MOLECULAR IDENTIFICATION AND DRUG SENSITIVITY OF AFRICAN TRYPANOSOME STABILATES FROM LIVESTOCK IN LAMU COUNTY, KENYA

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EGERTON UNIVERSITY

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DECLARATION AND RECOMMENDATION

Declaration

This thesis is my original work and has not been submitted or presented for examination in any institution.

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Recommendation

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DEDICATION

This work is dedicated to my beloved mom, Fraciah Wambui and my loving dad, Gathogo Macharia not forgetting my eight sisters: Betty, Nancy, Grace Ann, Jane, Anne, Wathanu, Emma, Tabby and three brothers: George, Njehuri and Maleby for their love for education and unconditional moral, financial and spiritual support. It is also dedicated to my son, Reagan Jones and his loving dad Kihungi who played a motivational role all through my study. God bless.

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ABSTRACT

Animal African Trypanosomiasis (AAT) causes economic losses estimated at US $5 billion per annum. Records from the veterinary department indicate high levels of trypanocidal drugs use in Lamu County. The objective of this study was to identify the trypanosome species causing infections in domestic animals in Lamu using parasitological and molecular techniques and to determine their drug sensitivity in white Swiss mice. Fifteen trypanosome stabilates and 92 whole blood samples collected from parasitologically negative animals were randomly retrieved from cryobank freezer at Biotechnology Research Institute (KALRO-BioRI), Kenya, and characterized. Human serum resistance associated (SRA) gene present in *T. b. rhodesiense* was used to differentiate *T. brucei* positive stabilates. Four stabilates identified as *T. b. brucei* were used for drug sensitivity study. Groups of six white Swiss mice were infected with these stabilates and treated with single doses of Homidium Bromide (1mg/kg), Isometamidium Chloride (1mg/kg) and Diminazene Aceturate (20mg/kg). Pathogenicity and virulence determination for one drug sensitive and two drug resistant stabilates was also carried out. Changes in packed cell volume (PCV), parasitaemia and body weights of mice were monitored. Results showed that 10/15 (67%) trypanosome stabilates and 13/92 (14%) whole blood samples from cattle, donkeys and goats were positive by PCR. Positive *T. congolense* 5/23 (22%) gave a product size of 700bp using ITS1 primers. Brucei group 7/23 (30%) and *T. vivax* 11/23 (48%) amplicons were 480bp and 250bp, respectively. Identified trypanosome stabilates were *T. b. brucei* (7), *T. vivax* (2) and *T. congolense* Savannah (1). Whole blood PCR profiles revealed *T. vivax* (9) and *T. congolense* Savannah *(4)*. *Trypanosoma b. brucei*, *T. vivax* and *T. congolense* Savannah were the etiological agents for AAT in donkeys. In contrast, *T. vivax* and *T. congolense* Savannah caused the disease in cattle, and *T.b.brucei* in goats within Lamu County. KETRI 4028 stabilate was sensitive to Isometamidium and Diminazene. KETRI 4032, KETRI 3985 and KETRI 3984 stabilates were resistant to the three drugs used in this study. Importance of including molecular and parasitological methods when carrying out epidemiological disease surveys was highlighted. The results also indicate presence of more *T. b. brucei* sub populations circulating in Lamu that exhibit multiple resistance to Homidium, Isometamidium and Diminazene and presence of about 25% of sub population of *T. b. brucei* in Lamu that may be sensitive to Isometamidium and Diminazene and recommends that use of Homidium should be discouraged while use of Diminazene and Isometamidium should be used only in cases that proper diagnosis of the disease has been done.

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ABBREVIATIONS AND ACRONYMS

AAT	Animal African Trypanosomiasis
BCT	Buffy Coat Technique
BioRI-KALRO	Biotechnology Research Institute-Kenya Agricultural and Livestock Research Organization
Bp	Base pairs
CNS	Central Nervous System
DNA	Deoxyribonucleic acid
DNTPs	Deoxynucleotide- triphosphates
EDTA	Ethylendiaminetetra acetic acid
ESG	EDTA Saline Glucose
FAO	Food and Agriculture Organization
HAT	Human African Trypanosomiasis
HCT	Hematocrit Centrifugation Technique
ITS	Internal Transcribed Spacer
KETRI	Kenya Trypanosomiasis Research Institute
$MgCl_2$	Magnesium Chloride
NTDs	Neglected Tropical Diseases
PCR	Polymerase Chain Reaction
PCV	Packed Cell Volume
RNA	Ribonucleic acid
SRA	Serum Resistance Associated
TBR	*Trypanosoma brucei*
T_m	Melting temperature
VAT	Variable antigen types
VSG	Variant Surface Glycoprotein
WHO	World Health Organization
μ_g	Microgram (10^{-6} of a gram)

CHAPTER ONE

INTRODUCTION

1.1 Background information

African trypanosomes (genus *Trypanosoma*) are protozoan parasites transmitted by an insect, tsetse fly (genus *Glossina*), that is found in 37 countries in sub-Saharan Africa. These elongated eukaryotic cells are biologically transmitted by blood feeding invertebrates especially tsetse flies of the *Glossina* from a mammalian host to the other (Cecchi *et al*., 2010). Mechanical transmission of *Trypanosoma equiperdum* and *T. evansi* by tsetse is possible. *T. equiperdum* can be transmitted through coitus. Vertical and horizontal experimental transmissions are also possible. Diseases involved with trypanosome infections include Animal African Trypanosomiasis (AAT) also known as nagana in cattle, sheep and goats caused by *Trypanosoma* (Nannomonas) *congolense, T.* (Duttonella) *vivax* and *Trypanosoma brucei brucei* (Jean-Marc *et al*., 1997; Prashant *et al.,* 2005; Sanchez *et al*., 2015); trypanosomiasis in donkeys and camels referred to Surra which is predominantly caused by *T. evansi* (Prashant *et al.,* 2005). HAT or sleeping sickness (Brun *et al.,* 2010) also occurs in sub-Saharan Africa (Van den Bossche *et al.,* 2006; Mpho, 2008) and virtually all (>95%) cases are due to *T. b. gambiense,* which is indigenous to West and Central Africa (Cecchi *et al*., 2008) while the remaining less than 5% are caused by *T. b. rhodesiense* in eastern and southern Africa.

Notably, most within the affected regions main economic activity is pastoralism, characterized by continuous movement in search of pasture and water for their livestock. All domestic animals including cattle, donkeys and goats can be affected by nagana and through illness advancement the animals weaken more and more, eventually becoming unfit for traction activities, hence the disease "N'gana" a Zulu word meaning "powerlessness/useless" (Van den Bossche *et al.,* 2006; Mpho,2008). The pathogenic effects of trypanosome infections are mainly characterized by intermittent fever, anemia, lymphadenopathy and weight loss, loss of body condition (cachexia) and, in the absence of treatment, death in infected animals. AAT causes morbidity, unthriftness and death leading to economic losses estimated to amount to US $5 billion per annum. An estimated 50 million cattle and 70 million sheep and goats are at AAT risk while 3 million cattle die from the disease annually (WHO, 2012). It is one of the Neglected Tropical diseases (NTDs) which restrict economic development in Africa. Cattle distribution has been altered by tsetse flies causing widespread of trypanosomiasis, local overstocking in grazing areas enforcing nomadism on breeding herds and continued losses in slaughter cattle

and goats along the trade routes (Cecchi *et al.*, 2008). Trypanosomosis reduces meat and milk production in cattle by at least 50% (Ashiembi, 2013). AAT prevents use of draft oxen for cultivating land and donkeys for transport in tsetse belts. It also deters upgrading at low-yielding local breeds of cattle and programs that control trypanosomiasis (Murilla *et al.*, 2010). When agricultural communities are denied use of animals for productivity, only small areas of land can be ploughed by hand leading to a huge impact on food security and nutrition as well as livelihoods across Africa continent (Mpho, 2008).

Molecular tools such as PCR have been shown to be more sensitive (Liliana *et al*., 2014) than immunological and parasitological techniques in characterizing parasites that have similar morphologies but different AAT symptoms. Internal Transcribed Spacer 1 (ITS1) PCR was the first method to be developed which targets multi species organisms like trypanosomes. Serum Resistance Associated gene from a strain of *T. b. rhodesiense* has been shown capable of conferring the human infectivity trait on *T. brucei* by genetic transfection and therefore spreading the SRA gene and hence the resistance to human serum trait leading to human infectivity (Prashant *et al.,* 2005; Peacock *et al.,* 2011). In Uganda, the immense risk posed by domestic cattle and other livestock harboring *T. b. rhodesiense* has been revealed by the extensive Isoenzyme and RFLP analyses (Enyaru *et al.,* 1993).

Chemotherapy of HAT solely relies on a few drugs including; Pentamidine, Eflornithine and Melarsoprol. For treatment of Trypanosomosis in cattle, goats and sheep, Diminazene, Homidium and Isometamidium are primarily used. All these drugs have been available for at least 50 years in which Isometamidium was introduced in 1961. Since then, the development of new trypanocidal drugs has made minimal progress (Korir *et al*., 2013). Continued trypanocidal drug resistance, failure of treatment and that a handful of trypanocidal drugs are currently available in the market calls for more research especially in sub-Saharan Africa.

Occurrence and distribution of trypanosomiasis in sub-Saharan is known to generally mirror that of the vectors - tsetse flies. In Africa, tsetse flies infest 36 countries with a total combined area of between 9 and 10 million km^2 (Murilla *et al.*, 2010). African Trypanosomes are transmitted by tsetse flies (*Glossina spp*) with exception of *T. evansi* which is transmitted mechanically by biting flies including *Tabanus spp*., *Stomoxys spp* and *Liperoxia spp* (Gibson, 2003; Prashant *et al.,* 2005); for this reason the disease has spread outside Africa to Middle East, Southern Asia, Latin America, Australia and Europe (Hoare, 1972). In Kenya, eight species of tsetse flies have been identified to spread across much of the country.However, HAT

has only been identified in Western Kenya bordering Uganda and to a much smaller extent, the Maasai Maara ecosystem adjacent to the Tanzanian border where sporadic cases of HAT have been reported in Tourists (Kagira *et al*., 2011). As a result, studies on the epidemiology of HAT in humans, animal reservoirs and vector tsetse flies have traditionally focussed on these geographic regions (Musa *et al.*, 2005). The potential presence of the human infective parasite *T. b. rhodesiense* in other regions where tsetse fly vectors are present has not been adequately investigated.

Lamu County is located on the Kenyan Coast, which is one of the 47 administrative units that were created when Kenya adopted a devolved system of Government in 2010 (Refer to the 2010 new constitution). Notably, Kenyan coast has the largest tsetse habitat, infested with *G. austeni, G. pallidipes, G. brevipalpis* and *G. longipennis* (KENTTEC, 2016), and *T. congolense, T. vivax* and *T. evansi* infections (Mdachi *et al.,* 2006) reported. In addition, wild life in the Boni game reserve (Fig. 4) act as an important source of tsetse blood meals, thus increasing the risk of trypanosomiasis to local livestock. Most of the Lamu County is inhabited by Bajunis and Somalis who are pastoralists and hence livestock movement within the border is a risk factor. Recent research has shown that control of AAT in coast region is mainly by use of chemoprophylactic and chemotherapeutic drugs including Diminazene aceturate, Isometamidium and Homidium bromide. Farmers in this region have been experiencing treatment failure attributable arguably to improper trypanocidal drugs use, resistance development and poor qualities of trypanocidals (Ashiembi, 2013) with which farmers administer themselves.

1.2 Statement of the problem

In Lamu County, the various species of animals kept include cattle, goats, donkeys, sheep and camels. *Glossina pallidipes* and *G. austeni* species of tsetse fly are commonly found causing trypanosomiasis in livestock in this region. There is paucity of data on infection of livestock in Lamu County and consequently potential role in AT transmission dynamic. From an ongoing project, anemia has been found in livestock and preliminary data shows there is persistent high usage of trypanocidal drugs. Sub-clinical trypanosome infections in donkeys, goats and cattle are difficult to identify using the available parasitological methods while definite clinical symptoms may not be obvious as such. Ineffective control of animal trypanosomiasis has led to persistent socio-economic losses caused by the disease.

1.3 Objectives

1.3.1 General objective

To carry out molecular identification and drug sensitivity of African trypanosome stabilates, from livestock in Lamu County, Kenya to enhance sustainable management of Trypanosomiasis in livestock for livelihood improvement in Lamu community.

1.3.2 Specific objectives

1. To identify trypanosome species infecting livestock in Lamu County, Kenya.
2. To determine presence of Serum Resistance Associated (SRA) gene related to human trypanosomiasis in *T. brucei* trypanosome stabilates from Lamu County.
3. To determine the drug sensitivity of trypanosomes isolated from livestock in Lamu County.
4. To determine the correlation between drug sensitivity/resistance to virulence and pathogenicity.

1.4 Justification

Literature shows that animal trypanosomiasis has great economic consequences to livestock keepers and that inhabitants suffer substantive economic losses due to persistent trypanocidal use. Infection in cattle causes losses in milk and meat production. Donkeys are used as traction animals while cattle and goats provide meat and milk and they act as a source of income, for bride price and traditionally they were a symbol of physical wealth. Kiunga and Lamu archipelago where some of the samples used in this study were collected are adjacent to the Boni forest and Dodori conservancy which are ideal habitats and breeding sites of tsetse flies. Determination of drug sensitivity will enable livestock farmers in Lamu County use the available effective drugs properly. Determination of circulating trypanosome species will be useful to veterinary department to plan management and control measures of trypanosomiasis.

CHAPTER TWO
LITERATURE REVIEW

2.1 Classification of trypanosomes

Trypanosomes are scientifically grouped in phylum sarcomastigophora, order kinetoplastida, family trypanosomatidae and Genus *Trypanosoma (*Adams *et al.*, 2006). Other selected species include: *T. suis* which causes Surra, *T. equiperdum* in horses, *T. theileri* in ruminants, *T. avium* in birds, *T. hosei* in amphibians, *T. lewisi* in rats and *T. simiae* in primates (Earl *et al.,* 1999). Trypanosomes which affect mammals can be divided into two groups depending on transmission namely Stercorarian and Salivarian. Most stercorarians are non-pathogenic except *T. cruzi* that causes Chaaga's disease whose vector is kissing bug (Klotz *et al.,* 2010). This group infects insects, develop in their posterior gut and infective organisms are released in feces and deposited on the skin of a host. The Salivarian group which is transmitted through saliva of tsetse flies, is subdivided into four subgenera; *pycomonas, nannomonas, duttonella (*Gibson, 2009*)* and the *trypanozoon* (Osório e*t al*., 2008). The Salivarian group has a characteristic of antigenic variation unlike the Stercorarian group (Marchalonis, 1984).

T. congolense species belongs to subgenera *nannomonas* (Mpho, 2008) and is made of four genotypic groups; the Savannah, Kilifi, Tsavo and West Africa Riverine forest. The subgenus *Trypanozoon* consists of 3 species; *T. equiperium*, *T. evansi* and *T. brucei* and the latter is subdivided into 3 subspecies including; *T. b. gambiense, T. b. rhodesiense* and *T. b. brucei* (Shimelis and Melkamu 2015*). T. b. rhodesiense* causes East African trypanosomiasis in man. It is morphologically indistinguishable from *T. b. brucei* and the two are referred to as Brucei group. The latter is distributed across the tropical countries and it occurs in dogs, equines, sheep and goats (Gutierrez *et al.,* 2010). *T. b gambiense* causes Gambian sleeping sickness, predominant in West Africa (Franco *et al*., 2014). The rhodesiense form is a zoonosis, with the occasional infection of humans, but in the gambiense form, the human being is regarded as the main reservoir that plays a key role in the transmission cycle of the disease (Stijlemans *et al.,* 2018).

2.2 Geographical distribution of trypanosomes

Trypanosomes distribution mirrors that of tsetse flies and comprises currently an area of 8 million km^2 between 14°North and 20°South latitude (Dietman, 2008), from southern edge of Sahara desert to Zimbabwe, Angola and Mozambique (Fig. 1). Trypanosomes, particularly *T. vivax*, have been found to spread beyond the tsetse range through mechanical vectors

transmission (Mpho, 2008, Samuel *et al.*, 2010) for example in South and Central America and the Caribbean.

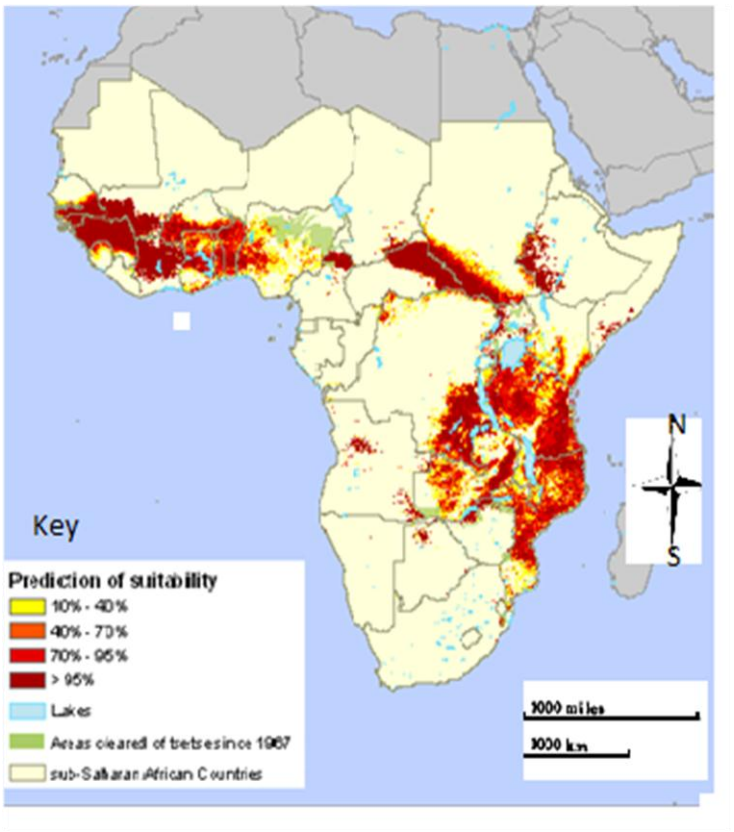


Fig. 1: Distribution of *G. pallidipes* and *G. longipalpis* groups in Africa (Courtesy: FAO, 2015)
In Kenya, areas infested by tsetse flies include Lake Victoria region and Coastal region, parts of the Rift Valley and parts of Western, Eastern and N. Eastern.

2.3 Morphology of trypanosomes

Salivarian trypanosomes are leaf shaped; streamlined and unicellular organisms whose sizes vary depending on the species. Their sizes range between 8-50µm (Mpho, 2008), (Fig**.** 2). The smallest is *T. congolense* followed by *T. vivax* and *T. brucei* the longest. Under an electron microscope, a trypomastigotes has cellular organelles including; kinetoplast, parabasal body, nucleus, undulating membrane and a flagellum (Sharma *et al*, 2008). Kinetoplast is majorly involved in metabolism, reproduction (Sanchez *et al*, 2015) and cyclic transmission of trypanosomes. Flagellum is essential for viability and has emerged key in numerous facets of development, transmission and pathogenesis (Gerasimos and Kent, 2014).Notably, *T. brucei* has a single flagellum which is present during all stages of development.

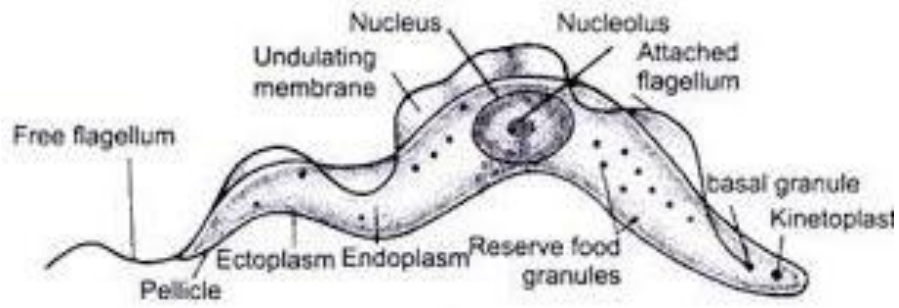


Fig. 2: Morphology of *T. b. gambiense* (Courtesy: Mpho, 2008)

2.4 Life cycle (of Brucei group) and transmission of trypanosomes

When an infected host is bitten by a tsetse fly (teneral fly) during a blood meal, blood stream trypomastigotes are taken up into the mid gut, and here the short stumpy forms differentiate into procyclic trypomastigotes (Fig.3). These undergo restructuring, together with an asymmetric division; generate a long and a short epimastigote (Gerasimos and Kent, 2014). Epimastigotes multiply in salivary glands transforming into metacyclic trypomastigotes (metatrypanosomes). As tsetse fly feeds on a blood meal, they inject metacyclic trypomastigotes into the target mammalian host (CDC, 2009).

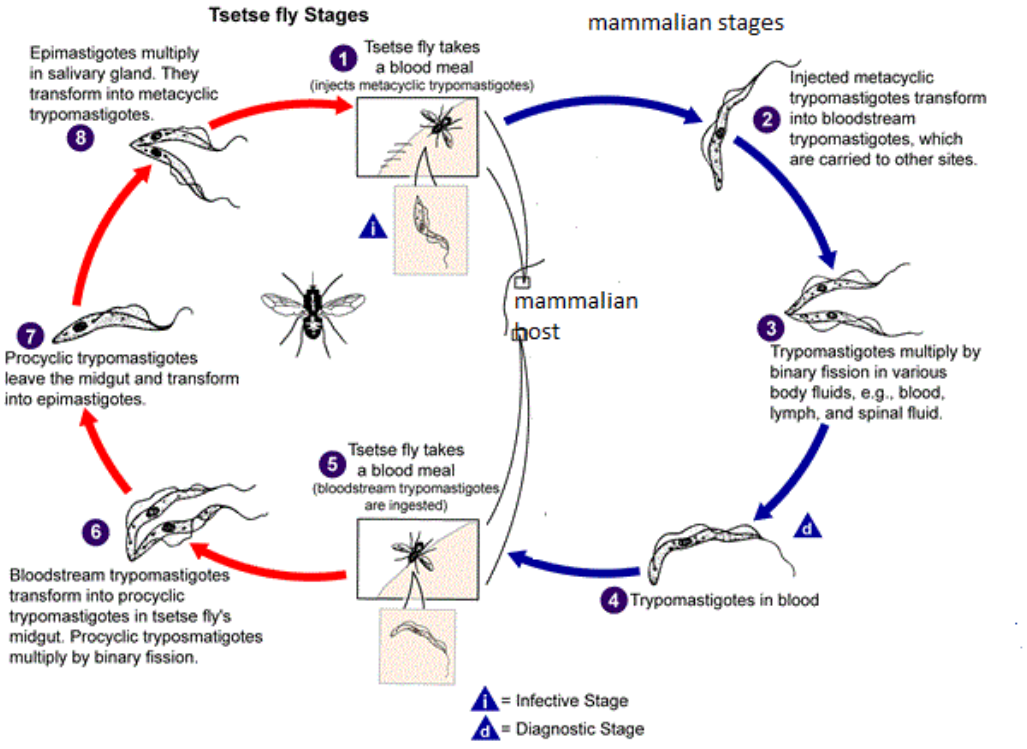


Fig. 3: Life cycle of *T. brucei* (Courtesy: CDC, 2009)

Transmission can either be through common insect vectors, mechanically or cyclically (Mpho, 2008). Most trypanosomes undergo 'cyclical development' where lifecycle alternates between the insect vector and mammalian host. As in the case of *T. brucei*, an insect host is infected from a mammal through blood sucking, a cycle of development and multiplication takes place which involve a development stage (WHO, 2014). Apart from blood sucking insects, transmission can also occur mechanically where there is no development stage (Cecchi *et al.*, 2008).

2.5 Pathology and symptoms of trypanosomiasis

Parasites multiplication is characterized by lymph nodes enlargement, diarrhea with blood stains, fever and sometimes death (Batista *et al.*, 2009). In humans, the disease is characterized by fever and shivering, increased pulse rate, swollen lymph nodes and splenomegaly (Mpho, 2008). In AAT the primary clinical signs are an intermittent fever, signs of anemia, lymphadenopathy and weight loss. Milk yield decreases in dairy animals (WHO, 2013). On reproduction, abortions, premature and still births in females while in males, testicles swell up. Sudden deaths have been reported in small ruminants found infected with *T. vivax* (Makokha *et al.*, 2011). As defined, pathogenicity is the ability of a parasite to multiply and be sustained in a given host while virulence is the ability of a parasite to cause a disease to a host (Bengaly *et al.*, 2001, Korir *et al*., 2013).

2.5.1 Diagnosis of trypanosomiasis

Diagnosis of AAT in field samples is currently based on suspicion clinically and less frequently on traditional parasitological methods which were characterized by low sensitivity, especially in sub-acute and chronic infection (Liliana *et al*., 2014). In identification of trypanosomes circulating in cattle, light microscopic examination of buffy coat is a reliable technique (WHO, 2008). Microscopy has a disadvantage in that it is more labor intensive and less sensitive compared to recent techniques (Cox *et al*., 2005). Molecular tools have been shown to have the highest sensitivity percentages (Liliana *et al*., 2014) compared to immunological and parasitological techniques and can differentiate parasites with similar morphologies but different disease outcomes.

2.5.2 Treatment and control of trypanosomiasis

Common chemoprophylactic drugs are Isometamidium chloride, Homidium bromide and Diminazene commonly used for treatment and prophylaxis of trypanosomosis in sheep, cattle and goats (Michael *et al.,* 2014). Reduced drug sensitivity of *T. b. rhodesiense* stabilates to trypanocidals used to treat cattle has been reported in Uganda and Kenya (Kagira and Maina, 2007). In an earlier study, despite presence of drug resistant trypanosomes in Lamu District, prophylactic and therapeutic drugs are still effective and hence, by use of quality drugs correctly, trypanosomosis can be effectively controlled (Mdachi *et al*., 2006a). Pentamidine and Melarsoprol represent classes of drugs, arsenicals and Diamidines, historically used to treat AT (Nicola *et al*., 2013). In a another study, Diminazene at 14mg/kg was not sufficient to cure mice (Enyaru *et al.,* 1998), while another study showed that management of trypanosomiasis by treatment of reservoir animal could face serious problems since drug resistant parasites would not be eliminated by Diminazene and Isometamidium administered at their recommended dosage levels (Kagira and Maina, 2007). Trypanotorelant breeds of livestock farming should be considered in case the disease is widespread (WHO, 2014). Resistance to one or more trypanocides used in livestock has been reported in 13 countries in Africa which is associated with prolonged, improper and widespread use of trypanocides especially Diminazene and Isometamidium which acts as a selection pressure for resistant trypanosomes development (Kagira *et al.,* 2005). The outstanding methods according to FAO (2006) include; reducing breeding grounds for tsetse flies, use of impregnated targets and nets which has recently been used to reduce tsetse populations by 90% and bovine trypanosomiasis from over 30% to below 5% in a 6 month period (Samuel *et al.*, 2008).

2.6 Molecular characterization of trypanosomes

It is difficult to identify a parasite on the basis of morphological characteristics while differentiating *T. simiae* from *T. congolense* or *T. b. brucei* from *T. evansi* because they are identical. Generally, the polymerase chain reaction (PCR) is a sensitive and specific diagnostic tool that has been widely used in trypanosomes detection in many countries (Waren *et al*., 2011b). During detection and characterization of trypanosomes, PCR is an ideal tool because of its sensitivity compared to immunological and parasitological techniques and it can also differentiate parasites with similar morphologies but different disease results in a host (Geysen *et al*., 2003; Mpho, 2008). PCR is a relatively simple technique by which DNA templates are amplified million folds quickly and reliably to generate enough material for subsequent

analyses (WHO, 2008). PCR amplification of SRA gene has formed the basis of a new test used to identify *T. b. rhodesiense*. Gel electrophoresis which proceeds DNA amplification can be used to identify amplified PCR products.

2.7 Recent work on Trypanosomiasis in Lamu County

The coastal region of Kenya is a tsetse and animal trypanosomosis endemic area (Murilla *et al.,* 2014). The common trypanosomes in Lamu identified morphologically are *T. Congolense* and *T. vivax* (Mdachi *et al.,* 2006a). Control of trypanosomiasis in coast region is mainly by use of chemoprophylactic and chemotherapeutic drugs including Diminazene, Homidium and Isometamidium (Murilla *et al.,* 2010). Farmers in Lamu have been experiencing treatment failures attributable to improper use of trypanocidals, resistance development and poor quality of drugs used (Mdachi *et al.,* 2006b). Another study was carried out to determine the role of trypanocidal use in treatment failure in cattle in five points within Lamu district (Andy and Patrick, 2000). Another recent study has proven that drug levels can be used to evaluate failure of treatment and drug use which contributes to efficacy of trypanocidals (Ashiembi, 2013). Studies have also shown that frequent exposure of trypanosomes to drugs in the field promotes mutation among trypanosomes leading to resistance to drug in the Kenyan market (Kagira and Maina, 2007). Records from the veterinary department indicate that there is a high level of trypanocidal drug use in coastal belt. The disease prevalence is still high in domestic animals; however, it is not clear whether this is due to drug resistance which has been demonstrated elsewhere in the coast region (Murilla *et al.,* 2014). Some other factors which relate to the farmer and the parasite have been identified as contributing to treatment failure (Mdachi *et al*., 2006b) which is key in Coastal region. Reduced sensitivity of cattle infected by these strains to trypanocidal drugs has been observed over years of drug use. A monitoring study is necessary to identify genetic variation and dynamics of sensitivity and resistance in this region.

CHAPTER THREE
MATERIALS AND METHODS

3.1 Study area

The stabilates that were used in the current study were recently collected from Lamu County in the Coast region of Kenya. Kiunga marine reserve and Boni forest create a suitable habitat for tsetse flies. The area is humid with mean temperature ranging from 22-34˚C and an annual rainfall of about 500mm. Economic activities like marine conservation; pastoralism, deep sea fishing and trade in Lamu have been sustained by a marine reserve (Ashiembi, 2013). Livestock kept in Lamu County in economic importance order of species include donkeys, sheep, goats, cattle and local poultry. Additionally, the Boni and Dodori game reserves (Fig**.** 4) are a home to a wide range of wild game ranging from large herbivores like elephants and buffalos to carnivores like lions and leopards as well as a whole range of reptiles. These game reserves are important sources of tsetse blood meals and potential reservoir for trypanosomes.

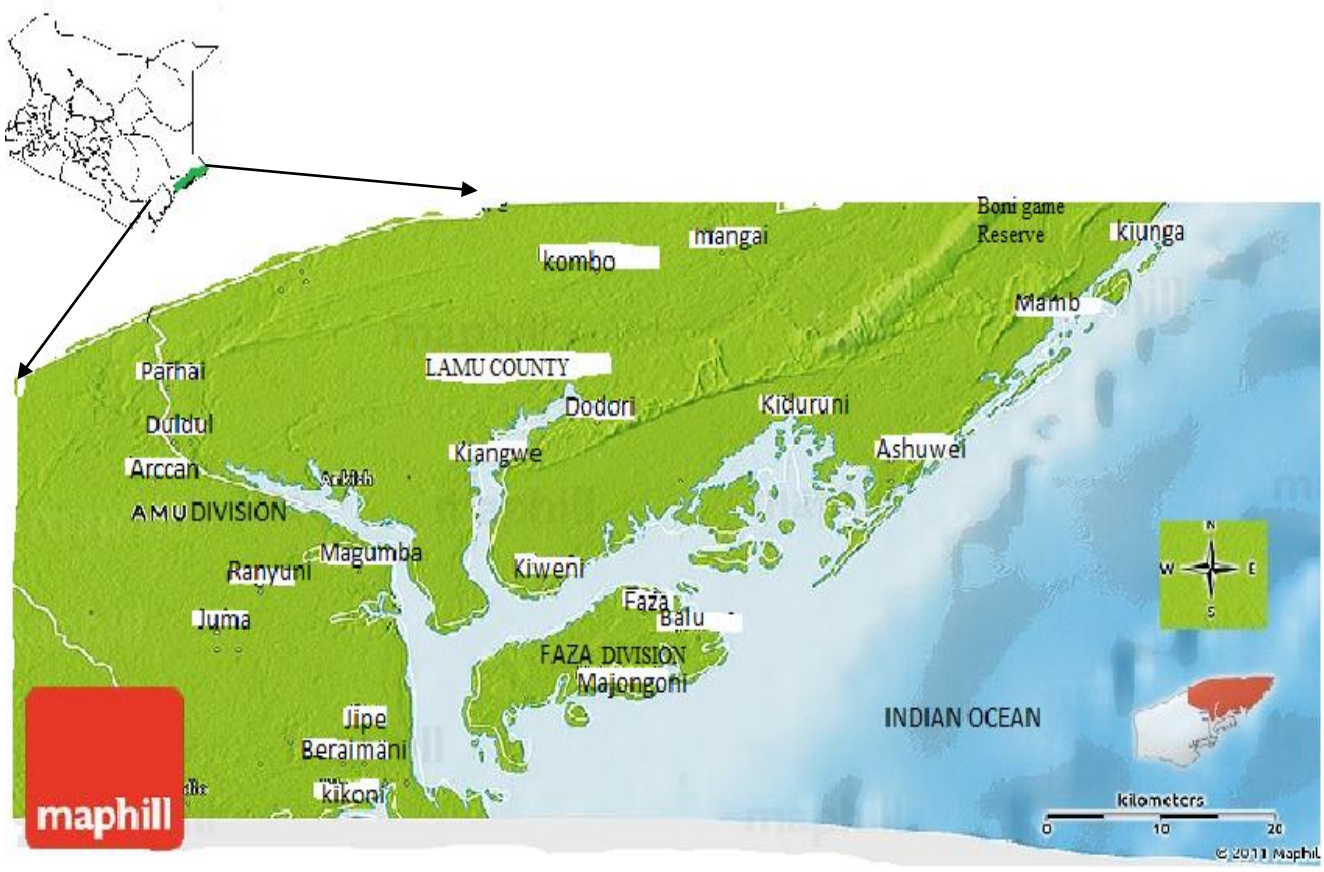


Fig.4: Location of Lamu County, Kenya. (Courtesy: Map graphics revolution, 2013).

3.2 Objective 1: To identify trypanosome species infecting livestock in Lamu County, Kenya.

3.2.1 Trypanosome stabilates and blood samples

3.2.1.1 Trypanosome stabilates

A total of 15 trypanosome stabilates stored at the Kenya Agricultural and Livestock Research Organization-Biotechnology Research Institute (KALRO-BioRI) trypanosome bank were used for this study. The stabilates had been collected from infected cattle, donkeys and goats in the Lamu County during 2007–2014 epidemiological survey. More than 26 trypanosome stabilates were prepared directly from blood and subsequently preserved in liquid nitrogen as previously described by Murilla *et al*, 2014, the bulk from cattle. The 15 trypanosome stabilates used in this study were randomly selected which included 9 from donkeys, 5 from cattle and 1 from a goat. Eight were secondary stabilates (prepared after passaging in mice, re-stabilated and given the KETRI code) and seven were primary stabilates (not passaged in mice).

3.2.1.2 Whole blood samples

Ninety two whole blood samples in EDTA randomly collected from Donkeys (40), Cattle (5) and Goats (47) were used in this study. These samples were part of blood samples collected from cattle, donkeys and goats parasitologically negative for trypanosomes that had been brought for observation during the epidemiological study in Lamu County in 2014. All the whole blood samples were prepared in EDTA and stored at -20°C.

3.2.2 Examination of stabilates for viability and infectivity to white Swiss mice

Two capillaries of each of the 15 stabilates were retrieved from liquid Nitrogen in trypanosome cryobank and placed in a beaker containing ice cubes to thaw slowly. The thawed stabilates were aspirated into 1ml syringe and made up to 0.4ml using EDTA Saline Glucose (ESG) buffer, pH 8.0. After confirming presence of trypanosomes through microscopic examination of wet smears, tentative identification based on morphological characteristics was carried out on the viable stabilates. Wet blood smears on slides were prepared, and then Giemsa® (Medic Diagnostic Reagents) stained. These slides were examined under a microscope at high power (40x) and oil immersion (100x) objectives (Waren *et al*., 2011a) to identify motile trypanosomes in the 15 positive stabilates. These wet smears were used to observe motility characteristics of different trypanosome species; thin

blood smears were made to see morphological characteristics used to tentatively assign each stabilate to a species.

Each stabilate was then inoculated at 0.2ml of motile trypanosomes in each of two immunosuppressed white Swiss mice intraperitoneally for multiplication. Monitoring for parasitaemia in a drop of tail blood placed on a clean slide was done daily via microscopic examination of a minimum of 20 fields at 400x magnification until parasitaemia reached antilog 8.1-8.4 (Korir *et al*., 2013). Estimation of trypanosome numbers was carried out using the matching method of Herbert and Lumsden (1974). At peak parasitaemia, blood was drawn from the tail vein using capillary tubes which was then dispensed in a microcentrifuge containing 200µl Tris EDTA buffer (pH 8.0) and this was subjected to PCR analysis. The stabilates that did not grow in mice were directly subjected to PCR analysis.

3.2.3 Extraction of DNA from Trypanosomes tabilates and blood samples

The protocol for amplifying nucleated cells (with some modifications) was derived from the Qiagen DNeasy blood and tissue handbook (07/2006). Into a 1.5 ml microcentrifuge tube, 20µl proteinase K was transferred. This was followed by adding 100µl of the blood and 200µl Buffer AL (Lysis buffer) to the tube and the mixture was agitated thoroughly by vortexing and then incubated at 56˚C for 10 min. To this sample, 200µl Ethanol (96%) was added and mixed by vortexing. The mixture was pipetted into a mini spin column placed in a 2ml collection tube, centrifuged at 8000 rpm for 1 min. The mini spin column was placed in a new 2ml collection tube, 500µl Buffer AW1 (Wash buffer) added which was then centrifuged at 8000 rpm for 1 min. The mini spin column was then placed in a new 2ml collection tube, 500µl Buffer AW2 (Wash buffer) added and centrifuged for 3min at 14000 rpm to dry the membrane. The mini spin column was placed in a clean 2ml microcentrifuge tube and 30µl Buffer AE (Elution buffer) directly pipetted onto the centre of the column. This was incubated at room temperature for 1 min. Discs were then separated from elutes containing DNA through centrifuging at 12000 revolutions/min for 1 min (Mpho, 2008). The DNA was stored either at 4˚C if it was to be analyzed within 3 days or at -20 ˚C for analysis after 3 days of extraction.

3.2.4 PCR amplification of extracted DNA

All samples were first analyzed using ITS1 CF, 5’ (CCG GAA GTT CAC CGA TAT TG) and ITS1 BR, 5’ (TTG CTG CGT TCT TCA ACG AA) primers (Njiru *et al*., 2005) of the

ribosomal DNA which is known to be a suitable target for PCR based detection of all trypanosomes. The ITS1 positive samples were then analyzed for presence of *T. brucei, T. congolense* and *T. evansi.* The latter was further analyzed to detect presence of *T. evansi* A using specific primers EVA A, 5' (ACA TAT CAA CAA CGA CAA AG) and B, 5' (CCC TAG TAT CTC CAA TGA AT) for type A which were earlier designed for the mini-circle DNA sequence (Njiru *et al*., 2006). Positive control sample *T. b. rhodesiense* KETRI 2537 was used during ITS1 PCR.

Screening for *T. congolense* involved using species-specific primers TCS1 , 5' (CGA GAA CGG GCA CTT TGC GA), TCS2, 5' (GGA CAA ACA AAT CCC GCA CA): TCK1, 5' (GTG CCC AAA TTT GAA GTG AT), TCK2, 5' (ACT CAA AAT CGT GCA CCT CG) and TCF 1 , 5' (GGA CAC GCC AGA AGG TAC TT), TCF 2, 5' (GTT CTC GCA CCA AAT CCA AC) (Njiru *et al*., 2005) to further classify it into Savannah, Kilifi and Forest subtypes respectively. *Trypanosoma congolense* Savannah positive sample was used as a positive control.

Trypanosoma brucei was analyzed using TBR 1, 5' (GAA TAT TAA ACA ATG CGC AG) and TBR2, 5' (CCA TTT ATT AGC TTT GTT GC) primers (Hoare *et al*., 1972). The DNA was amplified using GoTaq® Green Master Mix (Promega Co. USA) in a 10 µl total volume. Each reaction included 0.2 µl GoTaq® (5 U/ µl), 1 µl PCR buffer (10X), 0.2 µl dNTPs (10 Mm), 0.6µl $MgCl_2$ (25 mM), 1 µl of 10 mM of each primer (10 µM), 4 µl RNase-free water and 2 µl extracted template DNA.

Thermocycling for ITS1 PCR profile started with initial hold for 5 min at 94°C, followed by 35 cycles of 94°C for 40 sec, 58°C for 40 sec and 72°C for 90 sec and final extension step of 5 min at 72°C (Njiru *et al*., 2005). These repeated cycles of temperature shifts were done automatically in a thermo cycler, a machine that sequentially shifts between the desired temperatures and remains at each temperature for a specified length of time (Michael *et al.*, 2014).

Thermocycling *T. evansi* A PCR profile started with initial hold for 3 min at 94°C, followed by 30 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 1 min and final extension step of 5 min at 72°C. A positive and a negative control for *T. evansi* A was used during the analysis.

Cyclic conditions for *T. congolense* Savannah, *T. congolense* Kilifi and *T. congolense* Forest subtypes using a standard PCR profile started with initial hold for 1 min at 94°C, followed by 30 cycles of 92°C for 30 sec, 60°C for 45 sec and 70°C for 45 sec and final extension step of

4 min at 72°C. *T. congolense* Savannah-specific satellite DNA gives a band at approximately 316 bp with species-specific-primers TCS1 and TCS2 which distinguishes it from other *T. congolense* subtypes (Masiga *et al*., 1992, Thumbi *et al.*, 2008, Clement *et al*., 2016).

For *T. vivax*, TV East Africa type universal primers were used with thermocycling starting with pre-denaturation at 94°C (3 min) followed by 35 cycles of denaturation at 94°C (45 s), annealing at 58°C (30 s), extension at 72°C (60 s) and further extension at 72°C (10 min) (Mwandiringana *et al.*, 2012).

PCR products were electrophoresed through 100mls of 2% agarose gel in Tris-acetate EDTA buffer containing 5µl Ethidium bromide dye (10 mg/ml) at 60V for 1hr before being visualized under a UV light trans illuminator and photographed in an UVTEC gel imager and results recorded. A negative control and a positive control for each genomic DNA were included in all PCR.

3.3 Objective 2: To determine presence of Serum Resistance Associated (SRA) gene related to human trypanosomiasis in *T. brucei* trypanosome stabilates from Lamu County.

3.3.1: PCR analysis and gel electrophoresis

Samples that were positive for T. *brucei* group were further analyzed using SRA specific primers SRA A, 5' (GAC AAC AAG TAC CTT GGC GC) and SRA E, 5' (TAC TGT TGT TGT ACC GCC GC) (Njiru *et al*., 2008). These samples were amplified using GoTaq® Green Master Mix (Promega Co. USA) in a 10 µl total volume. Each reaction included 0.2 µl GoTaq® (5 U/ µl), 1 µl PCR buffer (10X), 0.2 µl dNTPs (10 Mm), 0.8 µl $MgCl_2$ (25 mM), 1 µl of 10 mM of each primer (10 µM), 3.3 µl RNase-free water and 2.5 µl extracted template DNA. Thermocycling of SRA PCR profile started with initial hold for 3 min at 95°C, followed by 35 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 1 min and final extension step of 2 min at 72°C. A positive control *T. b. rhodesiense* (KETRI 2537) was used during the analysis to compare with the results from samples. The amplicons were identified through gel electrophoresis, visualized under an imager and the results recorded.

3.4. Objective 3: Determination of drug sensitivity of trypanosomes isolated from livestock in Lamu County.

3.4.1 Preparation of experimental mice and treatment groups

Two hundred normal healthy experimental Swiss white mice weighing between 20-30g were obtained from BioRI-KALRO Small Animal Breeding Unit (SABU). They were dewormed

using subcutaneously injectable ivermectin® at 0.01ml per mouse and were housed in 4 cages designed for mice with 6 mice per cage and wood chippings as bedding material. The mice were acclimatized for 14 days during which they were maintained on mice pellets (mice pellets®, Unga Ltd, Nairobi, Kenya) and water provided *ad libitum* at room temperature. These mice were either treated as donor mice or experimental mice in this study. Body markings were made on mice for experimental identification to avoid errors during recording of data and ensure accurate records for analysis. Different anatomical areas in the body were marked using dipsticks with cotton soaked in picric acid solution. All experimental procedures and protocols involving mice were accordingly reviewed by Institutional Animal Care and Use Committee (IACUC) of BioRI-KALRO and approved.

3.4.2 Trypanosome stabilates

Four trypanosome stabilates (KETRI 4032, KETRI 4028, KETRI 3984 and KETRI 3985) previously identified by PCR as *T. b. brucei* isolated from livestock in 2007 and 2014 at different villages in in Lamu County (as described in 3.2.1) were used in this study.

3.4.3 Multiplication of trypanosome stabilates in donor mice

For each of the stabilates, two donor mice were immunosuppressed using 200mg/kg cyclophosphamide at 0.2ml per mouse for 3 days consecutively before infection (Kagira *et al* 2005).On the third day of the cyclophosphamide injection, capillaries containing stabilates were obtained from the Trypanosome Bank and placed in a beaker containing ice and allowed to thaw slowly. The thawed stabilates were aspirated into a 1ml syringe and made up to 0.4ml using EDTA Saline Glucose (ESG – pH 8.0). Each of the two donor mice were injected intra-peritoneally with 0.2 ml of this inoculum. The donor mice were monitored for parasitaemia from day 2 post infection via microscopy (Korir *et al*., 2013). Parasitaemia was scored using the matching method of Herbert and Lumsden 1974 (Kobo *et al.,* 2014). When parasitaemia level reached antilog 8.1-8.4**,** blood enough to fill a capillary tube was harvested individually (for each mice) from the tail vein and diluted appropriately to make an inoculum for infecting experimental mice.

3.4.4 Infection and treatment of experimental mice for drug sensitivity

At peak parasitaemia, blood was drawn from tail vein of donor mice which was diluted ten times (1:10) with ESG at pH 8.0.The number of parasites in the inoculum was quantified

using a hemo-cytometer. Further dilutions were made to 5 x 10^5 trypanosomes/ ml. There were 4 groups of 6 mice each per stabilate (Table 1). Each experimental mouse was intra-peritoneally injected with 0.2 ml of this inoculum containing 1×10^5 trypanosomes/ml. Drug dosage used was as described by (Geerts *et a*l., 2001). Experimental mice were treated on day 1 post infection with a single dose of Isometamidium Chloride (Samorin®), Diminazene Aceturate (Veriben®) or Homidium Bromide (Novidium®) at 1.0 mg kg^{-1}, 20mg $kg^{-1}$and 1.0 mg kg^{-1} respectively (Table 1).

Table 1: Dose regimens for drug sensitivity evaluation

Group	Drug	Dose (mg/kg body weight)	No of mice
1	Isometamidium	1.0	6
2	Diminazene	20.0	6
3	Homidium	1.0	6
4	Controls	distilled water	6

As described by (Korir *et al.,* 2013), a trypanosome stabilate was considered drug sensitive if at least 80% of treated mice were cured. In this study if fewer than five mice were cured, the stabilate in question was considered drug resistant. Resistant stabilates were from 0/6 – 4/6 while sensitive stabilates are from 5/6–6/6 (Kagira and Maina, 2007).

3.4.5 Monitoring the PCV, parasitaemia, body weight and mortality in experimental mice

Pre-infection levels of packed cell volume (PCV),body weight and clinical observations on fur state, feed intake and emaciation for both experimental and untreated control mice were collected once a week for two weeks prior to infection. After the mice were infected and subsequently treated 24 hrs. later, parasitaemia was monitored daily by microscopic examination of wet smears (Kobo *et al.,* 2014)which involved using a drop of blood from the mouse tail placed on a clean slide and covered with a cover slip. Parasites were counted under a microscope for the first 14 days then weekly for the rest of 60 days through Buffy Coat Technique (BCT).Parasitaemia was scored according to the commonly used matching method of Herbert and Lumsden 1974 (Kobo *et al.,* 2014).PCV of experimental mice was determined through hematocrit centrifugation technique (HCT) (Shimelis *et al.,* 2008). Blood enough to fill ¾ of a capillary was collected from the tail vein using a heparinized capillary tube and completely sealed with plasticin (Korir *et al*., 2013). The sealed capillaries were centrifuged in a hematocrit centrifuge at 10,000rpm, for 5 minutes. PCV was read using a hematocrit

reader then expressed as percentage (%) of the total blood volume (Naessens *et al*., 2005). Body weight of experimental mice was monitored weekly using an analytical balance (Mettler Tolendo PB 302 r, Switzerland) (El-arab *et al*., 2006) and expressed to the nearest grams. Mortality of mice was monitored daily. Surviving mice were monitored for 60 days (Korir *et al*., 2013) while those that died were recorded with the date. Post infection data collected was subsequently entered in Ms Excel spreadsheets and cleaned. Graphs were used to show how trends of PCV, parasitaemia and how body weights unfolded.

3.5 Objective 4: To determine the correlation between drug sensitivity/resistance to virulence and pathogenicity.

Pathogenicity and virulence of 1 drug-sensitive and 2 drug-resistant trypanosome stabilates identified from the sensitivity test (3.4.4) as shown in Table2were investigated using groups of 10 mice each; there was also a group of 10 mice which were used as un-infected controls.

Table2: Drug resistant and sensitive stabilates used in the pathogenicity studies

Stabilates classification	Stabilates ID
Drug-sensitive	KETRI 4028
Drug-resistant	KETRI 3985
	KETRI 3984

The mice were infected as described in 3.4.4using a trypanosome inoculum of 0.2ml of 5×10^5 trypanosomes/mouse. Parameters for virulence and pathogenicity monitored were parasitaemia level, pre-patent period, body weight, packed cell volume and survival period. Parasitaemia was recorded daily starting 2 days post infection as described in 3.4.5 to determine parasitaemia patterns. However, PCV and body weight changes determined as described in 3.4.5 were assessed once every week. Surviving mice were monitored for a minimum of 60 days post infection.

3.6 Statistical analysis

Data from this study was managed using Microsoft excel. Data was analyzed using GENSTAT 16th edition (Alvey, 1983) statistical software. Parasitaemia levels were analyzed using Generalized Linear Models. Log-Linear Regression were run to determine if there was a significant difference in PCV level of the stabilates when subjected to different drugs. Analysis of variance was run to determine if there was a significant difference in body weights of mice treated with different stabilates. Comparisons carried out were considered

significantly different at p values <0.05. Descriptive charts were used to show the progress of PCV, parasitaemia and body weights of the batch of the untreated infected and control mice for the pathogenicity study. To ascertain if there were differences in the survival of mice infected with resistant and sensitive isolates Kaplan-Meier method was used. Log Rank test was used to compare survival times of the three groups of mice.

CHAPTER FOUR

RESULTS

4.1 Objective 1: Identification of trypanosome species infecting livestock in Lamu County, Kenya.

4.1.1 Morphological characterization of stabilates

The identity of these stabilates is shown in Table3. Based on motility, 7 out of 15 stabilates were viable. The species identities of four of the seven viable stabilates were similar to their species identity during isolation in the field (Table 3). The difference in species observed in the 3 of the seven stabilates is attributed to the initial passage in mice which selected for *T. brucei*. This is an indication that the 3 stabilates may have been prepared from multi infection primary stabilate. Of the 7 viable stabilates only four grew in mice (KETRI 4032, KETRI 4028, KETRI 3984 and KETRI 3985).

Table3: Stabilates identity, host and Trypanosomes tentatively identified by microscopy in stabilates recovered from livestock in Lamu.

Stabilates identity	**Year of Isolation**	**Host**	**Stabilate species during isolation based on morphology**	**Examination of samples in 2016 by microscopy**
				Stabilate species based on morphology
KETRI 4028*	2014	Donkey	*T. congolense*	*T. congolense*
KETRI 3984*	2007	Donkey	*T. congolense*	*T. congolense*
KETRI 3986	2007	Donkey	*T congolense*	*T. brucei*
KETRI 3985*	2007	Donkey	*T. congolense*	*T. congolense*
KETRI 3987	2007	Donkey	*T. congolense*	*T. congolense*
D3	2007	Donkey	*T. congolense*	No trypanosome seen
D4	2009	Donkey	*T. congolense*	No trypanosome seen
C28	2014	Bovine	*T. vivax*	No trypanosome seen
C18	2007	Bovine	*T. vivax*	No trypanosome seen
KETRI 4033	2014	Bovine	*T. congolense*	*T. brucei*
C 98	2014	Bovine	*T. congolense*	No trypanosome seen
C99	2014	Bovine	*T. vivax*	No trypanosome seen
KETRI 3982	2007	Donkey	*T congolense*	No trypanosome seen
D 5	2014	Donkey	*T. congolense*	No trypanosome seen
KETRI 4032*	2014	Goat	*T. vivax*	*T. brucei*

Legend: C. cattle; D, Donkey; ID, identity, T: Trypanosoma, *-stabilates that grew in mice

4.1.2 Results of molecular characterization of 4 stabilates that grew in mice

a) ITS 1 PCR

Results from ITS 1 PCR analysis using ITS1 BR and ITS 1 CF primers established that all the four stabilates were trypanosome positive, and whose DNA gave a product size of 480 bp on the ITS1 PCR, similar to that for the positive control sample, *T. b. rhodesiense* KETRI 2537 (Fig.5), indicating that these stabilates belonged to subgenus Trypanozoon group of trypanosomes.

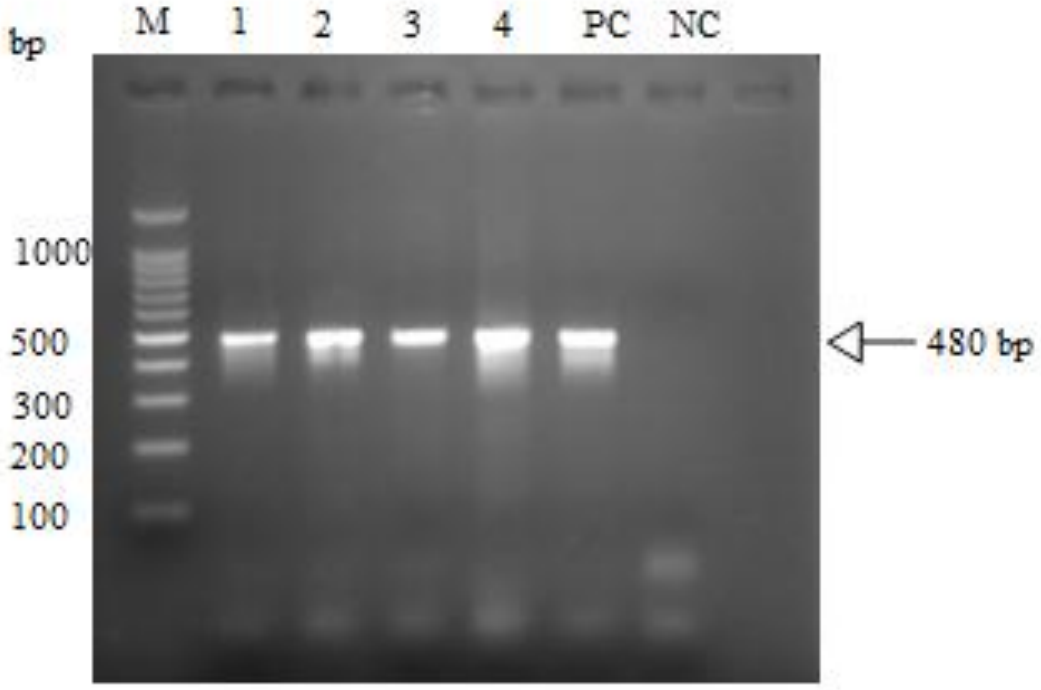


Fig.5: Agar gel electrophoresis of ITS1 PCR products of Lamu trypanosome stabilates that grew in Swiss white mice. M- DNA molecular marker of 100 Bp. Lane 1, KETRI 4032; Lane 2, KETRI 4028; Lane 3, KETRI 3984; Lane 4, KETRI 3985 samples; PC, positive control, NC, negative control.

b) *T. evansi* analysis

ITS1 PCR was also used to screen for presence of *T. evansi* using ITS CF and ITS1 BR primers which revealed that the samples were negative. *T. evansi* positive sample was used which showed a band at 150 bp (Fig. 6).

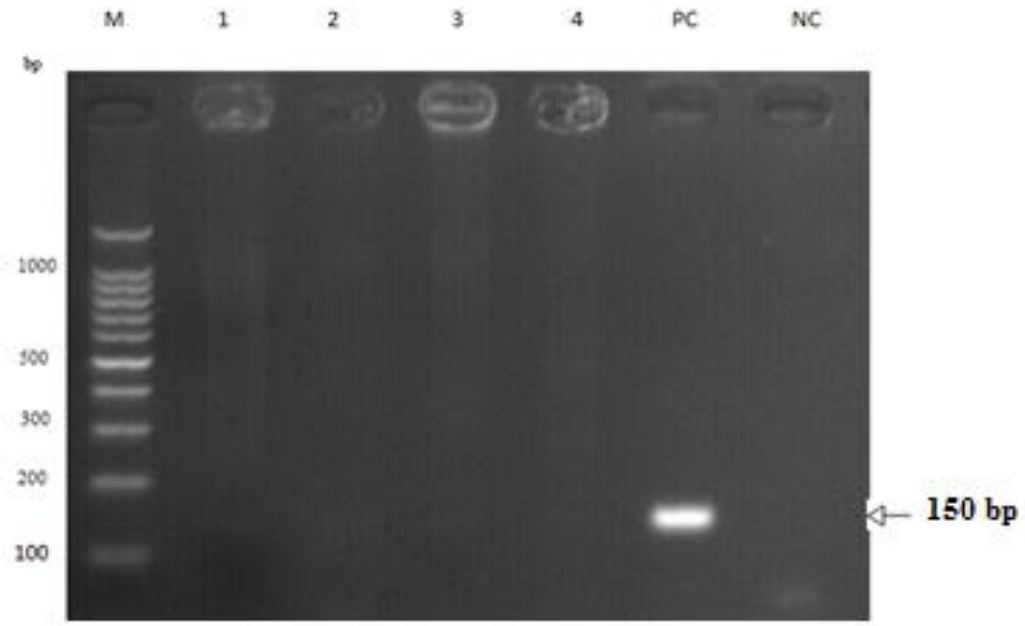


Fig. 6: Identification of PCR products using Agar gel electrophoresis. M- DNA molecular marker of 100 Bp. Lane 1, KETRI 4032; Lane 2, KETRI 4028; Lane 3, KETRI 3984; Lane 4, KETRI 3985 samples showing negative *T. evansi* products. Included are a positive control (PC) whose image showed one band at 150bp and a negative control (NC).

***c) T. brucei* analysis**

The four positive samples (in 'a' above) were further analyzed for presence of *T. brucei* using species specific primers TBR1 and TBR2. All the four samples were positive for *T. brucei* with gel electrophoresis image showing four bands at 177bp (Fig.7). The primers TBR1 and TBR2 target two sites in the satellite DNA and therefore there were two bands but the main one was at 177 bp.

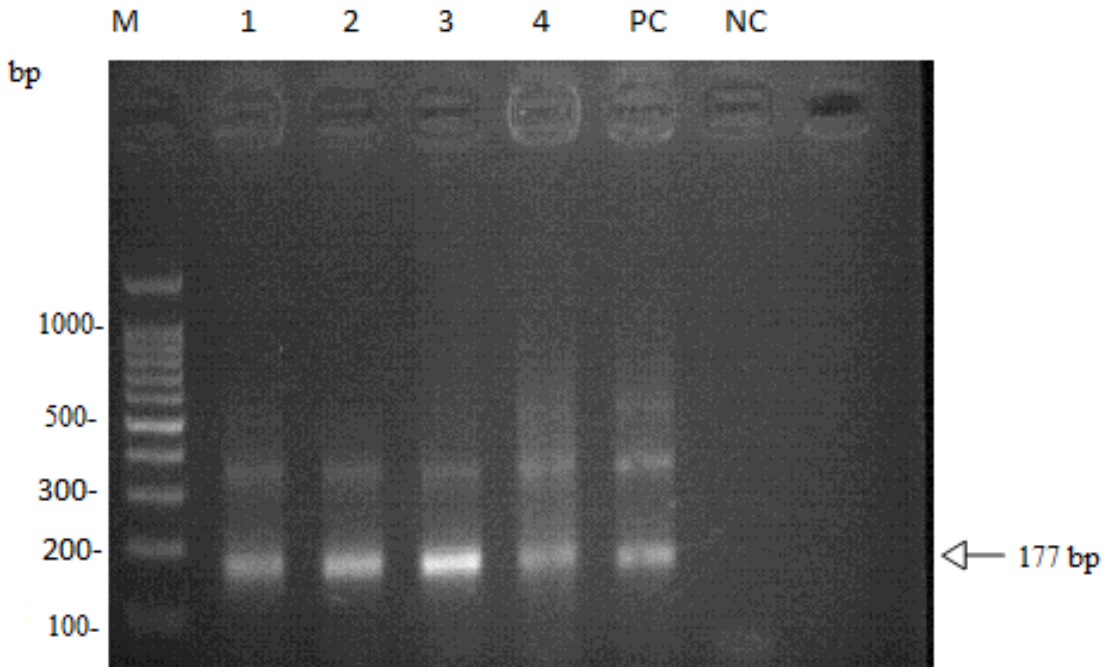


Fig. 7: Identification of PCR products of Lane 1, KETRI 4032; Lane 2, KETRI 4028; Lane3, KETRI 3984; Lane 4, KETRI 3985 samples using Gel electrophoresis. Image showed the four samples were positive for *T. brucei*. Also included are M- Molecular marker of 100 bp, PC- positive control and NC- negative control.

4.1.3 Analysis for 11 samples that did not grow in mice

a) ITS1 PCR

For the other 11 stabilates which did not grow in mice, six (55%) were positive for trypanosomes when analyzed using ITS1 CF/BR primers (Table 4). Of the positives, 3 (50%) had been stabilated from donkeys and three (50%) had been stabilated from cattle. The results for the positive stabilates were as follows; (1/6) 17% were *T. congolense* (3/6) 50 % Brucei group while (2/6) 33% were *T. vivax*. All Brucei group stabilates were from donkeys while *T. congolense* and *T. vivax* were from cattle

Table 4: Trypanosome identity and respective product sizes of stabilates that did not grow in mice.

Lane serial no.	Sample ID	Product size bp	Trypanosome identity
3	KETRI 3982	480	Brucei group
5	KETRI 3986	480	Brucei group
8	KETRI 3987	480	Brucei group
11	KETRI 4033	700	*T. congolense*
6	C 18	250	*T. vivax*
7	C 28	250	*T. vivax*

Legend: C-cattle, T- Trypanosoma

Fig.8 is an electrophoresis image showing bands at different base pairs of samples that failed to grow in mice. The positive control used was a *T. congolense* positive sample.

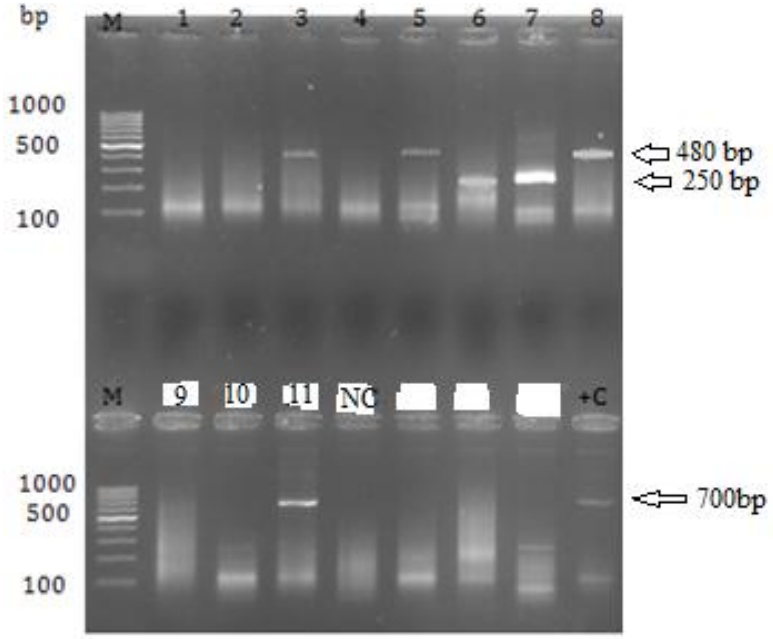


Fig. 8: Gel electrophoresis of Trypanosome DNA of samples that did not grow in mice amplified with ITS1 CF& BR primers. Brucei group gave a product of approximately 480 bp, *T. congolense* approximately 700 bp *T. vivax* approximately 250 bp. M- Molecular marker of 100bp, +C- positive control (*T. congolense* at approximately 700 bp) and NC- negative control. Lane 3, 5 and 8 showed samples KETRI 3982, KETRI 3986, KETRI 3987 stabilated from donkeys being positive for Brucei group, lane 6 and 7 showing samples C18 and C28 stabilated from cattle being positive for *T. vivax* while KETRI 4033 stabilated from cattle was positive for *T. congolense* in lane 11.

4.1.4 Comparing morphological and molecular characterization of viable stabilates

Comparison of morphological versus molecular characterization of viable (microscopically) stabilates was carried out and results indicated in Table 5. There was agreement in only 2 out of the seven stabilates (28.6%) between the two methods used.

Table 5: Comparison between morphological and molecular characterization of viable stabilates

Stabilate identity	**Host**	**Morphological characterization**	**Molecular characterization**
KETRI 4032*	Goat	*T. brucei*	*T. brucei brucei*
KETRI 4033	Cattle	*T. brucei*	*T. congolense* Savannah
KETRI 4028*	Donkey	*T. congolense*	*T. brucei brucei*
KETRI 3984*	Donkey	*T. congolense*	*T. brucei brucei*
KETRI 3986	Donkey	*T. brucei*	*T. brucei brucei*
KETRI 3985*	Donkey	*T. congolense*	*T. brucei brucei*
KETRI 3987	Donkey	*T. congolense*	*T. brucei brucei*

Legend- *stabilates that grew in mice, T- Trypanosoma

4.1.5 PCR size products for the 92 whole blood samples

a) ITS1 PCR analysis

Ninety two EDTA preserved blood samples were subjected to ITS1-PCR using CF and BR primer pairs, 13/92 samples were trypanosome positive as follows: 9/13 (69%) *T. vivax*, 4/13 (31%) *T. congolense* Savannah as shown in Table 6. *Trypanosoma vivax* was only found in donkey blood. Nine out of the eleven positive donkey samples (82%) had *T. vivax* while two (18%) had *T. congolense*. All the trypanosome positive bovine blood (2) had *T. congolense*. No trypanosomes were detected in blood from 47 goats.

Table 6: Trypanosome identity of positive whole blood samples subjected to ITS1-PCR

Sample No.	Animal of Origin	Trypanosome Identity	Sample No.	Animal of Origin	Trypanosome Identity
2	Donkey	*T. congolense*	43	Donkey	*T. vivax*
26	Donkey	*T. vivax*	44	Donkey	*T. vivax*
29	Donkey	*T. vivax*	45	Donkey	*T. vivax*
30	Bovine	*T. congolense*	46	Donkey	*T. vivax*
32	Bovine	*T. congolense*	48	Donkey	*T. vivax*
34	Donkey	*T. congolense*	49	Donkey	*T. vivax*
42	Donkey	*T. vivax*			

The infection rate of different trypanosome species identified in different animals whose whole blood samples ware as shown in Table 7.

Table 7: Infection rate of different trypanosome species in whole blood samples of livestock in Lamu.

Host	Trypanosome spp.	No. positive	Infection rate (%)
Donkeys	*T. congolense* Savannah	2	5
	T. vivax	9	22.5
	T. brucei	0	0
Cattle	*T. congolense* Savannah	2	40
	T. vivax	0	0
	T. brucei	0	0
Goats	*T. b. brucei*	0	0
	T. vivax	0	0
	T. congolense Savannah	0	0

The electro-micrographs taken after gel electrophoresis are as outlined in Fig.9.

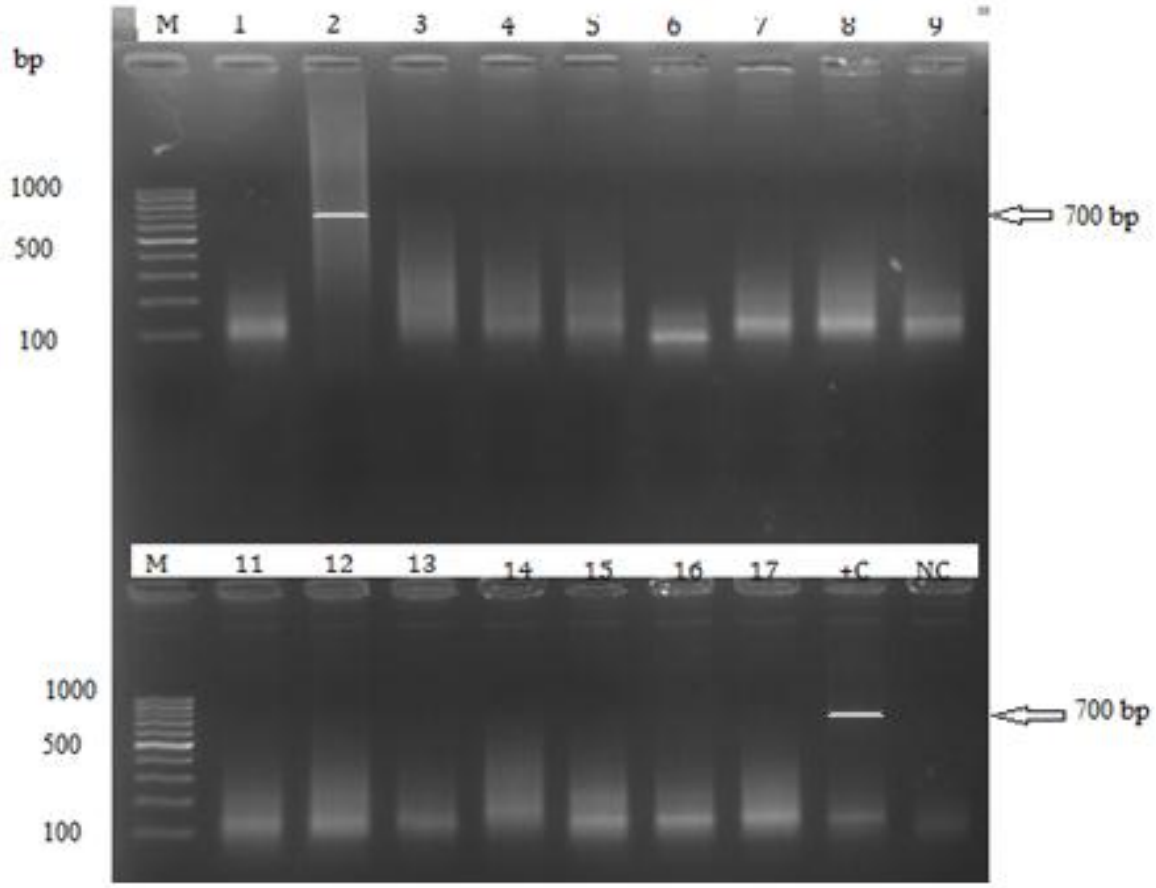


Fig. 9: Identification of PCR products of samples 1-17 in Lane 1-17 using Gel electrophoresis. Electro micrograph showed sample number 2 being positive for *T. congolense* at 700 Bp. M-Molecular marker of 100bp, +C- positive control (a *T. congolense* positive sample) and NC-negative control. All the other samples were negative for trypanosomes.

Samples number 26 and 29 were positive for *T. vivax* at 250 Bp while sample numbers 30, 32 and 34 were positive for *T. congolense* at 700 Bp (Fig. 10). All the other samples in this gel were negative for trypanosomes.

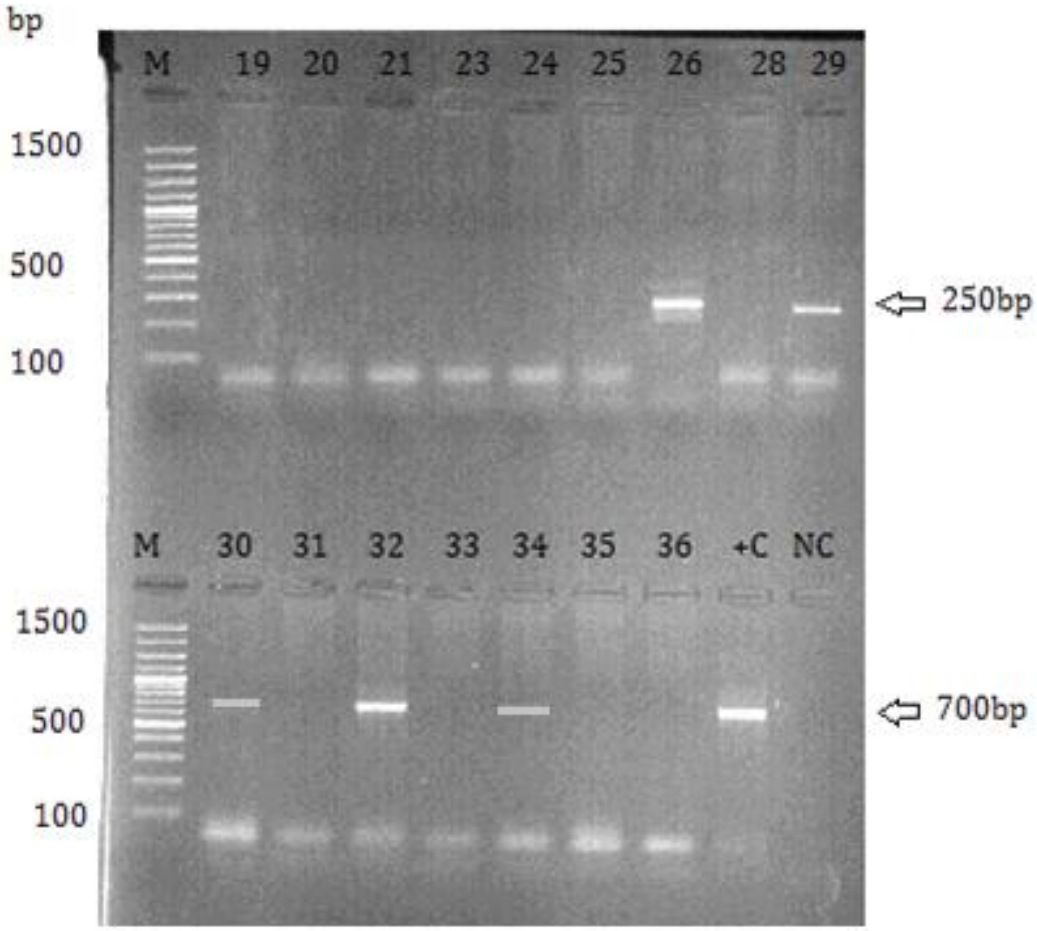


Fig. 10: Identification of PCR products of samples 19-36 in Lanes 1-16 using Gel electrophoresis. Image showed samples 26, 29 and 30, 32, 34 positive for *T. vivax* and *T. congolense*respectively. M- Molecular marker of 100 bp, +C- positive control (*T. congolense* positive sample) and NC- negative control.

Sample numbers 42-46,48 and 49 were positive for *T. vivax* at 250 Bp. All the other samples in this gel were negative for trypanosomes as electro micrograph (Fig. 11) shows.

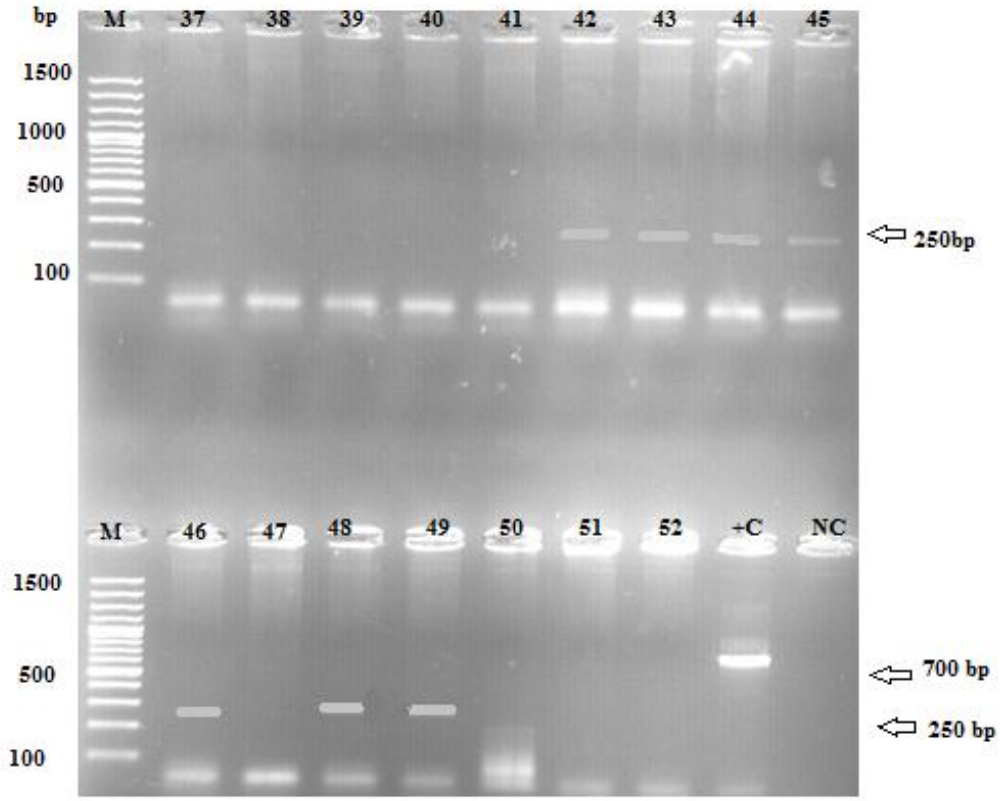


Fig.11: Gel electrophoresis identification of PCR products of samples 37-52 in Lane 1-16 using ITS1.Image showed samples 42-46,48 and 49 being positive for *T. vivax*. M- Molecular marker of 100bp, +C- positive control (*T. congolense* positive sample) and NC- negative control.

After ITS1 PCR, further analysis was carried out on *T. congolense* and *T. brucei* samples to classify them using species-specific primers. The image showed bands at approximately 310 bp and 177bp products representing *T. congolense* Savannah and *T. b. brucei* respectively.

***b) T. vivax* analysis**

Further analysis was carried out on the trypanosome positive samples using species-specific primers to confirm the presence of *T. vivax* using universal primers for *T. vivax* East Africa type which gave (Fig.12) electro-micro graph with all bands at approximately 700bp. The positive control used (KETRI 2501) gave a product size of 150 bp.

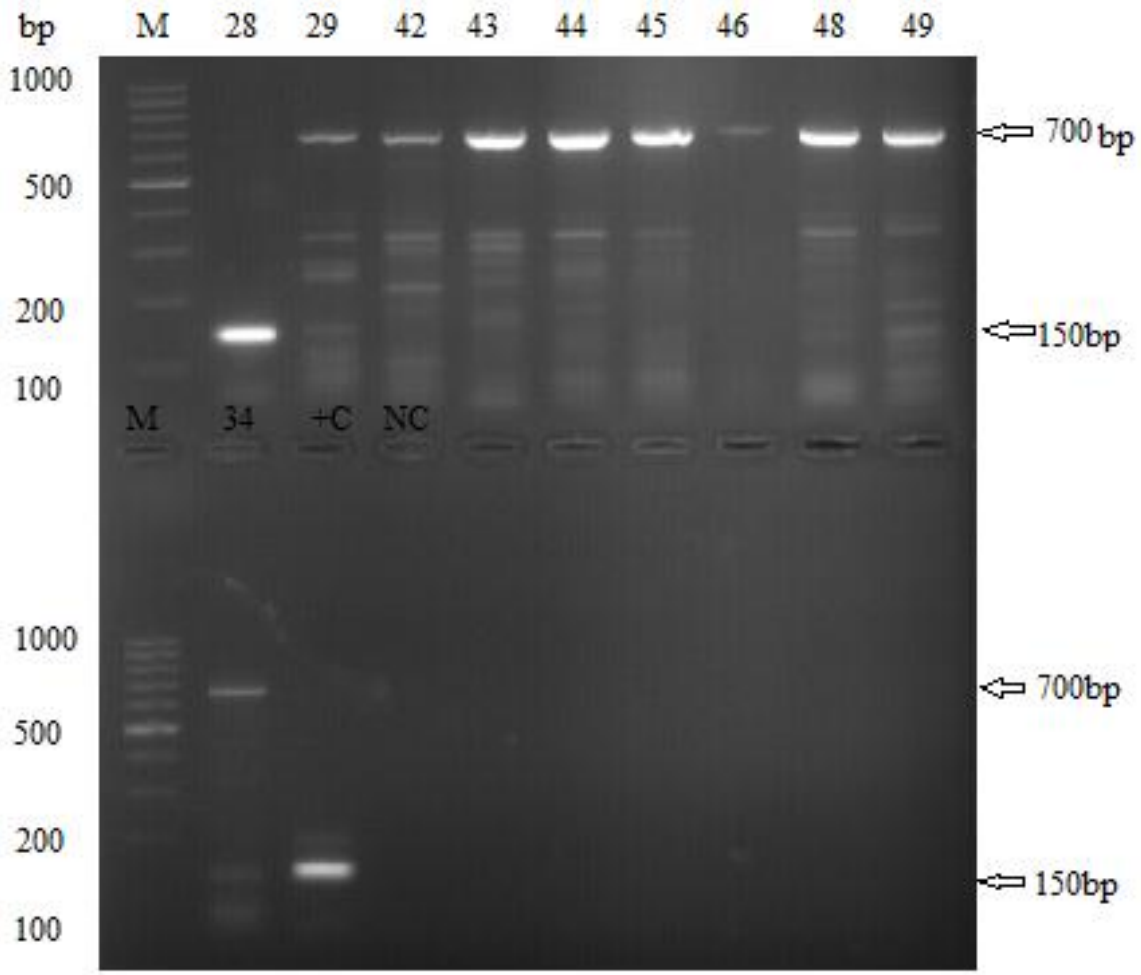


Fig. 12: Detection of *T. vivax* positive samples using Universal primers. Representative gel image of electrophoresis of reference DNA samples (+v), test samples 29, 42-46,48,49 and 34 with bands at approximately at 700 bp**,** negative controls (NC) and 100 base pair ladders labeled 'M'. No bands for the negative control.

c) *T. congolense* Savannah analysis

The samples positive for *T. congolense* in ITS1were further analyzed using species-specific primers TCS1 and TCS2 for Savannah subtype, TCK1 and TCK2 for Kilifi subtype and TCF1 TCF2 for forest subtype. All tested positive for *T. congolense* Savannah at approximately 310bp as shown in Fig.13.

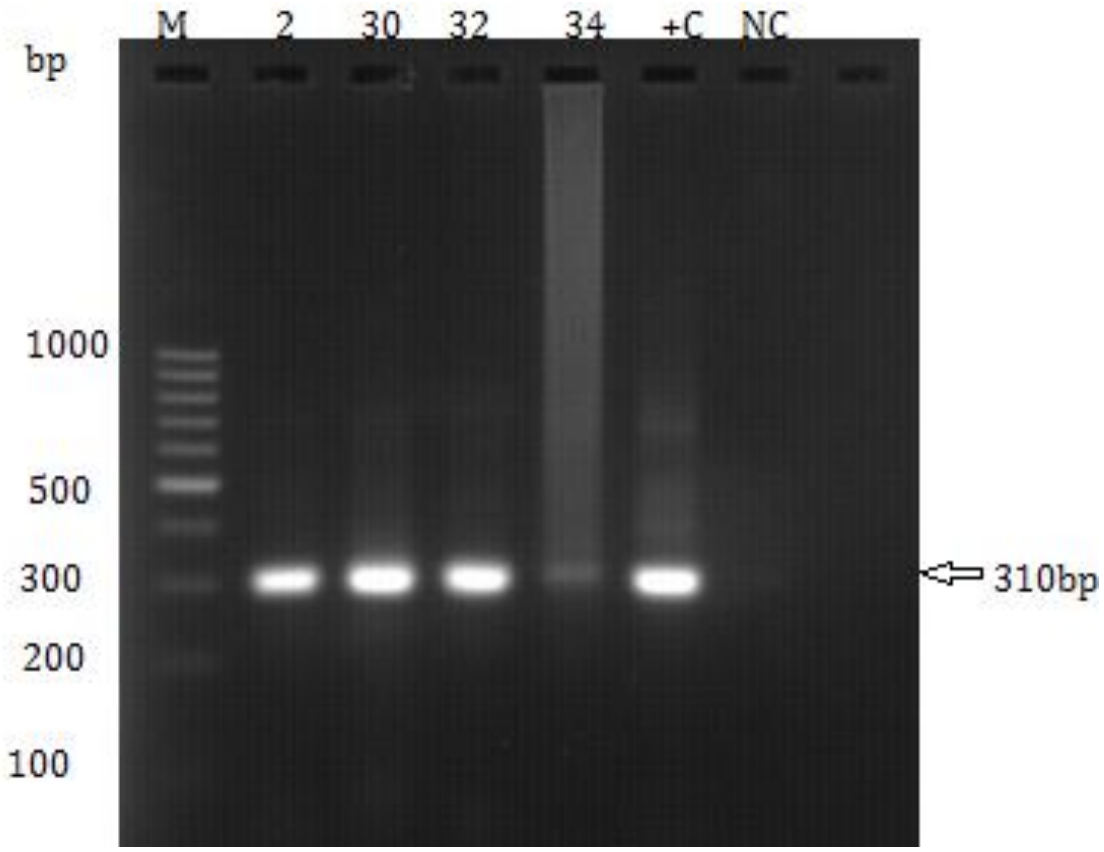


Fig. 13: Detection of *T. congolense* Savannah genomic DNA amplified with TCS 1 and 2 primers in trypanosome stabilates. Representative gel image of electrophoresis of reference DNA samples (+C), test samples 2, 30, 32 and 34 being positive for *T. congolense* Savannah with bands at approximately 310 bp**,** negative control (NC) and 100 base pair ladder labeled 'M'. No bands for the negative control.

4.2 Objective 2: Determination of presence of Serum Resistance Associated (SRA) gene related to human trypanosomiasis in *T. brucei* trypanosome stabilates from Lamu

Analysis of SRA gene aimed at identifying *T. b. rhodesiense* in all Brucei groups identified. Primers used were SRA A and SRA E and *T. b. rhodesiense* reference DNA (+C) (KETRI 2537 DNA). All samples analyzed were negative for *T. b. rhodesiense* as shown in Fig.14. The electrophoresis image showed one band at 460bp representing the reference DNA (+C).

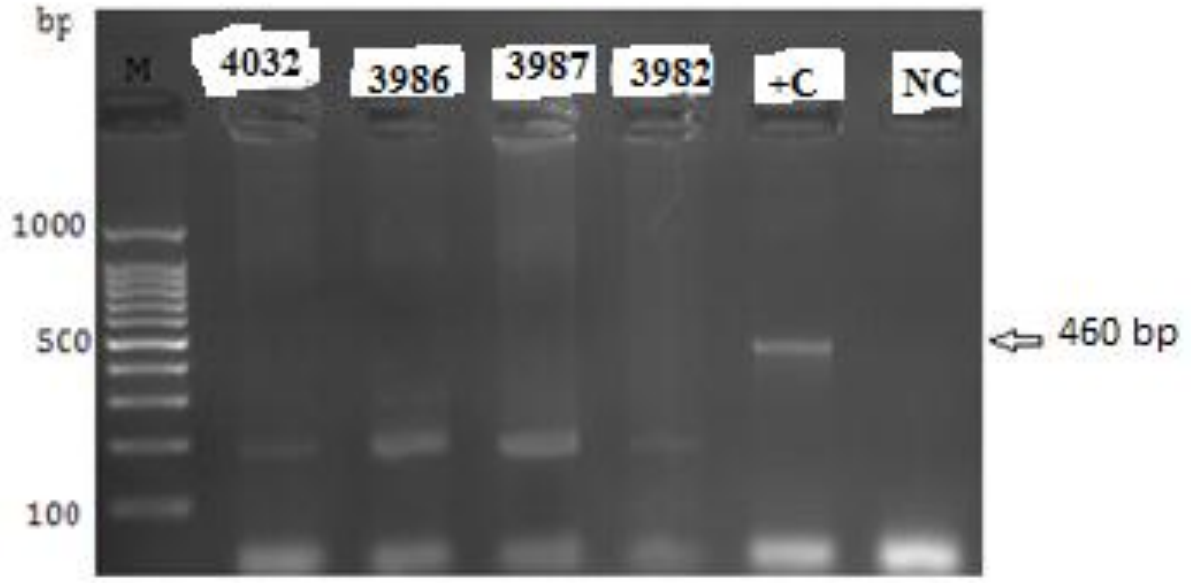


Fig.14: Gel electrophoresis for detection of SRA genomic DNA amplified with SRA A and SRA E primers. Representative gel image of electrophoresis showing some Brucei group-positive samples results with a band at 460bp of reference DNA samples (+C), test samples in lane 1-4 labeled KETRI 4032, KETRI 3986, KETRI 3987, KETRI 3982 respectively being negative for *T. b. rhodesiense*, negative control (NC) and 100 bp ladder labeled 'M'.

4.3 Objective 3: Determination of drug sensitivity of trypanosomes isolated from livestock in Lamu County

4.3.1 Pre-infection results and wet blood films of donor mice infected with stabilates used for drug sensitivity study

During pre-infection, generalized increase in all parameters used in drug sensitivity tests including body weight, PCV and parasitaemia was observed. When Wet Blood Films (WBF) were examined 24 hours post infection in donor mice, there was absence of trypanosomes until 48 hours post infection for KETRI 4028, KETRI 3984 while the rest of the trypanosome species studied, motile trypanosomes were observed 3 days and 4 days post infection for KETRI 3985 and KETRI 4032 respectively. The following WBF captions (Fig.15) were taken under microscope (Olympus 1 X 51 UTV05XC-3 –Japan and Nikon UFX-DX- Japan).

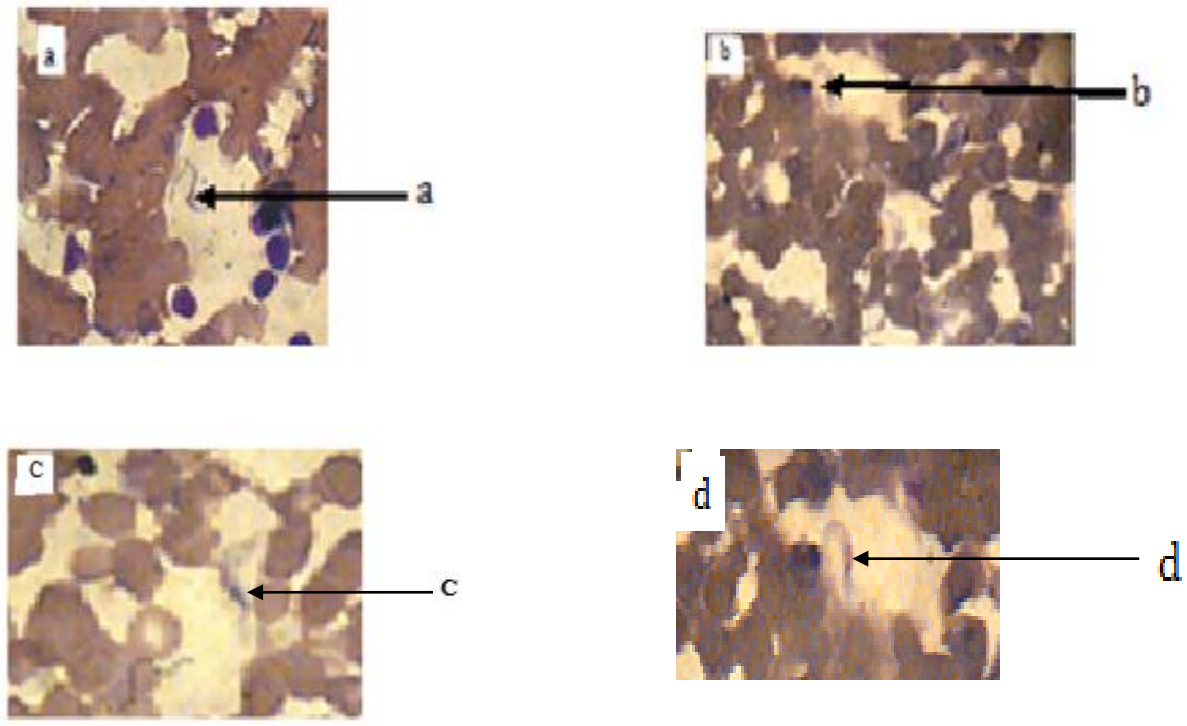


Fig.15: Wet Blood smears of four viable trypanosome stabilates used for drug sensitivity test. Key: a. KETRI 3985; b. KETRI 4032; c. KETRI 4028; d. KETRI 3984.

4.3.2. Drug sensitivity testing

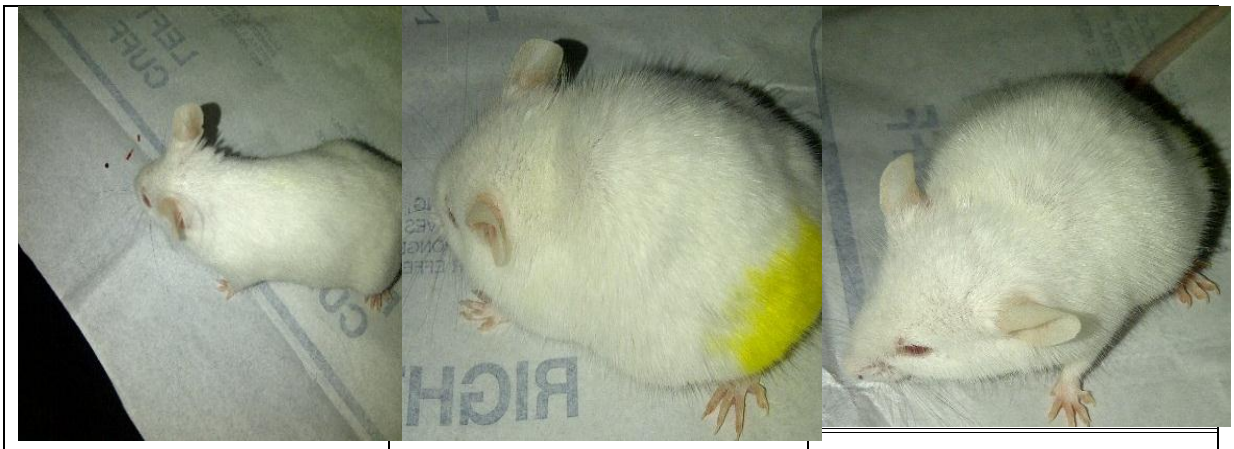
Results on drug sensitivity test in mice are summarized in Table8. Mice infected with KETRI 4028 were cured by Isometamidium at 1mg/kg and Diminazene at 20mg/kg. These two drugs cured 100% of the mice while Homidium cured less than 80% of the mice infected with KETRI 4028. The other three stabilates (KETRI 4032, KETRI 3984 and KETRI 3985) were resistant to all the three drugs. Mice that were not cured following treatment with the three drugs relapsed on different days as shown in Table 8. Homidium treated mice that were infected with KETRI 4028 took longer to relapse (34 days) than mice infected with the other three stabilates (7, 13 and 20 days).

Table 8: Sensitivity of different isolates to different trypanocidal drugs.

Isolate identity	**Number of mice cured**			**Relapse time (days)**		
	Isometami-dium1mg/kg	Homidium 1mg/kg	Diminazene 20mg/kg	Isometami-dium 1mg/kg	Homidium 1mg/kg	Diminazene 20mg/kg
KETRI 4032	2/6	1/6	1/6	13	7	14
KETRI 3985	0/6	0/6	0/6	13	20	14
KETRI 3984	2/6	0/6	0/6	41	13	27
KETRI 4028	6/6	4/6	6/6	0	34	0

4.3.2 Clinical signs

Prior to infection, mice showed normal body conditions but after infection, treatment and relapsing depending on the stabilate and the drug used for treatment, the mice showed varying body conditions. Mice that were cured by the drug showed no clinical symptoms of t trypanosomiasis. The following plates show captions taken in the course of the study. Mice infected with KETRI 3985 and KETRI 3984 (Fig. 16) stabilates showed raised hair coat, lethargy, facial and scrotal edema as the disease progressed and also reduced feed intake was observed. Mice infected with KETRI 4032 and KETRI 4028 and treated with different drugs manifested minimal clinical signs of the disease. Isometamidium 1mg/kg treated mice infected with either of the four stabilates exhibited the least clinical signs of the disease studied as shown in Fig.16. The infected mice showed no clinical signs until 96- 120 h post infection.



a). Mouse showing no symptoms of trypanosomiasis	b). Mouse manifesting moderate symptoms of trypanosomiasis	c). Mouse manifesting severe symptoms of trypanosomiasis

Fig.16: Caption a. Mouse infected with KETRI 4028 and treated with Isometamidium 1mg/kg manifesting no symptoms of trypanosomiasis. Mouse showed a smooth hair coat, and general

body condition appeared normal. The mouse was active and feed intake was high. Caption b. Mouse infected with trypanosome stabilate (KETRI 3985) and treated with Homidium 1mg/kg. The mouse showed shivering, listlessness and huddling in a corner. Caption c. An experimental mouse infected with KETRI 3984 and treated with Diminazene 20mg/kg during a breakthrough infection stage at day 12. The mouse showed raised hair coat, facial edema and emaciation. Feed intake was very low. Mice showed minimal movements inside the cage at advanced stage of trypanosomiasis. Severe clinical signs included shivering and muscle tremors. The mouse died later in the day.

4.3.3 Parasitaemia, PCV, and body weight scores in experimentally infected mice

a) Parasitaemia

There was significant difference (p-value <.001 (<α=0.05) between KETRI 4028 and KETRI 3985 stabilates and control but the difference between KETRI 3984 and KETRI 4032 was not significant. On average, the first day of parasitaemia detection in blood for KETRI 4028, KETRI 3985 and KETRI 4032 was 12th, 5th and 6th day respectively (Fig. 17 and Fig.18). When treated with Homidium 1mg/kg, KETRI 3985 stabilate had the highest mean parasitaemia levels followed by KETRI 4032. KETRI 4028 stabilate had the lowest mean parasitaemia.

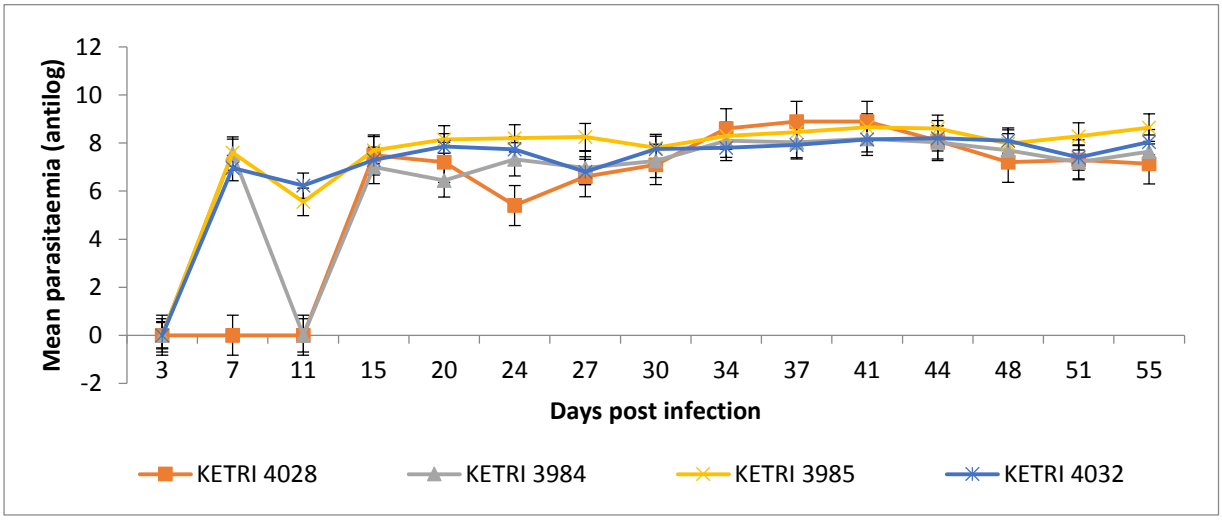


Fig. 17: Mean parasitaemia levels against DPI for the different trypanosome stabilates treated with Homidium 1mg/kg.

When treated with Isometamidium 1mg/kg, KETRI 4032 stabilate had the highest mean parasitaemia followed by KETRI 3985 and KETRI 4028 stabilate had the lowest (Fig.18).

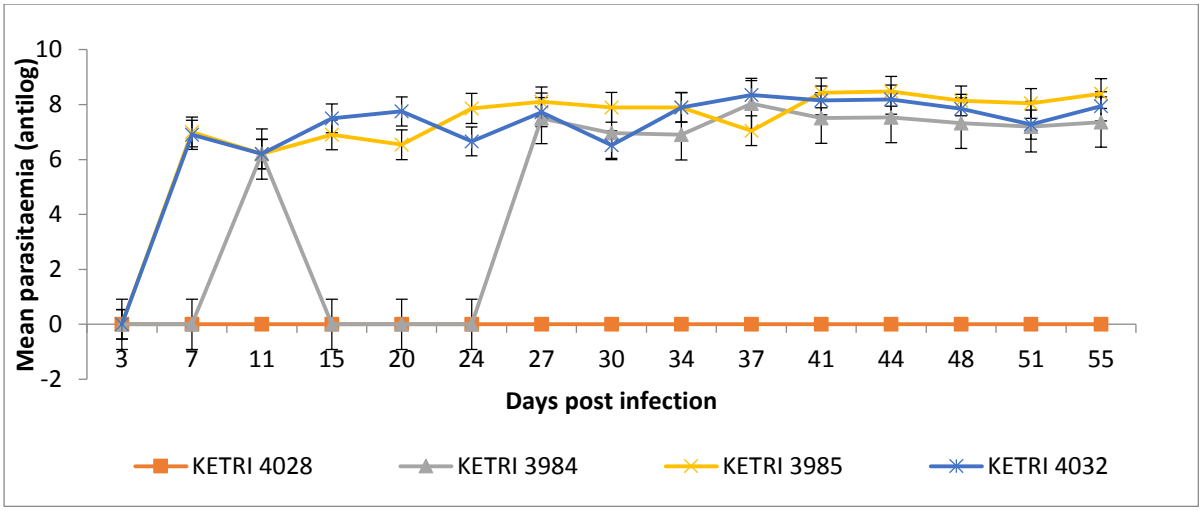


Fig. 18: Mean parasitaemia levels against DPI of the different stabilates treated with Isometamidium 1mg/kg

When treated with Diminazene 20mg/kg, KETRI 3985 stabilate had the highest mean parasitaemia throughout the trial followed by KETRI 4032. KETRI 4028 stabilate had the least (Fig.19).

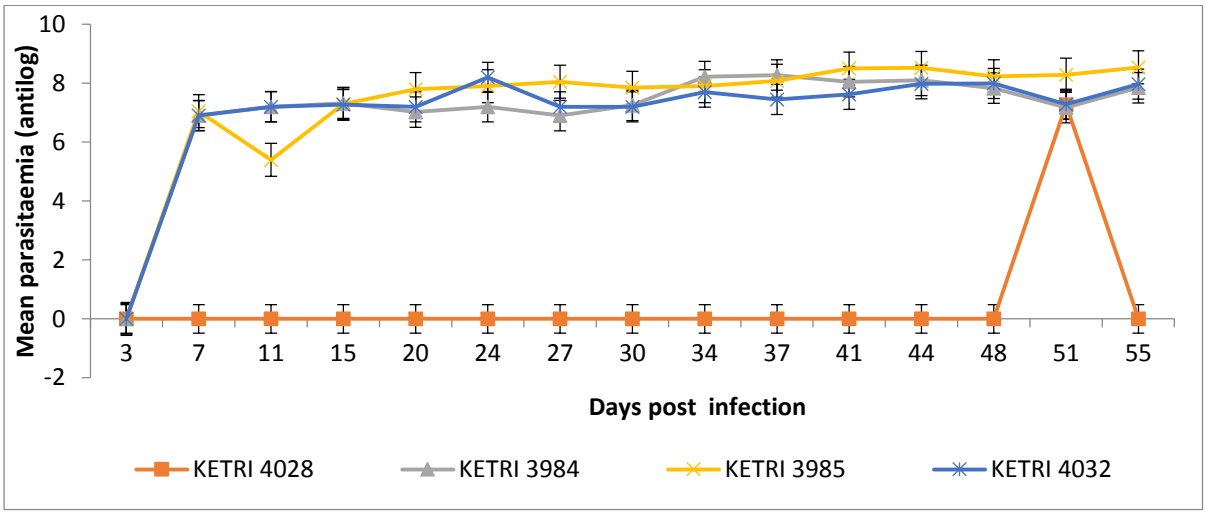


Fig.19: Mean Parasitaemia levels against DPI of the different stabilates treated with Diminazene 20mg/kg

When comparing parasitaemia levels of different stabilates in response to different drugs; the control group was most susceptible as it had the highest mean parasitaemia followed by groups treated with Homidium 1mg/kg and Diminazene 20mg/kg. Isometamidium 1mg/kg treated group was the least susceptible to KETRI 4028 isolate infection (Fig.20).

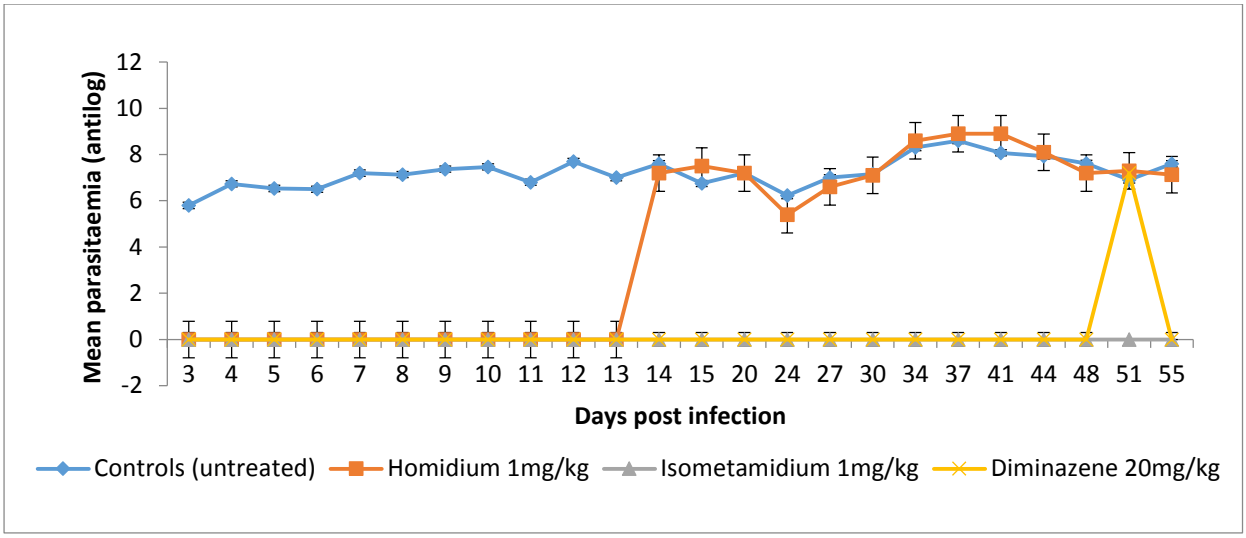


Fig. 20: Mean parasitaemia levels against DPI of KETRI 4028 isolate treated with different drugs and control group

Isometamidium 1mg/kg treated group was the least susceptible to KETRI 3984 stabilate infection (Fig.21). The control group and Diminazene 20mg/kg treated group were most susceptible as they had the highest mean parasitaemia followed by group treated with Homidium 1mg/kg.

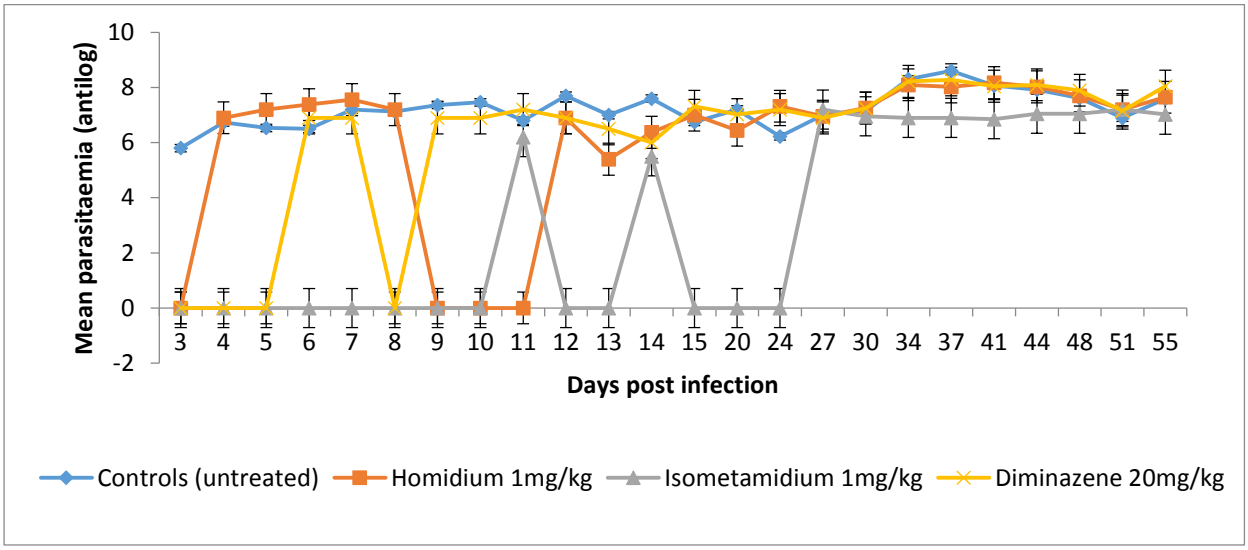


Fig. 21: Mean parasitaemia levels against DPI of KETRI 3984 stabilate treated with different drugs and control group

The group of mice treated with Homidium 1mg/kg was the most susceptible to KETRI 3985 stabilate as it had the highest mean parasitaemia on average throughout the trial followed by Diminazene 20mg/kg while Isometamidium 1mg/kg treated group was the least susceptible (Fig.22).

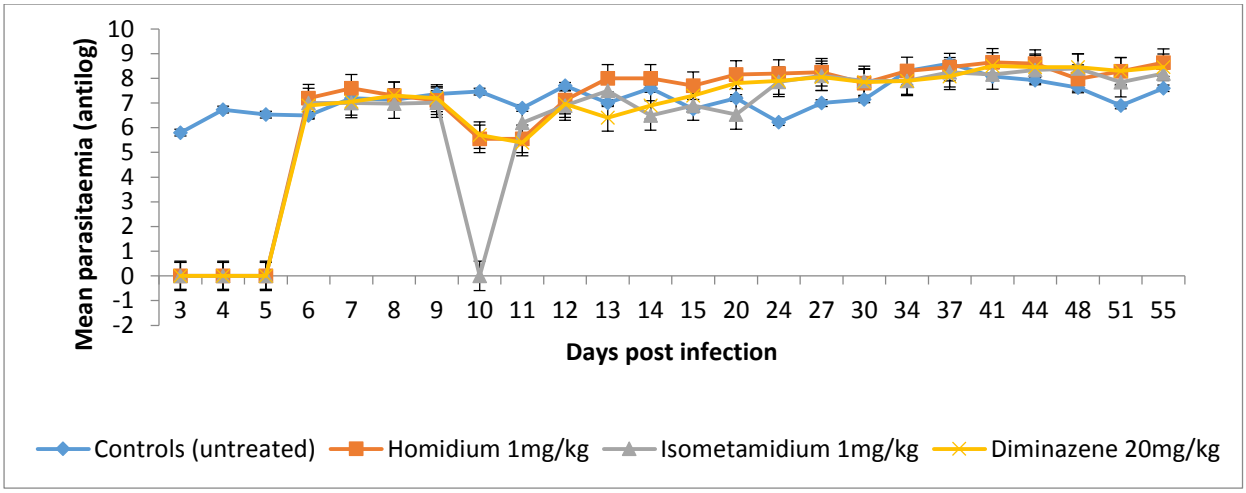


Fig.22: Mean parasitaemia levels against DPI of KETRI 3985 stabilate treated with different drugs and control group

The group of mice treated with Homidium 1mg/kg was most susceptible to KETRI 4032 stabilate as it had the highest level of parasitaemia followed by the groups treated with Isometamidium 1mg/kg and Diminazene 20mg/kg (Fig.23).

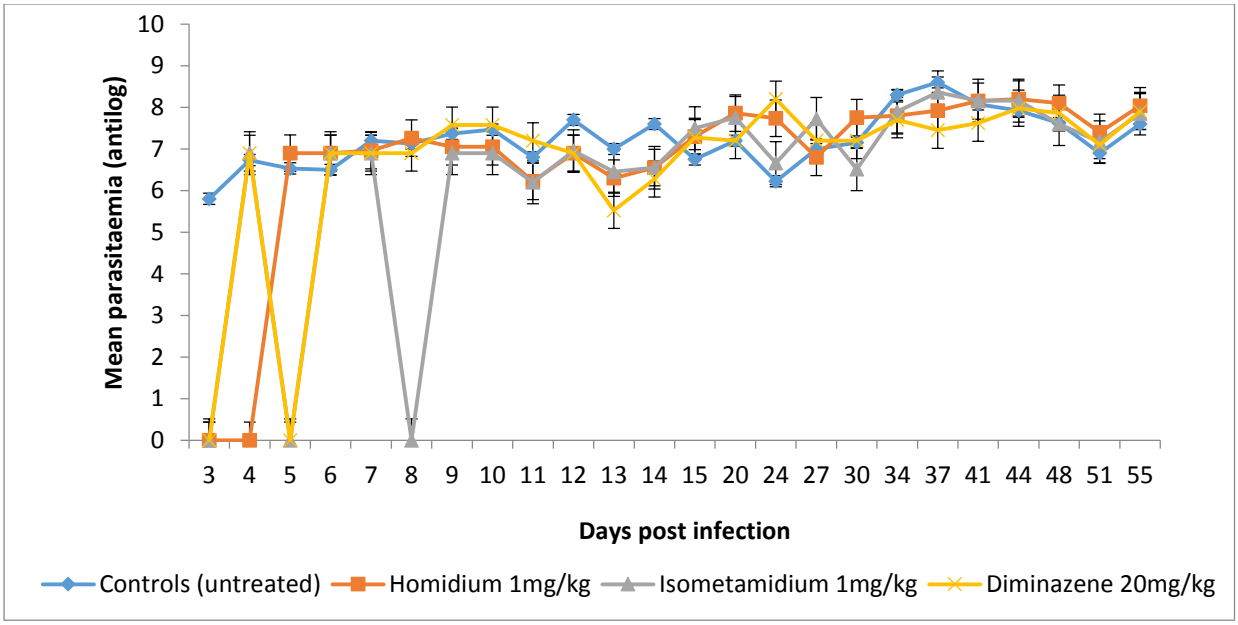


Fig.23: Mean parasitaemia levels against DPI of KETRI 4032 stabilate treated with different drugs and control group

b) PCV level

There was significant difference in PCV levels of different stabilates, p-value <.001 (<α=0.05). There was a significant change of PCV with time in mice infected with all the four stabilates. When infected with KETRI 3985, KETRI 4028, KETRI 3984 and KETRI 4032,

Homidium1mg/kg and Diminazene 20mg/kg treated mice had lower mean PCV compared to those treated with Isometamidium 1mg/kg which had the highest mean PCV during the evaluation period (Fig. 24, 25 and 26).

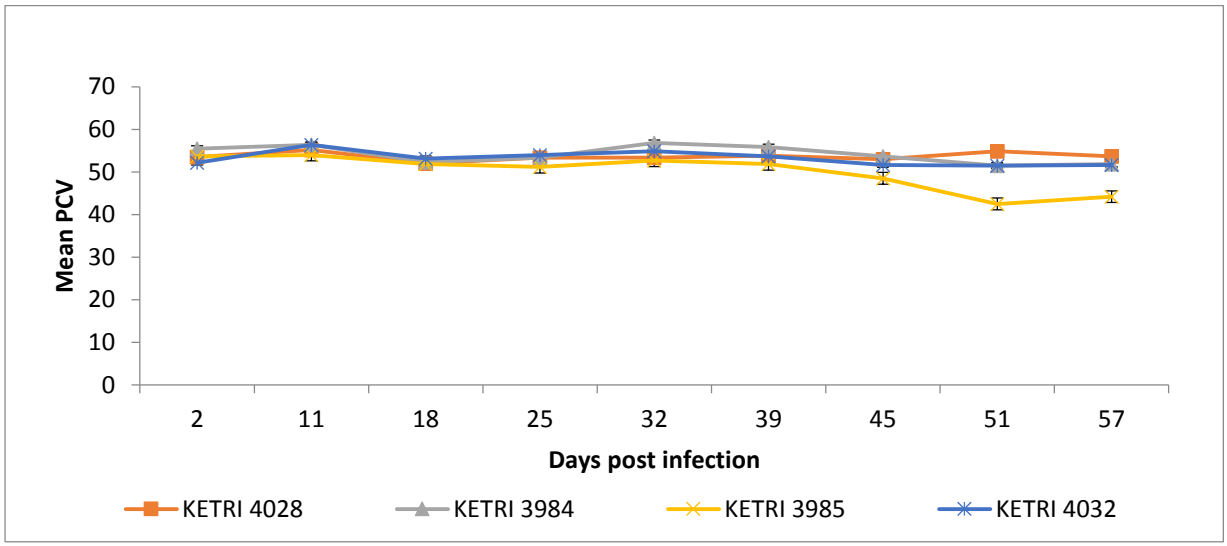


Fig. 24: Mean PCV levels against DPI of the four stabilates treated with Homidium 1mg/kg

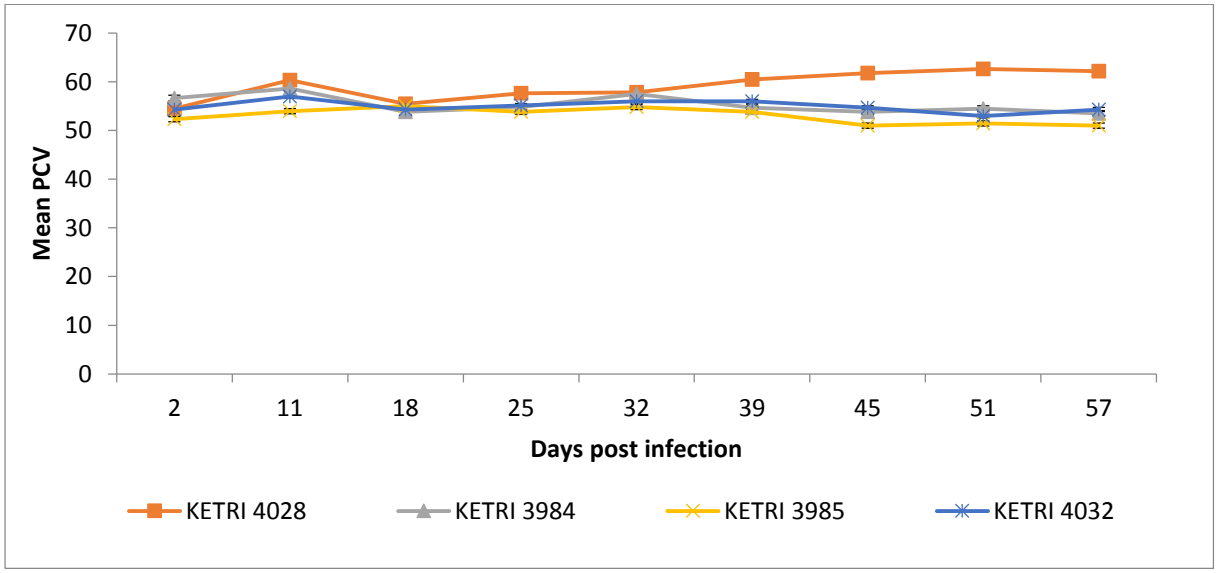


Fig.25: Mean PCV levels against DPI of the four stabilates treated with Isometamidium 1mg/kg

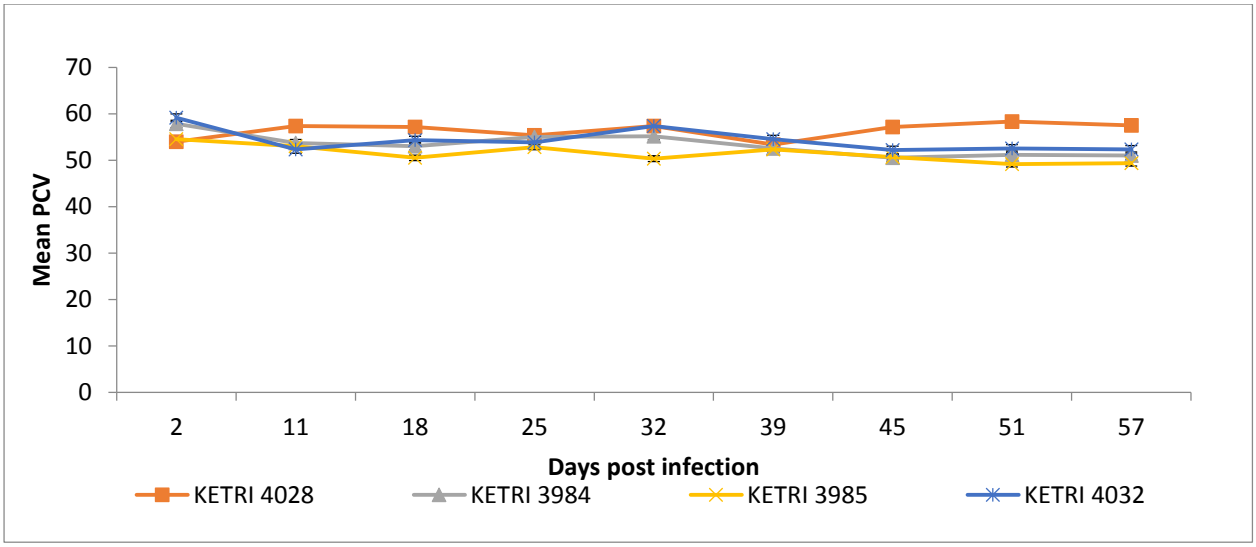


Fig. 26: Mean PCV levels against DPI of the four stabilates subjected to Diminazene 20mg/kg

The PCV levels of mice infected with different stabilates and treated with the three drugs showed p-value 0.049 (<α=0.05) which indicated a significant difference in PCV levels within groups of mice infected and treated with different drugs. After treatment with all the three drugs, KETRI 3985 and KETRI 4028 infected mice PCV levels were highest followed by KETRI 3984 and KETRI 4032 (Fig.27, 28, 29 and 30). The following graphs show that mean PCV in controls was significantly different from mice infected with different stabilates p-value <.001 (<α=0.05), as they had the lowest mean PCV.

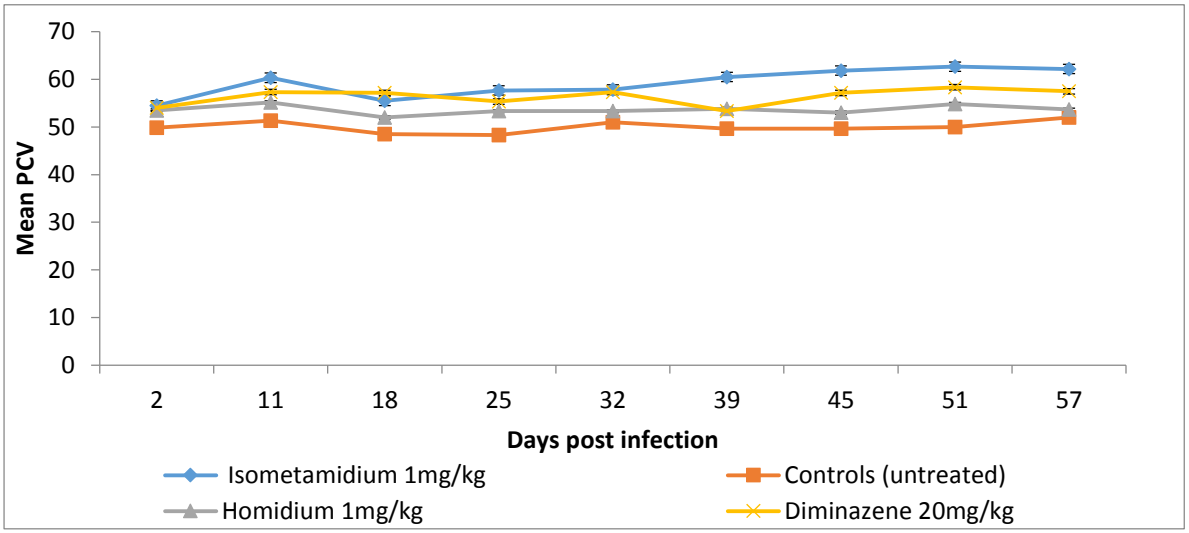


Fig. 27: Mean PCV levels against DPI of KETRI 4028 stabilate treated with different drugs and control group

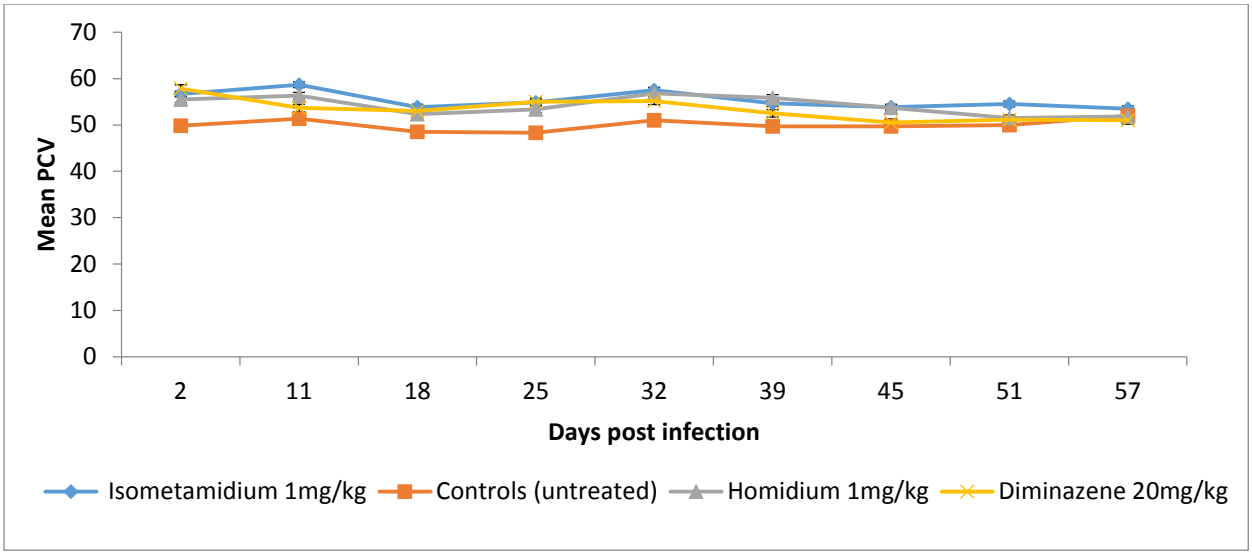


Fig. 28: Mean PCV levels against DPI of KETRI 3984 stabilate treated with different drugs and control group

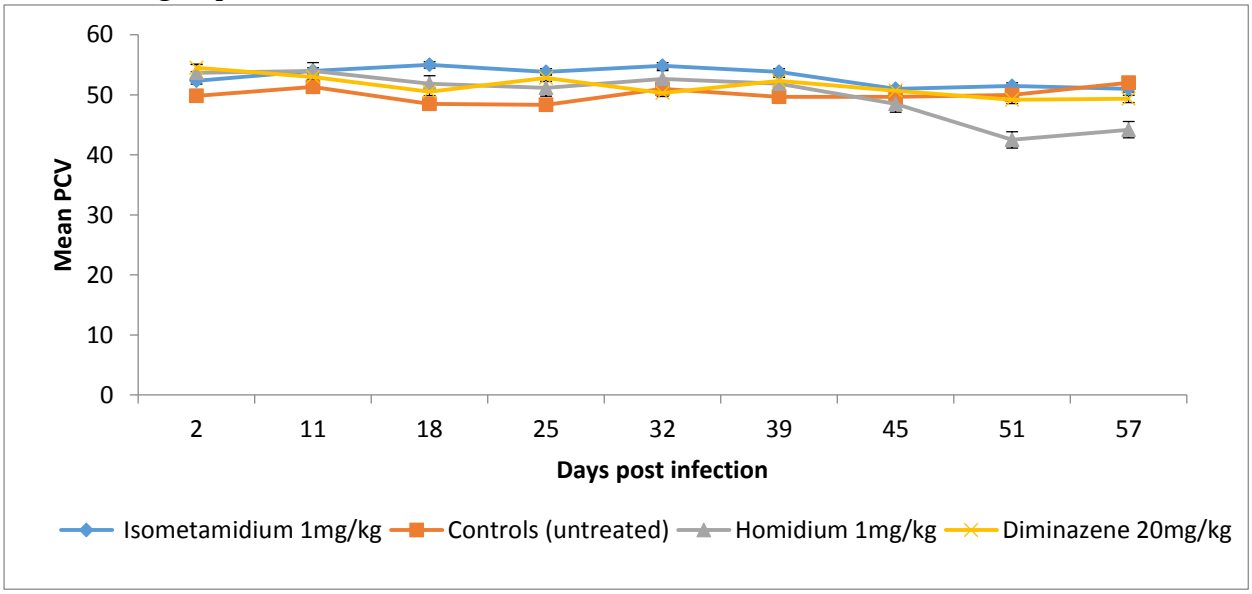


Fig. 29: Mean PCV levels against DPI of KETRI 3985 stabilate treated with different drugs and control group

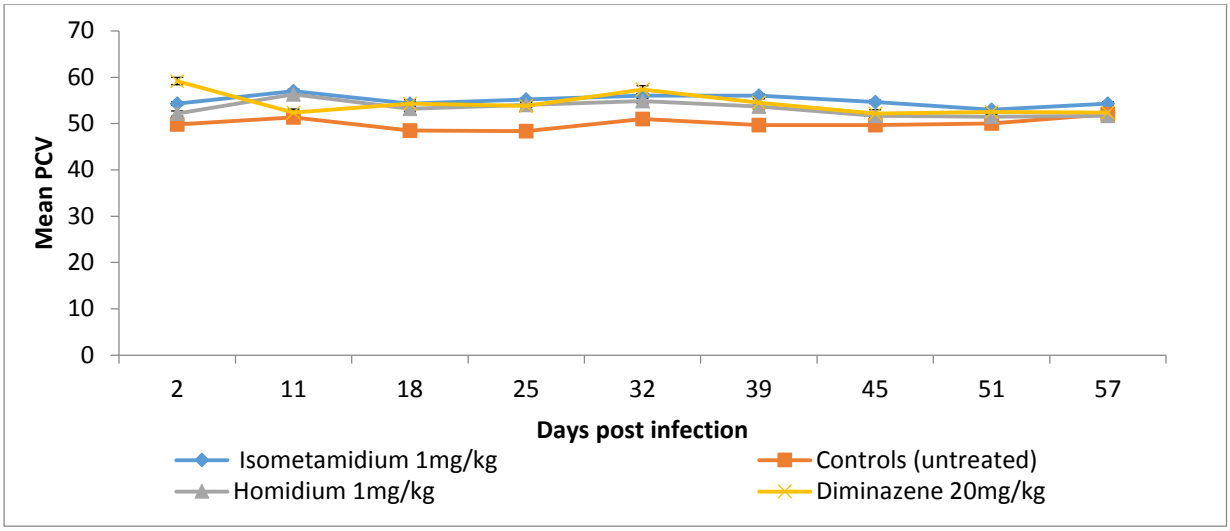


Fig. 30: Mean PCV levels against DPI of KETRI 4032 stabilate treated with different drugs and control group

c) Body Weight

There was no significant difference in body weights between mice infected with the different stabilates (p-value 0.306 (>α=0.05). The body weights for all stabilates were similarly increasing during the trial except after ten days when KETRI 3985 had a significantly higher mean than the other stabilates when treated with the three drugs, as shown in Fig.31, 32 and 33.

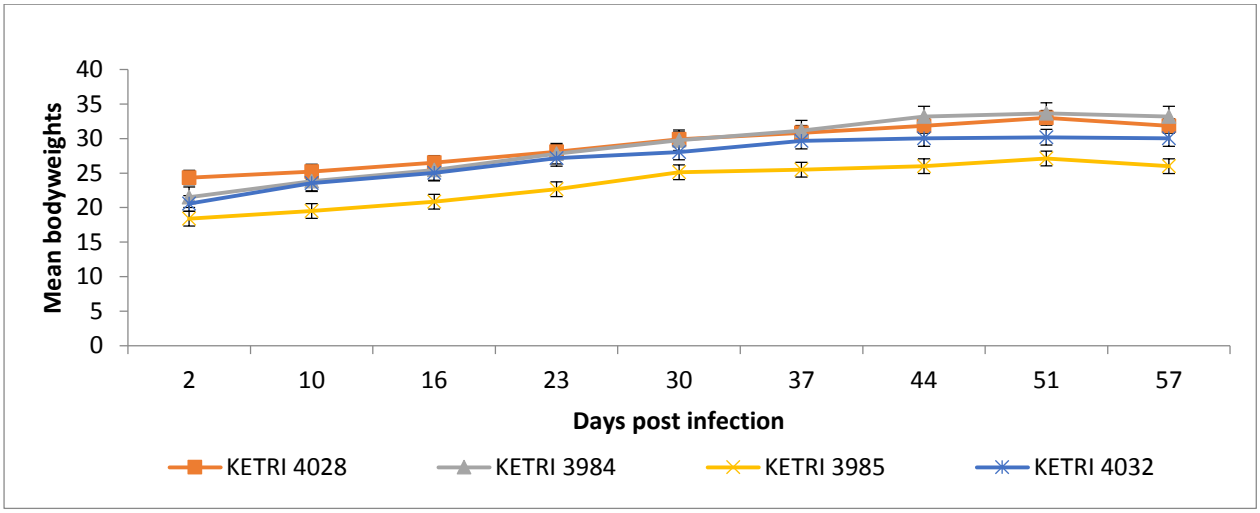


Fig.31: Mean body weights against DPI of the different stabilates treated with Homidium 1mg/kg

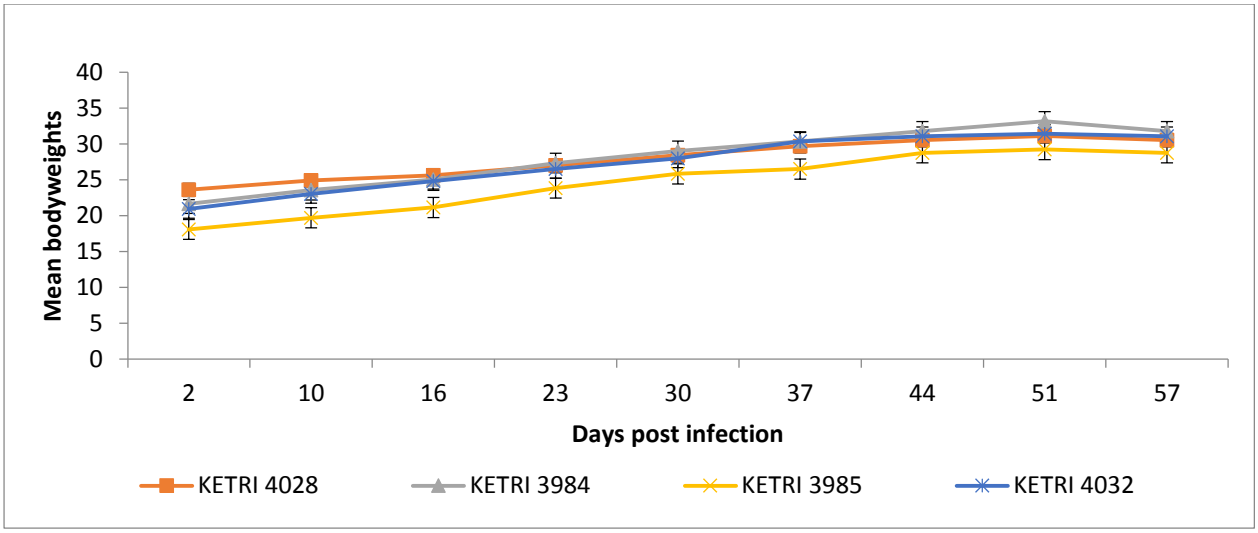


Fig. 32: Mean body weights against DPI of the different stabilates and treated with Isometamidium 1mg/kg

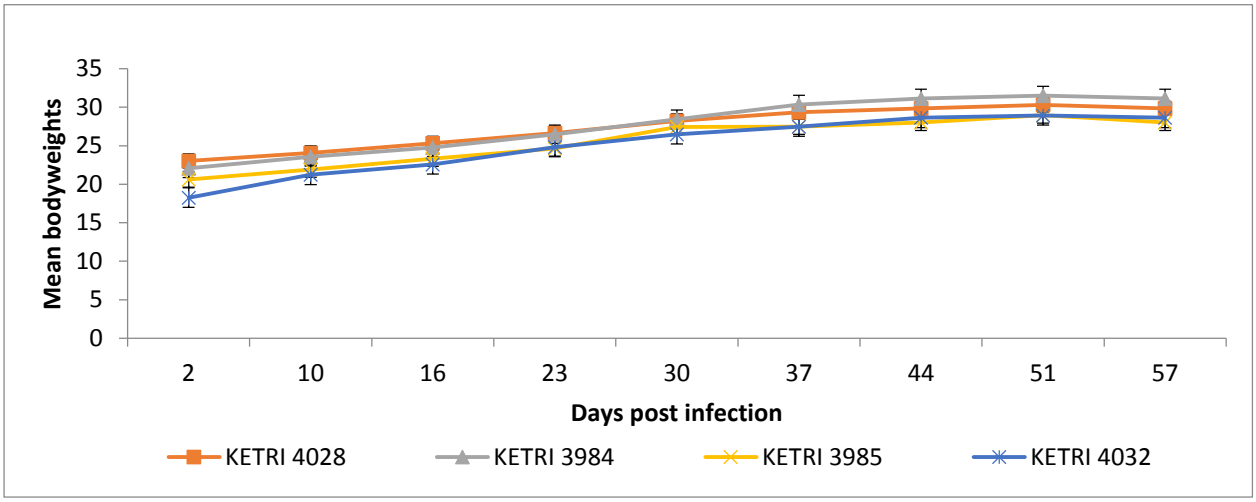


Fig. 33: Mean body weights against DPI of the different stabilates subjected to Diminazene 20mg/kg

When comparing the four stabilates against different drugs; body weight changes within the time of study p-value was 0.161 (>α=0.05) which means that there was no significant difference in levels of body weights of the different drugs. The body weights of different groups of mice infected with KETRI 3985, KETRI 4028, KETRI 3984 and KETRI 4032 stabilates and treated with Homidium 1mg/kg, Isometamidium 1mg/kg and Diminazene 20mg/kg had a similar increasing trend during the trial period, which was not significantly different from control group (Fig.34, 35, 36 and 37)

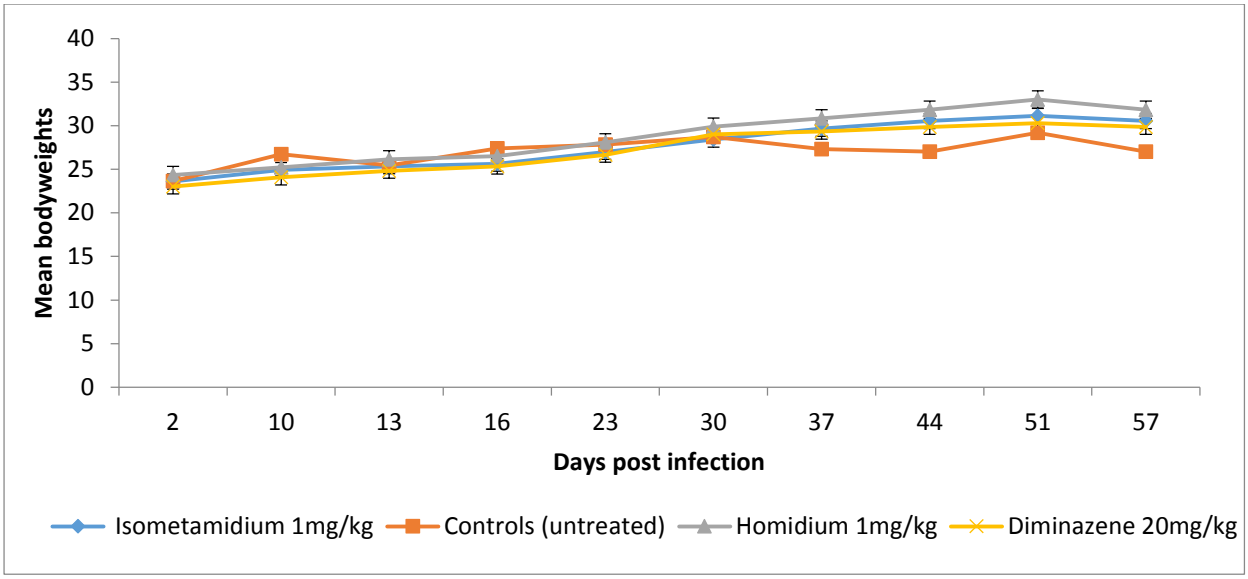


Fig.34: Mean Body weights against DPI of mice infected with KETRI 4028 stabilate treated with different drugs and control group

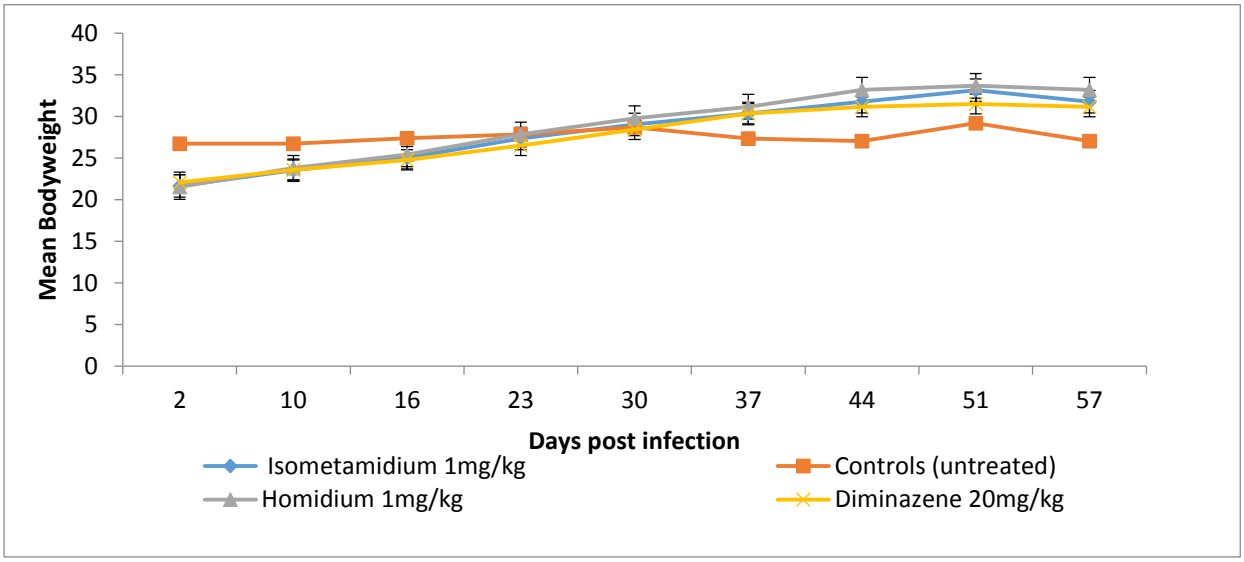


Fig.35: Mean body weights against DPI of mice infected with KETRI 3984 stabilate treated with different drugs and control group

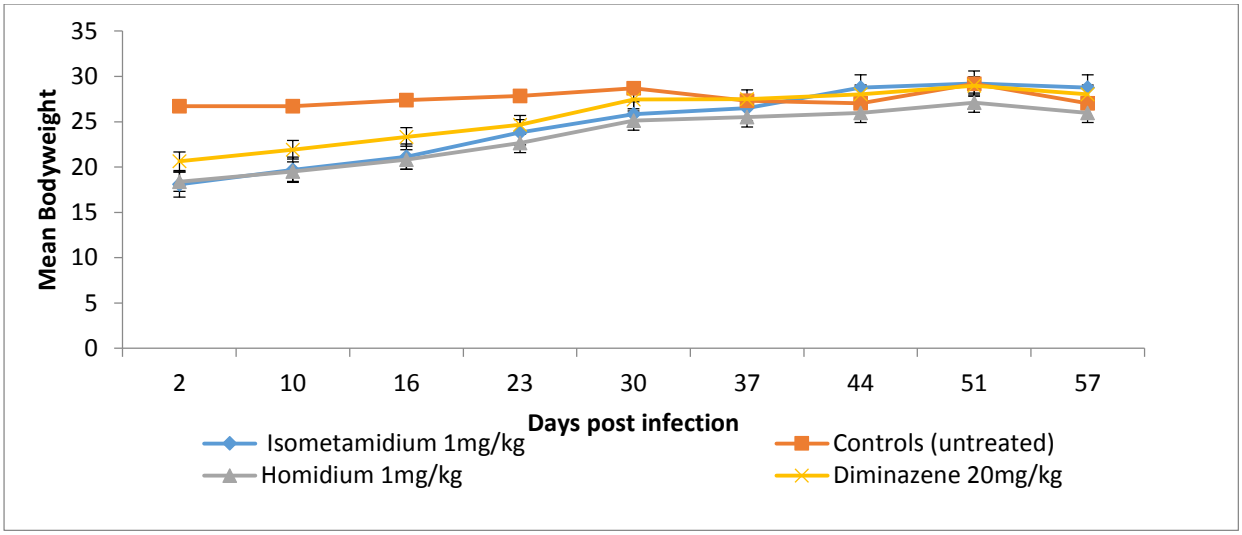


Fig.36: Mean body weights against DPI of mice infected with KETRI 3985 stabilate treated with different drugs and control group

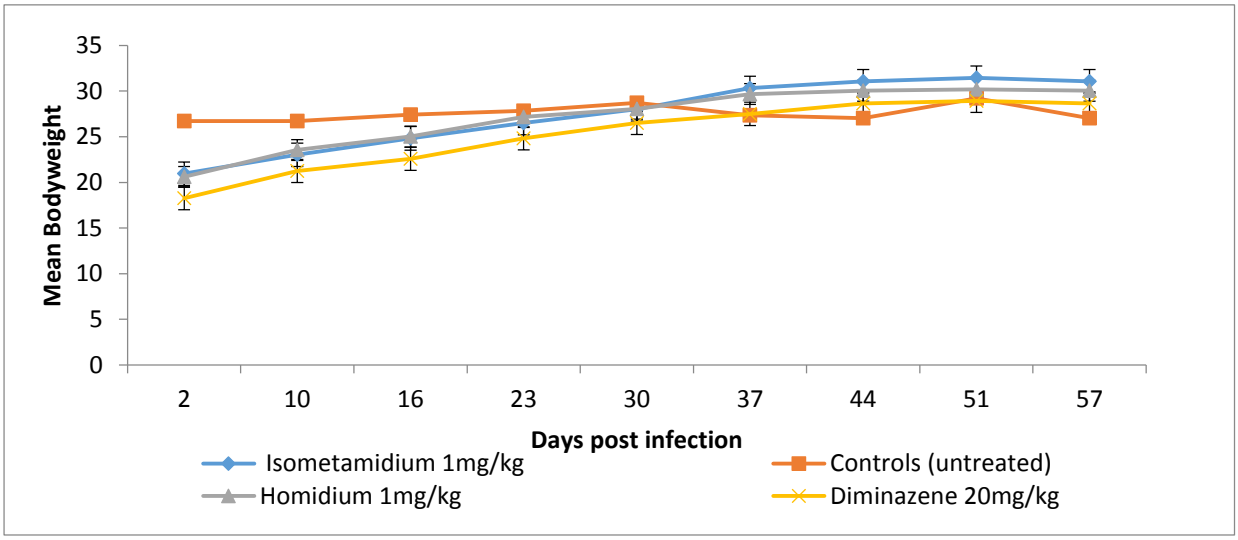


Fig.37: Mean body weights against DPI of mice infected with KETRI 4032 stabilate treated with different drugs and a control group

4.4 Objective 4: Determination of correlation between drug sensitivity/resistance to virulence and pathogenicity.

4.4.1 Selection of stabilates for use in pathogenicity and virulence determination

This was carried out to identify two drug sensitive and two drug resistant stabilates to determine their pathogenicity and virulence. Mice infected with KETRI 4028 were cured by Isometamidium1mg/kg and Diminazene 20mg/kg unlike KETRI 4032, KETRI 3985 and KETRI 3984 which were resistant to all the trypanocidals at the tested dosage rates of 1.0 mg/kg, 20 mg/kg, and 1.0 mg/kg respectively. Relapse to various drugs used in this study

occurred on different days (Table 8) on wards until the end of the experiment. As established in a previous study, the sensitive trypanosome strains usually grow faster and develop an infection earlier compared to their counterpart slower growing resistant strains (Kagira and Maina, 2007), which may result in selecting out the latter.

When Wet Blood Films (WBF) were examined 24 h post infection in pathogenicity studies, there was absence of trypanosomes until 72 h for KETRI 3984 while the rest of the trypanosome stabilates studied, motile trypanosomes were observed in WBF at 96 h post infection.

4.4.2 Parameters studied during pathogenicity and virulence determination

a) PCV levels

There was a significant difference in PCV levels in mice infected with KETRI 3984, KETRI 3985and Uninfected controls (p-value<0.001) while there was no significant difference between PCV levels of mice infected with KETRI 4028 and KETRI 3985 (Fig**.** 38).

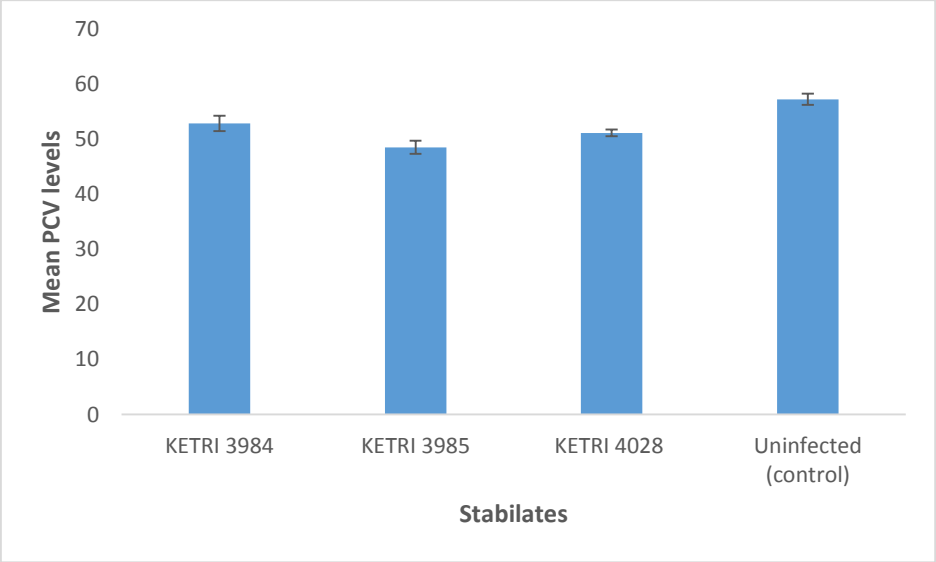


Fig.38: Mean PCV levels of mice infected with different stabilates and an uninfected control group

b) Parasitaemia

There was a significant difference in parasitaemia levels in groups of mice infected with the stabilates, p-value<0.001 (<α=0.05). When investigating the results further it was found that stabilate KETRI 3984 and KETRI 4028 were significantly different from KETRI 3985 (Fig. 39).

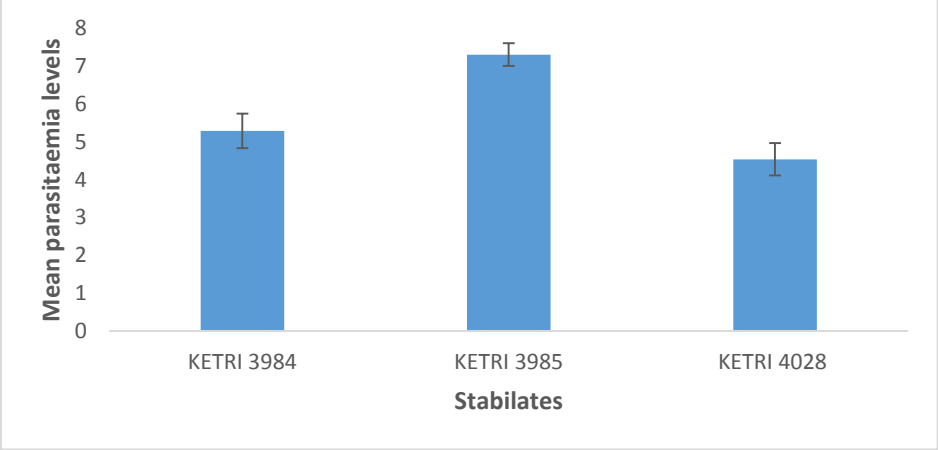


Fig. 39: Mean parasitaemia levels of mice infected with different stabilates.

c) Body weight

There was no significant difference in body weights of mice infected with the three stabilates (p-value0.090 (>α=0.05). There was a general increase in body weights with increase in DPI. KETRI 4028 infected mice had their body weights fairly constant up to day 30 which then started dropping to the end of the study. Uninfected controls had the lowest mean body weight throughout the study (Fig.40).

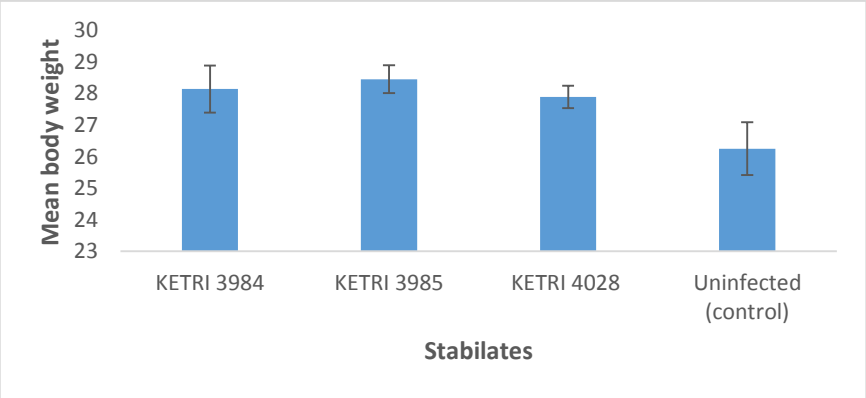


Fig. 40: Mean body weight of mice infected with different stabilates and an uninfected control group.

d) Survival analysis

The difference in survival for mice infected with drug sensitive and drug resistant stabilates was not significant since the p-value was 0.534 using Log rank test as shown in Fig.41. The mean survival times for mice infected with different stabilates were as shown in Table9.

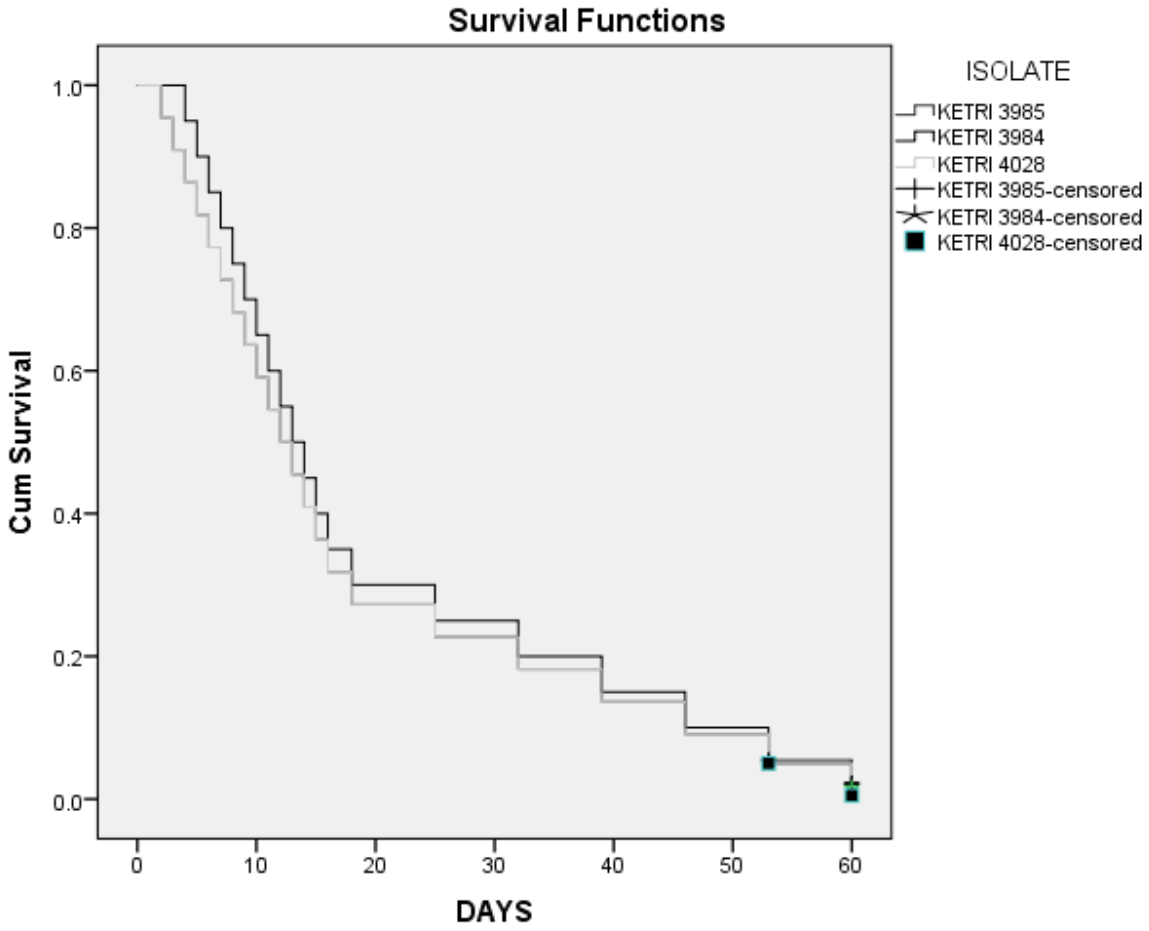


Fig. 41: Survival analysis for mice infected with drug sensitive stabilate (KETRI 4028) and drug resistant stabilates (KETRI 3984 and KETRI 3985).

e) Pre-patent period

There was a significant difference in the mean pre-patent periods between the stabilates. More specifically, there was a significant difference in the mean pre-patent periods between KETRI 4028 and KETRI 3985 (p= 0.002), as shown in Table 9**.**

Table 9: Mean pre-patent period and survival times of mice infected with different stabilates

Stabilate identity	Pre-patent period (range)	Mean pre-patent period	Survival time (range)	Mean survival time
KETRI 4028	(3-4 days)	2.9	(53-60 days)	59.4
KETRI 3984	(2-4 days)	3.4	(58-60 days)	58.4
KETRI 3985	(3-4 days)	3.8	(53-60 days)	59.3

CHAPTER FIVE
DISCUSSION

In this study, the characteristics of trypanosomes circulating in selected areas of Lamu County in 2007 and 2014 are described. Trypanosome isolates and blood samples collected from different livestock species over that period were analyzed by PCR and mouse sensitivity test to determine the infecting trypanosome species and their sensitivity to key trypanocidal drugs. Most of the parasites showed a predominantly slender morphology, a free anterior flagellum with their posterior end being narrow and a sub terminal kinetoplast.

Out of the analyzed, 10/15 (67%) trypanosome stabilates and 13/92 (14%) whole blood samples collected from parasitologically negative animals were positive with trypanosomes by PCR. No mixed infections were observed in whole blood analysis. This is in contrast to earlier work done in Lamu West division of Lamu County where 11 parasitological negative blood samples were analyzed by PCR and of the positive samples 67% of them had mixed infections (Mukiria *et al*., 2010). This could be attributed to the 5 year difference in the collection of the blood samples between the two studies during which regular Isometamidium prophylaxis program was in place and this may reduce the incidence of mixed infections. The overall infection rate of trypanosomes in donkey blood was 27.5%, 40% in cattle blood and zero in goats.

ITS 1 was able to detect *T. congolense*, *T. vivax* and Brucei group in the analyzed samples. A challenge comes when ITS-based tests are carried out singly since ITS has approximately 100-200 copies compared to species-specific tests which usually targets satellite DNA with over 10,000 copies (Njiru *et al.,* 2004) but in this study, species-specific primers were used which identified trypanosomes to subtypes level. Evansi B has only been found in dromedary camels in Kenya and Ethiopia (Njiru *et al.,* 2005) and hence was not characterized in this study. SRA gene present in *T. b. rhodesiense* was not detected in all samples analyzed. We were unable to employ species-specific primers in *T. b. brucei* characterization hence it was identified through elimination method. Identification of *T. vivax* was successful during ITS1 characterization where 11 *T. vivax* were identified with bands at 250bp but using the TV East Africa type universal primers, bands showed at 700bp. Results from this study support other studies suggesting that TV primers target certain DNA sequences that are not conserved in all *T. vivax* stabilates, which resulted to false negatives (McOdimba, 2006; Thumbi *et al*., 2008). In addition, low sensitivity of *T. vivax* observed in this study could be due to the TV primers

targeting molecules that are low in copy numbers as compared to ITS-PCR whose target gene could be higher in copy numbers (Jean *et al.,* 1997; Morlais *et al.,* 2001*).* In a recent study, it was suggested that the species-specific primer sets for *T. vivax* may not accurately assess the level of infection in wild animal's stabilates from Tanzania (Auty *et al.,* 2012). This agrees with another recent study where ITS PCR identified nine *T. vivax* samples that were not identified by species-specific primer set (Ahmed *et al*., 2013). In this study, *T. simiae* which does not usually infect ruminants (Jean *et al.,* 1997) was not considered.

This study highlighted poor resolution in microscopy to detect various trypanosome species in some animals while on the other hand, PCR was not able to pick trypanosome DNA from suspect animals and some samples which were positive by microscopy in the field. Inadequate PCR sensitivity was also observed which could have been caused by factors like loss of DNA during extraction, and low copy numbers of targeted DNA. Low parasitaemia also could have led to low sensitivity which is a characteristic of most trypanosome infections as in the case of *T. evansi* and *T. vivax* which develop chronic forms without demonstrable parasites in peripheral blood (Njiru *et al*., 2004). Amongst some samples that showed a negative result through microscopy gave weak PCR amplification which was in tandem with results from Jean *et al.,* 1997while negative controls of each sample had no bands. Some trypanosome strains were detected by microscopy in the field but not by PCR when they were inoculated in mice. This can be explained by the fact that they could have been more virulent in livestock but not in mice and therefore not mouse infective. It was therefore difficult to stabilate some trypanosome strains due to host selection pressure in rodents which eliminated them. Furthermore, since rodents are refractory to *T. vivax,* and not all *T. congolense* and *T. brucei* infections become established in a new host (in this case mice) could have also caused the low detection of trypanosomes in mice (Connor and Van den Bossche, 2004).

On the other hand, PCR was able to detect infection in whole blood samples that could not be detected microscopically. This increased the overall prevalence of trypanosome infection observed in Lamu County in 2014 survey (data not included) from 10% to 17% and in donkeys significantly from 0 to 24%. Previous PCR analysis of blood negative by microscopy donkey samples showed an infection rate of 81% compared to 3% infection rate found by microscopy in Lamu west Division (Mukiria *et al* 2011). Contamination by heme known to inhibit PCR could have led to the low detection of trypanosomes (14%) in whole blood samples from suspect animals in this study. *Trypanosoma evansi* previously stabilated from animals in the

Kenyan Coast, and which are key in donkeys was not identified in this study. Trypanosome species identified in cattle were also identified in donkeys. The differences in morphological and molecular characterization were evident as established in this study.

Clinical signs were characterized by raised hair coats in mice, poor body conditions (lethargy), facial and scrotal edema, loss of appetite, fast short breaths and pus from injured tails (Korir *et al.*, 2013) which would reduce survival times of mice. Experimental mice infected with KETRI 4028 and treated with Isometamidium 1mg/kg showed no clinical signs. This is because the isolate was sensitive to Isometamidium and the mice did not acquire the disease, unlike in the case of KETRI 3985 where mice showed mild clinical signs after treatment with Homidium 1mg/kg. Mice infected with KETRI 3984 and treated with Diminazene 20mg/kg were severely affected by trypanosomiasis as the disease progressed. This was further indicative of the resistance of the two isolates to Homidium and Diminazene.

Parasitaemia development in KETRI 3984 was faster compared to that in KETRI 4032, KETRI 4028 and KETRI 3985. Mean parasitaemia was the highest in mice infected with KETRI 3985 stabilate and treated with Homidium unlike in all other stabilates treated with the same drug. This could have suggested that KETRI 3985 stabilate was more resistant to Homidium at 1mg/kg than the other stabilates. When treated with Isometamidium 1mg/kg, KETRI 4032 infected mice mean parasitaemia was highest compared to those infected with other stabilates and treated with the same drug showing that it was more resistant to Isometamidium 1mg/kg than the other stabilates. KETRI 4032 was resistant to Homidium 1mg/kg as it equally showed a high parasitaemia. Diminazene 20mg/kg treated mice and infected with KETRI 3985 stabilate had the highest mean parasitaemia which suggested that this stabilate was more resistant to Diminazene 20mg/kg compared to the other two stabilates (KETRI 3984 and KETRI 4032). The control group was most susceptible to all stabilates followed by mice groups treated with Homidium 1mg/kg and Diminazene 20mg/kg. Parasitaemia levels of KETRI 3984, KETRI 3985 and KETRI 4032 were higher compared to KETRI 4028. Using Homidium 1mg/kg, KETRI 4028 took 15 days before parasitaemia developed. KETRI 3984 showed waves oscillating from day 7 through day 11 in which it was observed to drop to zero in day 15 when it relapsed. Using Isometamidium 1mg/kg, KETRI 3985 and KETRI 3984 stabilates parasitaemia developed in day 7 and day 11 respectively. The latter had parasitaemia waves oscillating to 27th day and in some instances it dropped to zero. Using Diminazene 20mg/kg, oscillation was observed in KETRI 4028 at 51st day when parasitaemia developed which later

dropped to zero in day 55. In KETRI 3984, slight oscillation was observed which did not drop to zero all through the 60 days of study. Different responses of immune system of mice infected with stabilates used in this study gave the difference in waves of parasitaemia. Different variable antigen types (VATs) of trypanosome stabilates to which immune responses is elicited especially in KETRI 3984, KETRI 3985 and KETRI 4032 could also be associated with the many waves observed in the graphs.

PCV has been defined as measure of anemia level (Kagira *et al*., 2005) in an animal. There was significant difference in PCV levels in the different stabilates treated with different drugs. There was a significant change of PCV with time in mice infected with all the four stabilates. The group of mice infected with KETRI 4028 and treated with the three drugs used in this study had the highest mean PCV indicating that it was sensitive to the drugs and blood PCV remained high. A high PCV level indicates that the parasitaemia levels were lowered by the drugs and blood cells kept high. When infected with KETRI 3985, KETRI 4028, KETRI 3984 and KETRI 4032, control group of mice had the lowest mean PCV followed by mice treated with Homidium1mg/kg and Diminazene 20mg/kg drugs.

The body weights of all groups of mice increased during the study even in the case of controls. Results from the analysis showed that body weights of mice unexpectedly increased with time despite being infected but were generally lower in KETRI 3984 and KETRI 3985, which is in tandem with findings from other studies (Korir et al., 2013). This has been explained by the fact that the mice were still young at the beginning of the experiment when they had just been weaned and now were growing to maturity by the progression of the experiment, thus went on gaining weight (Korir et al., 2013). In the previous studies, general decrease in body weights 12 days post infection (Celine *et al.,* 2005) have been shown which can be related to entry of trypanosomes in the CNS known to control body weight (Darsaud *et al*., 2004), unlike the case in this study. It was observed that infected mice whose parasitaemia levels were high had higher body weights but their PCV levels on the other hand were very low.

The relapse of infection by trypanosomes could occur majorly because the drugs used in this study were not able to target the relevant parts of the body where trypanosomes moved from the blood stream and sequestrated and death occurred in some cases. Where resistance was not a problem, a possible reason for infection relapse would be related to inaccessibility of the drugs to trypanosomes tissue stages of development (Al-Mohammed, 2008). Trypanosomes

usually reside in structures including blood vessels of all organs, brain extravascular spaces, lung interstitials (Sudarto *et al.*, 1990) and other extravascular sites (Matovu *et al*., 2003). Sites in which Diminazene aceturate (Diminazene 20mg/kg) in particular is not able to penetrate according to previous studies is the brain spaces (Peregrine, 1993) where relapse was possible. Relapse and death were also seen by some authors in their studies which was also the case in stabilates from Lamu. This study concurs with findings by Dargantes (2010), in which treatment with Diminazene 20mg/kg was found to be ineffective because relapse occurred on the 27thday post-treatment when used in rats and goats (Macaraeg *et al.,* 2013) which was the case in this study. Reduced sensitivity to drugs could have been associated with change in genetic constitution, mutation and selection. Drug resistance in *T. brucei* has been shown in other studies (Matovu *et al*., 2001) which were associated with the parasite surviving in high concentrations not tolerable to the host, or the parasite may be naturally resistant due to other factors like host, vector or parasite. KETRI 4028 stabilate was sensitive to Isometamidium and Diminazene while stabilates KETRI 4032 from goat, KETRI 3985 from donkey and KETRI 3984 from donkey were resistant to the three drugs used in this study. This study has demonstrated the presence of *T. b. brucei* sub populations that exhibit multiple drug resistance in mice. Earlier studies in Lamu, Kilifi and Kwale counties of the coastal Kenya have only demonstrated presence of *T. vivax* and *T. congolense* isolated from cattle that have shown multiple resistances to isometamidium, Diminazene, Homidium and Quinapyramine (Ashiembi, 2013, Mdachi, 2014).

Incubation period for AAT ranges from 4 days to approximately 8 weeks and infections with more virulent stabilates have an even shorter incubation period (Magona *et al*., 2008) and in this study, death in mice infected with KETRI 3984 and KETRI 3985 occurred earlier compared to KETRI 4028. Mice infected with KETRI 4028 were moderately affected by clinical signs and survived the longest to the end of the experimental period though the mice were weak with low PCV and high parasitaemia, which indicated a chronic infection from this stabilate. The mean PCV of uninfected controls maintained above 50% which in comparison with that of the stabilates. The control group of mice was found to have a significantly higher PCV levels compared to other groups of mice. Mice infected with KETRI 3984 and KETRI 3985 recorded a significant drop in PCV levels. The body weights of mice studied for pathogenicity steadily increased throughout the study although at a lower rate compared to infected mice in drug sensitivity study.

There has been found a correlation between anemia and death (Korir *et al.,* 2013) and hence death in mice in this study might have been contributed by anemia amongst other factors. Mice infected with KETRI 4028 stabilate were probably able to control infection and destruction of red blood cells (Kariuki *et al.,* 2008) hence they had a higher survival time, which suggested lower virulence. The variable surface glycoproteins (VSG) genes switch has been found to be similar to the rate at which body immune system develops an effective antibody response (Turner and Ormerod, 1984). The immune system of mice infected with stabilates KETRI 3984 and KETRI 3985 was unable to control the parasite infections, and hence the mice died earlier which would suggest a higher virulence.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 Conclusions

The results from this study indicated that;

1. Out of the species of trypanosomes in Lamu found to infect cattle, goats and donkeys, there were three species identified with a higher prevalence of *T. vivax* observed, followed by *T. b. brucei* and *T. congolense* Savannah.
2. Serum Resistance Associated gene (SRA) related to human infective *T. b. rhodesiense* species was not identified in trypanosome isolates from livestock in Lamu.
3. Trypanosomes isolated from goats were resistant to Isometamidium 1mg/kg commonly used in Lamu market in the recommended dosages according to manufacturers. There is presence of *T. b. brucei* sub populations circulating in Lamu that may exhibit multiple drug resistance and only 25% of the sub population may be sensitive to Isometamidium 1mg/kg and Diminazene 20 mg/kg.
4. There were more deaths in mice infected with the drug resistant stabilates which suggested more virulence.

6.2 Recommendations

1. Molecular markers such as microsatellite loci and sequence analysis of the rRNA and gGAPDH genes could be used to characterize the trypanosomes in livestock from Lamu. This will give a clear indication whether the results are unique or whether the stabilates have evolved. Use of PCR in epidemiological surveys for disease mapping and development of effective control strategies for trypanosomiasis control in livestock is important.
2. More studies to identify possible presence of SRA gene in reservoir animals and livestock in other parts of Kenya where tsetse flies are found. It is important to carry out buffy coats DNA extraction to improve detection of trypanosomes.
3. Use of Homidium should be discouraged while use of Diminazene and Isometamidium should be used with caution in cases that proper diagnosis of the disease has been done.
4. Integrated trypanosomiasis control strategies should be advocated in Lamu County.

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