dogs, sheep, cattle, rodents, monkeys and a range of game animals. These animal reservoir hosts have been identified as one of the principal factors associated with the persistence of sleeping sickness in endemic areas in-spite of chemotherapeutic campaigns (Noireau *et al*, 1989).

2.1.2 Rhodesian sleeping sickness

East African sleeping sickness differs from West African sleeping sickness in both its epidemiology as well as its clinical manifestation (Baker, 1974). The clinical symptoms of East African sleeping sickness are more severe and the onset of the disease is rapid. *T. b. rhodesiense* occurs with higher levels of parasitaemia in ungulates and with humans being the adventitious hosts (Molyneaux *et al,* 1982). The vectors of *T. b. rhodesiense* are the *G. morsitans* subspecies, *G. pallidipes* and *G. swynnertoni*, and to a lesser extent the peridomestic vectors from the *G. fuscipes* and *G. tachinoides*. Cases usually arise from among those in the population whose activities bring them into contact with the savannah woodland habitats of the *morsitants* group. Drought and political turmoil are known to increase the number of cases when entire communities relocate to hitherto unoccupied areas in search of safety or fertile lands and water (Molyneaux *et al*, 1982).

2.2 Life Cycle of *T. b. rhodesiense*

During a blood meal on the mammalian host, an infected tsetsefly injects metacyclic trypomastigotes into skin tissue. The parasites enter the lymphatic system and pass into the bloodstream inside the mammalian host, where they transform into bloodstream trypomastigotes. The trypamastigote are carried to other sites throughout the body, reaching other blood fluids such as lymph, spinal fluid and continue their replication by binary fission. The tsetse fly becomes infected with bloodstream trypomastigotes when taking a blood meal on an infected mammalian host. In the fly's midgut, the parasites transform into procyclic trypomastigotes, multiply by binary fission, leave the midgut and transform into epimastigotes. The epimastigotes reach the fly's salivary glands and continue multiplication

by binary fission. The cycle in the fly takes approximately three weeks. Humans are the main reservoir for *T. b. gambiense*, but this species can also be found in animals. Wild game animals are the main reservoir of *T. b. rhodesiense.*

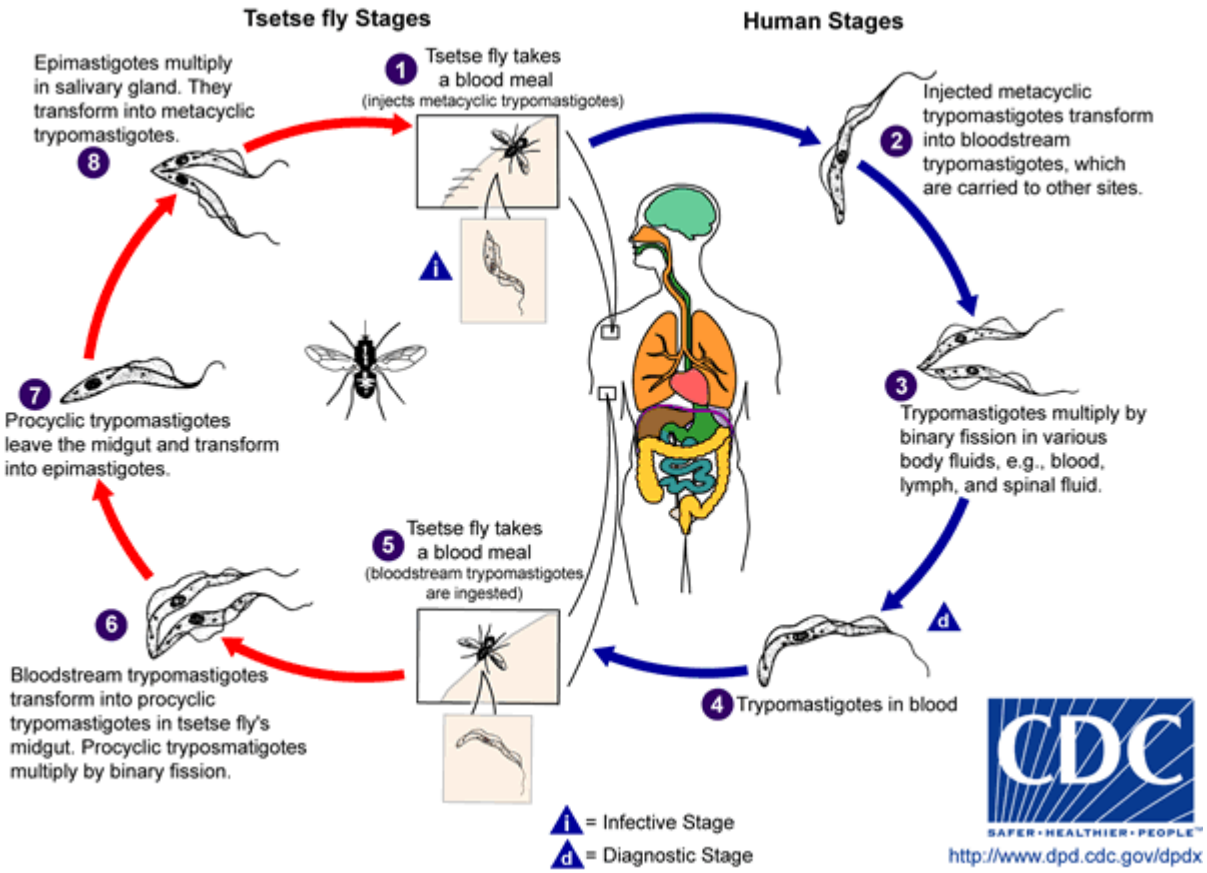


Figure 1: Life cycle of *Trypanosoma brucei rhodesiense*. Source: Centers for Disease Control and Prevention (CDC), 2000.

2.3 Trypanosomes

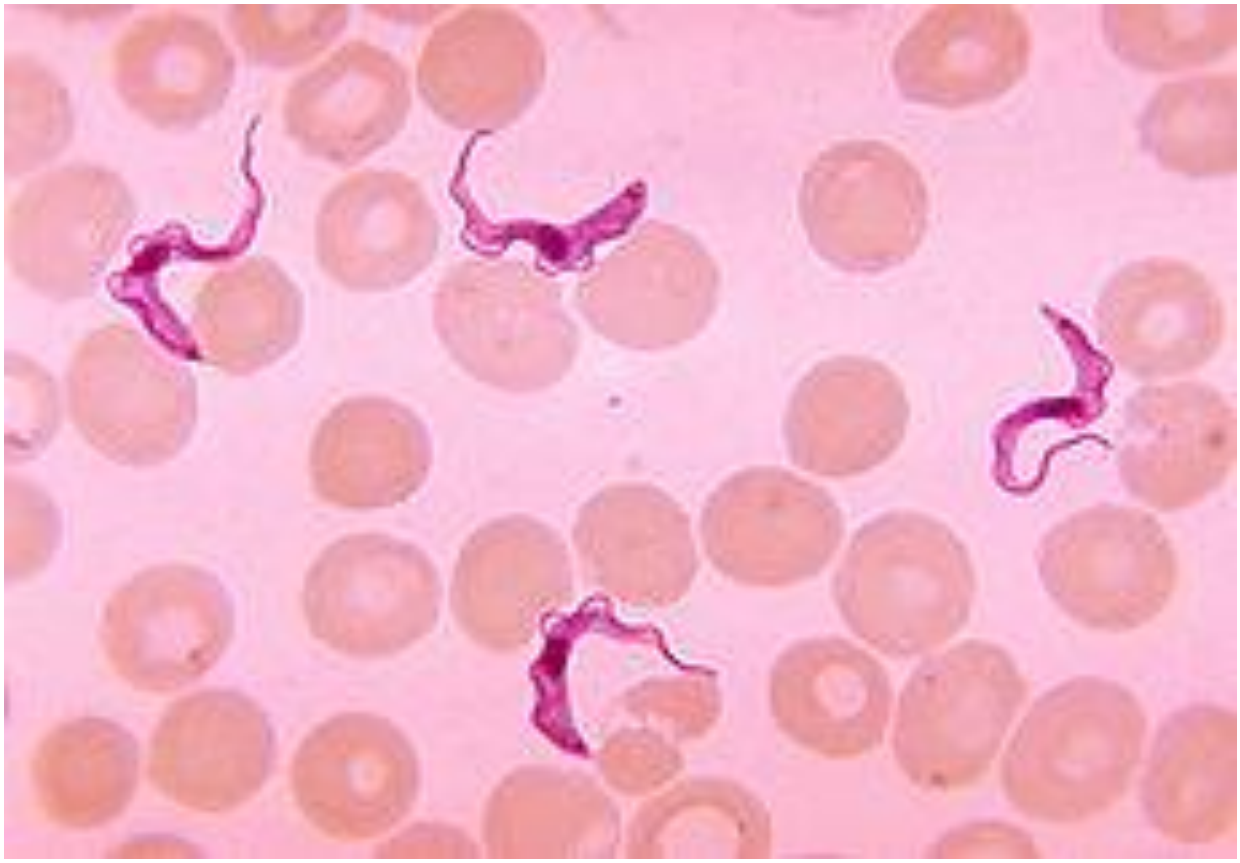


Figure 2: BloodstreamTrypomastigote forms of *T. b. rhodesiense* on peripheral thin blood smear. Source: Centers for Disease Control and Prevention (CDC), 2000.

African trypanosomes are flagellated protozoa that inhabit the extracellular compartment of host blood. The spindle-shaped parasites are about 20μm long and about 2μm in diameter at their widest point, have a single flagellum and are motile (Black & Seed, 2001). African trypanosomes are transmitted to mammals in saliva deposited by biting tsetseflies in which they undergo cyclic development (Hereld *et al*, 1986). Trypanosomes have a single specialised mitochondrion called a kinetoplast mitochondrion. One of its unusual features is that the entire deoxyribonucleic acid (DNA) of the mitochondrion, which can be up to 25% of the total cell DNA, is localized in the kinetoplast, adjacent to the flagellar pocket (Black & Seed, 2001). Kinetoplast DNA (kDNA) exists in two forms namely mini-circles and maxi-

circles (Gibson, 2002). Mini-circle DNA encodes guide ribonucleic acid (RNA) that direct extensive editing of RNA transcripts post-transcriptionally. Maxi-circle DNA contains sequences that, when edited, direct translation of typically mitochondrially-encoded proteins (Gibson, 2002). In the vertebrate host, trypanosomes depend entirely upon glucose for energy and are highly aerobic, despite the fact that the kinetoplast-mitochondrion completely lacks cytochromes. Instead, mitochondrial oxygen consumption is based on an alternative oxidase that does not produce adenosine triphosphate (ATP). When in the insect vector, the parasite develops a conventional cytochrome chain and tricarboxylic acid (TCA) cycle (Pays, 2006). The surface of the trypanosome has numerous membrane-associated transport proteins for obtaining nucleic acid bases, glucose and other small molecular weight nutrients (Borst, 2002). None of these proteins react well with antibodies, because although they lie in exposed regions of membrane, they are shielded by allosteric interference provided by the variant surface glycoprotein coat proteins (Pays, 2006).

2.4 Mechanisms for Evading Immune Responses

The observation that an extracellular parasite which is found in the bloodstream causes a long-lasting chronic disease poses a paradox. Normally the host immune system is quite efficient at generating antibodies against infectious organisms and eliminating them from the circulation (Takeda *et al*, 2005). In this regard, a characteristic of African trypanosomosis is a fluctuating parasitaemia. In other word the number of parasites in circulation dramatically rises and falls (Borst, 2002). Generally, fever and other clinical symptoms are associated with the peaks in parasitaemia (Burri & Brun, 2003). Further examination of parasites obtained from successive peaks reveals that they are antigenically distinct or exhibit variant antigenic types (VAT) (Cross, 1990; Borst & Rudenko, 1994).

The VAT is determined by a protein known as the variant surface glycoprotein (VSG). VSG is an abundant protein and is a major component of the 12-15 nm thick electron-dense “surface coat” covering bloodstream trypomastigotes (Blum *et al*, 1993). The parasite is estimated to have more than 1000 distinct VSG genes. Periodically the parasite will express a different VSG gene which is antigenically distinct from the previously expressed VSG with a

switch rate of approximately 10^2 per cell per generation (Pays, 2006). Comparing the sequences of the various VSG genes reveals an N-terminal variable domain and a C-terminal conserved domain. Also at the C-terminus is a glycophosphate inisitol (GPI) anchor which is embedded into the lipid bilayer of the plasma membrane (Gough & Gordon, 2000). The VSG molecules fold and pack together in a manner so that the conserved C-terminal region is not directly accessible and that only the variable N-terminal region is exposed to host immune system (Donelson, 2003; Pays, 2006). VSG is immunogenic and antibodies against VSG do lead to parasite elimination. Switching expression to another VSG, results in a new surface which is now not recognized by the host antibodies (Cardoso de Almeida & Turner, 1983; Hereld *et al,* 1986). These parasites will then rapidly increase in number until the host mounts an immune response against the new VSG. The large repertoire of antigenically distinct VSG proteins means that the parasite stays one step ahead of the host and avoids complete elimination by the immune system (Taylor & Rudenko, 2006).

2.5 Clinical Presentation

Human African trypanosomosis is a disease that evolves through clinically distinct stages, with a lethal outcome if left untreated (Dumas & Bisser, 1999).

2.5.1 Local symptoms

The first symptoms begin at the site of the tsetse fly's bite after a minimum of five days. This local skin reaction is called a trypanosomal chancre. It is typically accompanied by regional lymphadenopathy and is more common in *T. b. rhodesiense* than in *T. b. gambiense* infection (August *et al,* 2002).

2.5.2 First stage of disease

As the disease progresses from local symptoms to the first stage of a systemic infection, fever is one of the commonest, albeit non-specific sign. Usually the pattern of fever is irregular over many weeks, reflecting progressive waves of parasites multiplying in the blood (August *et al,* 2002).

In East African trypanosomosis, symptoms at this stage of infection can already be severe, around one tenth of patients without rapid access to treatment will die, often due to myocardial involvement (Dumas *et al,* 2000). Early symptoms in West African trypanosomosis are usually more inconspicuous. Lymphadenopathy, especially in the posterior triangle of the neck described as Winterbottom's sign, is characteristic (August *et al*, 2002). Hepatosplenomegaly and a faint rash are other common non-specific signs**.**

2.5.3 Second stage of disease

In the second stage of the disease, beginning after weeks in East African trypanosomosis and months in the West African form, trypanosomes cross the blood-brain barrier by unknown mechanisms to invade the central nervous system (Greenwood & Whittle 1980). Once trypanosomes have been detected in blood or lymph the disease needs staging by lumbar puncture. Increased lymphocyte counts of more than 20 cells/μl, increased protein levels of more than 35mg/dl, or the presence of trypanosomes in cerebrospinal fluid confirms the involvement of the central nervous system (Olivier & Legros, 2001). This differentiates between haemolymphatic stage and encephalitic stage, essential for the correct choice of treatment (Lean *et al,* 2004). This later stage results in a chronic encephalopathy, associated with headache and mental changes (Enanga *et al,* 2002). Patients often have reduced higher mental functions, difficulty in concentration, are increasingly unable to cope with their surroundings and eventually enter a terminal somnolent state (Lean *et al,* 2004).

2.6 Diagnosis of Trypanosomosis

The diagnosis of human African trypanosomosis requires a high degree of training and expertise. Initially, the parasite must be detected in blood or cerebrospinal fluid (Robays *et al,* 2004). This relies on conventional techniques such as lymph node puncture, blood film examination, or various more elaborate techniques to concentrate parasites in the blood (Bailey & Smith, 1992). As the concentration of trypanosomes in the blood undulates, often decreasing below detection levels in West African trypanosomosis, examinations may have to be repeated daily (Simarro *et al,* 2003). Molecular or serological tools have not replaced these classic parasitological procedures. For conditions in the field the card agglutination test for

trypanosomosis is a useful complementary screening tool in West African trypanosomosis (Truc *et al*, 1994).

2.7 Drug Treatment

The range of drugs that are used against human African trypanosomosis are limited (Burri *et al*, 2000; Chappuis *et al*, 2005). Only one of them is less than 40 years old. Until recently melarsoprol, an arsenic compound was the only treatment readily available for late stage (stage 2) disease, but it is associated with 4% to 12% mortality (Dumas *et al*, 2000). Its main adverse effect is an acute encephalopathy, occurring around 8 to 10 days of treatment (August *et al*, 2002). For adjunctive therapies, only prednisolone for stage 2 disease is supported by sufficient clinical evidence (Pepin *et al*, 1989). Treatment lasts a minimum of 10 days and at least for stage 2, requires admission to hospital, specialist nursing care and intensive monitoring (Van Nieuwenhove, 1999).

2.7.1 Early stage treatment

Early-stage HAT is successfully treated with pentamidine and suramin. Pentamidine is less toxic than suramin, however it is not effective against *T. b. rhodesiense* (Kennedy, 2004).

Suramin is a colorless polysulphonated symmetrical naphthalene derivative, which was first used against sleeping sickness in 1922 (Voogd *et al*, 1993). It is used against early stage rhodesiense form of the disease. Its mode of action against trypanosomes remains obscure although some workers speculate the uptake of this drug by the trypanosomes occurs via endocytosis bound to low-density lipoprotein (Vansterkernurg *et al*, 1993) and it accumulates slowly in the trypanosomes.

Pentamidine is an aromatic diamine that was developed after a related compound that induces hypoglycaemia in mammals, synthalin, had prolific anti–trypanosomal activity (Denise & Barret, 2001). It is active against early stages of the *gambiense* form of sleeping sickness and the drug is concentrated to high levels by the parasites. The route of entry into the cells is thought to be via P2 amino-purine transporter that also accumulates melanine-phenyl arsenicals (Matovu *et al*, 2003). Loss of this transporter can render parasites cross resistant to both diamidines and arsenicals. However, some parasites without P2 remain

sensitive to pentamidine, suggesting other routes of internalizing the drug and therefore the mode of action of this drug has however not been establish (Denise & Barret, 2001).

2.7.2 Late stage treatment

This late-stage of HAT, where the trypanosomes have invaded the cerebrospinal fluid (CSF), depends exclusively on the arsenical compound melarsoprol. The one new drug marketed in the past 40 years, eflornithine (DFMO, Ornidyl®), is only effective against *T. b. gambiense* and is very expensive. Eflornithine, difluoromethylornithine (DFMO), an analogue of ornithine, was developed as an anti-cancer reagent, but still under trial stage against neoplastic disease (Barret & Barret, 2000). The drug has prolific activity against *T. b. gambiense* sleeping sickness, even in the late CNS-involved stage (Pepin *et al*, 1994). The uptake of DFMO occurs in *T. brucei* via passive diffusion across the plasma membrane (Bitonti *et al*, 1986). DFMO inhibits ornithine decarboxylase (ODC), which is involved in polyamine metabolism leading to cessation of trypanosomes growth. A functional immune system is required to kill the growth arrested trypanosomes (Ghoda *et al*, 1990). Its drawback is that large doses are given to be effective and has no action against *rhodesiense* sleeping sickness (Denise & Barret, 2001). Lack of activity against *T. b. rhodesiense* is thought to be due to higher overall activity and a shorter half-life of ODC in this subspecies compared to *T. b. gambiense* counterpart (Iten *et al*, 1997).

Melarsoprol on the other hand is a melaminophenyl based organic arsenical that was introduced as anti-trypanosomosis reagent in 1949 (Friedheim, 1949). It is accumulated into trypanosome cells via P2 amino-purine transporter whose loss leads to drug resistance (Barret & Fairlamb, 1999). This is the drug of choice for treatment of late stage disease against both *T. b. rhodesiense* and *T. b. gambiense* forms of the disease. Trypanosomes exposed to arsenicals lyse very rapidly, but their mode of action is yet to be established (Denise & Barret, 2001).

2.8 Mechanisms of Drug Resistance

There is an alarming increase in treatment failures with melarsoprol, the principal agent used against late-stage sleeping sickness (Matovu *et al*, 2003). In *T. b. brucei*, the

uptake of melarsoprol as well as diamidines is thought to be mediated by the P2 aminopurine transporter, and loss of P2 function has been implicated in resistance to these agents (Carter & Fairland, 1993; Matovu *et al*, 2001a). The trypanosomal gene *TbAT1* has been found to encode a P2-type transporter when expressed in yeast (Carter *et al*, 1995). *TbAT1*-null trypanosomes were deficient in P2-type adenosine transport and lacked adenosine-sensitive transport of pentamidine and melaminophenyl arsenicals (Maser *et al*, 2003). However, the null mutants were only slightly resistant to melaminophenyl arsenicals and pentamidine, while resistance to other diamidines such as diminazene was more pronounced. Nevertheless, the reduction in drug sensitivity might be of clinical significance, since mice infected with *TbAT1*-null trypanosomes could not be cured with 2 mg of melarsoprol/kg of body weight for four consecutive days, whereas mice infected with the parental line were all cured by using this protocol. Two additional pentamidine transporters, high affinity component of pentamidine transport (HAPT1) and lower affinity component of pentamidine transport (LAPT1) were still present in the null mutant, and evidence is presented that HAPT1 may be responsible for the residual uptake of melaminophenyl arsenicals. High-level arsenical resistance therefore appears to involve the loss of more than one transporter (Matovu *et al*, 2001b).

2.9 Prevention and Control of Trypanosomosis

Prevention and control of trypanosomosis focus where possible on the eradication of the parasitic vector, the tsetse fly. Two alternative strategies have been used in the attempt to reduce African trypanosomosis (WHO, 1998). One tactic primarily targets the disease directly using monitoring, prophylaxis, treatment and surveillance to reduce the number of mammalian hosts which carry the disease (Roger & Randolph, 1985). The second strategy is generally entomological and intends to disrupt the cycle of transmission by reducing the number of flies (WHO, 1998). Incidences of sleeping sickness are being reduced by the use of the sterile insect technique and fly trapping (WHO, 1998). Regular active surveillance, involving case detection and treatment, in addition to tsetse fly control, is the backbone of the strategy for control of sleeping sickness (Swynnerton, 1925). Systematic screening of communities in identified foci is the best approach as case-by-case screening is not practically possible in highly endemic regions. Systematic screening may be in the form of mobile clinics

or fixed screening centers where teams travel daily to the foci (WHO, 1998). The nature of *T. gambiense* disease is such that patients do not seek treatment early enough because the symptoms at that stage are not evident or serious enough to warrant seeking medical attention, considering the remoteness of some affected areas (Priotto *et al*, 2009). Also, diagnosis of the disease is difficult and most health workers may not be able to detect it. Systematic screening allows early-stage disease to be detected and treated before the disease progresses, and removes the potential human reservoir (WHO, 1998). There is a single case report of sexual transmission of West African sleeping sickness but this is not believed to be an important route of transmission (Priotto *et al*, 2009).

CHAPTER THREE
MATERIALS AND METHODS

3.1 Study Site

The research work was carried at Kenya Agricultural Research Institute-Trypanosome Research Centre (KARI-TRC) where the trypanosome isolates of *T. b. rhodesiense* EATRO 1992 which is known to be resistant to melarsoprol and EATRO 2634 which is known to be sensitive to melarsoprol was used for propagation in Swiss white mice. Both parasites were isolated from sleeping sickness patients from Uganda and stored under liquid nitrogen for nearly 20 years at KARI-TRC trypanosome bank.

3.2 Experimental Animals

Eighty-five normal healthy males Swiss white mice of between 20 to 30g body weights were obtained from KARI-TRC small animal breeding unit. They were dewormed using injectable ivermectin at 0.1ml per mouse and were housed in cages designed for mice. The animals were acclimatized for 14 days during which the animals were maintained on a diet of commercial mice pellets (mice pellets®, Unga Ltd, Nairobi, Kenya) and water was provided *ad libitum.* For each stabilate, two donor mice were immunosuppressed using cyclophosphamide at 0.2ml per mouse each day for 3 days before infection to avoid immune response.

3.3 The Infectivity and Drug Sensitivity

For propagation of the isolate, *T. b. rhodesiense* EATRO 1992 and EATRO 2634 isolates were obtained from the Trypanosome Bank. The stabilates were each thawed at room temperature, the content suspended in cold phosphate-saline-glucose (PSG) buffer pH 8.0 and injected intraperitonially into immunosuppressed donor mice for multiplication. At the first peak of parasitaemia, donor mice were anaesthesized using carbon dioxide, blood was collected from the heart of the mice using a syringe containing ethylene diamine tetra acetic acid (EDTA) as anticoagulant. The parasitaemia was quantified using a haemocytometer and the experimental mice were intraperitonially injected using an inoculum dose of 1×10^4 trypanosomes/ml in PSG buffer.

3.3.1 Treatment of mice

After 24 hours of inoculation, 18 mice infected with each isolate of trypanosome were treated using melarsoprol at various dosages. One group of six mice was infected but not treated and thus served as untreated control for each isolate as shown in Table 1.

Dose rate / Trypanosome isolate	2mg/kg	5mg/kg	10mg/kg	Untreated control
Melarsoprol resistant isolate (EATRO 1992)	6	6	6	6
Melarsoprol sensitive isolate (EATRO 2634)	6	6	6	6

Table 1: Drug sensitivity test in Swiss white mice infected with either melarsoprol sensitive or melarsoprol resistant isolates and treated with melarsoprol at various dose rates.

3.3.2 Parasitaemia examination

A drop of blood was collected from the mouse tail every two days, placed on a clean slide, covered with a cover slip and the parasitaemia score correlated to a score sheet as outlined by Hebert and Lumsden (1976). For the treated groups, the mice were monitored by direct microscopy for trypanosomes for sixty days post-treatment to confirm the status of cure.

3.4 Molecular Characterization

3.4.1 Preparation of trypanosome DNA

Blood from positive mice were used to obtain trypanosome DNA. This was done by repeated centrifugation of the blood for 10 min at 12,000 g and the pellet washed with various buffers as described by Masiga *et al*, (1992). Briefly 50 µl of blood were mixed with 500 µl of lysis buffer (0.2% NaCl, 0.15% saponin and 1mM EDTA). The mixture was then

subjected to centrifugation for 10 min at 12,000 g in a microcentrifuge. The supernatant was discarded and the pellet re-suspended in lysis buffer and then centrifuged for 10 min at 12,000 g. The pellet was then re-suspended in polymerase chain reaction (PCR) buffer (50mM KCl, 1.5Mm $MgCl_2$, 10mM Tris HCl, pH 8.4), centrifuged for 10 min at 12,000 g and then pellet re-suspended in 50 µl of Tris EDTA buffer and then boiled for 20 min. 20 µl of the material were thereafter used for one PCR reaction.

3.4.2 Polymerase chain reaction

The polymerase chain reaction is the most widely used method for *in vitro* DNA amplification for purposes of molecular biology and biomedical research (Saiki *et al*, 1988). *In vivo*, DNA is replicated by DNA polymerases with various accessory proteins, including a DNA helicase that acts to separate the DNA by unwinding the DNA double helix (Vincent *et al*, 2004). Helicase-dependent amplification (HDA) was developed from this concept, using a helicase to denature the DNA.

3.4.2.1 PCR analysis for *Trypanosoma brucei* subgroup

Identification of trypanozoon subgroup was performed by PCR. The extracted DNA was tested individually with TBR1 (5'GAATATTAAACAATGCGCAG 3') and TBR2 (5' CCATTTATTAGCTTTGTTGC 3)' primer pair to identify the *brucei* group (Masiga *et al*, 1992). The final concentration of the primers was 0.4µM. Standard PCR amplifications were carried out in 25µl reaction volume containing the final concentrations, 10mM Tris-HCl pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, 200µm of each of the 4 deoxynucleoside triphosphates and 1 unit of *Taq* DNA polymerase (Sigma). The amplification conditions were 35 cycles of 94^0C for 1 min, 92^0C for 30 sec, 60^0C for 45 sec, 72^0C for 45 sec and 72^0C for 4 min (Masiga *et al*, 1992). PCR products were separated by electrophoresis in 2% agarose gel containing 2.5µg/ml ethidium bromide and visualized under ultraviolet light.

3.4.2.2 SRA gene amplification from genomic DNA

SRA A (5'GACAACAAGTACCTTGGCGC3') and SRA E (5' TACTGTTGTTGTACCGCCGC 3)' primer pair were used to screen the subspecies of

Trypanosoma brucei. The amplification was carried out using the method described by Gibson (2002). Typical conditions for SRA amplification included an initial denaturation done for 3 min at 98^0C, followed by a second denaturation at 98^0C for 30 sec, annealing at 60^0C for 30 sec, extension at 72^0C for 1 min and final extension at 72^0C for 2 min. PCR was carried out for a total volume of 20 µl for 35 cycles. The final concentration of each component in the reaction volume included 0.4 µM of each primer, 4µl of 5 times buffer, 2.5 mM of dNTPs, 5µl of DNA template and 1 unit *Taq* polymerase.

3.5 Pathogenesis Study

Pathogenesis study was carried out using Swiss white mice. Each trypanosome isolate was used to infect ten mice and one group of ten mice was used as uninfected control. The prepatent period, parasitaemia, body weight, packed cell volume and survival parameters were monitored for sixty days post infection.

3.5.1 Packed cell volume and body weight determination

Packed cell volume was determined as outlined by Naessens *et al* (2005). Blood from each mouse was collected from the tail vein every two days using heparinized capillary tubes sealed on one end with plasticin. The sealed capillaries were then centrifuged in a haematocrit centrifuge at 10,000 revolutions per minute (RPM) and the PCV read using a micro-haematocrit reader. Body weight for each mouse was determined every two days using an analytical balance (Mettle Tolendo PB 302®, Switzerland).

3.5.2 Pre-patent period and parasitaemia progression

Using the procedure described under section 3.3.2, parasitaemia examination was carried out daily until the parasites were detected in the blood and pre-patent period determined. After detection of parasitaemia, examination interval of animals was reduced to three days a week.

3.5.3 Survival time

The survival time for each mouse was recorded by daily checking the cages for cases of any mortality, the number of dead mice recorded until all the infected mice had died. The average survival time was then calculated.

3.6 Data Analysis

Data for every experimental variable including the parasitaemia levels, body weight and packed cell volume were then entered and managed using Microsoft Excel (version 2007). Statistical analysis was conducted using SPSS. The significant differences of the mean of parasitaemia, body weight, packed cell volume of mice from melarsoprol sensitive and resistant *T. b. rhodesiense* isolates and uninfected mice were compared by Student's t-test.

CHAPTER FOUR

RESULTS

4.1 Drug Sensitivity of Selected *T. b. rhodesiense* Isolates

The results of this study were interpreted as described by Eisler *et al* (2001). A trypanosome isolate was considered as drug sensitive if at least five out of six treated mice were cured. If fewer than five mice were cured, the isolate was considered resistant. The results for the sensitivity testing are as presented in Table 2.

All the six mice per group infected with melarsoprol sensitive isolate and treated using melarsoprol at 2mg/kg, 5mg/kg and 10mg/kg did not show any parasites in blood at any stage of infection and were thus considered cured. However in the drug resistant group of mice, all the animals except one became positive from 5-7 dpi and they remained positive in blood until the mice started dying of the infection from 29-35 dpi. Only one infected mouse treated at 5mg/kg bwt melarsoprol did not show parasites in blood during the entire 60 days period of study.

Trypanosome isolate	No of mice cured/treated at 2mg/kg	No of mice cured/treated at 5mg/kg	No of mice cured/treated at 10mg/kg
Drug sensitive group (EATRO 2634)	6/6 (100%)	6/6 (100%)	6/6 (100%)
Drug resistant group (EATRO1992)	0/6 (0%)	1/6 (16.7%)	0/6 (0%)

Table 2: Cure rate of *T. b. rhodesiense* infected mice at various dose rates of melarsoprol.

4.2 Molecular Characterization

4.2.1 Confirmation of *T. brucei* subgroup

Both melarsoprol sensitive and resistant isolates were first confirmed as *T. brucei* subgroup using *Trypanozoon* specific primers TBR1 and TBR2 by showing the presence of the expected band size of 600bp for the positive control, melarsoprol sensitive and melarsoprol resistant isolates. This confirmed that the isolates used were of the *brucei* group (Figure 3).

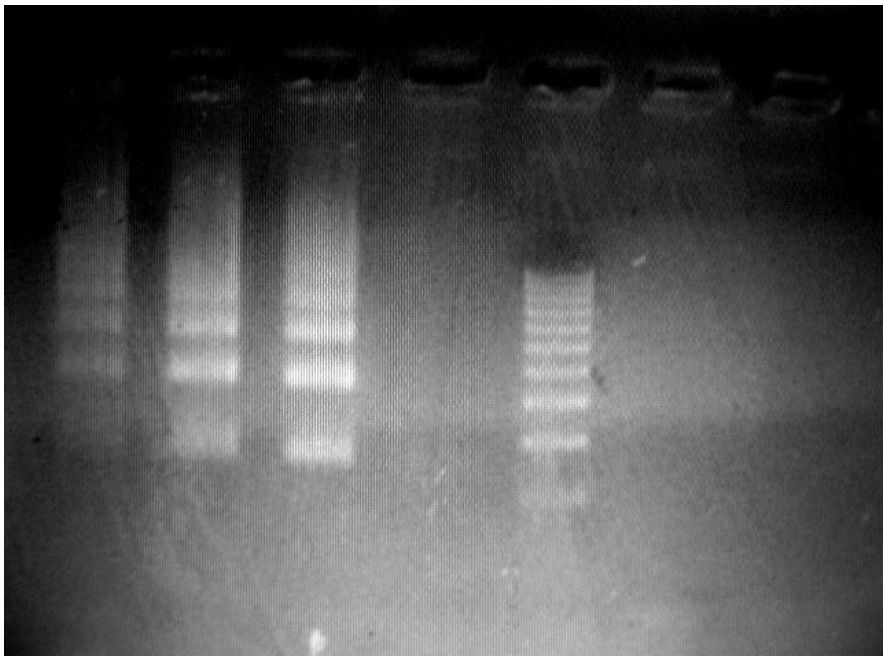
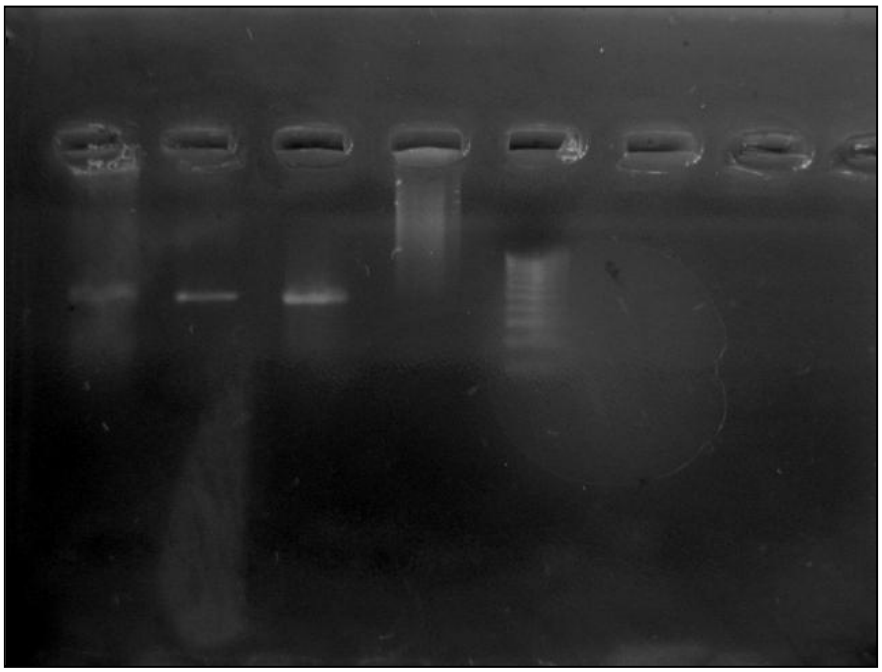


Figure 3: A 2% agarose gel electrophoresis showing DNA amplification of the *brucei* group using TBR1 and TBR2 primers. Lane 1 represents melarsoprol resistant isolate EATRO 1992, Lane 2 represents melarsoprol sensitive isolate EATRO 2634, Lane Pc represents positive control of *T. brucei rhodesiense* isolate (EATRO 693), Lane Nc represents Negative control as sterile water. Lane M represents molecular weight marker 100bp plus DNA ladder.

4.2.2 Amplification of serum associated resistance gene

The results for the gel for SRA gene amplification products are presented in figure 4. The two test samples showed a band size of 500bp which coincides with the band for the positive control indicating that the samples belong to *rhodesiense* subspecies. The PCR primers for SRA amplification were based on DNA sequence of SRA gene which is found in *Trypanosoma brucei rhodesiense* only.

500 bp →



1 2 Pc Nc M

Figure 4: A 2% agarose gel electrophoresis showing amplification of SRA gene using SRA A and SRA E primers. Lane 1 represents melarsoprol resistant isolate EATRO 1992, Lane 2 represents melarsoprol resistant isolate EATRO 2634, Lane Pc represents Positive control of *T. brucei rhodesiense* isolate (EATRO 693), Lane Nc represents Negative control as sterile water, Lane M represents molecular weight marker100bp plus DNA ladder.

4.3 Pathogenesis Study

4.3.1 Pre-patent period, survival time and parasitaemia progresion

The summary of mean±SD pre-patent and survival time are presented in Table 3. Mice infected with melarsoprol sensitive isolate EATRO 2634 had a mean pre-patent period (PP) of 4 days post infection (dpi) with a range of between 3 to 5 dpi. However the mice infected with melarsoprol resistant isolate EATRO 1992 had a longer PP of 6 days with a range of 5 to 7 dpi.

In terms of the mice mortality, the mean survival period of mice infected with melarsoprol sensitive isolate was 22 dpi with a range of 22-25 dpi. Of the group infected with melarsoprol sensitive isolate four animals died at 20 dpi, three at 22 dpi whereas the remaining three died at 25 dpi.

However, mice infected with melarsoprol resistant isolate, survived longer with a mean survival time of 32 dpi with a range of 29-35 dpi. Of these four mice died at 29 dpi, two at 31 dpi and the remaining four died at 35 dpi

Trypanosome isolate	Mean±SD (dpi) pre-patent period	Mean±SD (dpi) survival time
Drug sensitive isolate (EATRO 2634)	4±0.81	22±1.98
Drug resistant isolate (EATRO 1992)	6±0.87	32±2.21

Table 3: Mean±SD pre-patent-period and survival time (days) of Swiss white mice infected with melarsoprol sensitive (n=10) and melarsoprol resistant (n=10) *T. b. rhodesiense* isolates

The mean parasitaemia progressions are presented in figure 5. After the appearance of parasites in blood of mice infected with melarsoprol sensitive parasites from 3 dpi, the parasitaemia levels increased sharply reaching the first peak of 1x 10^8 parasites/ml at 7 dpi. Thereafter the parasitaemia levels continued to increase steadily and reached levels of 1x 10^9parasites/ml at 24 dpi when the experiment was terminated after all the mice in the group had died. Melarsoprol resistant infected group of mice showed a delayed appearance of parasites. The mice became parasitaemic from 5dpi with the parasite levels increasing sharply to reach the first peak of 1x 10^6 parasites/ml at 11 dpi. Thereafter, the parasitaemia levels decreased slightly with fluctuations being observed at 14 and 23 dpi. The parasite levels increased steadily to reach a second and third peak of 1x 10^7 and 1x 10^8 at 18 and 25 dpi, respectively. Thereafter the parasitaemia level increased steadily to reach levels of 1x10^9 trypanosomes/ml at 35 dpi when the experiment was terminated since all the infected mice had died.

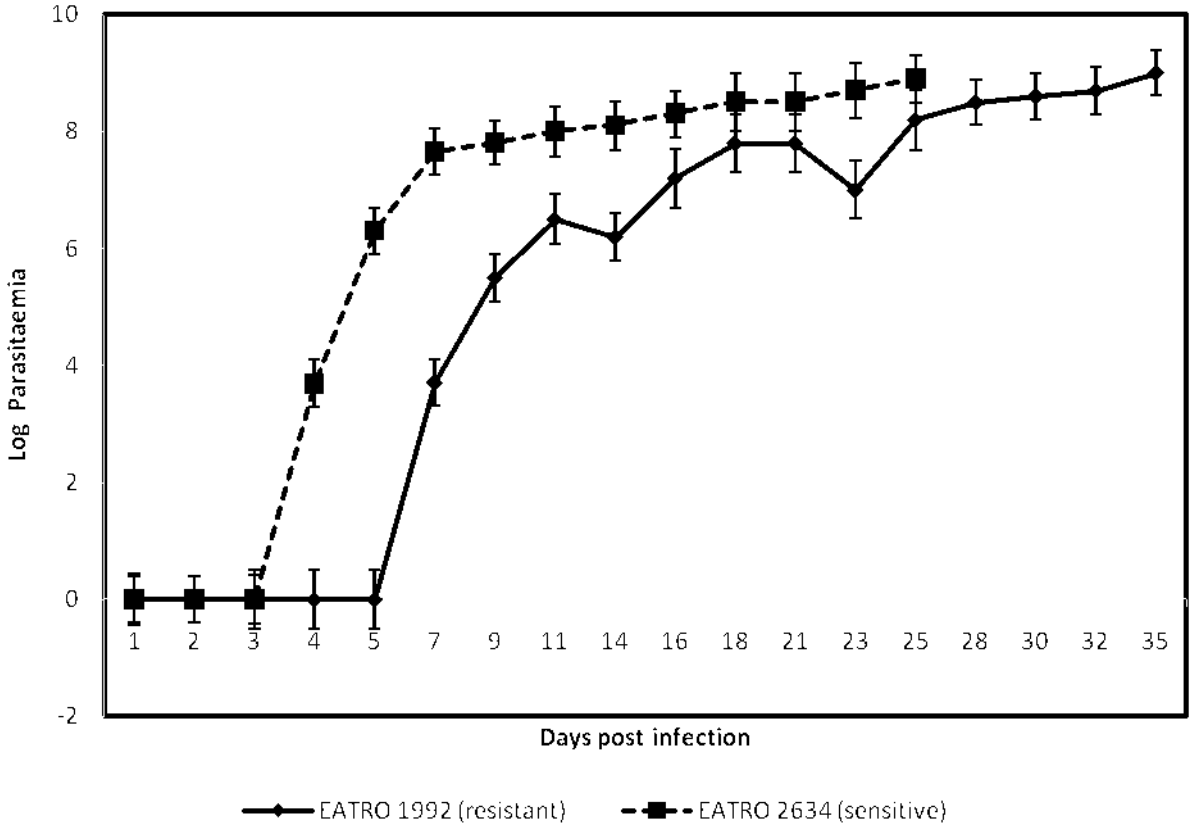


Figure 5: Parasitaemia progression in infected Swiss white mice infected with melarsoprol sensitive and melarsoprol resistant *T. b. rhodesiense* isolates.

4.3.2 Packed cell volume

The packed cell volume (PCV) values of the infected and control mice are presented in figure 6. The mean PCV for the uninfected mice were maintained around 51% throughout the experimental period of 60 days. These levels were significantly higher than those of infected mice ($p<0.05$). However the mean PCV levels for both groups of infected mice showed steady decline from 5 dpi throughout the time of infection and over the observation period. The rate decline in the mean PCV levels of animals infected with melarsoprol sensitive isolate EATRO 2634 produced a faster decline in PCV level compared to those infected with melarsoprol resistant isolate EATRO 1992 and at 7 dpi mean PCV were 45±4.01 and 46±2.49, respectively. Similar trends in the decline in PCV levels for

infected mice were maintained throughout the infection period but the levels were not significantly different ($p>0.05$) between the infected groups. The PCV levels reached 30% and 29% for the drug sensitive and drug resistant isolates, when the experiment was terminated respectively following the death of all the mice.

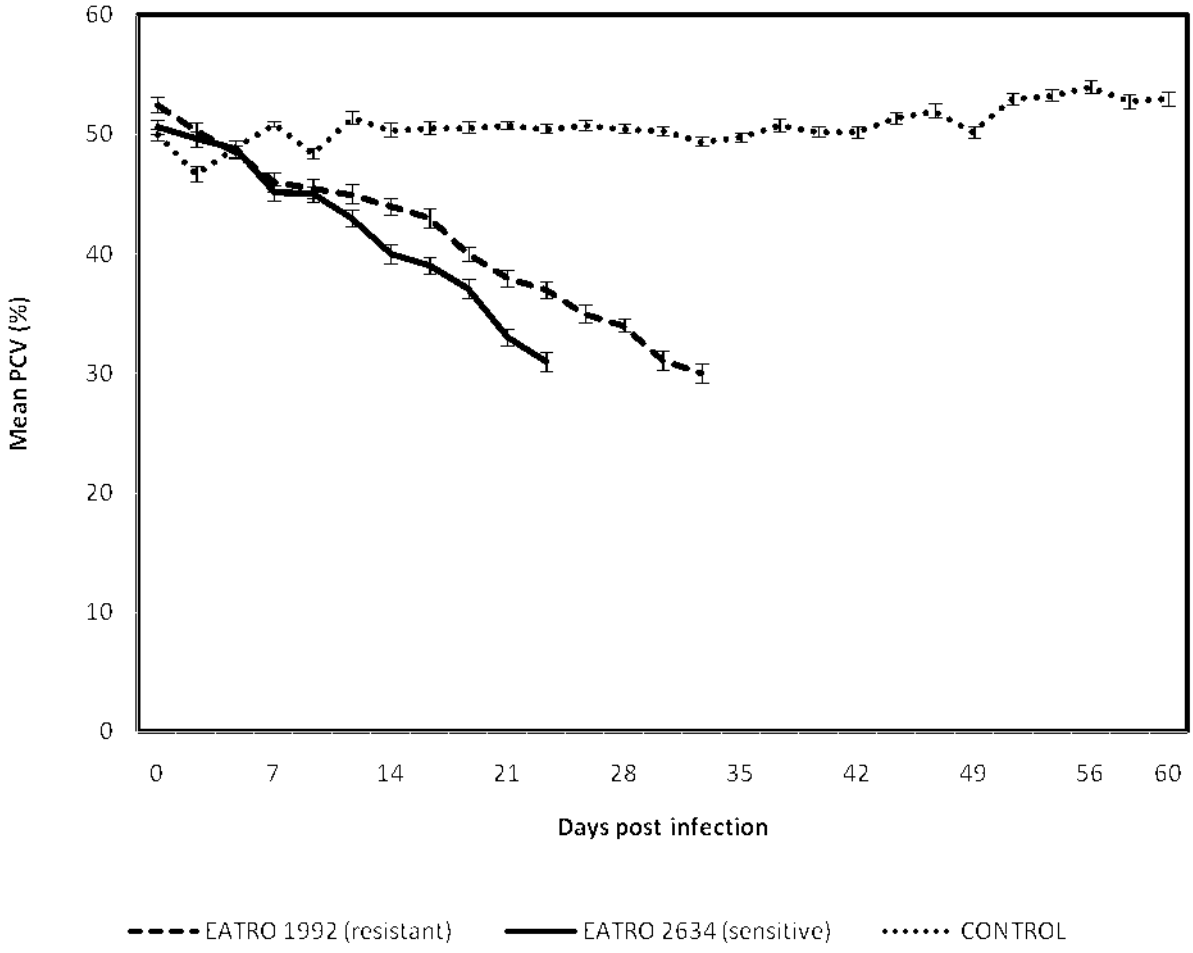


Figure 6: Changes in packed cell volume of Swiss white mice infected with melarsoprol sensitive and resistant *T. b. rhodesiense* isolates.

4.3.3 Body weight

The changes in body weight between the infected and uninfected mice are presented in figure 7. The body weigth of the uninfected mice remained within a norrow

range of 31.2±2.57g and 32.6±1.89g at 2dpi and 16 dpi, respectively. Thereafter the mice showed a gradual but consistent increase in body weight to reach mean weights of 38±0.97g at 60 dpi when the experiment was terminated. The mean weights of the infected groups of mice were not significantly different from those of the control mice upto 17 dpi. Therafter the weight of both groups of infected mice decreased gradualy to reach levels of 27±1.29g and 28±1.61g at 21 dpi for mice infected with melarsoprol sensitive and melarsoprol resistant isolates, respectively. The mean weight of the two groups of infected mice were significantly different from the control weight ($p<0.05$) but were however not significantly different from each other ($p>0.05$). The decrease in the mean weights of mice infected with melarsoprol sensitive isolate EATRO 2634 was however more marked than those of mice infected with melarsoprol resistant trypanosome isolate EATRO 1992. The mean weight between infected groups of mice were however not significantly different ($p>0.05$) and had reached weight of 24g by 35 dpi for the drug resistant infected group and 26g by 25 dpi for the drug sensitive infected mice, respectively when all the mice in the groups died.

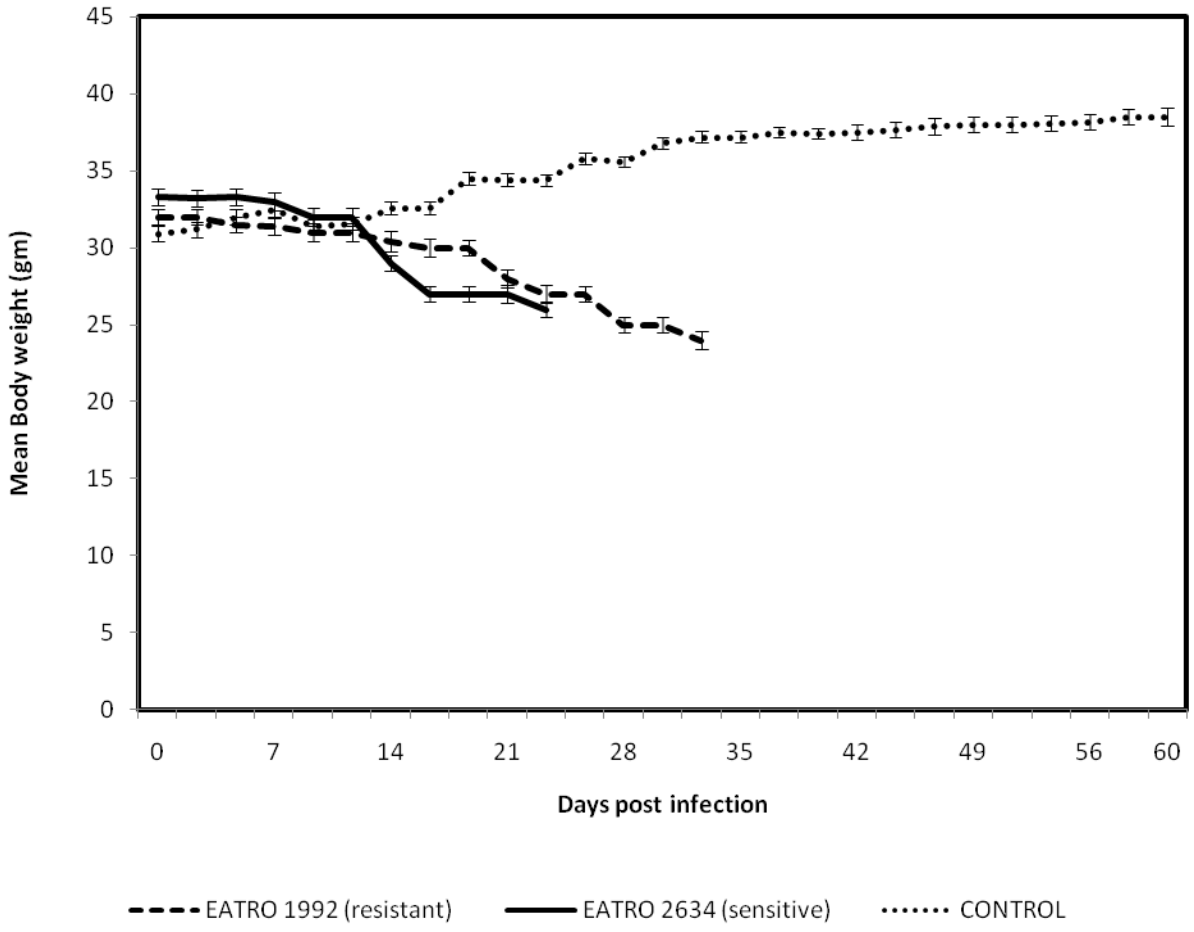


Figure 7: Changes in body weight of Swiss white mice infected with melarsoprol sensitive and melarsoprol resistant isolates.

CHAPTER FIVE

DISCUSSION AND CONCLUSION

5.1 DISCUSSION

Trypanomosis, caused by *Trypanosoma brucei ssp*., has become resurgent in sub-Sahara Africa (Matovu *et al*, 2003). Moreover, there is an alarming increase in treatment failures with melarsoprol, the principal agent used against late-stage of sleeping sickness (Matovu *et al*, 2003). The efficacy of melarsoprol was questioned leading to a change of treatment approach. In the recent past eflornithine became the first-line drug for the second stage disease (Brun *et al*, 2001). However, this was done without determining the cause of the melarsoprol treatment failures (Brun *et al*, 2001). To date, very little is known about the differences in pathogenesis between drug sensitive and drug resistant *T. b. rhodesiense*.

The present study was carried out to determine the molecular differences between melarsoprol resistant and melarsoprol sensitive *Trypanosoma brucei rhodesiense* isolates from Uganda and the effect of the difference in parasite pathogenesis. Results from this study demonstrated showed that all the mice infected with either melarsoprol sensitive isolate or melarsoprol resistant isolate and treated with up to 10mg/kg of melarsoprol were either cured or not depending on the nature of each isolate. The data indicate that the parasites that were known to be melarsoprol sensitive did not change their sensitivity status with preservation since in *T. brucei*, the uptake of melarsoprol as well as diamine is thought to be mediated by the P2 aminopurine transporter, and loss of P2 function has been implicated in resistance in these agents (Matovu *et al*, 2003). The procedures used at KARI-TRC to cryopreserve trypanosomes in liquid nitrogen have been demonstrated to be robust. However previous studies have shown that the *in vivo* testing of the viability using cryopreserved (ESG) trypanosomes showed reduced viability or resulted in complete loss of parasites especially *T. b. gambiense* (Matovu *et al*, 2001b).

Results also showed that there were no molecular differences between the two isolates since the fragment of DNA was amplified at the same size in both isolates for all the primers used. The two *T. b. rhodesiense* strains from Uganda analysed in this study were screened for the *T. brucei* subgroup and for the SRA gene and both showed an amplified fragment of the expected size of 600 bp and 500 bp respectively confirming that all these isolates were *T. b. rhodesiense* as reported by Kibona *et al* (2007) and by Njiru *et al* (2004). Since these isolates showed differential melarsoprol sensitivities, a correct differential diagnosis between melarsoprol sensitive and resistant *T. b rhodesiense* isolates from Uganda was essential for unambiguous diagnosis of drug resistance. Therefore, the use of a simple molecular diagnostic technique such as the amplification of SRA gene could not explain the real cause of this difference. Future research is necessary to investigate the genes which code for resistance. Also other molecular methods such as DNA sequencing or RFLP-PCR need to be applied in the investigations of the molecular basis for resistance.

Results from the pathogenesis studies indicated differences in the pathogenesis of melarsoprol resistant and melarsoprol sensitive trypanosome isolates in mice. Pre-patent periods observed in mice infected with melarsoprol sensitive isolate were different from the animals infected with melarsoprol resistant isolate. Mice infected with melarsoprol sensitive isolate had a shorter mean pre-patent period of 4 (range 3-5) days while mice infected with melarsoprol resistant isolate recorded a slightly longer mean of 6 (range 5-7) days. This was in agreement with previous reports (Thuita *et al*, 2008) which showed that the mean pre-patent period of *T. b. rhodesiense* was between 4-10 dpi.

The survival time of mice infected with *T. b. rhodesiense* has been estimated between 3 and 12 weeks (Kagira *et al*, 2007). In this study the mean survival time of mice infected with melarsoprol sensitive trypanosome isolate was 3 weeks (range 20-25 dpi) however, for the drug resistant trypanosome isolate, it was longer with a mean of 5 weeks (range 29-35 dpi). The mean prepatent period and survival of both isolates showed that melarsoprol sensitive isolate rendered the host more susceptible to infection resulting in reduced pre-patent period and survival time. This finding is consistent with the finding of Sacks & Askas (1980) who found a correlation between pre-patent period, survival time and

pathogenesis of trypanosomes. No significant differences were observed in parasitaemia progression between the two groups of mice, although the parasitaemia level of mice infected with melarsoprol sensitive isolate was higher and demonstrated fewer waves of parasitaemia than the mice infected with melarsoprol resistant isolate. Expression of several waves of parasitaemia is an indicator of different variable antigen types (VATs) of trypanosomes to which immune response is elicited (Brancroft & Askonas, 1985). The rate at which VSG gene switch occurs is very similar to the rate at which the immune system develops an effective antibody response (Turner & Ormerod, 1984). The lower level of parasitaemia demonstrated by mice infected with melarsoprol resistant isolate compared to the melarsoprol sensitive isolate indicates the ability of the immune system to control this parasite. The higher parasitaemia observed in the mice infected with melarsoprol sensitive isolate compared to the melarsoprol resistant group may suggest that this parasite is more virulent. In a previous study (Murray & Morrison, 1979), it was observed that in both *T. brucei* and *T. congolense* infections, the higher the parasitaemia, the greater the degree of the pathogenesis. Turner *et al* (1995) also observed that trypanosome stocks that grow faster had higher parasitaemia and greater virulence.

The packed cell volume in mice infected with either melarsoprol sensitive or melarsoprol resistant isolates was marked by a drop, coinciding with the first peak of parasitaemia and continued to decline as the infection progressed. This is in agreement with observations from previous studies (Morrison *et al*, 1981; Murray & Morrison, 1979). The mean PCV of the control uninfected mice were maintained at 51% throughout the experimental period of 60 days. These levels were significantly higher than those of infected mice. The mean PCV as a measure of anemia indicated that there were no significant differences between melarsoprol sensitive isolate and melarsoprol resistant isolate in terms of mean packed cell volume changes.

Although melarsoprol sensitive isolate had a shorter pre-patent period and high parasitaemia, the mice appeared to have been able to control the infection and thus red blood cell destruction. Similar findings have been observed by Kariuki *et al* (2008).

The body weights of the control uninfected group of mice showed a gradual but consistent increase in body weight up 38g at the end of the experiment of period of 60 days. The two groups of mice infected with the two parasites reduced in body weights as the disease progressed. The body weight values of infected mice from 17 dpi were significantly different from controls ($p<0.05$). Although a decrease in weight is common in trypanosomosis infections (Toth *et al*, 1994; Nishimura *et al*, 2001), it was observed in the current study that the drop in weight of mice infected with melarsoprol sensitive isolate was higher than that of animals infected with a melarsoprol resistant isolate despite of the latter having a short pre-patent period and also high parasitaemia. Statistically the differences observed between melarsoprol sensitive stabilate and melarsoprol resistant isolates with regard of body weight were not significant ($p>0.05$). However, in both groups, the drop was observed between 10 and 12 days post infection. This is in agreement with results from a previous study where rats infected with *Trypanosoma brucei brucei*, showed reduction in body weight starting 12 dpi (Celine *et al*, 2005). Another study (Darsaud *et al*, 2003) explained the reduction in body weight to be associated with the entry of trypanosome into CNS because that body weight loss is controlled by the hypothalamus, and as such the effect could be caused by entry of parasites into the brain (Darsaud *et al*, 2004).

5.2 CONCLUSION AND RECOMMENDATION

Little is known about the differences in pathogenesis between drug sensitive *T. b. rhodesiense* and drug resistant *T. b. rhodesiense*. In this study, the procedures used at KARI-TRC to cryopreserve trypanosomes in liquid nitrogen have been demonstrated to be robust since the sensitivity/resistance status of each isolate did not change after several years of cryopreservation. No molecular differences were observed according the molecular approach used in this work.

Based on studies on pre-patent period, survival time, parasitaemia progression, packed cell volume and body weight, studied it may be concluded that the sensitive *T. b rhodesiense* isolate appeared relatively more pathogenic than the resistant isolate. Further studies should be carried out using a higher number of isolates from different countries and also using various molecular techniques such as DNA sequencing, RLFP-PCR which could shed light on the implications of genetic diversity in trypanosomes and pathogenesis in HAT control.

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