CHARACTERIZATIONOF AN ANOMALOUS *TRYPANOSOMA BRUCEI RHODESIENSE* ISOLATE FROM BUSIA COUNTY, A SLEEPING SICKNESS FOCUS IN WESTERN KENYA

MIRIAM JEMUTAI

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2019

DECLARATION

I Miriam Jemutai do hereby declare that this thesis is my original work and has not been presented elsewhere for the award of a degree. Where other people's work has been used, it has been referenced and acknowledged accordanly.

Signature.....**Date**....13/10/2020.....

Miriam Jemutai

I56/88557/2016

School of Biological Sciences

University of Nairobi

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

Dr David Odongo

Signature.....**Date**....15/10/2020.....

(School of Biological Sciences, University of Nairobi)

Dr Gloria Omosa Manyonyi

Signature.....**Date**.....15 OCT 2020.....

(Department of Medical Microbiology, University of Nairobi)

Dr Florence Wamwiri

Signature.....**Date**14 OCT 2020.....

(KALRO- Biotechnology Research Institute –Muguga)

DEDICATION

I dedicate this work to Almighty God, to my husband Micah Kipronoh Kiplagat who supported me throughout my studies. To you I say thank you and may God's blessings be your portion always.

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LIST OF ABBREVIATIONS

HAT	Human African Trypanosomiasis
Dpi	Days post infection
EDTA	Ethylenediaminetetra-acetic acid
PCR	Polymerase chain reaction
KALRO	Kenya Agricultural and Livestock Research Organization
	BioRI Biotechnology Research Institute
KETRI	Kenya Trypanosomiasis Research Institute
IUCAC	Institutional Animal Care and Use Committee
WHO	World Health Organization
Mls	Milliliters
IP	Intra-peritoneal
CSF	Cerebrospinal fluid
BSF	Blood stream forms
µl	Microlitre
ITS	Internal Transcribed Spacer region
Kb	Kilo base
$MgCl_2$	Magnessium Chloride
mM	Millimolar

VSG	Variable Surface Glycoprotein
dNTPs	Deoxynucleotide triphosphates
EATRO	East Africa Trypanosomiasis Research Organization
USAID	United States Agency for International Development
BLAST	Basic Local Alignment Search Tool
CNS	Central Nervous System
DNA	Deoxyribonucleic Acid
KB	Kilo Base
PCV	Packed cell volume
SIT	Sterile Insect Technique
SRA	Serum Resistant Associated gene
Taq	Thermus aquaticus
WHO	World Health Organization
PPP	Pre-Patent period
N	Number
Spp	*Species*
G	Glossina
Bp	base pair

CF	Forward primers
BR	Reverse primers
U.S.A	Uniited States of America
EU	European Union
TAE	Tris- acetate-EDTA
PSG	Phosphate saline glucose
IAEA	International Atomic Energy Agency
IP	Intra peritoneal
Mg	Milligramme
Kg	Kilogramme
ANOVA	Analysis of Variance
MST	Mean survival time
SD	Standard deviation
SE	Standard Error
NECT	Nifurtimox-eflornithine combination therapy

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ABSTRACT

This study was carried out to characterize an anomalous *Trypanosoma brucei rhodesiense* isolate (KETRI 3971) that was obtained from Obekai village; Busia County, Kenya and compared with three non-anomalous isolates (KETRI 2482, KETRI 2382 and KETRI 3799) that were obtained from the neighboring locality. All isolates were obtained from patients and later preserved at the parasite cryo-bank in KALRO- BioRI (Muguga). DNA was amplified to target the ITS (internally transcribed spacer) region and SRA genes. The virulence of the isolates was compared following *in vivo* infection of *Swiss* white mice(n=10) per isolate, which were regularly checked for pre-patent period (PPP), extent of parasitaemia, packed cell volume (PCV) and survival time for 30 days-post-infection (dpi) duration. The susceptibility of the isolates to Suramin was also compared following *in vivo* infection in *Swiss* white mice (n=5) per isolate. In addition, *Glossina pallidipes*(n=100) and *G morsitans*(n=100) per isolate were used to determine the infection and transmission ratesusing a mouse model. All the four isolates were successfully amplified using the trypanosome specific ITS marker confirming that these isolates were trypanosomes. Amplification of all the four isolates using serum resistance antigen (SRA) gene primers yielded specific PCR products, thus confirming the isolates as*T. b. rhodesiense*. All infected mice had pre-patent parasitaemia duration of 3-6 days. Mice infected with the anomalous isolate had low parasitaemia profile, unaffected PCV and they all survived to the end of the experiment compared to mice infected with the non-anomalous isolates whose parasitaemia levels were typically high with a significant decline

($p<0.01$) in PCV and a reduced survivorship (days) and high mortality. The observed low parasitaemia profile together with high PCV, low mortality in mice infected with the anomalous KETRI 3971 isolate is suggestive of a variant form of *Trypanosome brucei rhodesiense* that is possibly less virulent and causing a chronic form of infection in humans. All trypanosome isolates under study were susceptible to Suramin with no relapse detected following treatment of infected mice. *Glossina pallidipes* were the more susceptible species to infection and were more efficient in transmitting infective trypanosomes compared to *G morsitans.* This study reveals and reports for the first time, the existence of a *T. brucei rhodesiense* strain in Busia County that causes a chronic form of infection typically not associated with the *T. b. rhodesiense* infection. It also reveals that *G. pallidipes* was the suitable vector that competently pick, establish and transmit the anomalous KETRI 3971 isolate. In light of these findings, effective intervention measures will be necessary to control the possible transmission of the parasite strain through rapid diagnosis and treatment of infected persons as well as controlling the spread of the susceptible *Glossina* vector.

Key words:*Trypanosomabruceirhodesiense*; anomalous, *Glossina*, Busia, Kenya.

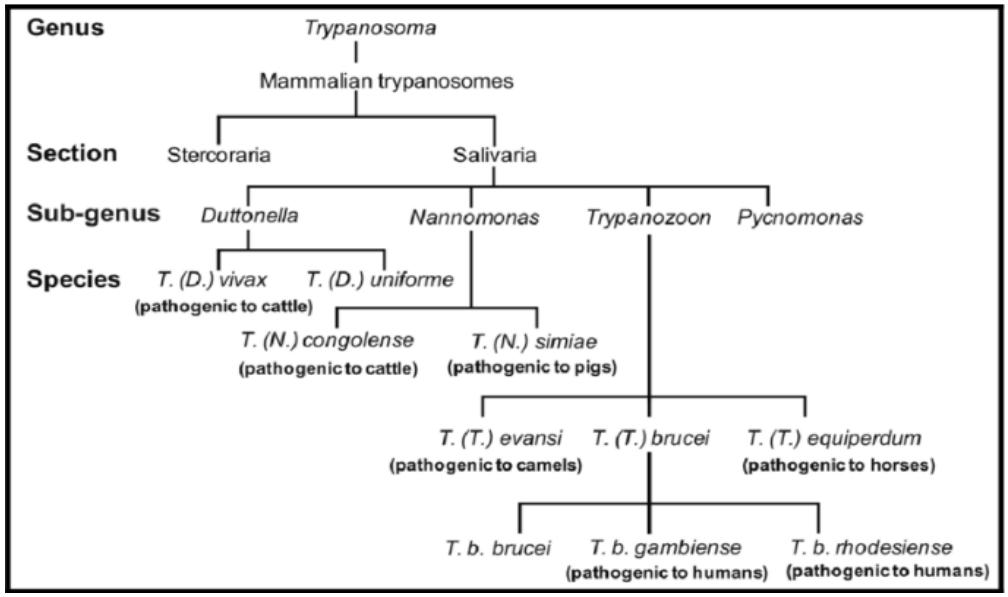
CHAPTER ONE:

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 General Introduction

Human trypanosomiasis is a disease caused by haemo-flagellated protozoan parasites known as trypanosomes belonging to the Phylum Protozoa, Order Kinetoplastida and Family Trypanosomatidae (Figure 1). The parasites are grouped into two based on the part of development to maturity stage within the vector; Salivarian trypanosomes whose vectors are tsetse flies (*Glossina* spp.) cause Human African Trypanosomiasis (HAT) and nagana in animals, its development takes place in the foregut of the tsetse fly (Radwanska *et al*., 2018),while Stercorarian trypanosomes which cause Chagas disease/American trypanosomiasis and whose main vector are triatomines (Stevens *et al*., 2011), develop in the posterior part of the insect gut and is transmitted through feces.

Figure 1: Classification of African trypanosomes (LEVINE *et al*., 1980)



HAT or African Sleeping Sickness is ranked as one of the known important disease after schistosomiasis and malaria (TIETZE P.E. and J.E., 1991) The two *Trypanosoma brucei* subspecies causing human infection, are *T. b. rhodesiense* which is present in Eastern Africa, and *T. b. gambiense* which is common in Central and Western Africa. Infection with these two parasites exhibiting variation in disease progression where *T. b. rhodesiense* causes severe and acute infection compared to a chronic form due to *T. b. gambiense*. *T. brucei brucei*, only infects animals and causes nagana. Other *spp* of trypanosomes that infect livestock include *Trypanosoma congolense*, *T. vivax* which are transmitted by several species of tsetse flies (*Glossina*)(Courtin et al., 2008)

African sleeping sickness was first reported in Uganda in early 1900 (Hide, 1999) and later on spread to Kenya in 1901 when it was first reported. The disease and the responsible vector was prevalent especially Nyanza region has been the disease focus particularly in Lambwe Valley (Baldry, 1972). In 2004, Busia and Bungoma counties in Western Kenya were considered as endemic areas. Human cases of sleeping sickness although not frequent, are still present within these regions particularly in Teso constituencies of Busia county (vonWissmann *et al*., 2011). HAT vectors found in Busia include *Glossina pallidipes* and *Glossina fuscipes fuscipes* species (Rutto *et al*., 2013).

Between the year1969-2009 several isolates of *T. b. rhodesiense* were obtained from various patients who attended Alupe Hospital in Busia County and presented with symptoms of HAT. In 2009, a *T. b. rhodesiense* isolate (KETRI

3971) was obtained from a patient from Obekai village who presented with clinical features like feeling sleepy most of the time, headache, loss of appetite and fever and these features had lasted for about 2 years. Upon diagnosis, a single motile but sluggish trypanosome was observed in the buffy coat preparation and a pre-patent period of 20 days in a mouse model.

From the foregoing case description, this species of *Trypanosoma rhodesiense* appeared to cause a chronic infection which has never been previously reported in Kenya. This study therefore undertook to carry out a thorough characterization of this anomalous isolate and determine the possibility of the presence of the chronic form of *T. b. rhodesiense* infection in Busia, Kenya.

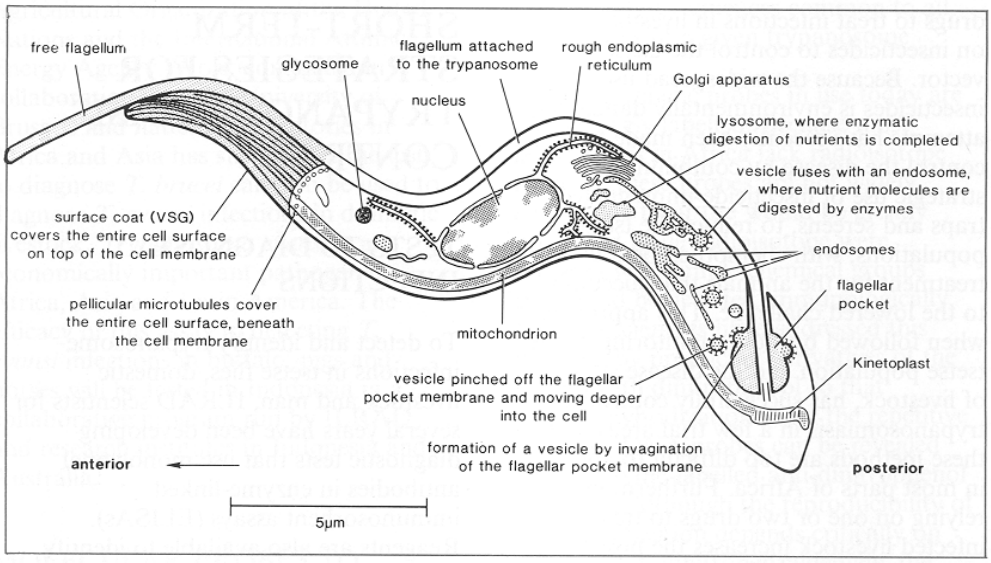
1.2 Morphology of trypanosomeand species characterization

The morphology of bloodstream trypanosome is illustrated in Figure 2. Trypanosome species are distinguished morphologically by features like shape, length, and the location, of organelles within the parasite such as the nucleus, basal body, flagellum and kinetoplast(Kreier and Baker, 1987). *T. congolense* have shapes which do not vary (monomorphic)while *T. vivax* and *T. brucei* species have marked pleomorphism (variable shapes) with blood stream forms appearing as slender, intermediate and stumpy forms(Kreier and Baker, 1987). The swimming patterns together with trypanosome morphology are important features used in the characterization and identification of different trypanosome species. This characterization is based on the waveform variations in relation to the parasites microenvironment (Bargul *et al*., 2016). The cellular waveform indicates the exact environment a particular parasite populates within the

definitive host (Bargul *et al*., 2016). For instance, *T. brucei* subspecies display waveforms more adopted to tissue spaces which is a more confined environment unlike *T. congolense* whose waveforms are small and stiff thus making the parasite a weak swimmer and enabling it adhere to cells confined in areas of low circulation (Bargul *et al*., 2016).

The parasite's locomotion is influenced by a single beat of a flagellum. The density of particles in the microenvironment influences the parasites maximum velocity; increase in the density of obstacles causes the parasite to reverse its flagellar beat and swims backwards to avoid getting trapped. The reversal is random resulting in cell tumbling and irregular wave forms(Bargul *et al*., 2016).

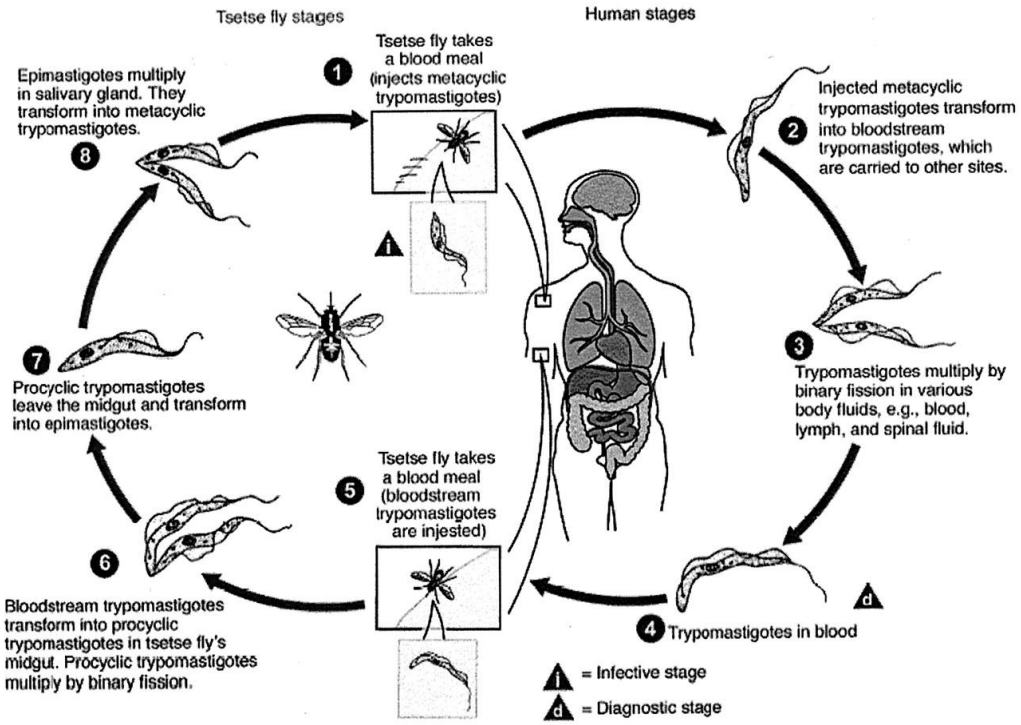
Figure 2: The trypanosome morphology



1.3 Life cycle of trypanosomes

The cycle which is illustrated in figure 3 is initiated when an arthropod vector ingests a blood meal containing trypomastigotes from an infected vertebrate. The transmitted parasites gets transformed into epimastigotes by shedding the antigenic coat typical of blood stream trypanosomes and establish in the midgut and begin to multiply, differentiate and eventually migrate to the mouthparts (*T. congolense* and *T. vivax*) or to the salivary glands (*T. brucei* spp.) as illustrated in figure 3 and transform into metacyclic forms (Matthews, 2005). Intra dermal injection of metacyclic trypanosomes through a tsetse bite begins new infection in a new mammalian host (Stijlemans *et al*., 2016) where the infective trypanosomes in the mammalian host transform into bloodstream forms (Barry and McCulloch, 2001). The host immune system begins to select the predominant blood stream form variants whenever the parasitaemia level goes high, resulting into parasitaemic waves which are continuous and therefore increasing the probability of the parasite spread to a new host.

Figure 3: Life cycle of *Trypanosoma brucei* in the human and the arthropod vector. Image credit: Alexander J. da Silva and Melanie Moser, Centers for Disease Control Public Health Image Library.



1.4 Epidemiology and Distribution of HAT

The HAT infections due to *Trypanosoma brucei* sub-species is common in 36 African countries and affects the health of many sub-Saharan inhabitants(Kennedy, 2004). Humans are regarded as the main reservoir for *Trypanosoma brucei gambiense* unlike *T. b. rhodesiense* which causes zoonotic infections. The presence of *Trypanosoma brucei gambiense* in human has been reported to be low and the duration of infection is long with few symptoms, these has been considered sufficient enough to maintain a human–fly–human transmission cycle(Baker, 1991). Therefore, the population of

people at risk is higher in areas where *Trypanosoma brucei gambiense* is found than in *T. b. rhodesiense* infected foci. The epidemiology of HAT in *T. b. rhodesiense* infected foci is influenced by the interaction of the animal and human hosts with the vectors (tsetse flies) and the parasite (trypanosome) within a given environment. These interactions are confined in rural remote areas mainly Sub-Saharan Africa where a disease foci has been created(Franco *et al*., 2014).

Previous study was conducted to estimate and map the population at risk of sleeping sickness in western part of Kenya(Simarro *et al*., 2012). This study reported the risk level of HAT as low and localized in the region. Part of Maasai Mara National Reserve is also estimated to be at risk of HAT infections as influenced by risk observed in the neighbouring Serengeti National Park(Simarro *et a*l., 2012).

A study was carried out to establish the factors associated with acquisition of human infective and animal infective trypanosome infections in a range of domestic animals in Busia County(vonWissmann *et al*., 2011). In their study, human infective *Trypanosome brucei rhodesiense* were reported in 21.5% of all sampled livestock with higher rates of infection in pigs than in cattle and other small ruminants. Pigs were reported to be reservoirs of infection for *T. b. rhodesiense* and present a risk to local communities.

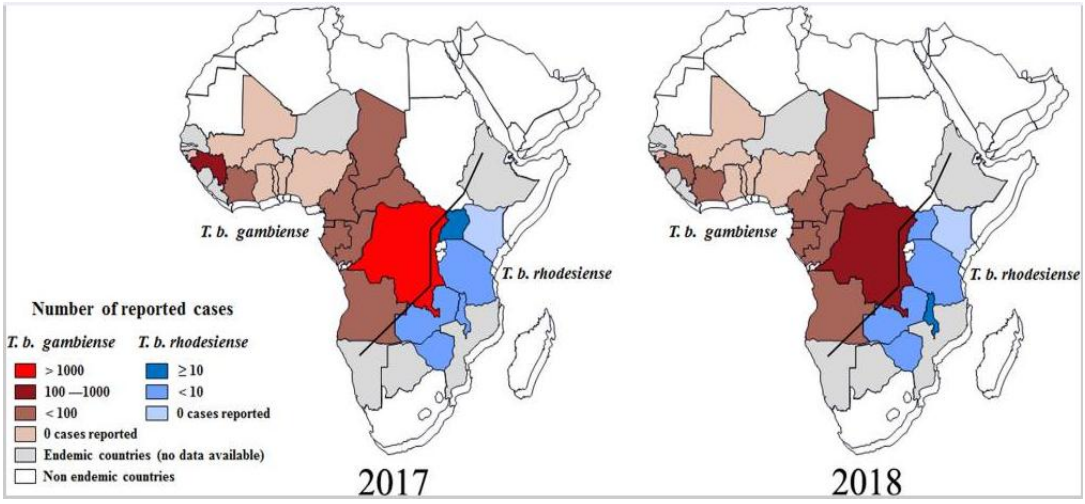


Figure 4: Distribution of human African Trypanosomiasis in endemic countries, 2017 and 2018. Data are taken from the World Health Organization (WHO) (Gao *et al*., 2020).

1.5 Pathogenesis of Human Africa Trypanosomiasis

The severity of Sleeping sickness is dependent on the pathogenicity of the parasite strain as well as the genetics of the human host (Courtin *et al*., 2008). The human host genetics differs across the different ethnic groups living in a given area and this causes variations in the susceptibility to infection(Courtin *et al*., 2008). The disease has two distinct stages, the first or early stage of the disease, also known as the haemo-lymphatic phase, is defined by the restriction of the trypanosomes to the blood and lymph system. The early clinical sign commonly associated with infection in humans is a chancre that develops due to tsetse fly bite (Gelfand, 1966). Following the initial bite, the inoculated trypanosomes multiply at the inoculation site forming a swelling. The parasites eventually spreads into the host bloodstream and invade various body organs (Kennedy, 2004; Kennedy, 2006). The early infection symptoms include

headache, itching, high temperature and joint pains while the late stage is identified by the presence of the parasites in the cerebrospinal fluid (Darsaud et al., 2003). If left untreated, *T. brucei rhodesiense* will cross blood brain barrier (BBB) within 3-8 weeks of infection (Kennedy, 2004; Odiit *et al*., 1997) while the central nervous system(CNS) involvement in *T. b. gambiense* infection occurs after several months to years (Büscher *et al*., 2017). The CNS involvement marks the last stage of the infection(Kennedy, 2006) with signs and symptoms such as severe motor and sensory problems, mental disturbances, and the classic reversal of sleep patterns (Kennedy, 2006) and in the absence of treatment the patient dies (Lorna MacLean, 2004). Domestic and wild animals like cattle, pigs and small ruminants like sheep and goats are important reservoirs of infections affecting human(vonWissmann *et al*., 2011). The infection is transmitted mainly via tsetse saliva when obtaining blood meals. However, in rare cases there are other modes of transmission like transmission via organ transplant, blood transfusion, sexual contact, and accidental laboratory exposure. Also a mother can occasionally pass the infection to her unborn child like in the case of *T. b. gambiense* infections (CDC., 2015).

1.6 Clinical presentation of HAT

Previous studies have described different clinical features of infections due to both *Trypanosoma brucei gambiense and Trypanosoma brucei rhodesiense*. *T. b gambiense* HAT has been estimated to take a mean time of over one year to reach the second stage (Haller *et al*., 1986) and the hallmark of this stage are

neurological signs and symptoms (Haller *et al*., 1986) which include severe motor and sensory problems, mental disturbances, and the classic reversal of sleep patterns (Kennedy, 2006). *Trypanosoma brucei rhodesiense* typically causes severe and acute infection in East Africa and other countries a (Lorna MacLean, 2004 ; Mwanakasale *et al*., 2014) with no specific signs and symptoms however; headache, lack of appetite and fever are common. Mild *T. b. rhodesiense* strains causing chronic infections have been documented in Zambia and Malawi(Mwanakasale *et al*., 2014) with existing reports of asymptomatic carriers (Bajyana Songa *et al*., 1991). In Zambia, two case reports were presented showing two varied strains of this parasite, one case showed a mild strain which was diagnosed with meningo-encephalitic stage of disease after two years and another case showed a highly virulent strain presenting with the known acute and severe infection diagnosed with CNS involvement after 3 weeks of infection. In Malawi, a milder case has also been documented(Lorna MacLean, 2004). In the study, two trypanosomiasis foci (Uganda and Malawi) with different virulence profiles were identified. Infections in Uganda had 86.8% of infected patients rapidly progressing to late stage while infections in Malawi presented with chronic form with only 7.1% of infected patients progressing to the late stage.

Previous studies were conducted in endemic areas of Tanzania and Uganda to described a clinical presentation of *T. b. rhodesiense* sleeping sickness in second stage patients from Tanzania and Uganda. In the study, 138 second stage patients from Tanzania and Uganda were enrolled and signs and symptoms observed. In endemic areas of Uganda, signs of infections which are

not specific to HAT were observed whereas in Tanzania neurological signs and symptoms associated with HAT were observed(Kuepfer *et al*., 2011).

1.7 Control and treatment of Human African Trypanosomiasis

Treatment of sleeping sickness primarily involves the use of few active drugs that have been in use for several decades (Kibona *et al*., 2006). Five drugs have been known to treat HAT this include; Suramin the only first stage drug for treating HAT due to *T. b. rhodesiense* and Pentamidine for early stage *T b gambiense* infections while melarsoprol, eflornithine and nifurtimox are use in treating second stage infection (Pépin J1 and F., 1994). However, there are challenges involved in the procurement and use of all these drugs;-They are expensive and their procurement process and approval for use has to go through WHO which is a long process, these drugs sometimes can fail to cure the disease due to inaccurate diagnosis of the parasite and staging of the disease and as a result, adverse side effects like toxicity of drugs are observed(Aksoy *et al*., 2017). Suramin has a number of negative effects which include Anaemia neuropathy fatigue, nausea, renal complications and anaphylactic shock. These negative effects occur when Suramin level is high therefore, its introduction into the blood stream should be 1 g/injection or less (Babokhov *et al*., 2013)Suramin can also treat *T. b. gambiense* infection however, it is recommended because it is contraindicated in co-infections involving *Onchocerca volvulus*(Büscher *et al*., 2017). Melarsoprol a 2nd stage HAT drug acts against both parasites that have invaded the cerebrospinal fluid (Blum J1 *et al*., 2001).

Nifurtimox-eflornithine combination therapy (NECT) is a recently developed drug reported to be effective, safe and tolerable in treatment of 2nd stage *Trypanosoma brucei gambiense*(Alirol *et al*., 2013). Controlling trypanosomiasis in mammalian host has been a challenge because currently there are no available vaccines due to the antigenic variations displayed by the parasite in the mammalian host, also there are no prophylactic drugs for the control of this disease(Balasegaram *et al*., 2009; Delespaux and de Koning, 2007). Therefore, proper coordination of available drugs should be put in place to effectively act on the developmental stages. Screening for infections due to *T. brhodesiense* is still a challenge due to unavailability of serological tests to be used in the field and the only way is through observation of the clinical features(Aksoy *et al*., 2017).

Insecticides have been used in attempt to reduce the population of tsetse vectors. This method involved the application of insecticides on screens or on odour-baited traps or ground spraying or air spraying by planes onto the tsetse infested area (MacLennan, 1980). Cattle dipping or use of deltamethrin as wash or pour-on on cattle is another method used to kill tsetse flies.

Sterile insect technique (SIT) is one of the genetic techniques used in controlling tsetse flies. Sterile male flies subjected to gamma radiation are released to the infested area to mate with wild female flies. This method has successfully worked in Zanzibar to eradicate *G. austeni*(Vreysen *et al*., 2009). However, application of the SIT method may not be practical in large infested areas due to the diversity of tsetse species transmitting the disease, the method

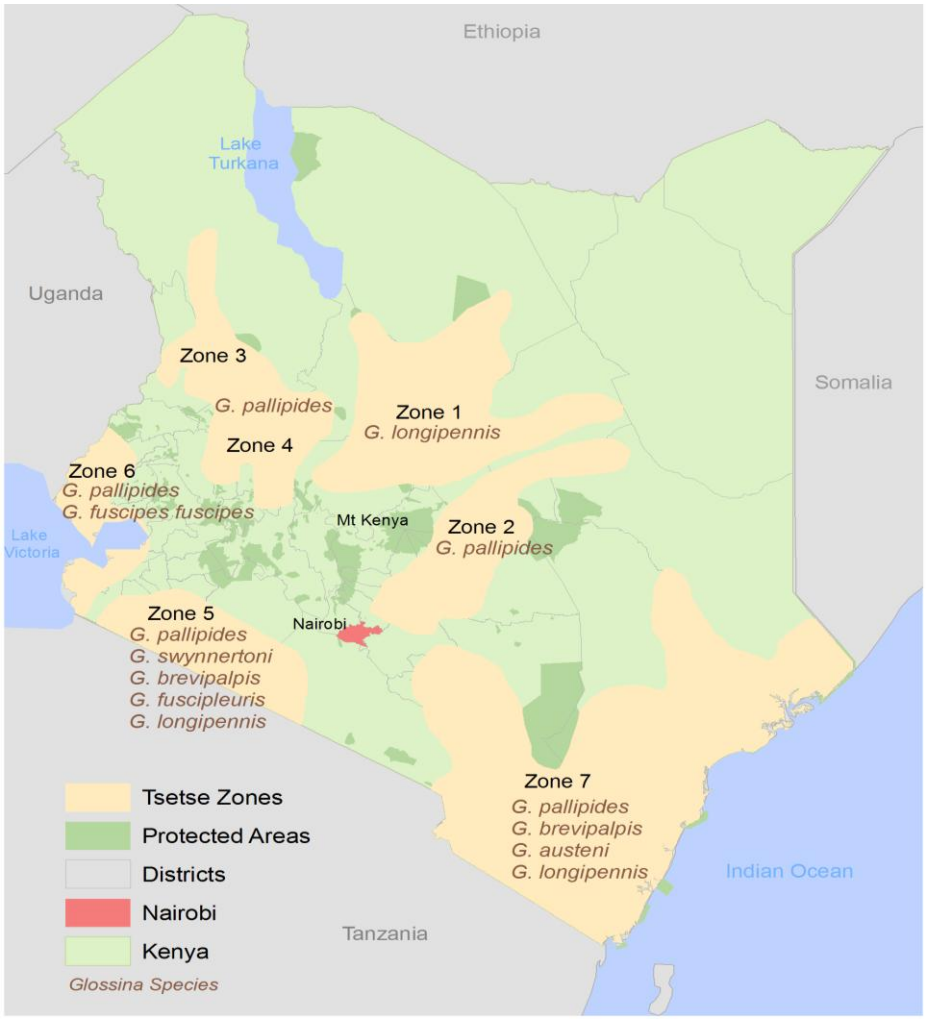
is highly costly, and it requires major infrastructures like big insectaries, iar planes and irradiation facilities(Aksoy *et al*., 2017).

Tsetse vectors can be controlled using other methods like elimination of wild animal hosts, bush clearing, and biological control using natural enemies of vectors which include parasites and predators like parasitic mites, bacteria and fungi.

1.8 Geographical distribution of tsetse in Kenya

Tropical African region has been reported to be a tsetse endemic area particularly the Southern edge of the Sahara desert to Angola, Zimbabwe, and Mozambique(Moloo, 1993). In Kenya eight species of tsetse flies have been reported(Grady *et al*., 2011). The species are distributed across the tsetse belts known as zones (figure 6) and HAT epidemics in Kenya have been reported in these zones (Grady *et al*., 2011).

Figure 6: A map of tsetse distribution in Kenya (Grady *et al*., 2011)



1.9 Animal models of HAT

Animal models have played a great role in basic research in understanding the virulence, pathogenicity and drug sensitivity of human trypanosome species (Christopher. *et al*., 2015). Animals like rats, mice, sheep, monkeys and also dogs have provided better understanding of the pathogenic mechanisms of HAT to neurological phase. These animal models have been used mostly for histological rather than behavioral or clinical studies (Darsaud *et al*., 2003).

1.10 Molecular characterization of trypanosomes

PCR method is a useful tool in identifying different trypanosomes (Cox *et al*., 2005) and a number of other parasite species like *Plasmodium* species (Padley D1 *et al*., 2003), *Schistosoma mansoni*(Jannotti-Passos *et al*., 1997), *Eimeria* species and *Cyclospora*(Orlandi *et al*., 2003). ITS-PCR is a useful screening assay used to distinguish trypanosome species based on the variation in the specific region in a parasite genome (Cox *et al*., 2005). For example, ITS PCR amplifies a product size of 250bp and between 600-720bp for *T. vivax* and *T. congolense* respectively while a band size of approximately 480bp is generated for Trypanozoon species (*T.brucei sub-group, T. evansi*) (Njiru *et al*., 2005). The serum resistance associated gene (SRA) is a conserved gene found in *T. b. rhodesiense* but absent in *T. b. gambiense*(Gibson *et al*., 2002) it is used to differentiate between the two species. The gene play a role of resistance to the trypanolytic factor in human serum thus providing protection of the parasite against destruction by human serum (Gibson *et al*., 2002).

1.11 Justification

T. b. rhodesiense typically causes acute infection in human. However, there have been documented reports in Zambia and Malawi of human chronic illness due to *T. b. rhodesiense* (Lorna MacLean, 2004 ; Mwanakasale *et al*., 2014). This implies that persons who are infected can be chronic carriers and this may contribute to transmission of the parasite in the presence of a susceptible vector. There is no documented report of chronic *T. b. rhodesiense* in Kenya. The data collected in this study hoped to provide information on whether there

is an emergency of new strains of trypanosome parasites spreading into Kenya which will necessitate effective intervention and control measures.

1.12 Objectives

1.12.1 General objectives

To characterize an anomalous *T. b. rhodesiense* isolated froma patient from Busia County, Kenyaand compare its characteristics, with other typical *T. b. rhodesiense* isolates.

1.12.2 The specific objectives were:

1. To determine the genetic identity of the anomalous KETRI 3971 *T. b. rhodesiense* isolate
2. To compare the virulence of KETRI 3971, with other *T. b. rhodesiense* isolates following *in vivo* infection in Swiss mice
3. To determine the susceptibility of KETRI 3971 to Suramin in comparison with other *T. b.rhodesiense* isolates
4. To determine the tsetse infectivity and transmissibility of the KETRI 3971 using *Glossina morsitans* and *Glossina pallidipes*.

CHAPTER TWO: MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Trypanosomes

Trypanosoma brucei rhodesiense stabilates were obtained from human in South Teso constituency, Busia County and its neighboring locality as shown in figure 5 between 1969 and 2009 identified as: KETRI 3971, KETRI 2382, KETRI2482 and KETRI 3799 (Table 1).

2.1.1.1 History of the isolates

KETRI 3971 was isolated from a 48 year old lady from Obekai village, Busia County in the year 2009 as shown in Table 1. She went to Alupe hospital on 4th Feb, 2009 complaining of a problem which had lasted for about two years and the symptoms were characterized by lack of appetite and being sleepy most of the time. She was diagnosed with one trypanosome which had as luggish movement in the buffy coat preparation. After two days a blood sample and lumbar puncture examination were performed, and a single sluggish trypanosome was once again observed in the buffy coat. In the CSF sample, there was no trypanosome observed and the white blood cells were in the normal range. Subsequent observations made on blood samples showed no trypanosomes. The patient was under observation for a period of 2 weeks waiting for Suramin to be availed from Geneva. When Suramin treatment commenced, the patient's condition improved and eventually recovered well. On 6th February 2009 two immuno-suppressed *Swiss* mice were injected with both blood and CSF samples from the patient. The mice were immuno-

suppressed using cyclophosphamide administered intra-peritoneally at a dose of 100 mg/kg per day for three continuous days before infection with trypanosomes. Blood infected mouse had apre-patent period of 20 days for one mouse which was high. The CSF infected mouse died negative at day 22 after infection and the cause of death was not identified. On the day parasitaemia was identified in the peripheral blood (20 dpi), a passage into clean mouse was done and was yet to become parasitaemic. However, the parasitaemia of the original mouse continued to rise for the next three consecutive days, but from the 24^{th} day after infection, the parasitaemia started to drop such that by $25^{th}$and 26^{th} day after infection, one single and sluggish trypanosome was observed. Another passage to a clean mouse was done at the30^{th} day after infection. Both the mice injected with human sample and the passage were transferred to KALRO- BioRI (Muguga) from Alupe, Busia where the immune system of the two infected mice were further suppressed using 300mg/kg of cyclophosphamide in an effort to enhance the establishment and multiplication of the infection. All the animals became parasitaemic and stabilates were made from the original mouse at day 33 after infection.

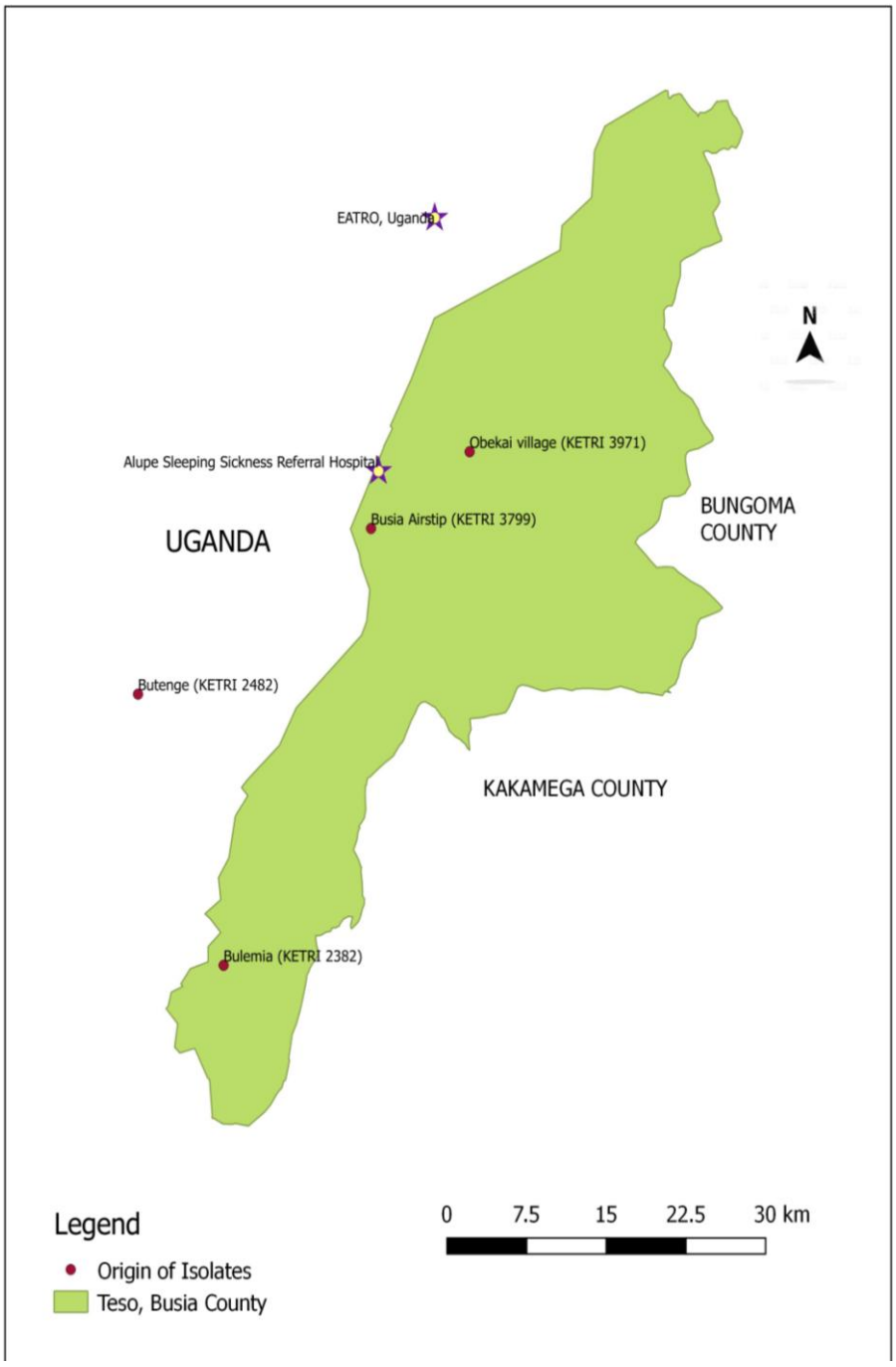
KETRI 2382 was isolated from a patient who came from Bulemia, Busia County in the year 1978 (Table 1). The patient had visited Alupe hospital where the blood was taken and upon injection into two donor mice became positive with trypanosomes after 6 days of infection. The blood was harvested at peak parasitaemia of about antilog 8.1trypanosomes/milliliter of blood and stabilates were made. This isolate had undergone 2 passages since isolation.

KETRI 2482 was obtained from a patient at East Africa Trypanosomiasis Research Organization (EATRO) hospital in the year 1969 (Table 1). The patient came from a locality called Butenge in Uganda. The isolate was established to cause fatal infection in mice and produced infection with a single peak parasitaemia followed by rapid death. This isolate had gone through 7 passages since isolation.

KETRI 3799 was isolated from a patient in Alupe hospital, Busia County who came from Busia airstrip in the year 1989 (Table 1). The harvested blood was inoculated into two irradiated mice whose pre-patent period was 10 days and at peak parasitaemia the blood was harvested and cryo-preserved in trypanosome bank. This isolate had gone through one passage only.

Each isolate was harvested from each mouse at peak parasitaemia of approximately antilog 8.1trypanosomes/milliliter of blood. The mouse was humanely euthanized using carbon-dioxide asphyxiation (Martinez-Gutierrez, 2014) and approximately 1ml of blood harvested by cardiac puncture (Frankenberg, 1979) and aspirated into a syringe containing anti-coagulated EDTA. Approximately 1ml of blood was mixed with equal ratio of 20% glycerol in phosphate saline glucose (PSG) and the mixture dispensed into capillaries. The capillaries were sealed at one end using plasticine and kept in well labeled perforated cryo-vials. The cryo-vials were placed in a cooling jacket and suspended for 2hrs in liquid nitrogen at a temperature of -60°C to -80°C and thereafter cryo-preserved in a canister at -196°C in KALRO-BioRI trypanosome bank with a room temperature of 24°C (Murilla *et al*., 2014).

Figure 5 A map of the study area



Isolate identity	characteristic	Year of isolation	Locality	Reference
KETRI 3971	Anomalous	2009	Obekai, Busia, Kenya	Uncharacterized/unpublished
KETRI 2382	Associated with acute sleeping sickness	1969	Bulemia, Busia, Kenya	Not published
KETRI 2482	Associated with acute sleeping sickness	2000	Butenge, Uganda	(Bacchi *et al*., 1990)
KETRI 3799	Associated with acute sleeping sickness	1987	Busia, Kenya	(Thuita *et al*., 2008)

Table 1: History of the trypanosome isolates used in this study

2.1.2 Laboratory mice

Male Swiss White mice (20-30g each) were acquired from mice breeding laboratory in KALRO-BioRI. The breeding unit always supplies infection free mice for various research purposes. Before the experiment began, the animals were acclimatized for seven days during which they were de-wormed using injectable Noromectin® at 0.2mg/kg (Ash and Oliver, 1989). The mice were fed on their pellets from Unga Feeds Ltd, Kenya and water ad libitum and maintained at room temperature in mouse cages that were supplied with wood-shaving as bedding materials. All animal experiments and procedures were carried out under the approval of KALRO-BioRI Institutional Animal Care and Use Committee (IACUC) number C/Biori/4/325/II/35. During acclimatization period, data before infection were collected for the packed cell volume (PCV).

2.1.3 Tsetse flies

One thousand male tsetse flies aged zero (newly emerged) to three day-old were obtained from the KALRO-BioRI tsetse rearing unit. The Institute rears both male and female tsetse flies for research purposes. The sex of the tsetse flies does not influence the vectorial capacity. During the study period, only male flies were available as female tsetse flies were used to build the colony size. Of this 500 were *G. pallidipes* and another 500 were of the *G.morsitans* species. The tsetse colony originally came from Tororo, Uganda in 1975, where pupae were obtained and first establish a colony in International Atomic Energy Agency (IAEA) Seibersdorf laboratory Vienna Austria. Subsequently pupae emerging from the IAEA colony were brought to Kenya and used to

establish the KALRO-BioRI tsetse colony (Ciosi *et al*., 2014). The tsetse insectary is maintained at a relative humidity of 75% ± 5% and temperature of 24°C ± 1°C and the colony is routinely fed artificially on defibrinated bovine blood at alternate days through a membrane feeding process(Feldmann, 1994b).

2.2 METHODS

2.2.1 Propagation of trypanosome isolates in Swiss mice

The trypanosome isolates described in Table 1 were obtained as stabilates from the KALRO-BioRI trypanosome bank and the viability confirmed through microscopic examination of motile trypanosomes. Thereafter, the stabilates were inoculated intra-peritoneally into mice and the trypanosomes allowed to propagate in immuno-suppressed donor mice. For each stabilate two mice were used and their tail snip blood smears examined from day 2 post inoculation to monitor parasitaemia. At peak parasitaemia (antilog 8.1trypanosomes/milliliter of blood) the mice were anaesthetized using carbon dioxide asphyxiation and the blood harvested from each mouse by cardiac puncture and aspiration.

2.2.2 Purification of genomic DNA from trypanosome isolates

Genomic DNA was purified from whole blood obtained in section 2.2.1 using Gene-JET ™ Genomic DNA purification kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. Briefly, twenty micro litre of proteinase k was added into 50 µl of anti-coagulated blood in a 1.5ml tube and the volume increased to 220 µl using phosphate buffer solution. To this, 200 µl of lysis buffer AL was added and the mixture incubated at 56°C for

10 minutes. Thereafter, 200 µl of ethanol (95%) was added and mixed thoroughly and the mixture pipeted into a DNeasy mini spin column in a 2ml collection tube and centrifuged at 8000rpm for 1 minute. The spin column was then placed in a new 2 ml collection tube and 500 µl wash buffer AW added and centrifuge for 3 minutes at 8000rpm. The flow through and the collection tube was discarded and the spin column was placed in a new tube. The column was rinsed using 500 µl of AW2 wash buffer after which the centrifugationdone at 14,000rpm for 180 seconds. The spin column was then transferred to a new tube and the bound DNA eluted using 50 µl elution buffer AE.

2.2.3 PCR amplification of ITS1 and SRA gene

The internally transcribed spacer 1(ITS1) and serum resistant associated gene (SRA) assays were used to characterize the trypanosomes. The primers flanking these gene sequences are presented in Table 2. The PCR amplification was performed in a volume of 10µl reaction mixture consisting 2.5µl of DNA template, 0.25 µl of Dream Taq DNA polymerase in Dream Taq Green buffer, 0.2mM dNTPs, 2mM $MgCl_2$ and SRA and ITS 1 specific primers at 1 µM each.

ITS 1	Sequence (5'-3')	SRA	Sequence (5'-3')
CF	¢CCGGAAGTTCACCGAT-ATTG	A	5'GACAACAAGTACCTTGGCGC-3'
BR	5¢TTGCTGCGTTCTTCAAC-GAA	E	5'-TACTGTTGTTGT ACCGCCGC-3

Table 2: Primers flanking the gene sequences

The ITS cycling conditions began with the first step of denaturation at 94ºC for 5min, followed by 35 cycles of 94ºC for 40 sec, 58 ºC for 40 sec, 72ºC for 90 sec and ends with an extension of 5 min at 72ºC. The cycling conditions for SRA involved a first step which takes 3 min at a temperature of 95ºC,second step of 35 cycles at 95ºC for 30s, 60ºC for 30s, 72ºC for a period of 60s and ends with extension of 2 minutes at 72ºC.

2.2.4 Agarose gel electrophoresis

Amplified products of PCR were resolved on a 2% agarose gel (Fermentas Life Sciences, EU) using 1x TAE as a running buffer. The gel was electrophoresed at 60 Volts for 1 hour. 100bp DNA ladder (Promega U.S.A) was run alongside the amplified products in order to confirm the size of the amplification. DNA was visualized under UV light following ethidium bromide staining.

2.2.5 Pathogenicity of trypanosome isolates following experimental infection in mice

2.2.5.1 Experimental infection in mice

Cryo-preserved stabilates (75µl each) corresponding to each of the four isolates were re-suspended in 0.2µl of phosphate saline glucose(pH 8.0) and 2 immuno-suppressed donor Swiss white mice per isolate were inoculated intra-pernitoneally (IP) using each diluted stabilate. The mice were immuno-suppressed using cyclophosphamide administered intra-peritoneally at recommended dose of 100 mg/kg per day for three continous days before infection with trypanosomes (Wu *et al*., 2015). At high parasitaemia of about antilog 8.1 trypanosomes/milliliter of blood, the donor mice were anaesthetized and then bled as previously described. Approximately 1 ml of blood was mixed with 1.5 mg/ml of ethylenediamine tetra-acetic acid (EDTA) in a tube. The blood was serially diluted and the parasite load quantified in order to achieve an inoculum of 0.2 ml per mouse corresponding to 1×10^4 trypanosomes(Kariuki *et al*., 2015). Ten mice were inoculated intra-peritoneally using each trypanosome isolate and served as the experimental group while another group of 10 mice were not inoculated and served as controls. After infection the mice were monitored and the following parameters were recorded; Parasitaemia progression and Packed cell volume(PCV). Parasitaemia was determined using the matching method (Herbert and Lumsden, 1976) by observing peripheral blood prepared from tail snip smears

and Packed cell volume (PCV) was checked once in every week (Naessens *et al*., 2005).

2.2.5.2 Survival time of mice following infection

Each mouse from the experimental and control group was monitored daily for 30 days post-infection and any mouse surviving beyond this day was humanely euthanized using carbon-dioxide asphyxiation (Martinez-Gutierrez, 2014). Their survival time was indicated as 30 days and recorded as censored data.

2.2.6 Susceptibility of trypanosome isolates to drug treatment

A group of five mice per isolate were infected with $1x10^4$trypanosomes/ml (As already described in section 2.2.5.1) and starved overnight before treating intra-dermally with a standard dose of Suramin (5mg/kg body weight) (WHO, 2015). Another group of five mice served as controls and were not treated. The treated mice were monitored on alternate days for relapse for sixty days. Any mice surviving after sixty days post infection and monitoring period were considered free from parasites (Brun *et al*., 2001) and were latter euthanized by carbon dioxide asphyxiation.

2.2.7 Trypanosome infection and transmission in *G pallidipes* and *G morsitans*

Glossina pallidipes and *G. morsitans* aged zero (newly emerged) to three day old males were obtained from the KALRO-BioRI insectary. For this experiment two mice per isolate were infected and at peak parasitaemia 50 clean flies (split in two cages of 25 flies per cage) were fed on each mouse to repletion. Upon feeding on the infective blood meal, any unfed flies were

removed from the cages and the remaining fed flies were then kept in the insectary and maintained at alternate days on parasite free bovine blood (Feldmann, 1994b) using *in vitro* artificial feeding system. Two groups of uninfected tsetse flies (one group for *G. pallidipes* and one group for *G. morsitans*) served as control and were fed *in vitro* on clean blood for 30 days. Mortalities of both the experimental and control groups of tsetse flies were checked and recorded at intervals of 48 hours for the entire study period.

Trypanosome transmission from infected flies were done using 48 hrs starved flies on flies surviving 30 days post infection to identify tsetse flies that harbors mature and infectious parasites (Thuita *et al*., 2008).The flies were applied tonaive mice and blood examined formotile trypanosomes. Surviving flies were killed, dissectedto find out if parasites were present in their mouth parts (labrum, hypopharynx, proboscis, salivary glands and midgut).

2.3 Statistical analysis

All the variables were measured and the response assumed a normal distribution. Data analyses were done using the General linear model (GLM) in SAS Release 8.02. The GLM tested the effects of DPI and isolate on the response and the interaction between the two variables. Tukey's Test was employed to test the significant differences between the isolates in parasitaemia progression pre-patent period, survival times, and Packed Cell Volume where $p \leqslant 0.05$ were considered statistically significant. The tsetse infectivity and transmissibility data analysis were carried out using proportions. Survival data analysis was carried using the Kaplan–Meier method on StatView (SAS

Institute, Version 5.0.1) statistical package for determination of survival distribution function. Rank tests of homogeneity were used to determine the effect of the infection on host survival time (Everitt and Der, 1998).

CHAPTER THREE: RESULTS

3.1 PCR amplification of *T. b. rhodesiense* using ITS1 gene primers

PCR primers targeting the ITS1 region that discriminates between different trypanosome species was used in amplification of DNA from all the 4 *T. b. rhodesiense* isolates. No PCR product was obtained in the negative control sample (Figure 7, lane 1) Amplification of the ITS gene yielded a product size of ≈ 500bp in all the 4 *T. b. rhodesiense* field isolates (Figure 7, lanes 2-5). A product of approximately 500bp was also generated using the ITS1 primers when used to amplify a previously characterized and confirmed *T. b. rhodesiense* that served as a positive control in this assay (Figure 7 lane 6). The ITS1 was able to discriminate *T congolense*, *T. vivax* and *T. Simiae* based on differences in their amplicon sizes (Figure 7, lanes 7-9).

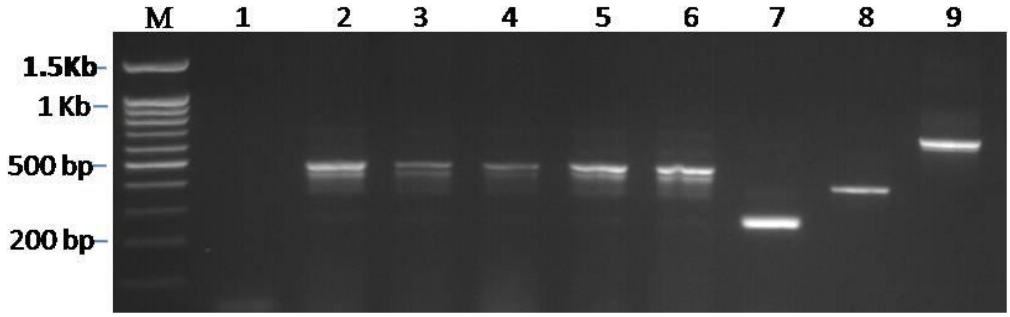


Figure 7: Agarose gel electrophoresis of trypanosome species amplified using ITS1 PCR primers. M-100bp DNA size marker, Lane 1 Negative control, lane 2- KETRI 3971 (anomalous *T. b. rhodesiense* isolate), lane 3-KETRI 2382, lane 4- KETRI 2482 Lane 5- KETRI 3799, Lane 6-positive control (confirmed and characterized *T. b. rhodesiense*-KETRI 3637), Lane 7-*T. simiae*, Lane 8-*T. congolense*, Lane 9-*T. vivax*

3.1.1 PCR amplification of the SRA gene

PCR primers flanking a partial sequence of approximately 480bp of the SRA gene were used to amplify target DNA from the four *T. b. rhodesiense* isolates. A product size of approximately 480bp was amplified from all the 4 isolates (Figure 8, lane 2- 5). The positive control also yielded a PCR product of the same size (Figure 8, lane 6). The negative control generated no PCR product when amplified (Figure 8, lane 1).

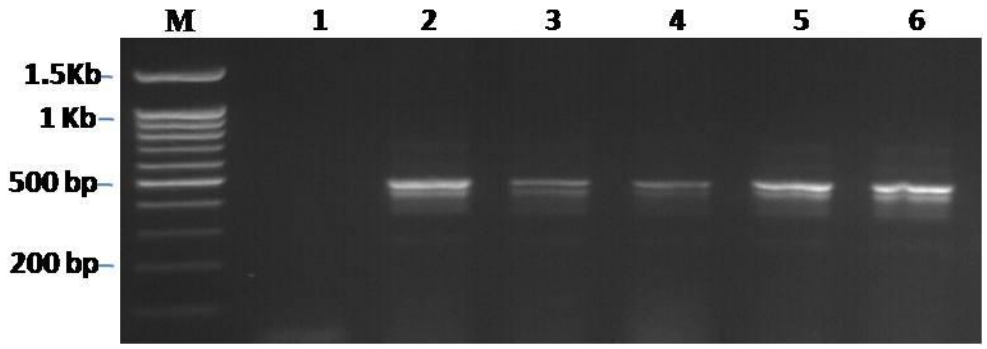


Figure 8 Agarose gel electrophoresis of trypanosome isolate amplified using SRA PCR primers. M-100bp marker, 1-NC-negative control, 2- KETRI 3971, 3-KETRI 2382, 4- KETRI 2482, 5- KETRI 3799, 6- PC-Positive control (*T. b. rhodesiense*)

3.2 Virulence of KETRI 3971 isolate following experimental infection in *Swiss* mice

3.2.1 Trypanosome parasitaemiain mice

Ten mice per group were infected with each of the isolate and the parasitaemia monitored with course of time. Results of the parasitaemia are shown in Table

3. Only 6 out of the 10 mice infected with KETRI 3971 developed detectable parasitaemia this in comparison with group 2, 3, and 4 where all infected mice developed parasitaemia (Table 3).Pre-patent parasitaemia ranged between 3 to 6 days being shortest in group 1 and was longest in group 3. All mice in group 1 and group 4 showed parasites on day 4 and day 5 respectively. Parasites were first observed between day 4 and day 6 and between day 3 and 5 in group 2 and 3 respectively. The mean pre-patent period of mice infected with KETRI 3971was significantly different (F-test $p<0.01$) when compared to the pre-patent period of mice infected with, KETRI 2482 and KETRI 3799 but not with mice infected with KETRI 2382. The peak parasitaemia was lowest while the duration to peak parasitaemia was shortest in KETRI 3971 mice compared to mice infected with other isolates and this differences were significant ($p<0.01$)**.**

Group	Isolate number	Mice /Group	Mice showing parasitaemia	Pre-patent parasitaemia (days)	Mean pre-patent parasitaemia	Mean peak parasitaemia	Duration to peak parasitaemia	Mean post patent parasitaemia
1	KETRI 3971	10	6	4	5.6±0.20	3.36±0.9	4.8±0.54	1.3
2	KETRI 2382	10	10	4-6	6.42±0.30	8.37±0.06	8.3±0.54	7.5
3	KETRI 2482	10	10	3-5	6.54±0.32	8.63±0.13	7.2±0.55	7.6
4	KETRI 3799	10	10	5	6.63±0.28	8.8±0.07	7.9±0.28	8.2

Table 3: Mean ±SE Pre-patent period and parasitaemia generated by four *T. b. rhodesiense isolates.* n= Number of animals with parasites.

3.2.2 Progression of parasitaemia.

The progression of parasitaemia following infection with each of the four *T. b. rhodesiense* isolate is shown in Figure 9. There was no significant difference ($p<0.05$) in the progression of parasitaemia in mice infected with KETRI 2382, KETRI 2482 and KETRI 3799 isolates. Mice infected with KETRI 3971 recorded lowest parasitaemia compared to the other isolates. Fluctuation in parasitaemia that is typical in trypanosome infection was detected in infections with all the isolates.

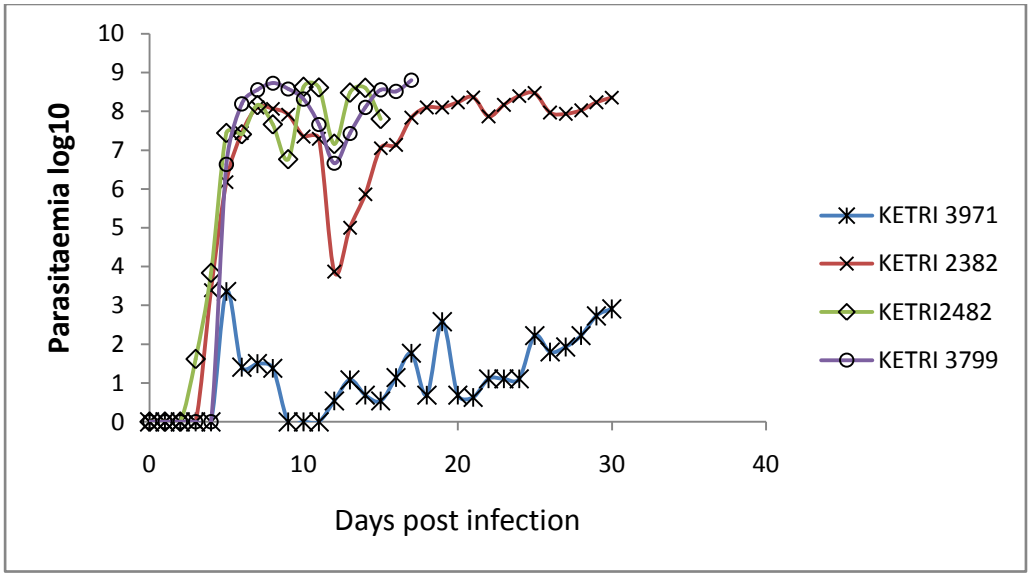


Figure 9 Mean ±SE Parasitaemia progression curves from mice infected with the four isolates of *T. b. rhodesiense*.

3.2.3 Effects of trypanosome infection on packed cell volume (PCV)

The mean PCV of mice following infection with trypanosomes is shown in Figure 10. The mean PCV range was 47% and 52% for KETRI 3971 and KETRI 2482 respectively. The PCV of mice infected with KETRI 2382,

KETRI 2482 and KETRI 3799 declined significantly ($p<0.01$) in comparison with the non-infected. However, from day 14 after infection the PCV of mice infected with the KETRI 2382 increased minimally with minor fluctuation to the end of the experiment. Mice infected with the anomalous KETRI 3971 isolate showed a significantly ($p<0.01$) higher PCV in comparison with the mice infected with the other isolates however; their PCV was slightly lower when compared to the control group.

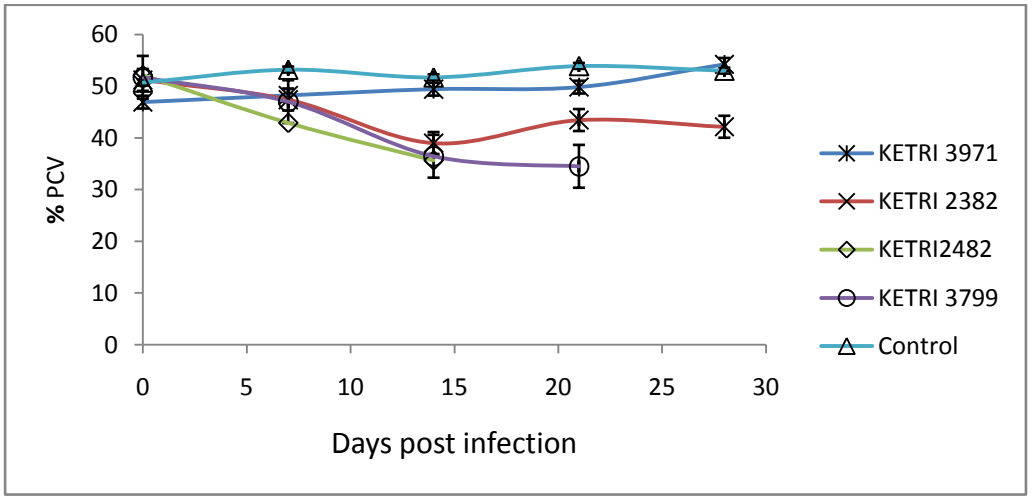


Figure 10 Mean ±SE packed cell volume (PCV) of mice infected with *T. b. rhodesiense* isolates

3.2.4 Survival of mice following infection

The survival times of all the infected groups of mice were recorded and compared to the control group and are shown in Figure 11. Mice infected with KETRI 3971 recorded longer mean survival time (MST) of 30±0 days with all the mice in this group surviving to the end of the experiment. Mice infected with KETRI 2382, KETRI 2482 and KETRI 3799 had mean survival times of 27.9±1.78, 13±0.86 and 16.8±0.71 days which was equivalent to 93%, 43%

and 56% survival rates respectively. The survival rates were generally higher for infected mice group which had low parasitaemia. All mice in the non-infected/control group survived beyond the 30 days experimental period. Any mouse that survived beyond the 30 days experimental period had their survival period data categorized as censored. The p value associated with Wilcoxon and Logrank test of homogeneity were 0.0001 suggesting that there was significant difference between the shorter and longer survival times.

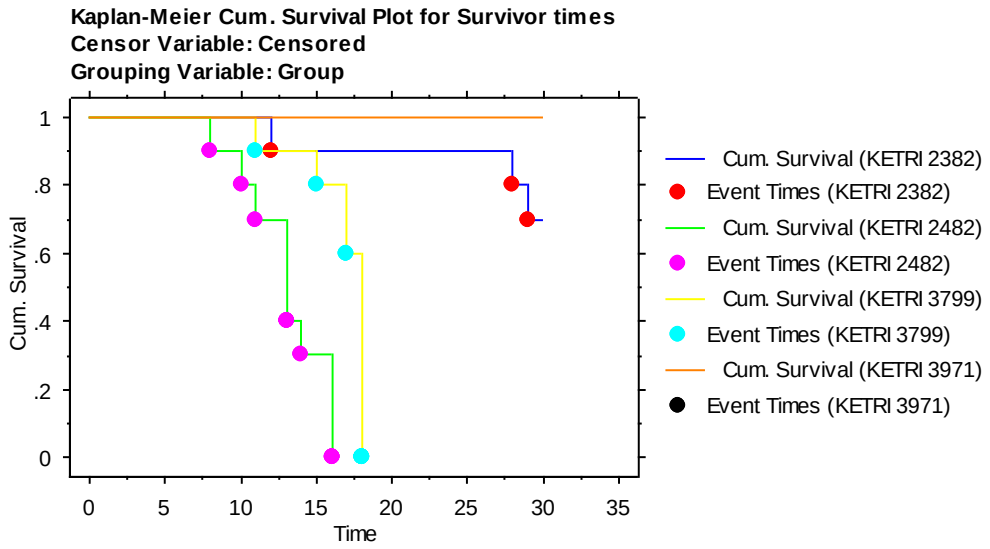


Figure 11 Mean survival time (days) ± SD of mice infected with different trypanosome isolates.

3.3 Parasitaemia profile of infected mice after treatment

Five mice per group were infected with each of the trypanosomes isolates and treated after 24 hours with 5mg/Kg body weight of Suramin. The control group were infected but not treated. All mice were monitored for the presence of trypanosome parasite over a period of 60 days. All the infected and treated

mice survived beyond the 60 day period with no evidence of trypanosome relapse. However, mice in the control group were only monitored for demonstration of trypanosomes and all of them had trypanosomes in the peripheral circulation.

3.4 Survival, probing success and trypanosome transmission following infection in tsetse

100 clean flies of *G. pallidipes* and 100 clean flies of *G. morsitans* were fed on infected mice per isolate and the trypanosome detected in saliva by probing on day30. The probing results are shown in Table 4 and Table 5. The survival rates of the infected flies ranged from 59% to 75% for *G. pallidipes* and from 60% to 75% for *G. morsitans* this in comparison with the control group whose survival rates were 71% for *G pallidipes* and 70% for *G. morsitans.* Of the surviving *G. pallidipes* flies infected with KETRI 3971 only 8.1% of the flies that probed had trypanosomes in their saliva, this compared to 6.8% of the KETRI 2382. The KETRI 2482 and KETRI 3799 infected flies probed successfully at the rate of 91.9% and 88% respectively but had no parasites in their saliva. In contrast with the *G. morsitans* only flies infected with KETRI 2382 had trypanosomes in their saliva. The KETRI 3971, KETRI 2482 and KETRI 3799 did not show any trypanosome parasites in their saliva despite their high successful probing rate of 90%, 92% and 76.1% respectively (Table 5).

Group	Isolate number	Tsetse flies /Group	Tsetse flies Survival(%) after 30 days	Number of flies that probed successfully		Number of flies that did not probe
				Trypanosomes seen in saliva	No trypanosomes seen in saliva	
1	KETRI 3971	100	62	5(8.1%)	55(88.7%)	2(3.2%)
2	KETRI 2382	100	59	4(6.8%)	50(85.7%)	5(8.5%)
3	KETRI 2482	100	62	0	57(91.9%)	5(8.1%)
4	KETRI 3799	100	75	0	66(88%)	9(12%)
5	Control	100	71	-	-	-

Table 4: Survival, probing success in *G. pallidipes*

Group	Isolate number	Tsetse flies /Group	Tsetse flies Survival after 30 days (%)	Number of flies that produced saliva		Number of flies that did not produce saliva
				Trypanosomes seen in saliva	No trypanosomes seen in saliva	
1	KETRI 3971	100	60	0	54 (90%)	6 (10%)
2	KETRI 2382	100	71	10 (14.1%)	51(71.8%)	10 (14.1%)
3	KETRI 2482	100	75	0	69 (92%)	6(8%)
4	KETRI 3799	100	67	0	51(76.1%)	16 (23.9%)
5	Control	100	70	-	-	-

Table 5: Survival, probing success in *G. morsitans.*

Transmission of infection was performed by xenodiagnosis using infected flies fed on naive mice and the results are shown on Table 6. Transmission experiments were not performed for flies that did not produce saliva as per Table 4 and Table 5. Only 4/5 (80%) of *G. pallidipes* infected with KETRI 3971 and that had successfully probed were able to transmit infection to clean mice. This in comparison with all the 4/4(100%) flies infected with KETRI 2382 which were able to transmit infection. Only 60% of the *G. morsitans* flies were able to transmit infection to naive mice.

Group	Isolate number	*G. pallidipes*		*G. morsitans*	
		No. of flies	Transmission (%)	No. of flies	Transmission (%)
1	KETRI 3971	5	4(80)	0	0
2	KETRI 2382	4	4(100)	10	8 (60)
3	KETRI 2482	0	-	0	_
4	KETRI 3799	0	-	0	_

Table 6: Transmission rates of trypanosomes from infected tsetse to uninfected mice

All surviving tsetse were dissected to find out if the trypanosomes were present and the results are shown in Table 7 and Table 8. For *G pallidipes* (Table 7),

trypanosomes were observed in the labrum, hypopharynx, salivary gland and midgut in all the infected flies except for flies infected with the KETRI 2482 isolate where trypanosome infection was only detected in the midgut. KETRI 3971 recorded relatively higher infections in all the organs compared to other groups. In contrast, *G. morsitans* flies infected with KETRI 2382 showed high infection in all their organs compared to the other groups as shown in Table 8. Salivary gland infections were higher in *G. pallidipes* infected with KETRI 3971 (Table 7) compared to *G. morsitans* (Table 8).

		Infection (%)			
Isolate	No. of flies	Labrum	Hypopharynx	Midgut	Salivary glands
KETRI 3971	62	7(11.3)	8(12.9)	10(16.1)	10(16.1)
KETRI 2382	59	3(5.1)	3(5.1)	5(8.1)	5(8.1)
KETRI 2482	62	0.0	0.0	3(4.9)	0.0
KETRI 3799	75	1(1.4)	1(1.4)	2(2.8)	1(1.4)

Table 7: Detection of trypanosomes in surviving *G. pallidipes* following infection with trypanosomes

.

		Infection (%)			
Isolate	No. of flies	Labrum	Hypopharynx	Midgut	Salivary glands
KETRI 3971	60	2(3.33)	0	13(21.7)	6(10)
KETRI 2382	71	8(11.3)	8(11.3%)	29(40.8)	13(18.3)
KETRI 2482	75	0	0	9(12)	0
KETRI 3799	67	1(1.5%)	0	11(16.4)	5(7.5)

Table 8: Detection of trypanosomes in surviving *G. morsitans* following infection with trypanosomes

CHAPTER FOUR: DISCUSSION AND CONCLUSION

4.1 DISCUSSION

*Trypanosoma brucei rhodesiense*is typically known to cause an acute and severe HAT infection in East Africa and in other foci in Africa (Lorna MacLean, 2004 ; Mwanakasale *et al*., 2014). Mild strains of *T. b. rhodesiense* causing chronic infections have also been reported in Malawi and Zambia(Lorna MacLean, 2004 ; Mwanakasale *et al*., 2014). However, no such cases have been reportedly diagnosed in Kenya. Establishing different severities of *T. b. rhodesiense* HAT in foci can help improve disease management via control and surveillance in the affected region. *T. b. rhodesiense* HAT can be present with relatively no specific signs or symptoms, the early sign of infection is a trypanosomal chancre which is an acute and localized skin inflammatory response at the inoculation site (Gelfand, 1966). The sign becomes visible 5–15 days after infection and persist for 2–3 weeks (MacLean, 2010). Early stage of infection is realized in one to three weeks after infection and is associated with weight loss, lymphadenopathy, fever, general malaise, weakness, hepatomegaly, tachycardia, skin rash, splenomegaly and pruritis (MacLean, 2010). The second stage of infection begins when the parasites enters the central nervous system (CNS). This stage is associated with mental problems, motor and sensory disorders, and change of sleep patterns which leads to coma and finally death (Kennedy, 2004).

This present study undertook to characterize an anomalous isolate of *T. b. rhodesiense* that was obtained from a patient by establishing its genetic

identity, virulence, drug sensitivity and tsetse transmissibility in comparison to characterized and confirmed *T. b. rhodesiense* isolates that had been collected from the neighboring locality.

Molecular characterization using the ITS and SRA markers confirmed the anomalous isolate as *T. b. rhodesiense*. This isolate demonstrated a chronic infection characteristic that was less virulent, easily tsetse transmissible by *G. pallidipes* than *G. morsitans* and sensitive to Suramin.

Variation pre-patent periods have been observed in previous studies involving mice and monkeys infected with *T. b. rhodesiense* via tsetse fly bite, consistent with what has been observed in HAT patients (Fèvre *et al*., 2004; Kennedy, 2004). These variations are probably because tsetse flies inject infective metacyclics with no defined inoculum dose, which may influence the virulence status (Caljon *et al*., 2016; Thuita *et al*., 2008). The minimal variation observed in pre-patent period in this study may be due to use of syringe passage and a well-defined inoculum dose and as such, may not explain the virulence status of the anomalous isolate.

In the current study, all experimentally infected mice showed a persistent fluctuating parasitaemia, consistent with trypanosome infection with the non-anomalous isolates demonstrating a characteristic features of a typically acute infection which progresses rapidly to late stage (MacLean, 2010). However, progression of parasitaemia following infection of mice with the anomalous isolate was characterized by many low-density waves with some of the infected mice failing to show parasites. This observation suggested a mild form of a

disease which can be easily cleared by the host immune system through phagocytosis and/or antibody-mediated trypanolysis (Stijlemans *et al*., 2016). Similar observation of a mild form was observed in the human patient from where the anomalous isolate was obtained. Mild forms of *T. b. rhodesiense* infection have been reported in Malawi and Zambia (Lorna MacLean, 2004 ; Mwanakasale *et al*., 2014). A study on severity of HAT established two trypanosomiasis foci (Malawi and Uganda) with variation in disease virulence (Lorna MacLean, 2004).

Development of anemia is an important feature of trypanosome infection in mice (Noyes *et al*., 2009), primates (Thuita *et al*., 2008) and humans (Chisi *et al*., 2004). This condition is attributed to enhanced erythrophagocytosis and suppressed erythropoiesis and to factors derived from both the parasite and the host (Naessens, 2006; Stijlemans *et al*., 2018). In the present study, anemia was observed in mice infected with non-anomalous isolates evidenced by a drop in PCV. However, the anomalous-infected group did not show any indication of anemia. This observation could be attributed to the parasite density. Indeed, the parasite density in mice infected with the anomalous isolate at peak parasitaemia was significantly ($p<0.05$) lower compared with the non-anomalous isolates indicating that there was a direct relationship between parasite density and anemia. Anemia in the non-anomalous infected mice was characterized by both the acute and the chronic phase where the PCV level of mice infected with some isolates declined drastically while in mice infected with KETRI 2382 isolate demonstrated a decline and then recovery. Indeed, KETRI 2482 and 3799 infected mice demonstrated acute anemia where all the

infected mice died before the end of the study period. This could be attributed to severe loss of erythrocytes in the infected mice as a result of high parasite density. However, anemia with KETRI 2382 was of the chronic nature characterized by acute followed by recovery phase (Stijlemans *et al*., 2018). The findings of this study are important as it has demonstrated variations in anemia in *T. b. rhodesiense* infections (Stijlemans *et al*., 2018). Survivorship of infected individuals may be used as a proxy for trypanosome virulence in humans (Lorna MacLean, 2004) and animals (Masumu *et al*., 2006; Van Den Bossche *et al*., 2011). Despite being isolated from a known acute trypanosomiasis foci, mice infected with the anomalous isolate survived beyond 30 days contrary to the short survival period observed in non-anomalous infected mice. This could be attributed to the low virulence of this isolate supported by data from a previous study which associated longer survival with isolates of low virulence (Masumu *et al*., 2009). The shorter mean survival time recorded by non-anomalous infected mice is a further demonstration that these were of a higher virulence than the anomalous isolate.

Natural transmission of trypanosomes via the tsetse fly is the main mode of transmission in the area of isolate origin. This study established *G. pallidipes* to demonstrate a higher infection rate and therefore higher vectorial capacity than *G. morsitans. G. Pallidipes* was very effective in picking up, establishing and spreading *T. b. rhodesiense* of the anomalous isolates to naïve mice. Similar studies have highlighted the higher compatibility of *G. pallidipes* for *T. b. rhodesiense* as compared to *G. morsitans* (Okoth.; *et al*., 2009). The higher vectorial capacity of *G. pallidipes* could be linked to the fact that this species is

one of the major vectors of trypanosomes in Busia (Rutto and Karuga, 2009). Therefore, unlike *G. morsitans* which is absent in the region, *G. pallipides* has evolved to be an efficient vector for these parasites and can spread the disease and create an endemic focus in the region.

In addition, this study found out that both species of tsetse flies infected with the highly virulent isolates had less mature trypanosomes suggesting a low probability of being transmitted possibly due to the effective intrinsic defense mechanisms in the tsetse fly against trypanosomes infection and migration and also a very low pick-up and transmission by tsetse vector (Brun *et al*., 2001; Gibson and Bailey, 2003; Peacock *et al*., 2012), this finding may be one of the reasons why currently HAT infections are not reported in Busia.

Drug resistance has been reported in major trypanosome species of animals (*T. congolense, T. evansi and T. vivax*) and some endemic areas of HAT due to *T. b. rhodesiense* (Kibona *et al*., 2006). For example, resistance of *T. b. rhodesiense* to diminazene, melarsoprol, and isometamidium was reported in HAT endemic areas of Tanzania (Brun *et al*., 2001; Kibona *et al*., 2006). Suramin is the only drug use in treatment of early stage infections due to *T. b. rhodesiense*, resistance of human pathogenic trypanosomes to this drug has not been reported (Pépin J1 and F., 1994), this report is consistent with the current study findings which showed that a curative dose of suramin could clear trypanosomes in an infected mouse. Therefore, proper coordination of available drugs should be put in place to effectively act on the developmental stages and avoid misdiagnosis.

4.2 CONCLUSION

This study describes a different clinical presentation of HAT due to *T. b. rhodesiense* infection in Kenya. In the current unavailability of sensitive diagnostic tools and safe drugs to identify and cure *T. b. rhodesiense, t*he following important actions will be required: Rapid diagnostic tools for early detection of the infection, treatment of infected persons as well as controlling the spread of the susceptible *Glossina* vector. A clear and comprehensive clinical manifestation of *T. b. rhodesiense* needs to be understood among the affected communities and personnel in the health sector

4.3 RECOMMENDATIONS

1. This study provides an indication that chronic strains of *T. b. rhodesiense* may be circulating in western Kenya. Therefore, active screening and surveillance should be intensified to enable diagnosis and treatment of chronically ill patients and carriers and also control the spread of the susceptible *Glossina* vector.
2. Proper coordination of available drugs should be put in place to effectively act on the developmental stages to avoid treatment failures.
3. HAT tends to affect poorest communities with a low degree of disease awareness. Therefore, greater sensitization of the general public, as well as health workers is required.
4. Further molecular genotyping is recommended to establish if there is anygenetic variation of this chronic strain of *T. b. rhodesiense.*

5. Indentification of possible animal reservoirs of infection is important in controlling the disease

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