TRANSMISSIBILITY AND VIRULENCE OF DRUG SENSITIVE AND RESISTANT *Trypanosoma brucei rhodesiense* ISOLATES FROM KENYA

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DECLARATION AND RECOMMENDATION

DECLARATION

I hereby declare that this thesis is my original work and has not been presented for award of a degree in any institution of learning to the best of my knowledge.

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RECOMMENDATION

We confirm that this thesis was prepared under our supervision and has our approval to be presented for examination as per the Egerton University regulations.

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DEDICATION

I dedicate this work to the Almighty God, my beloved parents Denis Edikalinde Mokeni and Josephine Afua Muzalia. You recognized the great need for education for a woman, and you chose the right path for me. To you I say, thank you. May God Bless you, always.

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ABSTRACT

Trypanosome parasite isolates are routinely collected within Human African Trypanosomiasis foci in the region and cryopreserved in a parasite cryobank at the Trypanosomiasis Research Center of the Kenya Agriculture Research Institute (KARI-TRC). Investigations were conducted to compare pathogenicity and transmissibility of drug sensitive (KETRI 2427) and resistant (EATRO 237) *Trypanosoma brucei rhodesiense* isolates obtained from the KARI-TRC bank. *Glossina pallidipes* (tsetse fly vector) was used for transmission in the mouse model. To determine transmission rates, two groups of tsetse flies (150 flies each) were separately infected with the drug sensitive and resistant *T. b. rhodesiense* isolates and were allowed to feed separately on Swiss white mice (one fly per mouse) in a controlled laboratory setting. The infected mice were regularly monitored for 1) pre-patent period (PPP), 2) parasitaemia level, 3) body weight, 4) packed cell volume (PCV) and 5) survival time for a period of 60 days-post-infection (dpi). The infection rates were significantly higher ($p<0.05$) in drug sensitive (67%) than in the resistant isolate (29%). The values obtained for survival were significantly lower in mice infected by either isolates than the controls. However, mice infected with sensitive isolate had significantly reduced survivorship (days) than those infected with the resistant isolates (days). Infections by either isolates did not significantly affect body weights of the experimental mice. Pre-patent periods among the isolates in mice were also similar. The observed significant differences in parasitaemia, survival time and PCV between the isolates in mice suggests differences in transmissibility and virulence between the isolates, which may be influenced by drug resistance gene in the parasite. Drug resistance appears to putatively reduce virulence and transmissibility of the parasite. This phenomenon can present major challenges in the management in the field.

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LIST OF ABBREVIATIONS

CNS	Central Nervous System
CSF	Cerebrospinal Fluid
EATRO	East African Trypanosomiasis Research Organization
EDTA	Ethylenediaminetetracetic Acid
ESG	EDTA Saline Glucose
HAT	Human African Trypanosomiasis
IACUC	Institutional Animal Care and Use Committee
RPM	Revolutions per minute
PCR	Polymerase chain reaction
PCR-RFLP	Polymerase chain reaction - Restricted fragment length polymorphism
PCV	Packed Cell Volume
PP	Prepatent Period
*Tb*ATI	*T. brucei* adenosine transporter1 (the transporter)
VSG	Variant Surface glycoprotein

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CHAPTER ONE
INTRODUCTION

1.1 Background Information

Human African Trypanosomiasis (HAT) is a major threat to the health of more than 60 million people in 36 countries in sub-Saharan Africa, and is the world's third most important parasitic disease affecting human health after malaria and schistosomiasis (Kennedy, 2007). Two subspecies of the protozoan parasite *Trypanosoma brucei* are the causative agents of HAT; *Trypanosoma brucei gambiense* leads to chronic development of the disease in Central and West Africa while *Trypanosoma brucei rhodesiense* establishes an acute form in Eastern Africa, which is fatal within months or even weeks (WHO, 2006). Relative prevalence of HAT within the regions is foci dependent, defined by species and distribution of tsetse fly vector (Hoare 1972). The Infestation by the tsetse fly covers 10 million square kilometers, one third of Africa's landmass (Kennedy, 2007). *Palpalis* (mainly *Glossina fuscipes fuscipes*) or *morsitans* (mainly *Glossina pallidipes*) tsetse flies are the vectors of *T. b. rhodesiense* (Richner *et al.,* 1988). Understanding the interactions between the trypanosomes and the respective tsetse vector is critical in developing and implementing effective control strategy to reduce disease transmission.

Endogenous and exogenous factors influencing infectivity of the vectors by the parasites have been identified (Leak, 1998). Apart from some endogenous biochemical and immunological factors (Aksoy *et al.,* 2003), infectivity of the tsetse vector is also dependent on the genetic make up of the parasite and tsetse host (Maudlin et al. 1986). Although the role of various parasite dependent factors in establishment of infection in the tsetse vector has been established (Dale *et al.,* 1995; Maudlin *et al.,* 1986; Reifenberg *et al*., 1997) that of drug resistance in *T.b. gambiense* on transmissibility by the vector and virulence in the human host of the parasite has not been elucidated, despite emergence of drug resistance in the parasite in many countries, (Balasegaram *et al.,* 2009; Delespaux and de Koning, 2007; Legros *et al.,* 2002). The resistance may influence fitness characteristics of the parasite (transmissibility and virulence), determining relative abundance of a particular strain (resistant vs susceptible) in nature with epidemiological implications on HAT (Masumu *et al.,* 2006). This study was conducted to determine the impact of multi-drug resistance in *T.b. rhosensiense* on transmissibility and

virulence of the parasite in mouse model, and impact of the infections on *T.b. rhosensiense* genetics.

1.2 Statement of the Problem

Strain of *T.b. rhosensiense* in nature has developed resistance to all drugs against the parasite. However, influence of the resistance on the transmissibility and virulence of the parasite has not been documented. The impact of the resistance on these parameters may impact on epidemiology of HAT.

1.3 Objectives

1.3.1 Main Objective

To compare transmissibility and virulence of drug sensitive and resistant *T. b. rhodesiense* isolates from Kenya.

1.3.2 Specific Objectives

1. To determine relative transmissibility of drug sensitive and resistant *T. b. rhodesiense* isolates from Kenya by *G. pallidipes* tsetse fly.
2. To determine differences in virulence of drug sensitive and resistant *T. b. rhodesiense* on Swiss white mice.
3. To determine genetic changes in *T. b. rhodesiense* before, during, and after infection through flies and mice.

1.4 Hypotheses

1. There are no similarities in transmissibility of drug sensitive and resistant *Trypanosoma brucei rhodesiense* isolates from Kenya *G. pallidipes* tsetse fly.
2. There are no similarities in virulence of drug sensitive and resistant *Trypanosoma brucei rhodesiense* isolates from Kenya on Swiss white mice.
4. There are no genetic changes in *T. b. rhodesiense* before, during, and after infection through flies and mice.

1.5 Justification

Use of animal model has contributed greatly to what is currently known about relationships between parasite invasion of different body systems and potential of resultant host clinical condition. This has played significant role in analyses of and biological changes, as diagnosis and disease staging markers in HAT. Strains/isolates of *T. b. rhodesiense* in nature have developed resistance to most of the conventional drugs against HAT. However association between drug resistance phenotype other phenotypes of epidemiological importance in HAT such as transmissibility and virulence of the parasite has not been established. Additionally, impact of the transmission on the genetics of the parasite (drug resistant vs susceptible) has not been determined, which might shed light on the molecular dynamics in the parasites as it adapts to different host (vertebrate vs invertebrate) which can be exploited in development of novel chemotherapeutic agents against the parasite.

CHAPTER TWO

LITERATURE REVIEW

2.1 Human African Trypanosomiasis (HAT) (Sleeping sickness)

Human African Trypanosomiasis (HAT) is caused by two morphologically indistinguishable yet bionomically distinct subspecies of the hemoflagellated protozoan parasite *Trypanosoma brucei*, transmitted to human host by a specific *Glossina* tsetse fly species. Both vectors inhabit distinct areas of Africa, leading to an epidemiological division of the causative agents of the disease. *Trypanosoma brucei rhodesiense* and *T.b. gambiense* are the causative agents of HAT in East/Southern Africa and Central/West Africa respectively (Anene *et al.*, 2001). The prevalence of the disease differs from one country to another as well as in different parts of a single country (WHO, 2006).

In first half of the twentieth century, HAT caused by *T.b. gambiense* decimated entire communities in central Africa (Pepin, 2007), but by the early 1960s the disease was controlled and had almost disappeared (Steverding, 2008), primarily as a result of highly effective surveillance programs. However, there was progressive resurgence in HAT, peaking in 1997 at an estimated 450 000 cases (Barrett, 2006) with an estimated 60 million people at risk in 37 countries of sub-Saharan Africa (corresponding to one third of Africa's total land area) (WHO, 2000). Factors causing this increase were primarily war and famine, which resulted in severe disruption of disease surveillance and treatment, especially in Uganda, Angola, Sudan, and the Congos where the disease occurred in epidemics (Kuzoe, 1993). Since that date, case detection and treatment, and surveillance have been increased (WHO 2006), and by 2007, the reported number of new HAT cases had dropped to 10,769 (WHO, 2007), which probably equates to 50-70 000 total human cases. The HAT has therefore demonstrated its ability to recur even after it had been virtually brought under control.

Major outbreaks were observed in Angola, the Democratic Republic of Congo and Sudan in 2005, and remain an important public health problem in most of Africa (Central African Republic, Chad, Congo, Côte d'Ivoire, Guinea, Malawi, Uganda and United Republic of Tanzania) (WHO, 2006). Both animals and humans act as reservoirs of parasites capable of causing HAT; however, the detailed mechanisms by which this occurs are not fully understood . Typically, the *T. b. rhodesiense* transmission cycle involves wild and domestic animals, but

intensified human-to-human transmission may occur during epidemics. An animal reservoir is considered essential to the long-term persistence of *T.b. rhodesiense* sleeping sickness (Heisch *et al.,* 1958, Onyango *et al.,* 1968). The *T. b. gambiense* transmission cycle is mostly from human to human, involving animals to a much lesser extent, with importance of a non-human reservoir being less certain (Mehlitz *et al.,* 1981). Animals are therefore the main reservoir for *T.b. rhodesiense* parasites while humans are the main reservoir for *T.b. gambiense* parasites (Kennedy, 2007). In humans, *T. b. rhodesiense* infections are acute, lasting from a few weeks to several months, while *T. b. gambiense* infections are chronic, generally lasting for several years and often without any major signs or symptoms (Kennedy, 2007). There are no prophylactic drugs or vaccines available to prevent HAT and without proper diagnosis and treatment, the outcome is fatal. However, all four drugs currently used to treat HAT exhibit toxicity and, in many countries, drug resistance is beginning to emerge (Balasegaram *et al.,* 2009; Delespaux and de Koning, 2007; Legros *et al.,* 2002). Disease severity is dependent on both the pathogenicity of the parasite strain and the genetics of the human host (Courtin *et al.,* 2008).

2.2 Trypanosome parasites

Two distinct groups of insect-transmitted trypanosomes are generally recognized: the Stercoraria (subgenus *Megatrypanum*, *Schizotrypanum* and *Herpetosoma*) and the Salivaria (subgenus *Nannomonas*, *Duttonell*a and *Trypanozoon*). Stercorarian trypanosomes develop in the hindgut of the insect and are transmitted in the faeces. The predominant arthropod vectors of stercorarian trypanosomes are tabanids, triatomines and ticks (Kennedy, 2007). It is only the salivarian trypanosomes that cause HAT. The salivarian trypanosomes develop in the anterior part of the tsetse fly alimentary canal and are transmitted via the mouthparts. Tsetse flies are the major vectors of *T. brucei* trypanosomes but mechanical transmission of several salivarian trypanosome species, by tabanids and *Stomoxys* vectors, also occurs (Kennedy, 2007). *Trypanosoma vivax* (subgenus *Duttonella*), believed to be the most ancient of the salivarian trypanosomes, produce a high incidence of infection in the tsetse proboscis (Haag *et al.,* 1998) and are pathogens of cattle. The *Trypanozoon* subgenus contains the trypanosomes causing HAT (*T. b. gambiense* and *T. b. rhodesiense*) and *T. b. brucei*, which is one of the parasites causing nagana. *T. b. brucei* is unable to infect humans since it is sensitive to a trypanolytic factor found

in human serum (Oli *et al.,* 2006), and is thus restricted to development in domestic animals and many species of wildlife.

2.3 Tsetse identification and distribution

Tsetse flies are the sole insect vectors responsible for cyclical transmission of HAT. In West Africa, *T. b. gambiense* is transmitted primary to humans by *G. palpalis*, whereas in East Africa *G. morsitans* play a role as a vector for incidental transmission of *T. b. rhodesiense* to humans, while generally infecting domestic mammals (Tanowitz *et al.,* 2000). The flies are obligate blood sucking Diptera in the family Glossinidae and genus Glossina and are restricted to sub-Saharan Africa except for two localities in the Arabian Peninsula. Twenty-three species and eight sub-species of tsetse fly are currently recognized (Krafsur, 2009; Leak, 1999). The 23 species in this genus belong to Fusca, Palpalis and Morsitans groups. Fourteen species, mainly inhabiting rainforest constitute the Fusca, while five species, predominantly inhabiting rainforests and savannah woodlands form the Papalis group. Morsitans group, consist of five species of which *Glossina morsitans, Glossina pallidipes, Glossina swynnertoni* and *Glossina longipalpis* (Colvin and Gibson, 1992) are restricted to the savannah while *Glossina austeni* occupy coastal forests. In Central and West Africa, the riverine species (Palpalis group) tend to feed predominantly on reptiles and ungulates. Humans regularly encounter these flies, particularly when visiting water sources and these species are important vectors of human sleeping sickness (Kennedy, 2007). The savannah-woodlands species (Morsitans group) are the most economically important, as they preferentially feed on livestock and wildlife and are the major vectors of nagana. Both the Palpalis and Morsitans groups are vectors of *T. brucei* spp. Most tsetse flies from the third clade (Fusca group) inhabit the damp, evergreen forests. The exception is *G. brevipalpis,* which is more regularly found in association with livestock (Leak, 1999). With the exception of *G. brevipalpis*, flies in the Fusca group are not considered to be medically or agriculturally important. Tsetse control has been hampered by development of resistance to conventional insecticides among other factors.

2.4 Tsetse feeding physiology

Tsetse flies are pool feeders and the repeated penetration of mammalian host tissue by the tsetse proboscis results in the formation of a sub-surface blood pool. Saliva is expressed into the wound and trypanosomes are transmitted to the mammalian host at this stage. Both male and female adult tsetse flies are obligate haematophages capable of transmitting trypanosomes. The bloodmeal is aspirated through the proboscis and oesophagus and propelled into the rest of the alimentary canal by the rhythmic pumping of the cibarial pump aided by the contraction of circular muscles that encompass the oesophagus (Lehane, 1991). The proventriculus (cardia) lies at the junction of the oesophagus, midgut and crop duct. Blood may pass directly into the midgut or into the extension of the foregut known as the crop, before regurgitation into the midgut (Moloo and Kutuza, 1970). The proventriculus acts as a valve regulating the directional flow of blood and is also the organ responsible for producing the peritrophic matrix (PM). The tsetse midgut is a simple tube, lacking diverticula, running from the proventriculus to the junction with the hindgut, which is marked by the entrance of the Malpighian tubules into the alimentary canal. Although more complex divisions exist (Boehringer-Schweizer, 1977), the midgut can be crudely separated into three functional regions: the anterior midgut, bacteriome (mycetome) and posterior midgut. The first part of the anterior midgut is a linear tube running though the thorax. Once it enters the abdomen, the anterior midgut becomes distended; here the blood is stored and dehydrated prior to digestion. Epithelial cells of the anterior midgut possess extensive infoldings of the basal plasma membrane with associated mitochondria, enabling the fly to achieve this rapid dehydration (Boehringer-Schweizer, 1977). The anterior midgut is interrupted approximately in its middle section by a region of cells called the bacteriome. The bacteriome houses the intracellular symbiotic bacteria *Wigglesworthia glossinidius.* Haemolysis and bloodmeal digestion commence at the very beginning of the posterior midgut, where haemolytic agents and digestive enzymes are produced. Virtually all proteolytic enzymes are restricted to the posterior midgut (Gooding, 1974).

2.5 Trypanosome (*T. brucei spp*.) life cycle: development and differentiation

Trypanosoma brucei has the most complex, but perhaps the best characterized, life cycle of all African trypanosome species. The trypanosome life cycle was first described in detail by Muriel Robertson who described the successive stages of parasite establishment and maturation within the insect and mammalian hosts, demonstrated the migration of parasites through the fly midgut and proved that only salivary gland forms were capable of producing a mammalian infection (Robertson, 1913). Since then, a more complete understanding of trypanosome development has been achieved, with an agreed parasite nomenclature adopted (Roditi and Clayton, 1999) and a consensus achieved on many of the barriers present in the fly that the trypanosome must overcome to survive and develop in order to complete cyclical transmission.

Within the vertebrate bloodstream at least two different major forms of trypanosomes are found; a long slender form, which replicates by asexual division, and a short stumpy, non-replicating form. These extracellular parasites are covered with an immunogenic surface coat composed of approximately 107 identical variant surface glycoprotein (VSG) molecules (Barry *et al.,* 2005; Barry and McCulloch, 2001;Vickerman, 1969). The VSG coat physically shields underlying membrane proteins from host immune responses and is central to antigenic variation and survival in the mammalian host. The consecutive, but mainly unpredictable, expression of a large repertoire of VSG genes permits expansion of antigenically distinct trypanosome populations within the host. After activation of host immune responses (in reaction to high parasitaemia), the majority of the parasite population is destroyed. A small number of trypanosomes survive because they express an antigenically distinct VSG coat, and proceed to expand in numbers. The continuous cycles of trypanosome replication and destruction result in waves of fluctuating parasitaemia. The differentiation of the long slender bloodstream forms (BSF) into the non-dividing stumpy BSF occurs in high-density populations of long slender BSFs (Seed, 2003; Vassella *et al.*,1997). The switch to stumpy BSF involves changes in metabolism within the trypanosome, but the molecular signals involved are not yet known. Short stumpy BSF are believed to be pre-adapted for survival within the insect midgut due to the presence of a functional mitochondrion. In the vertebrate bloodstream, trypanosomes utilize glucose as an energy source. However, in the fly midgut, glucose is limiting and a more efficient utilization of glucose and amino acids occurs via the Krebs cycle and oxidative phosphorylation

in the mitochondrion (Maudlin *et al.*, 2004). Characteristic for the African trypanosomiasis is that no intracellular form exists.

It is evident that differentiation of the BSF to the procyclic forms (i.e. the insect midgut-adapted form), which involves replacement of surface VSGs by procyclins, occurs rapidly after bloodmeal ingestion by the fly (Acosta-Serrano *et al.*, 2001; Gibson and Bailey, 2003; Vassella *et al.*, 2001). Most flies successfully kill all invading trypanosomes in a process termed self-cure. For the first three days, trypanosomes are mostly contained within the bloodmeal as it is being digested. The critical events in parasite establishment appear to occur approximately three days after infection, when the relatively small proportion of surviving trypanosomes (~10%) either die or rapidly multiply in number (Gibson and Bailey, 2003). Typically, from eight days after the infected bloodmeal, dissected flies can be confidently divided into two groups; the first in which most flies will have self-cured (having completed the clearing of ingested trypanosomes from their midguts) and the second, which have established midgut infections. Trypanosomes in an established infection migrate to the ectoperitrophic space three to five days post infection (Gibson and Bailey, 2003). It is believed that this occurs by direct penetration through the PM (Ellis and Evans, 1977; Gibson and Bailey, 2003) although an alternative but less likely, suggestion is that it occurs by circumnavigation around the open, posterior end of the PM in the hindgut. Typically the midgut population in an established infection reaches approximately 5 x 10^5 trypanosomes (Gibson and Bailey, 2003; Van den Abbeele *et al.*, 1999). From six to eight days post infection, large numbers of trypanosomes congregate within the proventriculus (Sharma *et al.*, 2008 ; Gibson and Bailey, 2003; Van den Abbeele *et al.*, 1999). They cease division, elongate to mesocyclic forms and later differentiate into long trypomastigotes (Van den Abbeele *et al.*, 1999). Trypanosomes then migrate back into the endoperitrophic space by actively penetrating the PM and move anteriorly in the lumen of the foregut to the opening of the hypopharynx at the tip of the proboscis. An alternative theory of migration involves the direct penetration of the tsetse salivary glands after trypanosomes have traversed the fly haemolymph (Mshelbwala, 1972). It is generally accepted that this is unlikely, as trypanocidal factors known to be present in the haemolymph (Croft *et al.*, 1982) would act as a major barrier for trypanosomes attempting to traverse it. Early positioning of trypanosomes in the anterior midgut

and proventriculus should also favour passage along the foregut to the salivary glands (Peacock *et al.,* 2007).

Asymmetric division of the proventricular epimastigote form generates both long and short parasites and it is either the asymmetrically dividing trypanosome or the short epimastigote that arrives at the salivary gland (Sharma *et al.*, 2008). Each tsetse fly has two salivary glands. Evidence suggests that each gland is invaded and colonized separately, with few epimastigotes constituting the founder populations (Peacock *et al.,* 2007). The short epimastigote forms are believed to attach to the salivary gland epithelium by interdigitation of their membranes. Upon binding, the non-infective epimastigotes complete several rounds of replication and differentiate into the metacyclic form (Sharma *et al.,* 2008). Differentiation (metacyclogenesis) includes the appearance of a VSG surface coat. Metacyclic VSGs display a specific VSG repertoire subset and their expression is regulated differently to bloodstream VSGs (Barry *et al.,* 1998; Graham *et al.,* 1999). Mitochondrial changes also occur, including loss of mitochondrial cristae and Krebs cycle enzymes. The biochemical changes accompany the posterior migration of the kinetoplast before the parasite detaches into the lumen as a mature, free form, and infective metacyclic trypomastigotes. At this point, each mature metacyclic parasite has undergone the transformations necessary for survival in a mammalian host.

The number of flies that develop a mature infection and the length of time required for an infection to establish and mature into a transmissible form can vary depending on several factors, including fly species (Welburn *et al.,* 1989; Welburn *et al.,* 1994), fly sex (Distelmans *et al.,* 1982; Dale *et al.,* 1995) and parasite strain (Dale *et al.,* 1995). Once a fly is infected it will produce infective metacyclics for the duration of its life, which can be 150 or more days for females and about half that for males (Lehane and Mail, 1985; Msangi *et al.,* 1998). Thus, there is potential for infective parasites to be transmitted every time a fly feeds on a new host. Sexual reproduction has been reported in *T. b. brucei* in the salivary glands and may exist in other trypanosome species (Gibson *et al.,* 2008; Jenni *et al.,* 1986; Peacock *et al.,* 2007). It is suggested that the unattached epimastigote is the mating stage (Gibson *et al.,* 2008). A fly remains infective for life, and the whole infective cycle is probably completed successfully in only 1 in 10 flies (Atouguia and Kennedy, 2000).

2.6 Tsetse midgut interactions

All tsetse species are susceptible, to some degree at least, to trypanosome infections. In general, the Palpalis group species tend to be poor vectors of congolense-type trypanosomes compared to the Morsitans group flies (Harley and Wilson, 1968; Moloo and Kutuza, 1988; Ndegwa *et al.,* 1992). Conversely, tsetse flies of the Morsitans group are poorer vectors of *T. b. gambiense* than the Palpalis group (Richner *et al.,* 1988). Care needs to be taken with much of the data on susceptibility, as often fly and trypanosome strains used in experiments are from widely divergent geographical origins. Many factors influence fly susceptibility to trypanosome infection. The understanding of these factors and their underlying mechanisms is still rudimentary.

2.7 Tsetse immune system and vector competence

Despite their obvious efficiency in maintaining large burdens of trypanosome-based disease in Africa, tsetse flies exhibit a considerable level of refractoriness to trypanosome infection. Additionally, intraspecific variations in vector competence have been reported among subpopulations of *Glossina* ssp., which may in turn contribute to the focal distribution of Rhodesian sleeping sickness. Field and laboratory studies on the infection rates of salivarian trypanosomes have shown that some species or subspecies of *Glossina* are better vectors than others (Harley and Wilson, 1968; Moloo and Kutuza, 1988 ;Roberts and Grays, 1972). This differential expression of vector competence may be directly dependent on environmental and intrinsic fly variables (Okoth, 2007).

Even under optimal laboratory conditions, where flies are fed at regular intervals, only a proportion of flies will establish midgut infections and the number decreases dramatically after the adult fly has taken three to four bloodmeals (Distelmans *et al.,* 1982; Kubi *et al.,* 2006; Welburn and Maudlin, 1992). Furthermore, less than half of the infections that become established in the midgut will mature (Gibson and Bailey, 2003; Peacock *et al.,* 2006; Van den Abbeele *et al.,* 1999). A key factor in this refractoriness is the fly immune system (Hao *et al.,* 2001).

Based largely on work on *Drosophila melanogaster*, it is known that insects possess a complex, interacting, and innate immune system. This system is comprised of physical barriers

(such as the cuticle and the PM), cellular responses (such as encapsulation and phagocytosis), and humoral responses, such as the generation of host defense peptides (HDP, previously called antimicrobial peptides), reactive oxygen species (ROS) and melanisation by the phenoloxidase pathway (Lemaitre and Hoffmann, 2007).

The immune response depends not only on the nature of the immune stimulus, but also the point of entry of the antigenic molecule/organism, with quite distinct epithelial (ingestion) and systemic immune (wounding) response profiles generated to the same pathogen (Hao *et al.,* 2001). Clearly, in tsetse trypanosome interactions, it is the epithelial immune responses of the alimentary canal and salivary gland tissues that are likely to be of major importance, as trypanosomes involved in the natural life cycle are exposed only to epithelial surfaces throughout the parasite life cycle. Attacin, defensin, and diptericin antimicrobial peptide genes have been described in tsetse fat body tissue obtained by subtractive cloning after immune stimulation with trypanosomes (Hao *et al.,* 2001). Differential regulation of these genes shows tsetse immune system can discriminate not only between molecular signals specific for bacteria and trypanosome infections but also between different life stages of trypanosomes. The presence of trypanosomes either in the hemolymph or in the gut early in the infection process does not induce transcription of attacin and defensin significantly. However, both antimicrobial genes are expressed at high levels in the fat body and viability of parasites in the midgut is not affected, after parasite establishment in the gut. Unlike other insect immune systems, *diptericin* is constitutively expressed in both fat body and gut tissue of normal and immune stimulated flies. In laboratory infections, transmission rates vary between 1-20%, depending on the fly species and parasite strain (Moloo and Kutuza, 1988; Moloo *et al.,* 1994), whereas in the field, infection with *T. brucei* spp. Complex trypanosomes are typically detected in less than 1–5% of the fly population (Lehane *et al.,* 2000; Woolhouse *et al.,* 1993).

Many factors, including lectin levels in the gut at the time of parasite uptake, fly species, sex, age, and symbiotic associations in the tsetse fly, apparently play a part in determining the success or failure of parasite infections. Tsetse flies have been shown to possess midgut lectin(s) that are capable of killing trypanosomes *in vivo* by a process resembling apoptosis (Welburn *et al.,* 1992). There is however no direct evidence that trypanosomes may be killed by an innate immune response in the fly (Croft *et al,* 2006; East *et al.,* 1983).

2.8 Pathogenesis in Human host

Approximately 5 to 15 days after infection, a painful skin lesion called a *trypanosomal chancre* may develop at the site of the bite (Atouguia and Kennedy, 2000). The parasites spread in the host bloodstream one to three weeks after the initial bite, and invade the lymph nodes and systemic organs including the liver, spleen, heart, endocrine system, and eyes in stage 1, or *hemolymphatic* stage. (Atouguia and Kennedy, 2000; Kennedy, 2004). If untreated, within a few weeks in the case of *rhodesiense* infection, or many months in the case of *gambiense* infection, the parasites will cross the blood–brain barrier (BBB) and enter the central nervous system (CNS), which marks the late, stage 2, or *encephalitic* stage of the disease. (Atouguia and Kennedy, 2000; Kennedy, 2005). The entire tempo of the disease is faster in the more aggressive *rhodesiense* infection compared with the chronic *gambiense* infection, probably as a result of the greater adaptation of the latter parasite to the host.

Immediately after inoculation into the humans, the trypanosome multiplies at the point of inoculation, producing a local inflammatory reaction. It then invades the whole body, and the central nervous system (CNS). The involvement of the CNS leads to an irreversible demyelinating process ending by death, in the absence of treatment. Apart from the initial stages, it is not easy to determine the phase of the disease that the patient is presenting (Greenwood and Whittle, 1980). The parasite can escape the host immune response by varying the surface glycoprotein coat. Variable surface glycoproteins (VSG) are strongly antigenic and lead to great antibody response with immune lyses (Greenwood and Whittle, 1980). But some heterologous antigenic variants can survive to repopulate blood and other tissue. The trypanosome can release numerous pathogenic substances, which cause alterations in cytokine/prostaglandin network. This disorder is classed as stage 1 or 2 depending on whether parasites have become manifest in the cerebrospinal fluid. The pathology has been reviewed elsewhere (Burri and Brun, 2003). First stage is inoculation whereby parasites proliferate at the site of infection leading to an inflammatory nodule or ulcer (Barrett, 2001). This trypanosomal chancre arises about 50% of all *rhodesiense* infection (Burri and Brun 2003). After 3-4 weeks, the chancre usually heals with altered pigmentation (Stich *et al.*, 2002). In the second stage, parasites spread to the draining lymph node and reach the blood stream, initiating the haemolymphatic stage of the disease

(Dumas and Bisser, 1999). This stage is characterized by general malaise, headache, and fever, pancarditis with congestive heart failure, pericardial effusion and pulmonary oedema (Hao *et al.,* 2003).

2.9 Pathogenesis in animal

Animal models of HAT have been developed to understand the pathogenic mechanisms leading to the passage into the neurological phase, most of them referring to histological aspects but not clinical or behavioral data (Darsaud *et al.,* 2003). Different animal models have been proposed, such as trypanosome-infected mouse, rat, dog, sheep, and monkey, using three pathogenic strains (human: *T. b. gambiense* and *T. b. rhodesiense*; rodents: *T. b. brucei*). However, physiological and clinical data have been seldom reported in rodent models because most of the studies were performed on larger animals, as they focused on the immunological reaction to the invading parasites (Losos and Ikede, 1972), and the pathogenesis of the CNS alterations. Available physiological data (e.g., body temperature, motor activity, food intake, etc.) describing the clinical state of the animals during the time course of the disease is therefore scarce.

2.10 Standard HAT chemotherapy

Treatment of HAT is challenging because drugs have to be efficient against the two subspecies of *T. brucei* and the two developmental stages of the disease, necessitating co-ordination of chemotherapy (Matovu *et al.*, 2003). Pentamidin is applied in the early stage of the disease (Mäser *et al.,* 2003), but its efficiency is restricted to *T. b. gambiense* (Delespaux and de Koning, 2007). Pentamidine possesses trypanocidal activity by binding to a minor groove of kDNA in the mitochondrion, thereby, inducing changes in DNA topology and inhibiting topoisomerases (Fox et l., 1990). As a result, the replication of the mitochondrion is impaired and DNA cleavage occurs. Pentamidin has also been identified as inhibitor of the S-adenosylmethionine decarboxylase (Bitonti *et al.,* 1986), resulting in inhibition of the polyamine synthesis and in turn lack of trypanothione.

Suramin is the second early stage drug applied in infections with *T. b. rhodesiense* (Vansterkenburgi *et al.,* 1993). Its trypanocidal action is probably constituted by inhibition of

LDL uptake, prohibiting the parasite's supply of cholesterol and phospholipids. For the treatment of late stage human African trypanosomiasis melarsoprol, is the drug of first choice by providing efficiency against *T. b. gambiense* and *T. b. rhodesiense* (Blum *et al.,* 2001). Application of melarsoprol is crucial to combat late stage infections, but the application of this drug is hampered by its adverse side effects (Blum *et al.,* 2001). Most prominently, encephalopathy causes mortality in five percent of treated patients (3). A newer late stage drug, α-difluoromethylornithine (DMFO, eflornithine) (Kuzoe, 1993), is safer, but only possesses efficiency against *T. b. gambiense* (Iten *et al.,* 1994).

2.11 Development of drug resistance in Trypanosomes

Development of drug resistance in trypanosomes is caused by different mechanisms. Altered metabolic pathways prevent the drug from reaching its target. Alternative pathways facilitate the parasites' independence on inhibited targets or detoxification of the drug. In addition, intracellular accumulation of the drug can be inhibited by decreased drug import or increased drug export (Weber and Courvalin, 2005). Due to the extensive use of drugs and their structural similarities, multiple drug resistance has developed (Kennedy, 2007). Resistance in *T. b. rhodesiense* and *T. b. gambiense* is frequently mediated by reduced net drug uptake. Reduced drug import is mediated primarily via loss of P2, also named TbAT1 (for *Trypanosoma brucei* adenosine transporter 1) (Mäser *et al.,* 2003). Additionally, silent and coding point mutations in the TbAT1 gene lead to changes in substrate specificity (Mäser *et al.,* 1999). Surprisingly, loss of P2 induces only a small increase of resistance towards melarsoprol and pentamidine (2-3-fold) (Matovu *et al.,* 2003). This might be due to a P2-independent uptake of melarsoprol and pentamidine by additional transporters. Another drug resistance mechanism for melarsoprol represents the over expression of the efflux pump TbMRPA (10-fold) (Mäser *et al.,* 2003; Shahi *et al.,* 2002). TbMRPA is one of three ATP-binding cassette transporters present in *Trypanosoma brucei* (Mäser and Kaminsky, 1998). It belongs to the sub-family of multidrug resistance-associated proteins (MRPs), characterized by the need of ATP and thiols to transport molecules

against a concentration gradient (Keppler *et al.*, 1997). Other resistance mechanisms, unrelated to drug transport, have also been observed emphasizing the multifactorial nature of drug resistance (Mäser *et al.*, 2003). Since DMFO is not effective against *T. b. rhodesiense*, there is no safe and efficient drug that crosses the blood-brain barrier to treat the late stage human African trypanosomiasis. The parasite has also developed resistance to suramin, pentamidine and eflornithine (Docampo *et al., 2002*).

CHAPTER THREE
MATERIALS AND METHODS

3.1 Test parasites

The research work was carried out at KARI-TRC using sensitive (KETRI 2427) and resistant (EATRO 237) *T. b. rhodesiense* isolates maintained at TRC Trypanosome bank. Drug sensitive parasite stabilate (KETRI 2427) was prepared from isolates originally collected from a male patient from Busia, Kenya in 1979. Infected patient celebral spinal fluid (CSF) was injected into mice and cryopreserved after first passage. The parasites in the stabilate were confirmed to be sensitive to suramin, Pentamidine, Diminazene and MelB at dose rates of 5mg/kg, 5mg/kg, 10mg/kg and 20mg/kg respectively (unpublished data Pharmacology Division of TRC). Drug resistant parasite (EATRO 237) stabilate was originally isolated from a sleeping sickness patient from Bulenia, Bunyolo, and Port Victoria Kenya in 1961.

3.2 Animals

3.2.1. Donor mice

Twenty-four adult female Swiss white mice (6-8weeks old) and weighing 25-30g were sourced from KARI-TRC and used in this study (20 for transmission and four for virulence). Mice were immuno-suppressed with cyclophosphamide at 300mg/kg daily for three days and inoculated with either drug sensitive (KETRI2427) or resistant (EATRO237) *T.b. rhodesiense.* A capillary tube of the stabilate from the bank was diluted in ethyl diaminetetracetic acid saline glucose (ESG) buffer and the parasitaemia adjusted to 1x 10^6/ml of *T. b rhodesiense.* All donor mice (ten for each stabilate) were inoculated intraperitonially with 0.2ml of the stabilate per mouse. The parasitaemia of the donor mice were monitored daily by bleeding from the tail vein. At the peak of parasitaemia, the mice were euthanized by exposure to concentrated carbon dioxide (CO_2) and bled by cardiac puncture. The blood samples were collected into a tube containing EDTA (anti-coagulant), and trypanosomes quantified using the improved Neubauer chamber. An inoculum dose of 1 x 10^4 trypanosomes was prepared using phosphate buffered saline plus glucose (PSG) pH 8.0 for the virulence study.

3.2.2 Experimental mice

Swiss White mice (6-8weeks old) and weighing 25-30g sourced from Trypanosomiasis Research Centre Animal Breeding Unit, Nairobi, Kenya, were used in this study. Pre-infection data (weights and packed cell volume (PCV) were collected three times a week for a period of two weeks prior to infection. The animals were maintained on commercial mice pellets (Mice pellets® Unga Ltd Nairobi, Kenya) and water *ad libitum* at 21–25^{o}C. These mice were acclimatized for seven days before the experiments commenced. Wood-chippings were provided as bedding material. All experimental procedures involving mice were reviewed and approved by Institutional Animal Care and Use Committee (IACUC) of Trypanosomiasis Research Center (TRC), Kenya.

3.3 DNA extraction

Trypanosome DNA was extracted using Saponin method of Mugittu *et al.,* (2001). Briefly, bloodstream forms of trypanosomes were expanded in mice and purified from the blood by di-ethyl-aminoethyl chromatography method of Lanham and Godfrey, (1970). This was followed by repeated centrifugation and sediment washes with phosphate-buffered saline. The DNA was extracted from purified trypanosomes according to the method described by Higuchi (1989). Purified trypanosome suspensions (250µl) were mixed with similar volume of lysis buffer (0.32 M sucrose, 0.01 M Tris, 0.005 M $MgCl_2$ and 1% Triton X-100, pH 7.5) and centrifuged at 12,000×g for 25 s. The pellet was washed three times with 500µl lysis buffer. The final pellet was re-suspended in 250µl of 1× PCR buffer. Then, 1.5µl of proteinase K (10 mg/ml) was added and mixed by votexing. The samples were incubated at 56°C for 1 h followed by 95°C incubation for 10 min. The DNA concentration was estimated by means of a spectrophotometer at a wavelength of 260 nm using Gene Quant Calculator (Amersham Pharmacia Biotech, Freiburg) and the samples stored at −20°C until required for use.

3.4 PCR validation *T. b rhodesiense* stabilates for species status and SRA gene

The DNA from drug sensitive (KETRI2427 and resistant (EATRO237)) stabilates was validated for *T. b rhodesiense* status and presence of SRA gene by PCR methods of Masiga *et*

al., (1992) and Gibson *et al.,* (2002). Briefly, 2µl DNA products were amplified with 0.04 unit of Phusion high fidelity DNA polymerase (Finnzymes Espoo, Finland) and 0.15mM dNTP in the buffer (provided by the manufacturer which contained 1.5mM $MgCl_2$) in the presence of *T. b rhodesiense* specific primers (forward 5`GAATATTAAACAATGCGCAG 3' and reverse 5` CCATTTATTAGCTTTGTTGC 3`) or SRA specific primers (forward SRA A 5`GACAACAAGTACCTTGGCGC3' and reverse SRA E 5`TACTGTTGTTGTACCGCC GC3) at 200 µM each. Reactions were carried out in PTC 100 thermocycler. The first cycle included 3 minutes at 98°C, 30 seconds at 60°C, and 30 seconds at 72°C. Subsequent cycles involved one minute at 98°C, 30 seconds at 60°C, and one minute at 72 °C for 35 cycles. The final extension was conducted at for two minutes at 72 °C. A no-sample negative control was used. The PCR products were loaded onto Ethidium Bromide 2 % agarose gels in a TAE buffer (Samrook *et al.,* 1989). On every gel, a 50 bp DNA ladder molecular weight marker (Life Technologies, Rockville, MD) was run to confirm expected molecular weights of the amplification products.

3.5 Infection of *G. pallidipes* tsetse fly vector by *T. b rhodesiense*

The species of tsetse flies to be used were *G. pallidipes* from the KARI-TRC tsetse-breeding unit. The flies were maintained in the insectary at a temperature of 24^0C and a relative humidity of 82.5% + 2.5%. Newly emerged (Teneral) flies starved for two days were used.

During transmission studies the flies were placed into a cage holding 25 flies each and fed on the infected mouse until all the flies were fed. The flies were then taken back into the insectary where they were fed *in vitro*, until the infection matured (for about 28days). Within this framework, the flies were fed on blood placed on an electrically heated PVC mat with a silicon membrane, at a temperature of 36-38^0C. In order to determine whether the flies had been infected, probing was done, by removing flies from the holding cage into the probing tubes (holding one fly per tube). The tubes were inverted on a glass slide on a warm plate to induce salivation in the flies. After the saliva had dried the slides were stained and observed under a compound microscope for trypanosomes. The fly was fed on a clean mouse after salivating, and the mouse monitored for parasitaemia. Thereafter the flies were dissected for assessment of trypanosome infections in the mid-gut, proboscis or salivary glands. This was accomplished by euthanizing the flies using chloroform and dissecting them under a dissection microscope. The

presence of parasites was determined through observation of the dissection slides under compound microscope.

3.6 Transmission of drug resistant and sensitive *T. b rhodesiense* to mice by *G. pallidipes* tsetse fly vector

Transmission studies on trypanosomes through mice and tsetse were conducted by methods of Fairbrain and Culwick, (1950) and Wijers, (1958). Briefly; emerged tsetse flies were starved for two days and allowed to feed on infected or uninfected mice. In general, 350 (150 for each stabilate and 50 control) *G. pallidipes* tsetse were infected by allowing them to feed on donor mice infected with sensitive or resistant isolates of the parasite at peak parasitaemia. Each of the individual infected fly from each treatment was fed on individual uninfected mouse. Control experiment was set where a group of 50 uninfected flies were fed on five uninfected mice. Twenty tsetse flies were allowed to feed on a single adult (>30g) mouse. The infected flies were maintained in the insectary for 28 days after which they were starved for two days and then probed to detect mature metacyclic trypanosomes. The flies were then singly fed on mice (one fly per mouse) to determine their ability to transmit the infection. The infected mice were monitored daily for the development of parasitaemia. Original Trypanosome stabilates, blood from infected mice, tsetse saliva obtained from probing and tsetse parts collected after dissections were examined by microscopy and/or PCR. Only 20 samples picked at random from negative mice (by microscopy test) for each stabilate were tested by PCR to confirm infection.

3.7 Virulence of drug resistant and sensitive *T. b rhodesiense* to mouse

Inoculums of $1x10^4$parasites were used to infect each of the 20 experimental mice (10 resistant, 10 sensitive). Control group of ten (non-infected) mice was used. The infected mice were monitored three times a week for 60 days post-treatment. Prepatent period, parasitaemia level, body weight, packed cell volume (PCV) and survival time were then determined. Blood samples from the mouse tail vein were collected three times a week, in a capillary tube for PCV determination and a drop placed on a clean slide, covered with a cover-slip, examined under microscope and the parasitaemia score correlated to a score sheet as outlined by Herbert and Lumsden, (1976). Packed cell volume was determined according to the methods of Naessens *et al., (*2005*).* Briefly, infected blood was collected from the tail vein three times a week using

heparinized capillary tubes sealed with plasticin. The sealed capillaries were then centrifuged in a haematocrit centrifuge at 10,000 revolutions per minute (RPM) and PCV read using the haematocrit reader. Body weights were assessed three times a week using analytical balance (Mettler Tolendo PB 30 ®, Switzerland). The infected mice were examined daily and death of animals recorded, for assessment of survival period.

3.8 Assessment of genetic changes in *T. b rhodesiense* following transition through *G. pallidipes* tsetse and mouse host

Assessment of the genetic changes in *T. b rhodesiense* following transmission through *G. pallidipes* tsetse and mouse (host) was assessed by PCR-RFLP method of Geysen *et al., (*2003). DNA was extracted by methods of Mugittu *et al.* (2001) from *T. b rhodesiense* samples from drug sensitive and resistant isolates obtained 1) before infection of the tsetse flies (original bank stabilates), 2) during infection in the fly (from dissection of infected flies) or 3) in the mice (from parasitemia in the mice). The DNA was amplified by PCR using SRA A and SRA E primer pair by methods of Gibson *et al.* (2002). The PCR products were electrophoresed in 2 % agarose gels in Ethidium Bromide in a TAE buffer (Samrook *et al.,* 1989). On every gel, a 50 bp DNA ladder molecular weight marker (Life Technologies, Rockville, MD) was run to confirm expected molecular weights of the amplification products. The bands were subsequently excised from the gels and transferred to sterile eppendorf tubes with a sterile scalpel. The DNA was subsequently extracted from the gel using QIAquick Gel Extraction Kit (Qiagen, CA, USA) according to the manufacturer's instructions. The ammplicons were restricted with Bam HI, Hae III, Mbo II, HnfI and Hind III separately or in combination in the appropriate NEBuffer (New England BioLabs). On completion of incubation, the whole 20µl volumes were separately loaded into an agarose gel 2% containing ethidium bromide and the electrophoresed gel submerged in TIX TAE buffer. At the end of the run, the gels were documented. The band patterns from both isolates and stages/phases of infection were compared.

3.9 Data analysis

Chi-square test and evaluation of % survivorship were used to assess the first hypothesis by comparing the efficiency of transmission between the two isolates. The second hypothesis

was assessed by analysis of mean parasitaemia, PCV and bodyweights by the student's t-test, which compared the differences between mean values for measured parameters of the control mice and those of the infected group for virulence. The third hypothesis was assessed by visual inspection of presence/absence of bands among isolates and between parasite infection stage/host. In all analyses, a 95% confidence interval ($p < 0.05$) was considered significant.

CHAPTER FOUR

RESULTS

4.1 Transmission of drug resistant and sensitive *T. b rhodesiense* to mice by *G. pallidipes* tsetse fly vector

The isolates in the stabilates (drug sensitive and resistant) were confirmed as belonging to *T. brucei* subgroup (Fig. 1). A total of 150 flies fed on immuno-suppressed mice infected with drug sensitive isolate, survived up to day 35, and when probed, 11 were found positive by microscopy and 62 by PCR, similarly of the 150 flies fed on mice infected with resistant isolate 62 survived up to day 35 and were also probed. When probed, 7 flies were found positive by microscopy and 53 by PCR. PCR analysis using *T. brucei* subgroup specific markers on blood samples from mice exposed to bites from infected *G. pallidipes* revealed presence of the parasite in 54.55% (n =22) mice exposed to drug sensitive, 40.91% (n= 22) of the mice exposed to drug resistant *T. b. rhodesiense* isolates (Fig. 2). The *T. b. rhodesiense* infected *G. pallidipes* infected had a lower survivorship than uninfected control (Table 1). The flies infected with drug resistant isolate had a higher survivorship than those infected by drug sensitive isolate (Table 1). However, more flies infected by drug resistant isolate were positive for infection as demonstrated by PCR while the reverse was observed from microscopy results (Table 1).

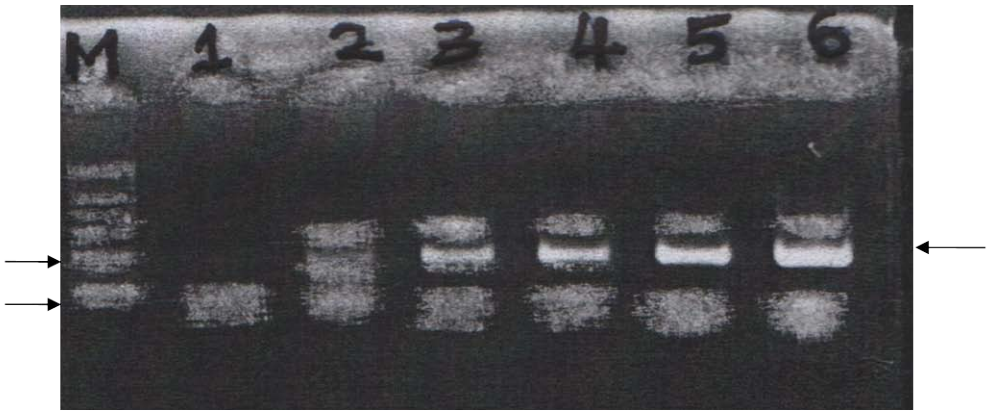
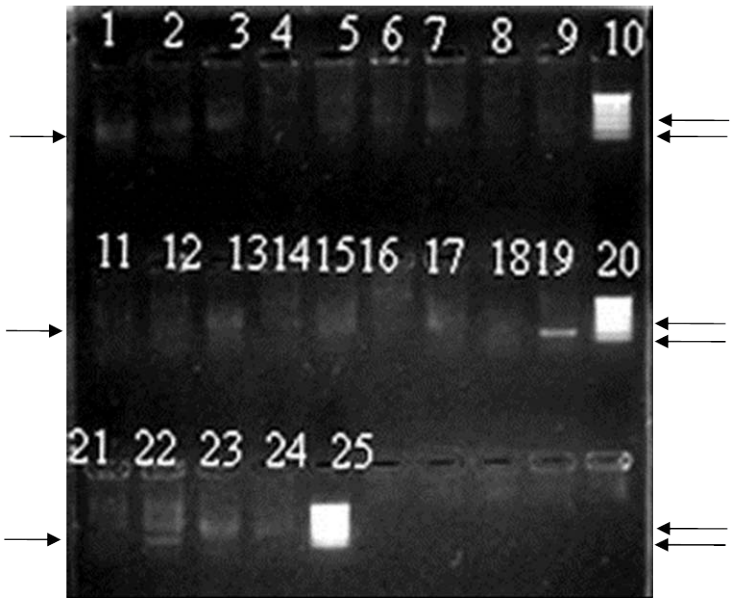


Figure 1. Validation of *T. b. rhodesiense* sub species status by species-specific marker

The lanes denote the following satellite DNA PCR products; 1= negative control, 2 = positive control, 3 and 4 =drug sensitive *T. b. rhodesiense* isolates, 5 and 6 = drug resistant *T. b. rhodesiense* isolates. The PCR was also carried out on *T. brucei* DNA as a positive DNA control, with distilled water as negative control. The amplicons were separated on 2% agarose gel and visualized through ethidium bromide staining.

Panel A



Panel B

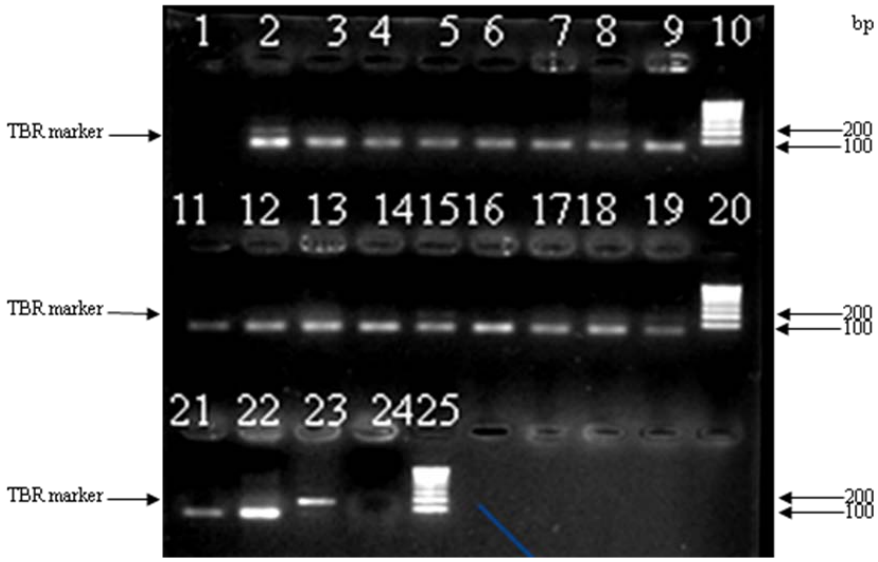


Figure 2. Detection of *T. brucei* parasites in Swiss white mice transmitted via infected *G. pallidipes* bites

Panel A presents PCR amplicons the trypanosome DNA present in blood of mice infected with drug sensitive isolates while Panel B represents drug resistant isolate. The amplicons were separated on the 2% agarose. Samples 1-22 represent PCR products from respective in vivo parasite while samples 23 and 24 represent positive and negative controls, respectively.

Table 1. Percentage survivorship of *G. pallidipes* and infection rates of the flies by drug sensitive and resistant *T. b. rhodesiense* isolate

Stabilates	No of flies infected	Mortality before dissection	No of flies infecting mice	Positive flies by PCR	Positive mice by PCR	Infection rate in % in flies
EATRO 237	150	72	78	53	18	0.67
KETRI 2427	150	82	62	18	15	0.29
Negative control	50	5	40	-	-	0

4.2 Virulence of drug resistant and sensitive *T. b rhodesiense* to Swiss white mice

Results of pre-patent and post-infection survivorship periods in mice infected with drug sensitive and resistant *T. b. rhodesiense* are summarized in Table 2. The mice infected with drug resistant isolate of *T. b. rhodesiense* had a significantly longer ($t_{(df = 9)} = -2.279$, $p = 0.049$) (but marginal) pre-patent period. Post infection survivorship was also significantly longer ($t_{(df = 9)} = -2.360$, $p = 0.043$) in drug resistant than in drug sensitive trypanosome-infected mice. Profiles of parasitaemia in mice infected with drug sensitive and resistant *T. b rhodesiense* isolates are presented in Fig 3. Mice infected with drug sensitive *T. b rhodesiense* isolate had consistently higher parasitaemia than those infected by the drug resistant isolate; however, these differences were not significant ($p<0.05$). On average, mice infected with drug sensitive isolate had a shorter survivorship (21 d.p.i) than their counterparts infected with the drug resistant isolate (38 d.p.i). Four animals of this group died on day 21, three on day 24 and three on day 26 due to the infection. Among the mice infected with drug resistant *T.b. rhodesiense* nine of 10 mice were parasitaemic between days 3 and 10. Increase in parasitaemia was exponential up to day 10 post-infection after which two waves of parasitaemia were recorded. One animal of this group died on day 21, three on day 24 and three on day 26, one each on days 35, 38 and 40. From day 14, the parasitaemia of animals infected with sensitive isolate, were more pronounced ($10^{8.9}$) than resistant isolate ($10^{7.2}$).

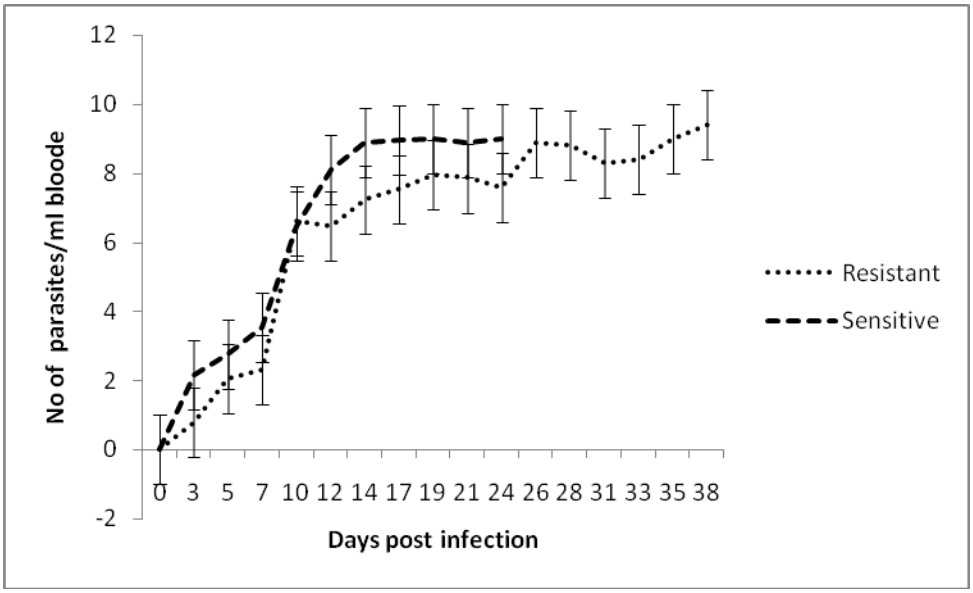
Results of PCV in Swiss white mice following infection with drug sensitive or resistant *T. b. rhodesiense* isolates, and in uninfected control are presented in Table 3. Mean PCV among

mice infected with both isolates, and the uninfected control were similar up to three days post infection. However, there was a consistent and significant ($P<0.05$) drop in PCV in the infected mice relative to the uninfected controls thereafter. Among the infected mice, those infected with drug sensitive isolates had consistently lower PCV than those infected with the resistant isolate. The differences were however not statistically significant ($P>0.05$). The PCV values of both drug sensitive and resistant trypanosome-infected mice dropped from 48.7 to 35 and 39.65 respectively at 12 dpi. The PCV values of the drug sensitive group increased up to 38.6% at 14 dpi and dropped again to 29.4% at 19 dpi, while those of drug resistant group of mice increased slightly to 41% and decreased to 36.7% at 19 dpi. The results of bodyweight measurements in response to infections by the drug sensitive and resistant isolates of the parasite are depicted in Table 4. Whereas the mean body weight of drug sensitive trypanosome-infected mice declined from by day 21 post infection, those of drug resistant trypanosome-infected mice gained slightly over the same period. However from day 21 post–infection significant differences were observed in body weight between the controls and mice infected with drug sensitive isolates and also between the two infected groups. All control mice survived up to the end of the experimental period of 60 days after which they were sacrificed.

Table 2. Mean (± SE, n=10) pre-patent and post- infection survivorship periods (days) in Swiss white mice infected with drug sensitive and resistant *T. b. rhodesiense*

	Pre-patent Period (days)		**Post infection Survivorship Period (days) in Mice**	
***T. b. rhodesiense* Isolate**	**Mean**	**Range**	**Mean**	**Range**
Sensitive (KETRI 2427)	7.50 ±1.19	3- 12	23.40 ± 0.70	21- 26
Resistant (EATRO 237)	11.50 ± 2.01	3 - 26	28.40 ± 2.11	21 - 40

Panel A



Panel B

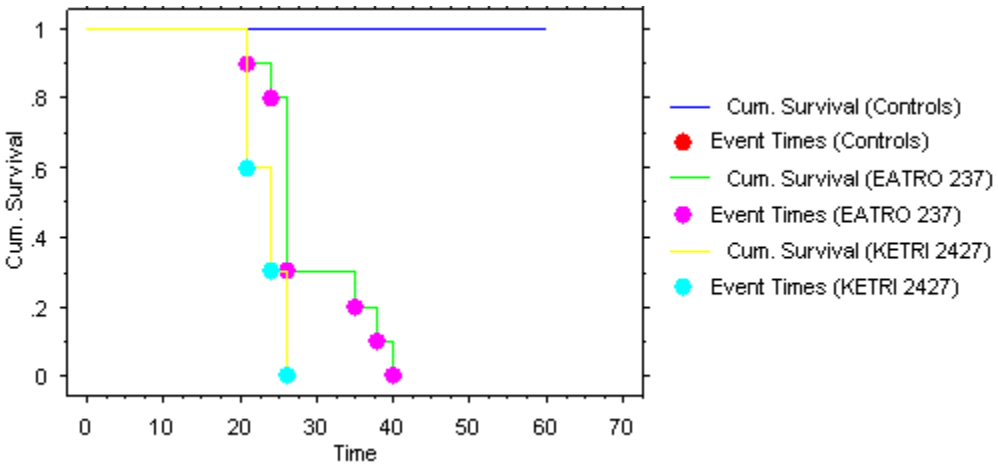


Figure 3. Mean (± SE) parasitaemia and Cumulative survival (days) of drug sensitive and resistant *T. b. rhodesiense* isolates in Swiss white mice

Panel A = parasitaemia profiles in infected mice; Panel B = Kaplan –Meier Cummulative Survival plot. Time = days, Censor variable = cemsored, Group variable = stabilate. Three replicates (ten mice each) were infected with drug sensitive or resistant *T. b. rhodesiense* isolates and parasitaemia and survivorship of respective mice, and also that of un infected control, monitored over 60 days post infection.

Table 3. Mean percentage (± SE, n=10) Packed Cell Volume (PCV) in Swiss white mice infected with drug sensitive or resistant *T. b. rhodesiense* isolates, and in uninfected control

Post Infection Days	Sensitive Isolate	Resistant Isolate	Uninfected Control
3	48.7 ± 0.53^{a}	48.0 ± 0.55^{a}	48.7 ± 0.62^{a}
7	43.5 ± 0.73^{a}	44.9 ± 0.51^{a}	48.6 ± 0.49^{b}
12	35.0 ± 0.46^{c}	39.6 ± 0.73^{c}	48.5 ± 0.55^{b}
14	38.6 ± 0.59^{c}	41.0 ± 0.66^{a}	48.5 ± 045^{b}
21	36.0 ± 1.95^{c}	36.7 ± 2.16^{c}	50.5 ± 1.01^{a}

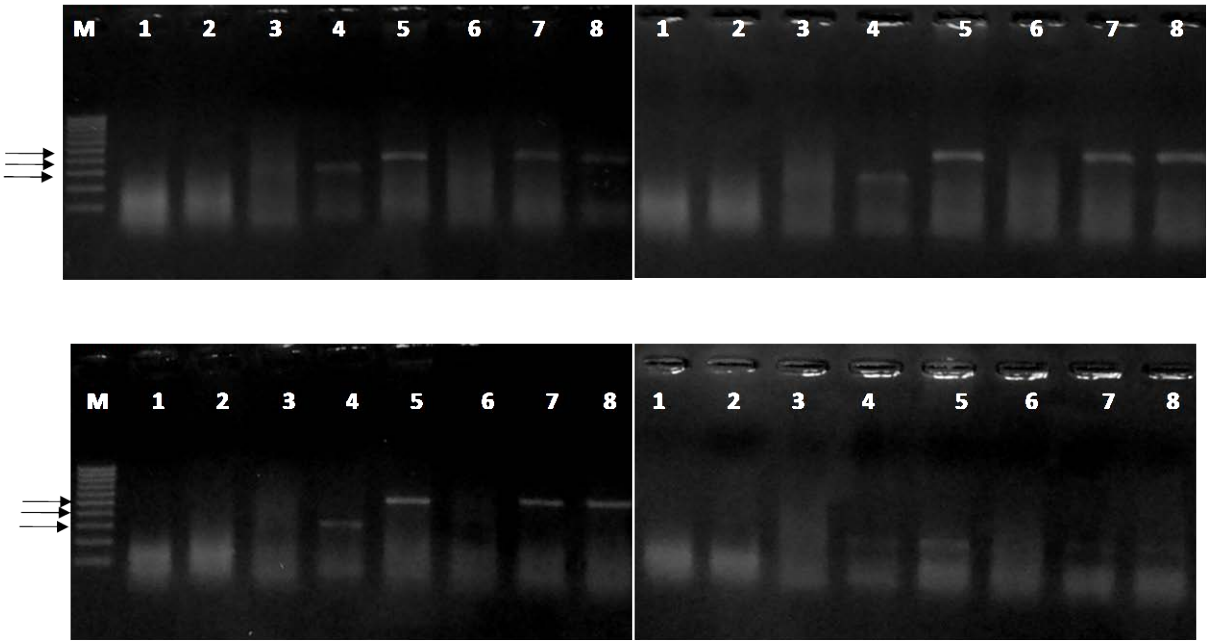
Three replicates (ten mice each) were infected with drug sensitive or resistant *T. b. rhodesiense* isolates and parasitemia of the parasites in the mice, and survivorship of respective mice, and that of un infected control, monitored over 60 days post infection. *Denote significant differences between uninfected control and infected groups ($P<0.05$) by ANOVA

Table 4. Mean (± SE, n=10) weekly bodyweight in Swiss white mice infected with drug sensitive or resistant *T. b. rhodesiense*

Post Infection Days	Sensitive Isolate	Resistant Isolate	Uninfected Control
0	28.20 ± 0.47	29.10 ± 1.08	27.10 ± 0.70
7	28.30 ± 0.58	29.90 ± 1.08	27.90 ± 0.62
14	28.60 ± 0.62	29.70 ± 1.05	28.30 ± 0.65
21	26.33 ± 1.17	29.10 ± 1.07	27.80 ± 0.61
28	-	29.33 ± 3.18	28.51 0.60

4.3 Genetic changes in *T. b rhodesiense* following transition through *G. pallidipes* tsetse and mouse host

Results of PCR-RLFP analysis of SRA A and SRA E in *T. b rhodesiense* revealed discernible polymorphism (MboII, BamHI and HindIII) between the genome drug resistant and sensitive of the parasite in the infected fly and those in the donor mice Fig 2. No polymorphism was observed within drug sensitive or resistant parasite DNA and also between the various stages of infection (mice and tsetse vectors) Fig 4.



The digest products were resolved in 2 % agarose gels stained with Ethidium Bromide. Numbers denote the following origin of *T. b rhodesiense* DNA samples; 1-2 = infected mice; 3-4 = flies; 5,6 = Donor mice; 7-8 = gene bank. All even and odd numbered samples denote drug sensitive and resistant *T. b rhodesiense*, respectively

Figure 4. PCR-RFLP of SRA A and SRAE genes in *T. b rhodesiense* during various transmission phases of the parasite between the *G. pallidipes* vector and Swiss white mice host

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMENDATION

5.1 Discussion

Treatment failures have been reported in human African trypanosomiasis, a disease that is fatal but can be treated. Drug resistance has been implicated as one of the causes of these treatment failures. This study has confirmed existence of drug resistance *T. b. rhodesiense* using PCR. The pathogenesis and transmissibility of the sensitive and resistance isolates were compared in studies carried out in Swiss White mice. The study has further confirmed the applicability of TBR marker to identify the *T. brucei* subgroup in concurrence with the findings of Masiga *et al.,* (1992). The findings further demonstrated viability of the technique, even on cryo-preserved samples, suggesting that the any genetic changes accompanying cryopreservation in *T. b. rhodesiense* do not affect the TBR gene. However further studies need to be conducted to establish if the marker would be applicable in *T. b. rhodesiense* by other methods, or if there are differences in performance of the marker between different field isolates. The results also highlight and confirm the relatively higher sensitivity of PCR technique in detection of *T. b. rhodesiense* relative to microcopy. The observed differences could be due to limitations of the field of view of the microscope, which may not be able to detect low parasitaemia in the mice. Alternatively, the higher sensitivity exhibited by PCR could be a product of false positive, which is common with the techniques in other applications as well. This suspicion makes the microscopy approach the gold standard. Reasons behind the higher parasitemia in drug sensitive than drug resistant isolates are not clear. However, this phenomenon could be related to strain to stain variations (Moloo and Kutuza, 1988; Moloo and Kabata, 1994) probably linked to biological cost of maintaining the resistance gene in the parasite adversely affecting the fitness of the parasite by reducing its proliferation in the insect vector. Some of the biological cost could be related to the enhanced susceptibility of the parasite to anti-microbial peptides (Hao *et al.,* 2001). Similar arguments can also explain the differences in survivorship between infected and uninfected tsetse, as well as among tsetse infected by different strains in this study.

Results established that the drug sensitive *T. b. rhodesiense* was more virulent than the resistant isolate. This phenomenon may also be linked to the biological cost of bearing the drug resistance gene in the parasite. The cost can be reflected in a decline of resistant parasite in

environments devoid of drug pressure (Agnew *et al.,* 2004). The average pre-patent period of the sensitive isolate in mice established in this study was similar to that reported by Thuita *et al.,* (2008), which showed that the mean pre-patent period of *T.b. rhodesiense* was between 4-10 days after infection. The results demonstrated that the resistant isolate took marginally longer than the sensitive to establish, multiply and cause disease, which could be reflecting populations at the initial stages of divergence. The significantly higher parasitaemia in drug sensitive than resistant isolate suggests that sensitive isolate is more pathogenic than the resistant, which concurs with the findings of Murray (1979) who established direct correlation between parasitaemia and pathogenesis/virulence in *T. brucei* and *T. congolense*. The differences in survivorship in mice infected with drug sensitive and resistant isolate potentially presents treatment challenges in the field. Sensitive methods therefore need to be developed to detect low levels of parasites in humans so that the treatment can be administered at the right time before these resistant parasites invade the brain during second stage of the disease.

The survival time of mice infected with *T.b. rhodesiense* has been estimated between 3 and 12 weeks (Kagira *et al.,* 2007). In our study the mean survival time of mice infected with the two stabilates was 3 and 4 weeks, respectively, (range 21-26) and (26-40), which is in agreement with previous studies (Morrison *et al.,* 1981; Murray *et al.,* 1981). For virulence test, the results from this study have confirmed that, by using body weight, parasitaemia and PCV as parameters, *T. b. rhodesiense* infections in the mouse model can be divided in two groups, sensitive and resistant, in concurrence with *T.b. brucei* infection divisions in cattle (Van den Bossche *et al,* 2006). There were no significant differences in body weight between the controls and resistant groups in terms of body weight loss due to infection. However, there were significant differences ($p<0.05$) between the group of mice infected with sensitive isolate and the controls from day 21 post-infection. These differences were also observed between the two infected groups during the same period. This phenomenon could be related to the differences in virulence between the different isolates, which could be related to the clinical manifestations such as anemia associated with trypanosomes. There were no significant changes observed in the genome of the parasite as it transited through the mouse host and tsetse vector. This is probably because there was no significant selection pressure sufficient for the parasite to modify its genome from the present

state to adapt to the pressure. Additionally, positive mutations in the genome normally take place over several generations of selection pressure, which was absent in the current study.

5.2 Conclusions

Drug resistance influences transmissibility in *T. b. rhodesiense* parasite by the *G. pallidipes* vector, with resistance decreasing transmissibility and reducing the survivorship of the tsetse vector. Drug resistance in *T. b. rhodesiense* reduces the virulence of the parasite to the vertebrate host and the transition through the tsetse and mouse host of the parasite do not significantly alter its genome.

5.3 Recommendations

1. Impact of drug resistance on transmissibility of *T. b. rhodesiense* parasite by the *G. pallidipes* vector, and on the fitness of the vector should be evaluated in nature.

2. The impact of drug resistance on transmissibility of trypanosomes by other vectors (other than the ones evaluated in this study) should be evaluated in the laboratory to establish if this phenomenon is a general feature of trypanosomes / vector interactions or unique to *T. b. rhodesiense* / *G. pallidipes* interactions. This can influence trypanosomiasis control initiatives.

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