TRYPANASOMA INFECTION RATES IN *GLOSSINA* SPECIES IN MTITO ANDEI DIVISION, MAKUENI COUNTY, KENYA

BY

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DECLARATION

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

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DEDICATION

I wish to dedicate this thesis to my family members and friends who supported me throughout my study.

LIST OF ABBREVIATIONS

µl	Microlitre
AAT	African Animal Trypanosomiasis
ANOVA	Analysis of Variance
Bp	Base pair
DNA	Deoxyribonucleic Acid
FAO	Food and Agriculture Organization
FFLB	Fluorescent Fragment Length Bar-coding
FTD	Fly Trap Density
HAT	Human African Trypanosomiasis
ILRI	International Livestock Research Institute
ITS	Internal Transcribed Spacer region
Kb	Kilo base
KEMRI	Kenya Medical Research Institute
LSD	Least Significance Difference
Mean AD	Mean Apparent Density
$Mgcl_2$	Magnessium Chloride
mM	Millimoles
PCR	Polymerase Chain Reaction
rDNA	Ribosomal DNA
SIT	Sterile Insect Technique
VSG	Variable Surface Glycoprotein

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ABSTRACT

This study was carried out to determine the infection rates of trypanosomes in *Glossina* species in Mtito Andei, Makueni County, Kenya. Tsetse species, *G. longipennis* and *G. pallidipes* were trapped and DNA isolated from their dissected internal organs (proboscis, salivary glands and mid-guts). The DNA was then subjected to a nested PCR assay using internal transcribed spacer primers and individual trypanosome species (*T. vivax* and *T. c*. Forest) identified following agarose gel electrophoresis. A total of 78 non teneral flies analyzed for trypanosomes infection revealed an average infection rate of 11.53% (9/78). The overall infection rates for the different trapping sites Kamunyu, Ngiluni, Kyusyani, Iviani and Nthunguni were 6.41%, 1.28%, 2.56%, 1.28% respectively. No infected tsetse were detected in Nthunguni and infection rates for the different sites were statistically different ($P<0.05$). *G. longipennis* was the most prevalent in Kamunyu while *G. pallidipes* was most prevalent in Iviani and Nthunguni. Kyusyani and Ngiluni areas had both tsetse species. Out of 78 flies analyzed, the prevalence of trypanosome infection in *G. pallidipes* and *G. longipennis* was 5.77% and 23.08% respectively and this difference was significant. Most of *G. pallidipes* were infected with *T. vivax* (3.85%) while the rest were infected with *T. congolense* (1.92%). This was in contrast to *G. longipennis* which were only infected with *T. vivax* (23.08%). The study showed overall prevalence of 10.26% for *T. vivax* and 1.28% *T. congolense* with *T. vivax* infections being higher in both tsetse species. The infection rates with these two trypanosome species was also statistically different ($P<0.05$) and were higher in males (13.95%) than in female flies (8.57%), however, this difference was not significant. There was a strong positive relationship between the mean apparent densities and infection rates confirming the importance of this parameter as an indicator of African animal trypanosomiasis.

These findings suggest the importance of designing control strategies targeting the biological vector (tsetse), mechanical vectors (*Tabanus* and *Stomoxys* spp), and the trypanosome parasites.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 General introduction

African Animal Trypanosomiasis (AAT) is a serious disease of livestock caused by a protozoan parasite of the genus *Trypanosoma* and transmitted cyclically by tsetse (*Glossina*) and mechanically by other haematophagous flies such as *Tabanus, Haomatopota, Stomoxys* and *Chrysops* (Kone *et al*., 2011). It is a major obstacle to livestock production on the Africa continent as it prevents full use of the land to feed the rapidly increasing population. AAT and its vectors occur in large areas of Sub-Saharan Africa and its occurrence parallels that of the biological vector (Taylor, 1998). The epidemiology and effects of this disease on livestock especially cattle production are determined largely by the prevalence and distribution of the disease and its vectors in the affected areas and also the proportion of infected flies in the tsetse population (Kubi *et al.,* 2006*)*. The eco-distribution of tsetse is determined by climate, presence of vegetation, water and presence of blood meals (humans and animals) but tsetse are mostly found in rural areas (Lutje *et al*., 2010). The limitations due to tsetse and trypanosomes problem continue to frustrate efforts and prevent progress in the livestock and crop production thus contributing to hunger, poverty and the suffering of entire communities in Africa. This disease is therefore a serious impediment to agricultural and economic advancement in the affected areas (Morrison and Macleod, 2011). The economic losses due to AAT are approximated to be over 1.5 billion dollars annually (Bitew *et al*., 2011).Tsetse infest about 10 million Km^2 of fertile land spread across 36 countries on the African continent with approximately 7 million Km^2 of the infested land being suitable for mixed agriculture if this disease was controlled (Bitew *et al*.,

2011). Out of 165 million cattle found in Africa only 10 million are found within the tsetse belt due to the disease constraint and these are the lowest producing breeds (Oluwafemi *et al.,* 2007).

1.1.1 Etiology

Trypanosoma species is a kinetoplastid parasite that infects a wide range of vertebrates including humans. The most important species infecting animals in Africa are *T. congolense* (Sub genus *Nannomonas*), *T. vivax* (sub genus *Duttonella*) and *T. b. brucei* (subgenus *Trypanazoon*) (Taylor, 1998). *T. congolense* can be classified into three subtypes which are savannah, forest and Kilifi types. Apart from *T. congolense,* other members of sub genus *Nannomonas* causing AAT include *T. simiae* (affecting domestic suids) and *T. godfrey* (Van Den Bossche *et al*., 2011)**.** Trypanosomes can infect all domesticated animals but in Africa, cattle are mainly affected. More than 30 species of animals including ruminants such as Antelopes (*Addax* spp, *Oryx* spp and *Hippotragus* spp), Buffalos (*Syncercus caffer*) and wild equidae are also susceptible and may serve as reservoirs of trypanosomes (Morrison and Macleod, 2011). These animals suffer syndromes ranging from mild chronic infection to acute fatal disease. Although host preferences of each *Trypanosoma* species may vary, *T. congolense, T. vivax* and *T. b. brucei* have a wider range of hosts among domesticated animals (Morrison and Macleod, 2011).

1.1.2 General life cycle of trypanosomes

Trypanosomes have a complex two-host life cycle involving a vertebrate host and an arthropod vector (Fig. 1). Tsetse ingest trypomastigotes present in the blood and lymph while feeding on an infected host. The trypomastigotes lose their glycoprotein surface coat and become elongated multiplying in the midgut before migrating forward to the salivary glands and proboscis. They then transform into epimastigote forms which multiply and then transform again into small typical

metacyclic trypomastigotes which are the infective stages and are introduced into the vertebrate host during feeding. These metacyclics have been shown to have a small repertoire of VSG genes and they multiply at the inoculation site for a few days before invading blood stream and lymphatics (Pays *et al*, 2001). This life cycle has variation in reference with the *Trypanosoma* species involved. The entire process is completed within the proboscis for *T. vivax* while in *T. brucei*, after the trypanosomes multiply they migrate forward to the salivary glands. In *T. congolense*, they migrate to the proboscis where they develop into long, slender forms and multiply by binary longitudinal fission at the site of inoculation (Prowse, 2005) eventually transforming into stumpy forms which invade the blood stream and lymphatics (Askoy, 2003)**.** The blood trypomastigote forms are taken up by tsetse along with its blood meal and undergo stages of complex biological development inside the insect host before becoming infective**.** Both male and female flies are capable of transmitting trypanosomes (Chatterjee, 2009).

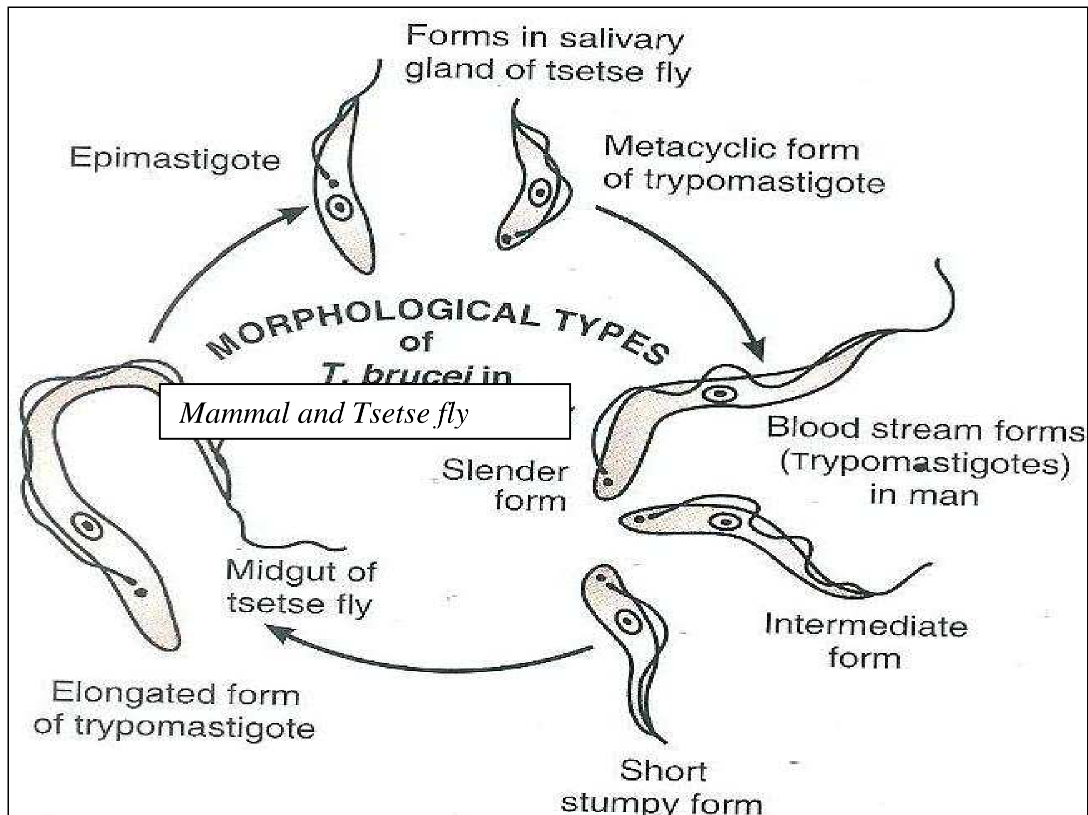

Figure 1 : Life cycle of trypanosomes

(Chatterrjee, 2009)

1.1.3 Biology of tsetse

Tsetse measure between 7.5mm to 14 mm in length and are brown-grey in colour and while at rest, their wings are cross like scissors. Their palpi are almost as long as the proboscis protruding from the front of the head and the base of the proboscis is swollen into a characteristic bulb. Tsetse are daytime feeders and are visually attracted to moving objects. Both sexes feed exclusively on blood of a wide variety of animals and humans.

Male and female tsetse are anatomically different with the distinction between sexes done by examining the end of the abdomen. Male flies are distinguished from the females by the possession of the hypogyium on the posterior tip which is folded on external genitalia (Fig 2). In front of the hypogyium is a hairy plate called hectors which together with hypogyium is used to hold onto the female's abdomen during mating (Bitew *et al*, 2011).

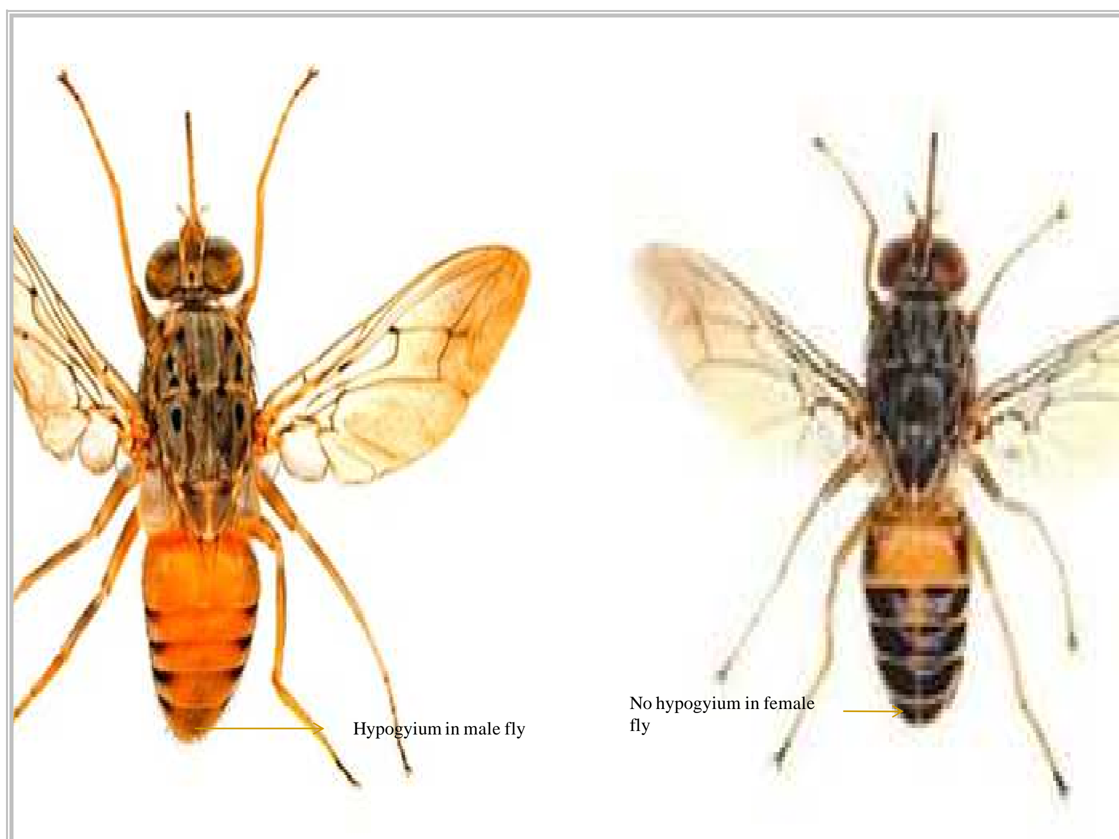

Figure 2: Distinguishing features between the male and female tsetse (Courtesy of Chatterjee, 2009). The above figure illustrates the difference between the male and female tsetse. The male fly on the left is distinguished by the presence of the hypopygium which is absent in the femaile fly.

1.2 Geographical distribution of tsetse

Tsetse are endemic to tropical Africa and they occur between latitudes 15ºN and 29ºS from Southern edge of the Sahara desert to Zimbabwe, Angola and Mozambique and with *T. vivax* even spreading beyond tsetse belt to South America where it is transmitted mechanically (The Centre for Food Security and Public Health, 2009). A total of 8 tsetse species occur in Kenya (Table 1), and are found distributed in various areas such as Narok, Transmara, shores of Lake Trukana, North of Mt. Elgon and in Lambwe valley (Fig. 3).

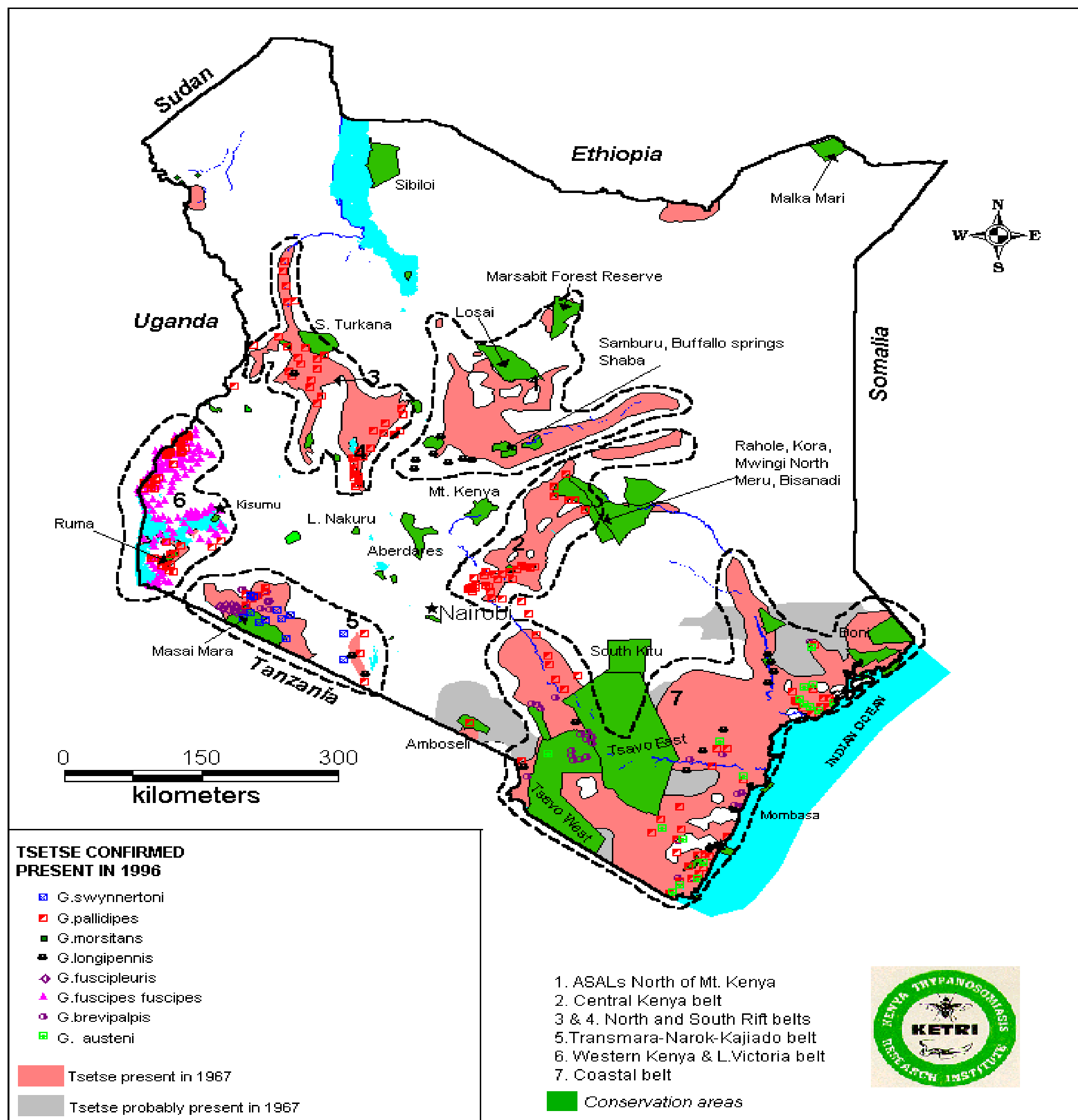

Figure 3: Tsetse distribution in Kenya

(Courtesy of Ministry of livestock development, 2011)

Table 1: Tsetse species distribution in Kenya

(Ministry of Veterinary Services, 2011)

Species	Spatial Distribution
G. brevipalpis	Coastal belt extending from Somalia to Tanzania and on the Kenya / Ethiopia border on the eastern shores of Lake Turkana
G. fuscipleuris	South –west Kenya (Narok and Transmara)
G. longipennis	Coastal belt extending into Kajiado
G. pallidipes	All tsetse belts in Kenya
G. austeni	Coastal belt extending from Somalia to Tanzania
G. swynnertoni	South-west Kenya (Narok and Transmara)
G. morsitans submorsitans	tsetse belt on Kenya Uganda border north of Mt Elgon
G. fuscipes fuscipes	Lake Victoria basins and its River systems

1.3 Classification of tsetse

Tsetse are the primary vector for *T. congolense, T. vivax* and *T. b .brucei* in Africa (Askoy, 2003). These trypanosomes replicate in blood stream and lymphatic vessels of veterbrate hosts and are transmitted through tsetse saliva when obtaining blood meals. There are 23 extant and 8 sub-species of tsetse (Rogers *et al.*, 1994) which are classified into 3 taxonomic groups according to their preferred ecological habitats**,** distributional behavioural, molecular and morphological features (Ajibade and Agbede, 2011). *Glossina fusca* group is mainly found in humid forested areas and they are important vectors of wildlife infection than for livestock trypanosomiasis. Eastern Africa has only *G. longipennis* as a fusca group species and is found in drier areas (Glasgow, 1963; Ford and Katondo, 1997). Other species in this group are found in countries such as Gabon and Zaire. The morsitan groups are largely found in open woodland savannah best suited for grazing livestock and is the most important group in the transmission of animal trypanosomiasis (Prowse, 2005). The species in this group include *G. swynertoni, G. pallidipes* and *G. morsitans submorsitans. Glossina palpalis* group of flies favor shaded habitats near rivers and lakes but can also occur in other ecological zones such as humid arid Savannah. *Glossina palpalis* group of flies are less efficient vectors compared to *G. morsitans* group (Ajibade and Agbede, 2011).

1.4 Economic impact of African trypanosomiasis

African Animal trypanosomiasis continues to be a serious health concern across large areas of sub-Saharan Africa despite several decades of research (Kubi *et al.,* 2006). It causes economic losses in livestock with many untreated cases being fatal**.** Globally, trypanosomiasis causes economic loss of approximately 1.5 billion dollars with African farmers spending 35 million dollars on treatment (Bitew *et al*., 2011).

It vastly affects meat and dairy production (Wille *et al*., 1996). Nagana has restricted agricultural development and nutritional resources in Sub-Saharan Africa, profoundly impacting the economy of much of the continent (Brian *et al.,* 2007). This disease decreases animal production on about 7 million km squares of land and leads to considerable financial losses due to high morbidity and mortality rates in susceptible but reproductive breeds as well as in the cost of trypanocide treatments (Kone *et al*., 2011). Moreover, the use of trypanocide and trypanocidal drugs is massive with nearly 50% of veterinary products sales in Africa thereby incurring risk of developing drug resistance. Livestock farmers continue to suffer losses resulting from reductions in fertility (due to abortion and testicular damage affecting sperm viability) and weight loss and mortality. It has been estimated that direct production losses in cattle alone are between US$ 1200 -6000 million per year in 36 tsetse infested countries of Sub-Saharan Africa. These estimates do not include indirect losses due to in-availability of livestock, manure and draught power (Taylor, 1998). It is probably the only disease which has profoundly affected the settlement and economic development of a major part of the continent and thus limits rural development in vast areas of tropical Africa (Cecchi *et al*., 2008). The cumulative effects of the various constraints due to trypanosomiasis to the agricultural communities are therefore enormous and the fly may also be preventing biodiversity across the continent by affecting movement of their livestock living in tsetse infested areas.

1.5 Literature review

1.5.1 Previous studies of trypanosomiasis in Kenya

Field studies have previously been conducted on prevalence of animal trypanosomiasis in Kenya. Thumbi *et al*., (2008) reported trypanosome infection rates of 41% and 29% in Suba and Teso districts respectively with *T. vivax* infections noted as the most dominant in both areas.

In their study, samples were screened for trypanosomes using species specific primers, multi-species detecting ITS BR and ITS CF, and nested ITS 1, 2, 3 and 4 primers designed to amplify the internal transcribed spacer of ribosomal DNA. The PCR based tests were adopted to overcome the low sensitivity limitations in microscopy.

In Tsavo West, Mihok *et al.,* (1992), reported an infection rate of 3.6% in *G. pallidipes* using species specific primers while Tarimo *et al.,* (1984) has reported trypanosome infection rates of 12.62%, 7.24%, 9.50% and 7.97% in Ukunda, Diani, Muhaka, Shimba hills and Mwalewa coastal areas respectively. This study was carried out between 1980 and 1982 using species specific primers. In Lambwe valley, Kenya, Njiru *et al*., (2004) reported trypanosome infection rate of 4.9% (118/3826) obtained microscopically. The 3638 microscopically negative samples were pooled together and further analyzed using species specific primers for the different trypanosomes revealing 9 more positive samples.

1.5.2 Detection of trypanosome infections in tsetse

1.5.2.1 Microscopy

Earlier studies done to determine the trypanosome infection rate in tsetse depended on direct examination of internal organs under a dissecting microscope (Lloyd and Johnson,1924). Infection rates of *G. pallidipes* and *G. fuscipes fuscipes* in Gojeb valley, Southwest of Ethiopia were determined by this method (Bitew *et al.,* 2011). In their study, a total of 384 flies were randomly selected and dissected and they comprised 200 (52%) *G. pallidipes* and 184 (48%) *G. fuscipes fuscipes*. Out of the dissected flies, 24.5% (49) *G. pallidipes* and 22.8% (42) *G. fuscipes fuscipes* were positive for trypanosomes. A study done by Mulaw *et al*, (2011) on the prevalence of major trypanosomes affecting cattle in Western Ethiopia employed microscopy

techniques for identification and reported an overall trypanosome infection rate of 28.1% (108/384). A major drawback of microscopy is that it fails to discriminate species that use the same developmental sites and is not able to discriminate mixed infections in tsetse (Malele *et al.*, 2003). Low sensitivity has also been reported with microscopy. For instance, Lefrancois *et al.,* (1999), identified only 31.5% of proboscis infections using microscopy. Further analysis of the microscopically negative proboscis using species specific primers revealed 9.7% more infections indicating that PCR based tests are more sensitive. Therefore, the use of DNA-based methods for identification of trypanosomes is being favored over microscopy although microscopy is still being used in low resource setting laboratories.

1.5.2.2 Molecular techniques

There are a number of PCR based techniques that have been developed for identification of trypanosome species (Masiga *et al.,* 1992 .; Masiga *et al.,* 1996) ; Majiwa *et al.,* 1993 ; Masake *et al.,* 1997 ; Cox *et al.,* 2005 ; Malele *et al* , 2003 and Thumbi *et al.*, 2008). Such studies have enabled important information to be obtained directly from the small number of parasites present in tsetse organs or blood samples thus overcoming the need to culture live parasites with the added risk of contamination and differential growth of mixed cultures.

Specific DNA probes and subsequently PCR primers have been developed for 11 different species, subspecies or subgroups of African trypanosomes. These species include: sub-genus *Trypanozoon*, *T. b. gambiense, T. b. rhodesiense, T. congolense* (savannah, forest and kilifi subgroups), *T. simiae, T. simiae* Tsavo, *T. godfreyi*, subgenus *Duttonella* and West African *T. vivax* (Hamilton *et al.,* 2008). A review done by Adam and Hamilton, (2008) explored the application of molecular markers for the identification of trypanosome species. According to

these authors there are three broad categories of molecular diagnosis, species specific identification techniques, generic PCR-based approaches and sequence analysis.

Earlier molecular techniques for detecting trypanosome infection rates involved the use of PCR analysis and DNA probes. Using this method, McNamara *et al.,* (1995) tested 70 of 124 trypanosome isolates obtained from trypanosome positive midguts of *G. palpalis, G. pallicera pallicera* and *G. nigrofusca nigrofusca*. This study revealed prevalences of : Trypanozoon 46%, Riverline forest *T. congolense* 86%, *T. congolense* Savannah 54% and *T. simiae* 29%. Thirty of the infection involved a single trypanosome species. Out of the 70 isolates analyzed, 30 of them were successfully amplified using PCR to provide materials for DNA hybridization. To a greater extent DNA probes confirmed the PCR results; 100% (28/28) of riverline forest and 82% (18/22) of *T. congolense* savannah. Only 8% (1/13) of the PCR positives for Trypanozoon were able to hybridize with the appropriate DNA probes. However, the range of primers and probes used in this study could not identify all trypanosomes known to develop in the tsetse midgut. This technique was also employed by Kukla *et al*., (1987). The major limitation of this method is that non specific PCR reactions can inhibit both capturing of PCR products to micro-titer plates and the correct hybridization of the specific probe thus leading to either false negatives or positive results. Furthermore, detection of multiple infections requires the use of multiple probes thus making the technique expensive. Therefore, the use of multiplex PCR techniques reduces the cost as each sample is screened in a single PCR unlike in species specifc PCR where each sample is subjected to at least five PCR reactions.

Species specific primers have also been used in the characterization of trypanosome species. These primers target specific genes in each trypanosome. (Table 2).

Table 2: Species specific primers

Primers	Specificity	Amplification size product (bp)	Reference
IL 344-345	*T. congolense* savannah type	320	Majiwa *et al.,* 1993
TCF 1, 2	*T. congolense* riverline-forest type	350	Masiga *et al.,* 1992
ILO 892, 3	*T. congolense* Tsavo	400	Majiwa *et al*., 1993
KOL 1, 2	*T. congolense* Kilifi type	520	Majiwa *et al*., 1985
TSM 1, 2	*T. simiae*	437	Masiga *et al*., 1992
VOL 1, 2	*T. vivax*	180	Dickin and Gibson 1989
TBR 1, 2	*T. brucei s.l*	177	Moser *et al*., 1989

In Tanzania, Malele *et al*., (2003), performed a species specific PCR on 1041 dissected positive proboscides and 24% (254/1041) of the tested samples were positive . In a similar study, Lehane *et al*., (2000) reported comparable findings where species specific PCR was able to identify 86.7% (352/643) dissected positive proboscis.

The use of species specific primers enable identification of only known species. This is explained by the higher number of infections that remain unidentified in surveys, 13% (Lehane *et al*., 2000),

17% (Njiru *et al.,* 2004) and 75% (Malele *et al*., 2003). The use of species specific primers is also expensive as each sample is screened for trypanosomes at least five times and is therefore not suitable for large scale epidemiological studies.

The use of generic primers provides an alternative to this problem. Generic PCR tests are based on internal transcribed spacers (ITS) and fluorescent fragment length barcoding (FFLB) and rely on detection of species specific size polymophisms in regions of ribosomal DNA, a locus which can be amplified using probes that are complementary to flanking conserved sequences (Adams *et al*., 2010). FFLB is based on length variations in regions of the 18s and 28s ribosomal DNA. PCR is carried out on the DNA samples and DNA probes used to determine and extend the PCR results. Using this technique, Hamilton *et al.,* (2008) examined 91 trypanosome samples from infected tsetse midguts in Tanzania, most of which had been identified using species specific PCR tests. The overall infection rate of trypanosome infected midgut samples increased from 78% to 96% confirming that FFLB is very sensitive and is able to identify previously unrecognized trypanosome species and mixed infections. A similar study done in Tanzania by Adams *et al*., (2010), revealed 61.9% (65 / 105) infected proboscides and 9 (13.5%) mixed infections. The use of FFLB based PCR test enabled identification of 2 new *T. vivax* genotypes. This study found that FFLB method was accurate, quick and had an extemely high level of identification. However, it requires expensive equipment and trained technical staff.

In a comparative study, Thumbi *et al*., (2008) evaluated the sensitivity and specificity of 3 PCR based diagnostic assays for the detection of pathogenic trypanosomes in cattle blood. A total of 103 blood samples obtained randomly from Teso and Suba districts of Western Kenya were screened for various trypanosomes using species specific primers, single ITS and Nested ITS PCRs'. Trypanosome infection rates detected using species specific primers, single and nested

PCR were 10.7%, 26.2% and 28.1% respectively. Unlike species specific primers, single and nested PCR picked mixed infections of 1.9% and 3.8% respectively. Both single ITS and nested ITS PCRs' picked higher *T. vivax* infections than species specific PCR which were 17.5%, 23.3% and 3.9% respectively, indicating the superiority of ITS multi species detection technique. The use of single ITS and nested ITS PCR also reduced labor, cost and time as compared to the five reactions carried out using species specific PCR.

In a similar study done in Tanzania, Njiru *et al*., (2005) reported that the diagnostic sensititivity using single ITS PCR was close to that obtained using species specific primers. In this study, 357 cattle and 185 camels blood were screned for trypanosomes using KIN, species specific and single ITS primers. Species specific primers detected trypanosome prevalence of 24.1% (86 / 357) and 25.9% (48 / 145) in cattle and camels respectively. Comparatively, single ITS primers detected 84.9% (73) while KIN primers (KIN 1 and KIN 2 primers designed to amplify ITS1region of ribosomal DNA) detected 67.4% (58). This study showed that ITS primers are more sensitive than KIN primers as they detected higher number of *T. vivax* infections. ITS primers target part of the ribosomal RNA gene locus. Ribosomal genes occur in tandem arrays of 100-200bp per trypanosome. Each gene has a number of non-coding spacer regions (ITS 1 and ITS 2) and conserved coding regions small sub-unit (SSU) and large sub-unit (LSU) (Cox *et al*., 2005).

1.6 Justification and significance of the study

The Kenyan economy is largely agricultural-based, with the sector accounting for about 24% of the Gross Domestic Product (GDP) and 70% of the employment (Ministry of Livestock Development, 2011). Only 7% of Kenyan land is suitable for crop production and a further 5% can sustain crops in years of adequate rains (Oniang'o, 2001). The rest of land is arid and semi-

arid and constitutes the range lands which are not suitable for crop production but rather good for livestock production. Mtito Andei comprises mainly these arid and semi-arid lands and livestock rearing is the mainstay of this region. This area also has a history of animal trypanosomiasis as it is heavily infested with *G. pallidipes* and *G. longipennis* and there is also a limited population of *G. brevipalpis* (Wilson, 1954). There is no accurate information that exists in regard to the current prevalence of trypanosomiasis in Mtito Andei. This is because previous data has been collected through microscopy on blood smears, dissection and microhaemotocrit techniques which are less sensitive. Moreover, these methods cannot identify immature and mixed infections too. Due to this gap in knowledge, this research hoped to provide information on the current prevalence and distribution of trypanosomiasis in this area. Assessment of prevalence vector hotspots and infection status can be instrumental in making decisions with regard to the formulations of suitable tsetse and trypanosomiasis control measures. This is also important in evaluating its financial, technical feasibility and to plan to maintain continuity of these control measures.

1.7 Objectives

The broad objective of this study was to determine the infection rates of trypanosomes in tsetse in selected villages in Mtito Andei Division using ITS nested PCR.

The specific objectives were:

1. To determine the prevalence and distribution of trypanosomiasis in the region.
2. To characterize different trypanosome species in the region.
3. To determine the distribution and densities of all potential trypanosome vectors in the region.

CHAPTER 2

MATERIALS AND METHODS

2.1 Study design

A cross sectional study was conducted to determine the infection rates of trypanosomes in *Glossina* species in Mtito Andei Division. Sample size was determined using the formula described by Thrusfield, (2005).

$$N=Z^2PQ^2/d^2$$

Where, **n** is the minimum sample size required, **Z** confidence interval**, P** expected prevalence from the study, **Q** 100% minus P and **D** being the significant level (0.05).

2.2 Study site

This study was carried out in Mtito Andei Division of Makueni County situated about 290 km South East of Nairobi. The study area was divided into five villages namely, Nthunguni, Iviani, Kyusyani, Ngiluni and Kamunyu. The altitude of the area varies from 600m to 1100m above sea level. The area was not inhabited until the 1930's due to its low agricultural potential and was heavily infested by tsetse. It is characterized by low and unreliable rainfall, marginal agricultural lands, dispersed populations and low fertility soils.

The principle means of communications in the area are the Nairobi -Mombasa road, railway line and many dry weather roads adjoining them. The Athi River is the main perennial river in the Division and its tributaries are Kambu, Kibwezi, Kiboko and Mtito Andei. The division is characterized by Savannah grassland with mostly low lying, gently eastward sloping plains towards river Athi, broken by occasional hills and seasonal and perennial rivers. The climate of

this area is typically semi arid and the average annual rainfall, evaporation and temperature are in the order of 600mm, 2000mm and 23°C respectively (Michieka and Van Der Pouw, 1977). Rainfall is bimodal with long rains occurring from March to May and short rains from November or December to early January. Short rains are more reliable in time than long rains and are therefore the most important. Highest mean temperatures (32-33°C) prevail during February to March, while the lowest (15-16°C) during July –August (Michieka and Van Der Pouw, 1977). The optimum temperatures that are suitable for physiological requirements within the tsetse belt are 20-28°C and relative humidity between 50-80% and rainfall 635-1524mm per year and therefore Mtito Andei Division best favours tsetse proliferation as it meets most of these requirements (Ajibabe and Agbede, 2011).

2.3 Collection and identification of tsetse

Tsetse trapping was done between the months of April and May 2012. This being a rainy season more abundant flies were expected, the possible reasons being their short developmental cycles during favorable periods and the fact that rains favor larval development (Kone *et al*., 2011). Trapping was done using 5 bi-conical traps in each village which were deployed near Mtito Andei River, Athi River and in the near vegetations types. The bi-conical trap was chosen for use in the collection of baseline data on tsetse distribution because of its effectiveness (Flint, 1985). The traps were set at georeferenced sites (Fig. 4)) and baited with acetone and cow urine to increase trapping efficiency (Malele *et al.*, 2003). Traps were set at regular intervals of 100 meters along various vegetation types and under shade to avoid undue fly mortality due to heat (Fig. 5). In order to prevent the ascent of ants on the poles towards the collecting cages, each pole was smeared with grease. All trapped flies were counted, identified to generic level using conventional identification keys (Goodwin, 1982) and sexed before dissection.

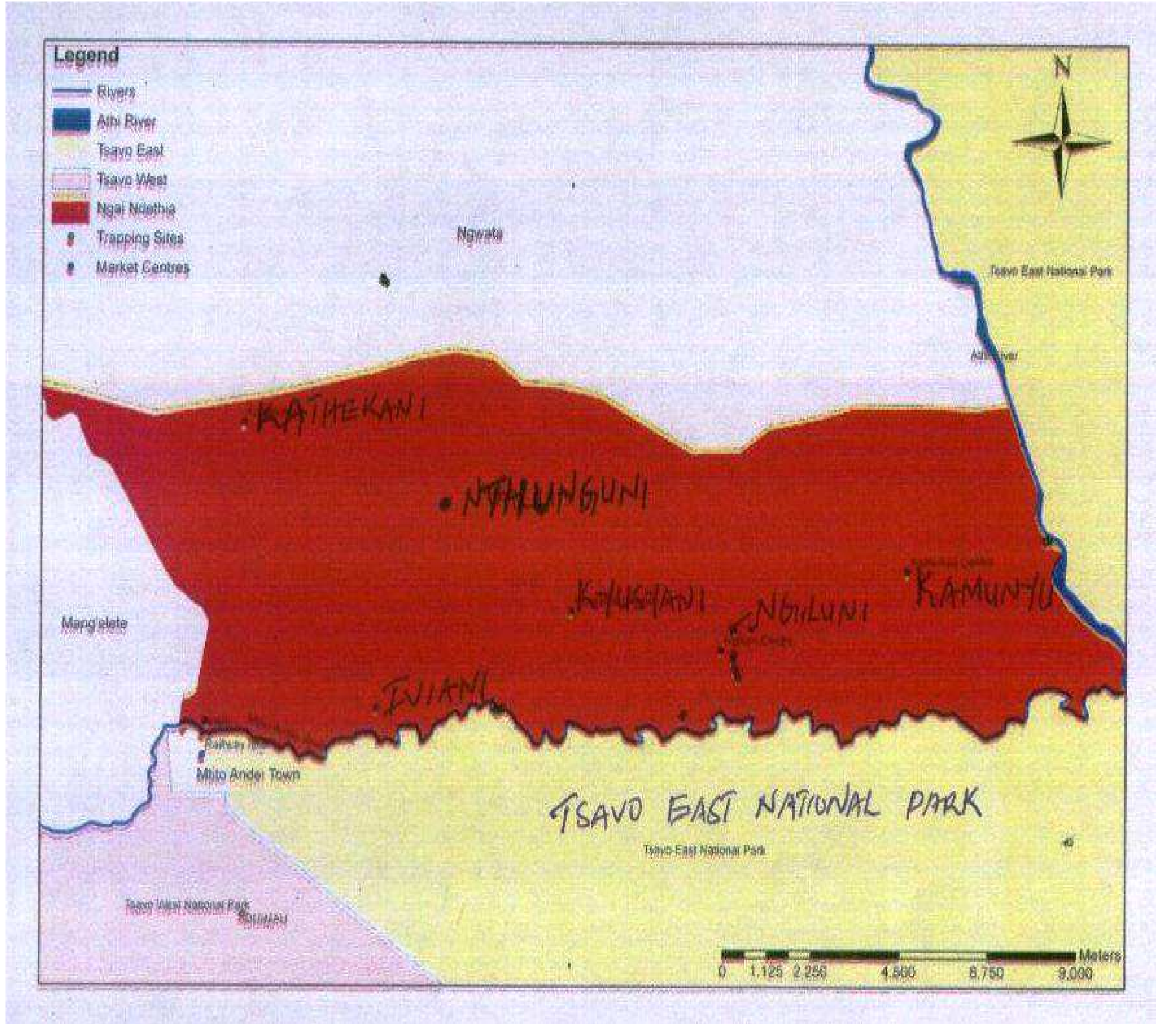

Figure 4: The map of Mtito Andei showing the georeferenced trapping sites

Figure 5: A bi-conical trap baited with cow urine and acetone to enhance trapping efficiency

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2.4 Tsetse Dissection

Dissection was carried out on freshly immobilized flies as trypanosomes are less likely to be found in dead dried flies. Flies were immobilized using ethyl acetate placed in a cotton wool at the bottom of a specimen tube. A total of 78 non teneral flies were dissected from which mid-gut, salivary glands and proboscis were removed and analyzed under a microscope for the presence of trypanosomes. In order to maximize the number of infected mid-guts encountered, mostly non teneral flies were examined prior to dissection. Non teneral flies were identified by the presence of dark or brown colour on their abdomen which indicated the last blood meal. Live flies were

surface sterilized by brief immersion in 70% ethanol then blotted dry on clean tissue paper and then dissected out in normal saline. The dissection was carried out based on FAO guidelines and manual for entomological techniques (FAO, 1982). In order to avoid cross contamination, the dissecting instruments were sterilized by immersion in 3-5% (w/v) sodium hypochrorite for approximately 2 min, followed by extensive rinsing in distilled water and final immersion in normal saline. Tsetse dissected organs (mid-gut, proboscis and salivary glands) from each fly were transferred into 1.5 ml micro-centrifuge tube containing 120 µl of ATL tissue lysis buffer (from Qiagen DNeasy blood and Tissue Kit) added. The tissues were then stored at -20°C till further processing.

2.5 DNA extraction from samples

DNA was extracted using the Qiagen DNeasy blood and tissue kit and according to the manufacturer's instructions. Twenty microliters (µl) of proteinase K was added to the dissected tissues and incubated at 56°C overnight in a thermocyler until the tissue was completely digested. The lysate was vortexed for 15 sec and 200 µl of Buffer AL added to the sample followed by vortexing to mix the contents. Two hundred µl of absolute ethanol was added, mixed thoroughly again by vortexing before pipetting the mixture into DNeasy mini spin columns placed in a 2 ml collection tube. The columns were centrifuged at 8000 rpm for 1 min and the flow through discarded together with the collection tube. The DNeasy mini spin columns where placed in a new 2 ml collection tube and 500 µl of wash Buffer AW1 added. It was then centrifuged at 8000 rpm for 1 min and the flow thorough together with the collection tube discarded. The spin column was again placed in a new collection tube and 500 µl of wash buffer AW2 added. The column was centrifuged at 14,000 rpm for 3 min. The flow-thorough and the collection tubes were discarded and the spin columns placed in a clean 1.5 ml micro-centrifuge tube followed by

addition of 30 µl of elution buffer (AE buffer) to the DNeasy column. The column was incubated at room temperature for 1 min and centrifuged for 1 min at 8000 rpm to elute the bound DNA. DNA extracted from samples was quantified using a nanodrop spectrophotometer and the purity determined by measuring the intensity of absorbance of DNA solution at wavelengths 260 and 280 nanometres.

2.6 Oligonucleotide primers

Primers used where synthesized at Bioneer and were kindly provided by International Livestock Research Institute (ILRI) (Table 3).

Table 3: Sequence of primers

Primer ID		Sequence (5'-3')
Outer primers	ITS1	GAT TAC GTC CCT GCC ATT TG
	ITS2	TTG TTC GCT ATC GGT CTT CC
Inner primers	ITS3	GGA AGC AAA AGT CGT AAC AAG G
	ITS4	TGT TTT CTT TTC CTC CGC

These primers were designed to amplify the internal transcribed region (ITS) region of ribosomal DNA (rDNA), a region which varies in size within trypanosome species, except for members of trypanozoon genus and are therefore capable of differentiating trypanosomes based on their amplified fragments. ITS 1 and ITS 2 (outer primers) amplify the conserved regions of ribosomal DNA (rDNA), small sub-unit(SSU) and Large sub-unit (LSU) represented by black arrows while ITS 3 and ITS 4 (inner primers) amplify the non coding spacer regions of rDNA and are represented by white arrows (Fig. 6).

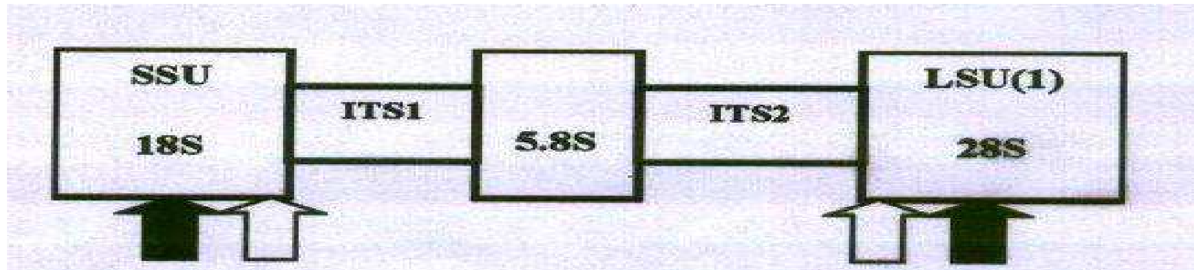

Figure 6: Schematic diagram of rDNA showing ITS 1 and ITS 2 annealing positions

(Cox *et al*., 2005)

2.7 PCR amplification and agarose gel electrophoresis

All PCR amplifications were done in 30µl reaction volumes containing final concentrations of 1× invitrogen® PCR buffer, 3.5mM $Mgcl_2$ (Promega), 0.2mM dNTPs mix, 1.25U/µl invitrogen® Taq, 100ng/µl of each forward and backward primer. All the DNA samples were screened for the various trypanosome species. A set of purified genomic DNA of *T. b*. brucei, *T. c*. Kilifi and *T. c*. Savannah, (strain IL1180) were included as positive controls during all PCR assays and distilled water as negative control. In the first round of reaction the outer primers (ITS1 and 2) were used. The reaction conditions were as follows: 1 cycle of 95°C for 7 minutes as initial denaturation followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. The final extension was done at 72°C for 5 min. The thermal cycling was carried out on a Bio-Rad thermocyler. In the second round of reaction the outer primers (ITS 1 and 2) were substituted with inner primers (ITS 3 and 4) and 5µl of round one PCR product used as DNA template. The reaction conditions were as detailed previously. In both reactions 5µl of DNA template was used. To analyse the amplicons 10µl of the PCR product was resolved in a 2% % agarose gel at 80 volts for 45 min and the gel visualised under UV following ethidium bromide staining.

2.8 Data analysis

Apparent density (AD) is an estimation of flies' density and is given in terms of the number of flies caught per trap per day (FTD). It was calculated using the formula:

$$\boldsymbol{FTD} = \frac{\sum F}{T \times D} \ where:$$

$$\sum F \ is\ the\ number\ of\ total\ flies,$$

$$T\ is\ the\ number\ of\ functioning\ traps$$

$$D\ is\ the\ number\ of\ \ days\ for\ which\ traps\ were\ operational$$ (FAO, 1982).

If a trap was not operational for some reason, either being blown down or cage being knocked off, it was excluded from the sum of trap days. After obtaining AD for each trap, mean AD for each trapping site was calculated. This statistic is important as it gives a detailed data on tsetse distribution in the area. One way ANOVA (analysis of variance) was then used to compare the mean AD for the five trapping sites in order to find out if they were statistically different.

Overall infection rates of trypanosomes in *G. pallidipes* and *G. longipennis* were calculated in percentages by taking the number of positive flies in each species divided by the number of dissected flies and multiplied by 100. The association of infection rates with fly's sex and vector species was tested using a Chi-square test. A correlation analysis was also done to establish whether there was a relationship between infection rates and apparent densities. The infection rates in the field sites were compared using one way ANOVA and all data were analyzed using SPSS version 16.0 software at 95% confidence interval.

CHAPTER 3

RESULTS

3.1 Nested PCR amplification of trypanosome DNA

PCR amplification of trypanosomes using ITS nested primers revealed DNA fragments of 611bp for *T. vivax* and 1513bp for *T. congolense* forest and were within the expected band sizes (Fig. 7). Positive controls were amplified from *T. brucei brucei, T. congolense* kilifi, *T. congolense* savannah and *T. congolense* forest, and the fragments obtained were within the expected sizes (Table 4).

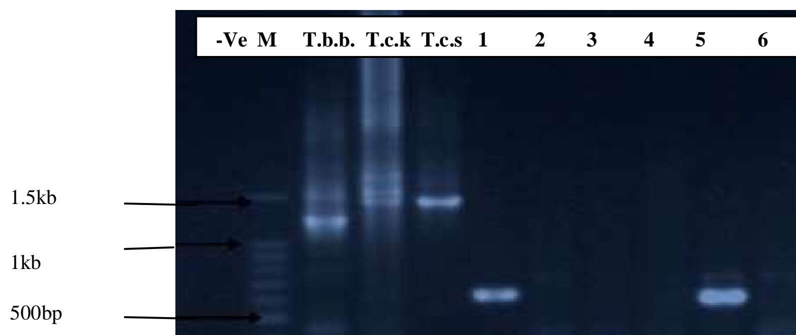

Figure 7: Image of an agarose gel showing PCR amplified DNA fragments using ITS nested PCR primers

The positive controls are *T. brucei brucei* (T. b. b), *T. congolense* Kilifi (T. c. k), and *T. congolense* Savannah (T. c .s). ‘M’ is 100bp molecular marker (Promega). Both samples 1 and 5 are positive for *T. vivax* while 6 is the negative control. Samples 2, 3 and 4 were negative for trypanosomes.

Table 4: Trypanosome species and the expected band sizes on amplification using nested ITS primers

(Cox *et al.*, 2005)

Trypanosome species	**Expected band sizes in base pairs (bp)**
T. congolense forest	1513
T. congolense Kilifi	1422
T. congolense Savannah	1413
T. congolense Tsavo	954
T. brucei	1207-1224
T. vivax	611

3.2 Vector distribution and densities

Out of the 117 flies trapped in the area, *G. pallidipes* constituted the largest percentage of 58% while *G. longipennis* had 42 %. Among the trapped tsetse 39 (33.3%) were tenerals while 78 (67 %) were non tenerals. In Kamunyu area, *G. longipennis* was the most prevalent species although a small proportion of *G. pallidipes* was also recorded. In Ngiluni, Kyusyani, Iviani and Nthunguni the most prevalent species was *G. pallidipes* while Ngiluni and Kyusyani had both tsetse species (Fig. 8). There were no flies trapped in Kathekani area which initially had been included as one of the study sites.

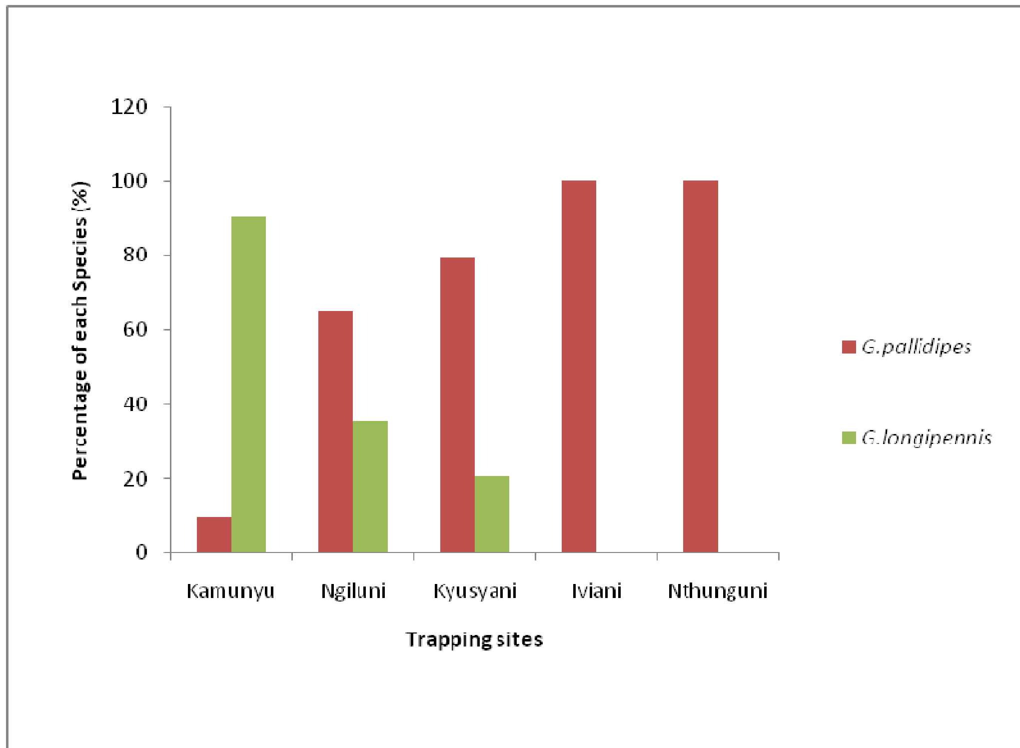

Figure 8: Distribution of *Glossina* species captured from study sites in Mtito Andei Division

The overall mean AD for Glossina species was 0.3 flies per trap per day with apparent densities (AD) ranging from 0.1 to 0.5 depending on the locality (Fig. 9). There was no statistical significance in the mean AD of *Glossina* species captured across the five trapping sites.

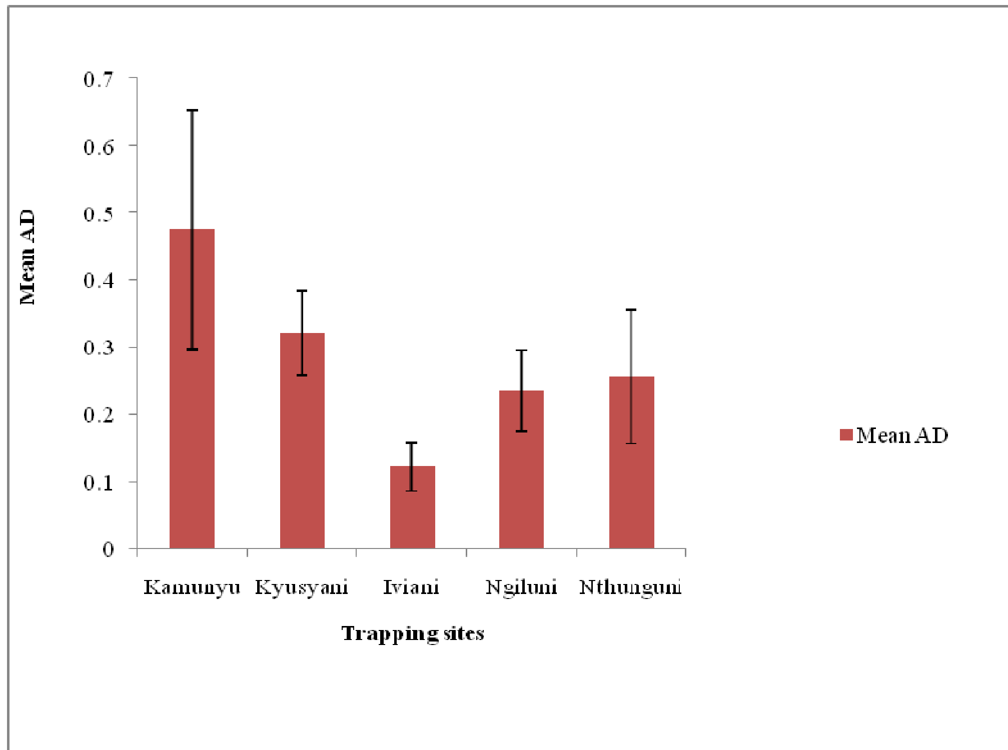

Figure 9: Comparison of Mean apparent densities within the five trapping sites

Other than *Glossina* spp, the area was also inhabited with mechanical flies such as *Tabanus* spp and *Stomoxys* spp. *Tabanids* were highly predominant over S*tomoxines* in all the trapping sites with an overall fly trap density (FTD) of 0.11 while *Stomoxys* spp had 0.02. Of all mechanical vectors, *Tabanids* accounted for 84% while *Stomoxys* had 16 % with the later being recorded only in Kyusyani area (Fig. 10).

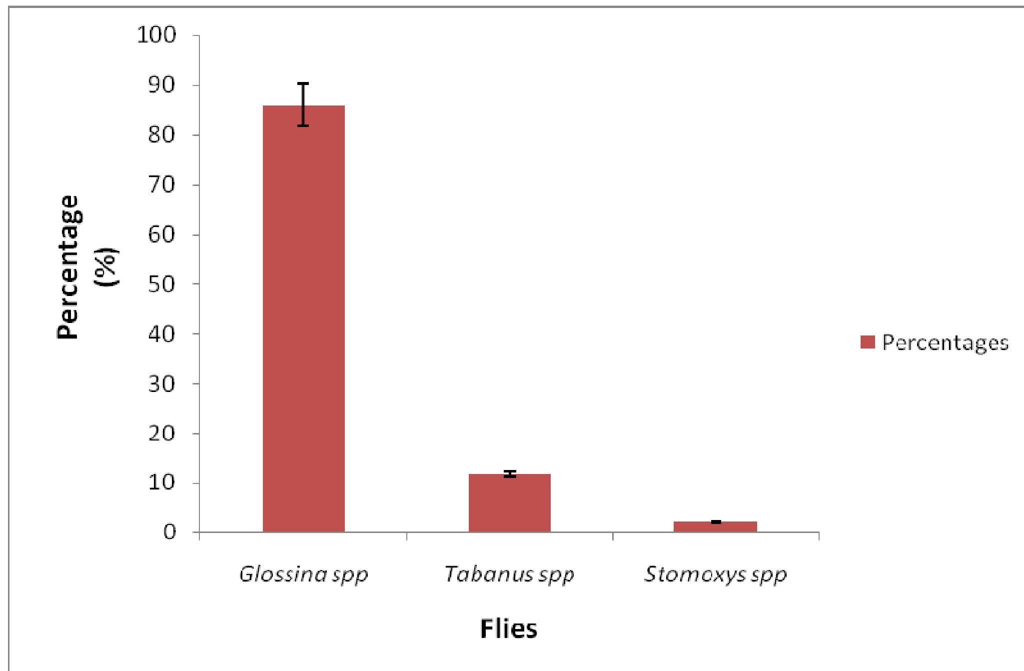

Figure 10: Relative distribution of total fly catch

3.3 Infection rates

Out of 78 flies analyzed using nested PCR and gel electrophoresis, the prevalence of trypanosomes infection in *G. pallidipes* and *G. longipennis* was 5.77% and 23.08% respectively (Table 5). Moreover, the overall infection rate in all *Glossina* spp was recorded as 11.53%. There was a significant difference in the trypanosomes infection rate with variation of *Glossina* species ($p<0.05$). Infections with *T. vivax* were the most prevalent and dominant (10.26%) in both species of tsetse compared to *T. congolense* with a prevalence of 1.28% (Table 5).

Table 5: Trypanosome infection rates in *Glossina* species

Count		Infection			Overall infection rates (%)	Total
		Uninfected	*T. vivax*	*T. congolense* forest		
Vector species	*G. pallidipes*	49	2 (3.85%)	1 (1.92%)	3 (5.77%)	52
	G. longipennis	20	6 (23.08 %)	0 (0%)	6 (23.08%)	26
Total		69	8 (10.26 %)	1 (1.28%)	9 (11.53%)	78

There was a significant difference ($p<0.05$) in *T. vivax* and *T. congolense* infections in males and females. *T. vivax* and *T. congolense* infections in males were 11.63% and 2.33% respectively while females had 8.57% infections with *T. vivax* only (Table 6).

Table 6: Comparison of infection rates in male and female flies

Glossina spp		Infection			Overall infection (%)	Total
		Uninfected	*T. vivax*	*T. congolense* forest		
Sex	Male	37	5 (11.63%)	1 (2.33%)	6 (13.95%)	43
	Female	32	3 (8.57%)	0 (0%)	3 (8.57%)	35
Total		69	8 (10.26%)	1 (1.28%)	9 (11.53%)	78

Although more males were infected than females with overall prevalence of 13.95% and 8.57% respectively, this difference was not significant

Infection rates for Kamunyu, Ngiluni, Kyusyani, Iviani and Nthunguni were 6.41%, 1.28%, 2.56%, 1.28%, and 0% respectively and ANOVA comparisons indicated that they were statistically different ($P<0.05$).

Least significance difference (LSD), a post hoc test for multiple comparisons showed that infection rates were only statistically different between Kamunyu and Ngiluni, Iviani and Nthunguni but not between Kyusyani and Kamunyu.

A correlation analysis between apparent densities and infection rates showed a strong positive relationship with a correlation coefficient of 0.95. For instance, Kamunyu area recorded the highest apparent density as well as the highest infection rates.

CHAPTER 4

DISCUSSION AND CONCLUSIONS

4.1 Trypanosome distribution and densities in Mtito Andei

This study found that there was variation in the distribution of *G. pallidipes* and *G. longipennis* in the area. The absence of tsetse in Kathekani which initially had been included as one of the study area, suggests that these flies may have shifted to other areas. This is probably due to higher human settlement, farming and deforestation which have destroyed their habitats.

G. longipennis was the only fuscan group of flies trapped in Mtito Andei and is known to occur in other Eastern Africa countries such Tanzania, Uganda, Sudan, Somalia and Ethiopia (Ford and Katondo, 1997). This species is found in drier areas more than any other tsetse of fusca group (Glasgow, 1963) and this is because the puparium contains large water reserves and a low permeability of its pupal membranes which helps it to survive harsh environmental conditions (Bursell, 1958). It is therefore suited in Mtito Andei as this is a semi arid area.The typical habitats for this fly is dry thorn bush riverline thickets near acacia woodlands and Mtito Andei is characterized by this type of vegetation. This species prefers shady sites of tree trunks, logs and underside of branches of multi stemmed trees as the resting sites (Langridge *et al.,* 1963).

G. pallidipes favor dense evergreen vegetation, heavier shade and humid habitats which are close to riverline thickets (Leak, 1999) and most of these flies were colllected in areas close to Mtito Andei River and Athi River. *G. pallidipes* is a morsitan group of flies which is widely distributed in Kenya and is a major vector of animal trypanosomiasis (Langridge *et al.*, 1963). It is also present in other Eastern African countries such as Uganda, Ethiopia and Somalia. Previous studies have shown that *G. pallidipes* might be a vector of *Trypanosoma b. rhodesiense* causing Human

African Trypanosomiasis (HAT) in Western Kenya and Busoga District of Uganda, as this parasite has been isolated from the fly (Ouma *et al*., 2011).

Among the mechanical vectors found in Mtito Andei, *Tabanids* were highly predominant over *Stomoxines* in all the trapping sites with the later being recorded only in Kyusyani area**.** This data shows that the species composition of mechanical flies is strongly dependent on site, substantial differences in climatic and environmental conditions as well as the eco-climatic area sampled. This observation is in agreement with Kone *et al*., (2011). Similar observations for *Tabanidae* have been made in other areas such as Brazil (Barros and Foil, 1999) and *Stomoxyinae* on La Re'union Island (Gilles, 2005 cited in Kones *et al*, 2011).

The presence of mechanical flies in the area cannnot be underestimated as they have been shown to mechanically transmit *T. vivax* and *T. evansi* (Desquesness *et al*., 2009) and can be responsible for periodical epidemic patterns in low tsetse density areas. Similar observations have been made in other areas such as Boromo in Burkina Faso where periodical epidemic patterns correspond to the annual peak of mechanical vectors such as *Atylotus aguestis* (Kone *et al*., 2011). Rahman, (2005) reported the same in tsetse free areas of Sudan where trypanosomiasis outbreaks coincides with high periods of *Tananids* and *Stomoxys* spp.

Despite the low mean AD recorded for tsetse in this area, it is possible that tsetse serve both as cyclical transmitters and reservoirs of the parasite while the mechanical vectors amplify it during high density periods. Other than amplifying the parasite, the mechanical flies can introduce it to other areas through immigration.

4.2 Trypanosome infection rates

This study determined the overall trypanosome infection rates of 11.53%. This result is in agreement with previous reports which showed the prevalence of trypanosome infections in the

field caught flies was typically low, at about 10% and was associated with interactions of various suppressive factors in the generally susceptible fly population (Askoy, 2003). Further, there are a number of barriers to both establishment and maturation process of trypanosomes and thus only a small proportion of these infections reach maturity (Maudlin and Welburn, 1989). It is also possible that these trypanosomes may lack differentiation triger or may be held back by the fly's immune response (Askoy, 2003). However, this study recorded a lower trypanosome infection rates as compared to Teso and Suba Districts of Western Kenya, where Thumbi *et al.,* (2008) has reported trypanosome infection rates of 41% and 29% respectively. In Coastal area of Kenya, Tarimo *et al.,* (1984) has reported trypanosome infection rates of 12.62%, 7.24%, 9.50% and 7.97% in Ukunda, Diani, Muhaka, Shimba hills and Mwalewa Coastal areas respectively in a study carried out between 1980 and 1982 using species specific primers.

The overall trypanosomes infection rates in *G. pallidipes* was higher than in *G. longipennis* and this difference was significant. Njiru *et al.,* (2004) studying *Glossina* infection rates in Coast Kenya has also reported comparable findings with infection rates of 5.7% in *G. pallidipes* and 0.2% in *G. longipennis* being recorded. However, the dominant trypanosome species was *T. congolense* forest type with a prevalence of 47.2%. The difference in infection rates in the two tsetse species in Mtito Andei could be due to variation in feeding preferences and host range differences (Bitew *et al.*, 2011). Jordan, (1974) reported that tsetse species respond differently to infections implying that there could be involvement of genetic differences. Within species infection rates may also vary due to individual host factors. For instance, the vulnerability of flies to *T. brucei* infections was shown to be due to maternally inherited features which are associated with the prescence of intracellular rickettsial like organisms (RLOs) (Maudlin *et al.*, 1989). Tsetse carrying these RLOs and other concurrent infections such as bacteria, fungi and

virus in the midgut were six times more likely to be infected with trypanosomes than those without. Moreover, the nutritional status of the tsetse at the time of infective bloodmeal can also affect their ability to acquire trypanosome infections (Kubi *et al.,* 2006).

The number of parasites available to infect tsetse, parasite infectivity to tsetse and the strain or subspecies have also been found to affect infection rates in tsetse (Jordan, 1974). Further, low vectorial capacity has also been reported and is more pronounced in *Palpalis* subgroup. The low vectorial competence in some *Glossina* spp is associated with higher levels of attacin expression in the proventriculus and midgut (Nayduch and Askoy, 2007).

Such a trait has also been reported in *G. pallidipes* and it could explain disparity of infection rates between *G. pallidipes* and *G. longipennis* (Welburn *et al*., 1989 ; Ingram and Molyneux, 1990)

T. vivax infection was the most prevalent and dominant in all trapping sites than *T. congolense* and this difference was significant. These results are comparable to Tesfaye, (2006) report where infection rates with *T. vivax* were 5.8%, 4.3% *T. congolense* and 0% with *T. brucei*. Similar findings were also reported in Luangwa Valley Zambia (Woolhouse *et al.,* 1994). Contamination can be precluded because contamination controls for each batch of experiments always gave negative results. The higher *T. vivax* infections can be ascribed to short developmental lifecycle of about 10 days which is entirely confined within the proboscis. For *T. congolense* and *T. brucei* development period has been estimated to be 14 days and 30 days respectively (Van den Bossche *et al*., 2010). Further, compared to proboscis, tsetse mid-gut is a hostile environment which contains mid-gut lectins which are able to kill trypanosomes in *vivo* (Welburn *et al*, 1989). Mid-gut also has proteolytic digestive enzymes, potent antimicrobial substances (Kaaya *et al*, 1987), prophenoloxidase cascade (Nigam *et al*, 1997), immune molecules such as agglutinins and

proteinous peritrophic matrix which help in expression of immune effector genes and mediating communication between the gut and the fat body contents when pathogens are encountered (East *et al,* 1980; Ibrahim *et al*, 1984; Ingram and Molyneux, 1990). The presence of all these immune molecules can prevent establishment and maturation of *T. congolense* and *T. brucei* as these parasites use midgut in part of their lifecycles hence the low infections rates detected. It is also known than *T. congolense* subtype parasites are generally low with low parasitaemia than those of *T. vivax* (Jordan, 1974 ; Stephen, 1986). It is therefore possible that such scanty parasites are rarely picked up by the flies during feeding which may account for the inability of the parasite to get established in the vector.

This study revealed more infection rates in males than in females. Samdi *et al*., (2011) in Nigeria, has also reported higher infection rates in males than females. Mihok *et al*, (1992) suggests that female flies should have higher infection rates than males as they live longer than males and thus they have higher chances of getting infection. However, this relationship has not been established. In contrast , Marlene and Mckean, (1996) explains that more males may be infected than females as they as they are involved in sex activities and competition than females. Whether more males or females are infected in field tsetse populations, this study found no statististical difference with variation in sex.

Apparent densities were strongly positively correlated with infection rates(r=0.95).This observation validates this parameter as an indicator of AAT transmission risk. Kone *et al*., (2011) reported comparable results in Mouhoun River basin, Burkina Faso where infection rates positively correlated to apparent densities (r=0.97).

4.3 Conclusion

The role of tsetse is important in maintaining the trypanosome parasite as they were dominant and prevalent in all trapping sites than mechanical flies. It is therefore possible that the sustainable reduction of tsetse, *Stomoxys* and *Tabanus* spp. from Mtito Andei Division can greatly reduce the prevalence of AAT. This study showed significantly higher trypanosome infection rates in *G. longipennis* than *G. pallidipes* with no difference in infection rates when males and females were compared. *T. vivax* was the most dominant and prevalent trypanosome species in all trapping sites. Mean apparent densities were also strongly positively correlated to infection rates implying that this parameter is a good indicator of AAT transmission risk.

4.4 Recommendations

1. Since both *T. vivax* and *T. congolense* are pathogenic in cattle and other ruminants, it would be important to conduct large scale integrated tsetse control in Mtito Andei by application of vector control strategies coupled with curative treatment of livestock as these animals could act as source infection to tsetse.
2. Further surveillance studies to determine trypanosome prevalence in livestock should be conducted in this area for effective trypanosomiasis control programs.
3. Entomological surveys should be conducted at different seasons to understand seasonal dynamics of the vectors and the associated trypanosomiasis risk. Data on seasonal variations of potential vectors can be integrated into epidemiological models to facilitate better understanding of the relative importance of cyclical and mechanical vectors in Mtito Andei.

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