MOLECULAR CHARACTERIZATION OF TRYPANOSOMES IN SMALL RUMINANTS AND PIGS FROM WESTERN KENYA

BY

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A thesis submitted in partial fulfillment for the award of the degree of Master of Science in Biochemistry of Kenyatta University, Nairobi, Kenya.

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DECLARATION

I declare that this thesis is my own original work and has not been presented for a degree in any other university or any other award.

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DEDICATION

This work is dedicated to my mother, Margaret Ng'ayo, brothers, Omondi, George, Richard and Gilbert and sisters, Mary, Joan and Josephine.

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ABSTRACT

Trypanosomosis a disease caused by protozoan parasites of the genus *Trypanosoma*. It is mainly transmitted by tsetse flies *Glossina* (*Glossinidae*; Diptera) in Africa. The disease causes over 40,000 deaths in human population and is also a major impediment to livestock production and economic development in many countries in sub Saharan Africa, where it is endemic. The role of small ruminants and pigs as reservoir of trypanosomosis is not well elaborated especially in Western Kenya. This study was designed to investigate trypanosomosis in small ruminants and pigs from Western Kenya, which is the only known active focus of sleeping sickness in Kenya. Microscopic and Polymerase Chain Reaction (PCR) based methods were used to determine diversity of trypanosomes infecting these livestock. Aneamia as a consequence of the disease in these livestock was assessed and expressed as packed cell volume (PCV). Further, isolates of *T. brucei* were and analyzed for the presence of Serum Resistant Associated (SRA) gene and genotyped using five microsatellites. Tsetse flies density and identity by species were also determined to assess their involvement in disease transmission. A total of 402 animals (255 goats, 95 sheep and 52 pigs) were sampled. Twenty one percent infections were detected by PCR, while 1.2% were detected by microscopy. PCR detection showed that 24% of the sheep, 21% pigs and 19.3% goats were infected. The main trypanosome species detected were *T. vivax* 6%, *T. simiae* 5.5 %, *T. congolense* 5.2 %, *T. brucei* 4.7 % and 17% of *T. brucei* stocks had the SRA gene. *Glossina fuscipes fuscipes* and *G. pallidipes* were caught together with stomoxys and tabanids. The results of genetic analysis revealed a diverse heterogeneity of genotypes. All microsatellite loci analyzed showed a high degree of size polymorphism. In particular one specific pattern clearly segregates the human pathogen *T. b. rhodesiense*. This study showed that PCR is a sensitive and reliable method of identifying infections, including those with no patent parasitaemia by microscopic methods. It was concluded that small ruminants and pigs in the area were infected and harbor various species of trypanosomes, including the human infective forms. Further, some of these livestock are infected with multiple genotypes of *T. brucei*, which may have implication on gene flow, and the transfer of genes that confer important traits, such as human infectivity.

CHAPTER ONE

INTRODUCTION

1.1 General introduction

Trypanosomosis is a term used to group together diseases caused by several species of the protozoan parasites of the genus *Trypanosom,* whose duration and symptoms vary with animal host and the trypanosome species. About 125 different species of trypanosomes have been reported from mammals alone, and are distributed among about 400 species of mammalian hosts (Hoare, 1972). In Africa, the human disease has been reported in 38 countries with some 55 million people at risk (Molyneux and Ashford, 1983; Kuzoe, 1993). The disease is cyclically transmitted by tsetse flies of the genus *Glossina.* In addition, mechanical transmission by biting insects or hemipteran blood-sucking bugs of the family *Reduviidae*, placental or blood transfusion occasionally occurs (Le Ray and Van Meirvenne, 1985).

Two disease groups can be distinguished; diseases due to typically African trypanosomes *T. vivax, T. uniforme, T. congolense, T. simiae, T. brucei, T. suis,* is generally termed Animal African Trypanosomosis (AAT) or Nagana. Surra is a trypanosomosis of Camelidae and equines caused by *T. evansi.* Dourine is a contagious trypanosomosis of equines caused by *T. equiperdium* (Shah-Fischer and Say, 1989). Human African Trypanosomosis (HAT) or sleeping sickness is caused by *T. b. rhodesiense* or *T. b. gambiense. Trypanosoma brucei gambiense*, the causative agent of the chronic or Gambian form of the disease is found mainly in Western and Central Africa. The more fulminant Rhodesian sleeping sickness is

restricted to eastern and southern Africa. American trypanosomosis or Chaggas disease caused by *T. cruzi*, is continental ranging from the Southern states of USA to Argentina (Le Ray and Van Meirvenne, 1985).

The epidemiology of trypanosomes in endemic areas, especially in sheep and goats was poorly understood (Griffin and Allonby, 1979 a). Initially it was believed that trypanosomosis hardly affected sheep and goats (Stephen, 1970). Goats have been observed to survive in an area of light to medium tsetse fly challenge (Griffin, 1978; Hecker *et al.*, 1993; Beably *et al.*, 1996). The apparent ability of goats to thrive under these conditions has been attributed to their agility, fecundity, and tolerance of trypanosome infections (MacLennan, 1970). Other studies indicate that not only are sheep and goats naturally infected by trypanosomes, but also that high mortality can occur (Masiga *et al.*, 2002). Trypanosomosis have also been shown to exert high cost of maintaining these livestock under high challenges (Irungu *et al.*, 2002). Pigs have been observed to be resistant to *T. vivax, T. congolense* and *T. brucei,* while they are susceptible to *T. simiae* (Hoare, 1972). They have been implicated as reservoirs of sleeping sickness caused by *T. b. gambiense* in Western Africa (Mehlitz *et al.*, 1982). Sheep, goats and pigs may, therefore, be important in the transmission of the disease to other livestock (Mahmuod and Eimalik, 1977).

In order to design rational control strategies, knowledge on the distribution of tsetse flies and detection of trypanosome species and subspecies across a study area is important (Mugittu *et al.*, 2001). In the past, many epidemiological studies of

trypanosomosis employed diagnostic criteria such as morphological appearance, vertebrate host range, development in the tsetse fly vector and mensural character described by Hoare (1972). Weaknesses of these parasitological criteria included; refractoriness of laboratory rodents to infection with some trypanosomes (Dirie *et al.*, 1993), inability to distinguish morphologically similar trypanosomes (Hoare, 1972), lack of specificity (Nyeko *et al.*, 1990) and inability to distinguish different subtypes especially among *T. congolense* isolates. The development of specific, sensitive and rapid DNA-based diagnostic methods have facilitated epidemiological studies of trypanosomes (Hide *et al.*, 1994; Solano *et-al.*, 1995). These techniques can rapidly detect and identify trypanosomes both in mammalian host and tsetse fly vector with high sensitivity and specificity (Ole-Moi Yoi, 1987).

The classification and distinction of trypanosomes within the *Trypanozoon* subgenus remained difficult. The five species and subspecies of this subgenus (*Trypanosoma b. brucei, T. b. rhodesiense, T. b. gambiense, T. evansi* and *T. equiperdum*) are morphologically identical and are generally classified according to host, disease type and geographical distribution. Exceptions to these criteria have been observed (Hide *et al.,* 1990) leading to the development of biochemical and genotypic techniques such as isoenzyme (Godfrey and Kilgour 1976), Restriction Fragment Length Polymorphism (RFLP) (Paindavoine *et al.*, 1986) and specific kinetoplast DNA probes (Mathieu-Daude and Tibaryrenc, 1994). The requirement for large amounts of parasite material was the major disadvantage for many of the above techniques especially for epidemiological surveys. The development of PCR based methods has

overcome these problems. Reliable molecular biological methods for genotyping *T. brucei* subspecies are now available (Masiga *et al.*, 2000). Recently genome analysis of *T. brucei* revealed a series of microsatellite (Biteau *et al.*, 2000) and minisatellite (MacLeod *et al.*, 1999) markers that are highly polymorphic and small enough to be amplified by trypanosome-specific PCR primers. They combine the convenience of PCR-based marker analysis with high levels of variability and sensitivity.

The ability of only certain subspecies of *T. brucei* to infect humans is important in the epidemiology of human sleeping sickness. In the past, the trait of human infectivity has been investigated by inoculating human volunteers with trypanosomes (Heisch *et al.*, 1958). This was replaced by *in vitro* tests (Rickman and Robson, 1970), which rely on the lyses of *T. b. brucei* by a trypanolytic factor in human blood (Raper *et al.*, 2001). The identification and characterization of human Serum Resistant Associated (SRA) gene from strain of *T. b. rhodesiense* (De Greef *et al.,* 1989) has solved the search for the molecular markers capable of distinguishing *T. b. brucei* and *T. b. rhodesiense* (Gibson *et al.*, 2001; Welburn *et al.*, 2001).

1.2 Justification

The economic impact of trypanosomes in sheep and goats was highlighted by the studies carried out by Griffin and Allonby (1979a; b). However, the role of small ruminants and pigs in the epidemiology of trypanosomosis is not yet well understood, especially in the study area. This area, which is endemic for sleeping

sickness as shown in Fig 1, is also an area with potential for increased productivity for both arable agriculture and livestock farming.

Sheep and goats provide milk, meat, skin fiber and manure for the local farmers as well as additional income. Pigs on the other hand are beneficial in terms of meat production and manure, they also act as a source of income to farmers. It is estimated that sheep and goats provide up to 30% of meat and 15% of milk in sub-Saharan Africa (Luckins, 1992). They also thrive in a wide range of ecological regions, often in conditions too harsh for the beneficial rearing of cattle. It is therefore important to understand the role of these livestock in the epidemiology of trypanosomes, which will lead to the development of more reliable control strategies.

Although it has been known for a long time that cattle are reservoirs of sleeping sickness (Onyango *et al.*, 1966), no data are available for sheep, goats and pigs in Western Kenya. However, in neighboring Uganda, *T. brucei* infections in small ruminants, have been reported (Katunguka-Rwakishaya, 1996), and in pigs on one smallholder farm in the proposed study area (Hide *et al.*, 1994).

Human African Trypanosomosis (HAT) is responsible for a significant disease burden in East Africa. To assess the risk presented to the human population by domestic livestock, it is vital to distinguish human infective *T. b. rhodesiense* from the non-human infective *T. b. brucei,* both of which may be present in livestock. In

the past, this was possible using laborious *in vitro* human serum growth inhibition tests combined with complex molecular analysis (Jenning and Urquhart, 1985). The identification and characterization of Serum Resistant Associated (SRA) gene isolated from *T. b. rhodesiense* and the now available PCR amplification procedures for this gene (Gibson *et al.*, 2001; Welburn *et al.*, 2001) provide an invaluable simple diagnostic test for *T. b. rhodesiense*. This will make it possible to identify the actual and potential reservoir hosts (especially sheep, goats and pigs), enabling targeted control of *T. b. rhodesiense* in the domestic livestock reservoir hence averting the public burden of HAT in East Africa.

An understanding of the extent and importance of genetic exchange in *T. brucei* populations will have practical implications. For example, sexual reproduction would facilitate the spread and inheritance of traits of medical and economic importance such as human infectivity, as well as generate a population with a high degree of genetic diversity. This would make the definition of common strains causing particular disease patterns difficult. The present work will therefore use microsatellite markers to assess the genetic exchange between *T. brucei* population.

1.3 Objectives

1.3.1 General objective;

The general objective of this study was to investigate the role of small ruminants (sheep and goats) and pigs in the epidemiology of trypanosomosis in Western Kenya.

1.3.2 Specific objectives;

1. To evaluate the prevalence of different species of trypanosomes in small ruminants and pigs.
2. To determine the genetic relationship between *T. b. brucei* and *T. b. rhodesiense* isolated from these livestock.

1.4 Hypotheses

- This study hypothesized that sheep, goats and pigs are not infected by different species of trypanosomes.
- There is no genetic relationship between *T. b. brucei* and *T. b. rhodesiense* isolated from small ruminants and pigs.

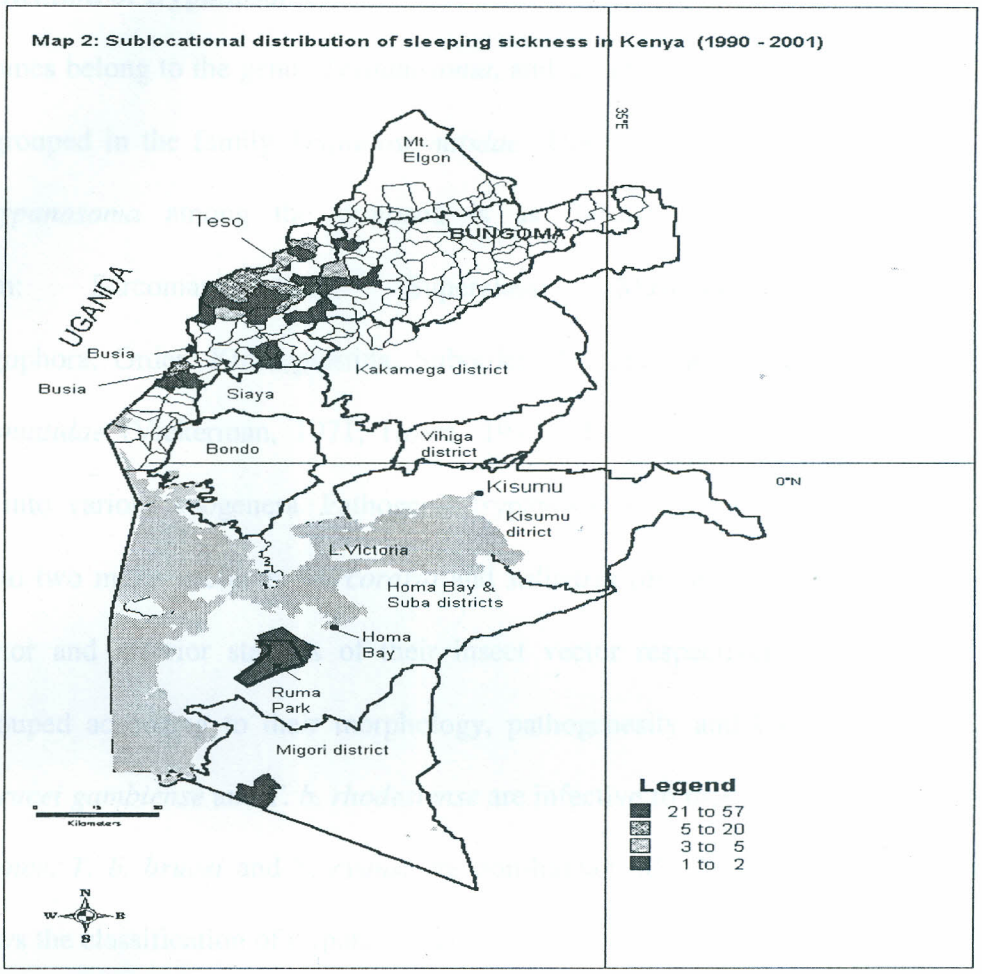

Figure 1: A map of Western Kenya showing the intensity of sleeping sickness. KETRI, 2001 poster.

CHAPTER TWO

LITERATURE REVIEW

2.1 Classification of trypanosomes

Trypanosomes belong to the genus *Trypanosoma*, and along with seven other genera they are grouped in the family *Trypanosomatidae*. The taxonomic position of the genus *Trypanosoma* among the protozoa is as follows: Phylum: Protozoa, Subphylum: Sarcomastigophora, Superclass: Mastigophora, Class: Zoomastigophora, Order: Kinetoplastida, Suborder: Trypanosomidae, and Family: *Trypanosomatidae* (Vickerman, 1971; Hoare, 1972). The genus *Trypanosoma* is classified into various subgenera. Pathogenic trypanosomes of mammals are also divided into two major sections: *stercoraria* and *salivaria* (those which develop in the posterior and anterior stations of their insect vector respectively). They are further grouped according to their morphology, pathogenesity and host range; *T. cruzi, T. brucei gambiense* and *T. b. rhodesiense* are infective to man while *T. vavax, T. congolence*, *T. b. brucei* and *T. evansi* are non-human infective (Hoare, 1972). Fig. 2 shows the classification of trypanosomes.

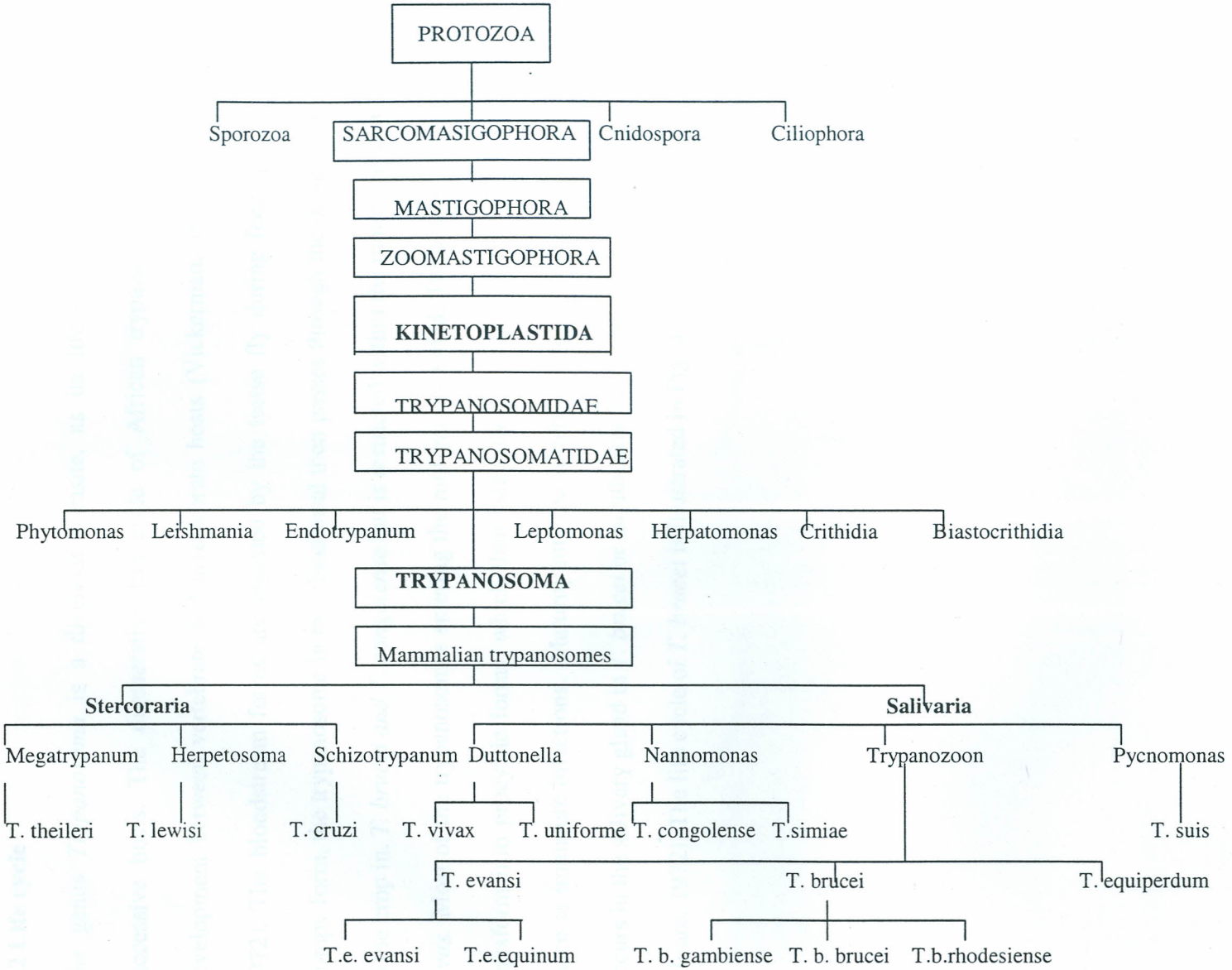

Figure 1: Classification of the order kinetoplastida and the genus *Trypanosoma* (Modified from Molyneux and Ashford, 1983).

2.2 Life cycle

The genus *Trypanosoma* is a dixenous parasite, as its life cycle requires two successive hosts. The digenerative life cycle of African trypanosome involves development between vertebrate and invertebrate hosts (Vickerman, 1971; Hoare, 1972). The bloodstream forms are ingested by the tsetse fly during feeding. The stumpy form, the trypanosome in the blood meal then passes through the oesophagus to the crop in, *T. brucei and T. congolense* but is restricted within the mouthpart in *T. vivax.* Most of the trypanosomes entering the mid-gut are killed. Those that survive transform into procyclic forms, which then establish themselves in the peritrophic space as immature infections. Maturity into the vertebrate infective metacyclic form occurs in the salivary gland in *T. brucei* or mouthparts for *T. congolense* and *T. vivax* (Hoare, 1972). The life cycle of *T. brucei* is illustrated in Fig. 3.

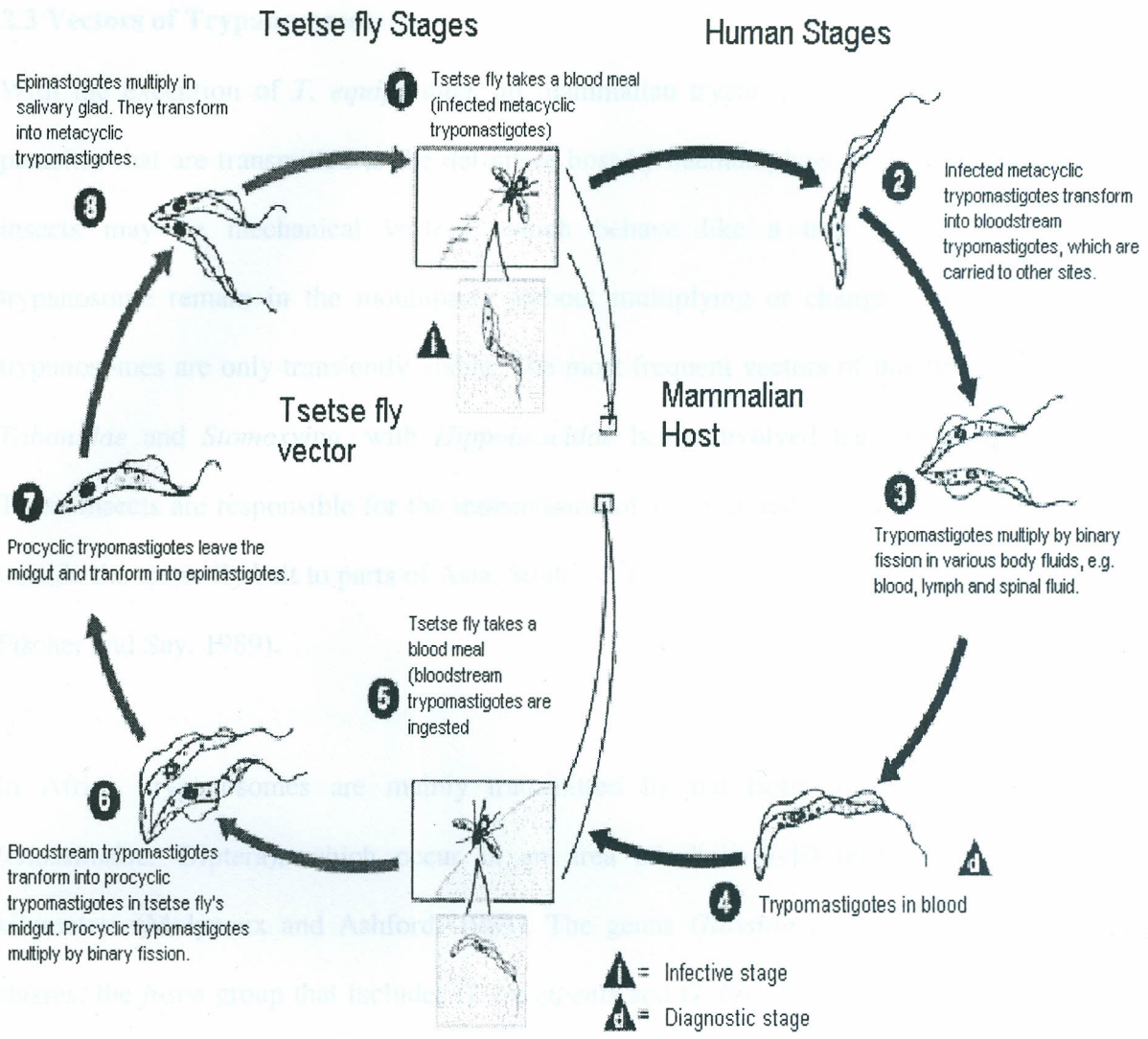

Figure 3: The developmental cycle of Trypanosome showing the infective and diagnostic stages (modified from Vickerman, 1986).

2.3 Vectors of Trypanosomes

With the exception of *T. equiperdum*, all mammalian trypanosomes are two-host parasites that are transmitted to the definitive host by haematophagous insects. The insects may be mechanical vectors, which behave like a true syringe, the trypanosome remain in the mouthparts without multiplying or changing and the trypanosomes are only transiently viable. The most frequent vectors of this type are *Tabanidae* and *Stomoxyina,* with *Hippoboscidae* being involved less frequently. These insects are responsible for the transmission of *T. vivax* and *T. evansi* in areas outside the tsetse fly belt to parts of Asia, South America and northern Africa (Shah-Fischer and Say, 1989).

In Africa, trypanosomes are mainly transmitted by the tsetse flies, *Glossina* (*Glossinidae:* Diptera), which occur in an area of about 7-10 million square kilometers (Molyneux and Ashford, 1983). The genus *Glossina* is divided into 3 classes; the *fusca* group that includes *G. longipenis* and *G. brevipalpis* dwells in the forest. The *morsitans* group that includes *G. m. morsitans, G. pallidipes* and *G. swynnertoni*, dwell in the savanna and the *palpalis* group that include *G. fuscipes*, *G. palpalis* and *G. trachninodes* have a riverine distribution (Maudlin, 1989). About 145 million head of cattle, 253 million other domestic livestock and 260 million people live in the tsetse fly belt (Griffin, 1978; ICIPE, 2001).

2.4 Impact of trypanosomosis in Africa

Throughout the African continent, trypanosomosis poses a major constraint to livestock and crop production. It has both direct and indirect impacts on agricultural production systems. The most direct economic impact is on cattle, where loses amount to an estimated 3 million deaths annually, affecting mainly the young stock (FAO, 1976). The infection impacts indirectly when sick animals produce lower milk and meat yields, and also experience reproductive losses. Estimates put the direct monetary losses at US$ 0.6 to 1.2 billion annually. Other valuable livestock such as camels also suffer from the disease (ICIPE, 2001). However, little information on the economic impact of the disease in small ruminants is available (Griffin and Allonby 1979b; Kanyari *et al.*, 1983; Masiga *et al.*, 2002 and Irungu *et al.*, 2002). The existence of numerous variables whose magnitude cannot be quantified hinders the accurate assessment of economic losses attributable to the disease in small ruminants (Jordan, 1986).

Crop production is indirectly affected by trypanosomosis in terms of lost draught power for ploughing, less manure and better access to markets through the adoption and use of cattle-drawn carts (DFID, 2001). The disease also affects land use, in areas of high tsetse fly challenge susceptible animals cannot be reared, although it is still possible to practice limited mixed farming where the challenge is low. For example, in Southwest Ethiopia, tsetse flies and trypanosomosis infect the lowlands restricting people to live and farm in the highlands. The consequence of this is high land pressure as compared to the fertile valley and lowlands, which are sparsely

populated (DFID, 2001). On the contrary, Human African trypanosomosis (sleeping sickness) is on the increase in Africa, with an estimated 300,000 new cases per year (WHO and Aventis, 2001). This report further states that the disease has great influence on the economy, health, workforce and food production and distribution in Africa.

2.5 Control of Trypanosomosis

Since the recognition of the adverse effects of trypanosomosis were determined, several methods have been developed to control it is spread. Among the most commonly used methods are discussed below.

2.5.1 Vector control

Techniques aimed at reducing contact between the vector *Glossina* and potential hosts have developed concomitantly with the increasing knowledge of the biology of the vector and the development of insecticides. The goal in controlling the vector is to maximally reduce its densities in areas populated by susceptible hosts (Wery, 1990; DFID, 2001). During the pre-insecticide era, techniques involved measures directed either against the fly's resting sites or the use of attractants that aggregates and retained the flies such as black screens impregnated with glue (Martin, 1920). The insecticide era initially involved the chlorobenzene derivative, then halobenzene insecticides and finally synthetic pyrethroids. These were extensively used by aerosol application from airplanes over savanna regions, and from helicopters in forested areas. Due to the problems associated with the use of massive insecticides (the appearance of resistance, toxicity to other fauna), spraying was selectively done

on the resting sites of tsetse flies. In endemic zones this method had produced encouraging results using considerably less quantities of insecticides, with the additional reduction in the risk of resistance development by the flies (DFID, 2001). Techniques using traps and targets, sterile insect technique (SIT) and insecticide treated cattle have been used considerably to disrupt the transmission cycle in Africa (Laveissiere, 1988).

2.5.2 Treatment

This strategy for controlling trypanosomosis involves the use of drugs to combat the parasite itself. Therapeutic drugs such as suramin, pentamidine, diminazene aceturate, melarsoprol, nifurtimox and eflornithine have been used to kill the parasite once they have infected an animal. Whilst the use of drugs proved to be very effective when first introduced, resistance is now becoming a significant problem. Besides, the drugs are toxic and the margin between a safe and lethal dose is small. Despite this, farmers use 35 million doses per year at a cost of US$ 1 each to protect about 10 million cattle (Dumas *et al.*, 1999; DFID, 2001).

2.5.3 Prophylatic strategies

Prophylysis concerns all measures that can be undertaken to prevent the onset or transmission of a disease. In trypanosomosis, these prophylatic measures involve action taken against the vector population and health care for both human and animal populations. Two management approaches are possible in the human population; firstly, routine screening for the disease and treatment of infected persons, and

secondly, drug prophylysis of exposed populations (Mattern, 1968 Stangehellini *et al.*, 1994).

2.5.4 Development of vaccines

Historical studies on vaccine development has proved unreliable in the field due to antigenic variation (Hide, 1999). Trypanosomes have over 100 possible surface coat proteins (Van der Pleog *et al.*, 1982) and many more can be regenerated by recombination. It is, therefore, generally accepted that a vaccine is unlikely to be available in the near future (DFID, 2001; Hide, 1999).

2.5.5 Tolerant animals

The use of animals that are inherently less susceptible to the effect of the disease or trypanotolerant and are able to remain relatively productive even when infected is a method that allows keeping of animals productively in the infested areas. There are about 10 million such cattle, mostly in West Africa, although they have not been widely adopted in other regions, possibly because they are not as large as the preferred Zebu-type cattle (ICIPE, 2001). These tolerant breeds include N'dama and Muturu cattle that have been bred in areas endemic to trypanosomosis and showed considerable resistance (Weits, 1970). In East Africa, the Orma Boran and the Maasai Zebu have been shown to be trypanotolerant. The larger Orma Boran has greater potential for introduction in other endemic areas from southeastern Kenya where it is traditionally kept (Mwangi *et al.*, 1998). Investigations on the

trypanotolerant quality of the small ruminants have received little attention (Griffin and Allonby, 1979a; FAO, 1976).

2.6 Trypanosomosis in sheep, goats and pigs

For decades trypanosomosis has been recognized as the greatest obstacle to increased productivity of livestock in Africa, and has prevented vast areas of potentially good land to be utilized for grazing. However, until recently limited research on the effect of trypanosomosis has been recorded in sheep and goats (Griffin, 1978; Masiga *et al.,* 2002). Stephen (1970) writes that sheep and goats are not often infected with trypanosomes under natural conditions. MacLennan (1970) dismisses the disease in small ruminants as unimportant. Goats were shown to survive in areas with light to medium tsetse fly challenge (Bealby *et al.*, 1996).

A survey conducted in Lambwe valley, Kenya, showed that 5% of sheep and 2.1% of goats were infected with pathogenic trypanosomes (Robson and Askar, 1972), while in a separate study in Kiboko, Kenya, showed that 42% of sheep and 44% of goats were infected (Zwart *et al.*, 1973). Leach (1973) also reported heavy loses among sheep and goats infected with trypanosomes, while Mackenzie *et al* (1975) observed *T. simiae* as infecting sheep naturally. Other studies by Griffin and Allonby (1979b) reported infection rates of up to 80% and 50% in sheep and goats respectively, with *T. congolense*. They further reported that while sheep and goats were easily infected with *T. congolense* they were rarely infected with *T. vivax*. The reports of natural *T. brucei* infections in sheep and goats are however rare. Studies

have now showed that *T. congolense, T. vivax, T. brucei,* and *T. simiae* infect sheep and goats (Makumyaviri *et al.,* 1989; Fakae and Chiejina, 1993, Katunguka-Rwkishaya, 1996; Kayanga *et al.*, 1997; Masiga *et al.*, 2002). More recent surveys and epidemiological studies of the diseases in sheep and goats however, indicate that these animals can be, and are often, severely affected by the disease (Masiga *et al.*, 2002). The disease in these animals may therefore be of greater economic importance (Irungu *et al.,* 2002).

The situation of trypanosomosis with regards to pigs is probably similar to those of ruminants but is less well documented (Murray and Gray, 1984). Domestic or wild pigs can be parasitized but are not susceptible to *T. brucei* or *T. congolense*. They are rarely infected by *T.* vivax. Pigs are however, susceptible to *T. simiae* through out tropical Africa, and to *T. suis* in East Africa (Shah-Fisher and Say, 1989). Survey done in West Africa showed that pigs are infected and harbor *T. b. gambiense,* the parasite that causes Gambian form of sleeping sickness (Mehlitz *et al.*, 1982). Other studies by Makumyaviri *et al* (1989); Asonganyi *et al* (1990); Hide *et al* (1994) and Katunguka –Rwakishaya (1996), have observed high infection rates and hence mortality in pigs as compared to sheep and goats.

2.7 Identification of trypanosomes

To measure accurately the challenge of trypanosomosis to livestock and man, it is important to distinguish between different trypanosome species and subspecies. The assessment of the extent of geographical distribution of each species would provide a

basis for their rational control. This section examines the methods available for the identification of African trypanosomes in tsetse flies and the mammalian hosts.

2.7.1 Identification in vertebrate host

In view of the fact that clinical signs are not diagnostic on their own, it becomes mandatory to confirm infection by direct or indirect demonstration of the presence of trypanosomes. The standard approaches for parasitological diagnosis is meticulous microscopic examination of lymph aspirate and blood (Murray *et al.*, 1977; 1979). For most practical purposes, trypanosomes are identified in blood smears, by their morphology and movement by microscopic examination of wet and stained blood films. For example, *T. vivax* is a large trypanosome characterized by rapid movement in wet blood film. In stained blood films, it exhibits a large terminal kinetoplast. The flagellum is present while an undulating membrane is not well developed (Hoare, 1972). The subgenus *Nannomonas* (*T. simiae* and *T. congolense*) are small trypanosomes with sluggish movement and are monomorphic. They lack a free flagellum and are often observed adhering to red blood cells (Hoare, 1972). *Trpanosoma brucei* is large and active, but lingers in the microscope field longer than *T. vivax*. The subgenus *Trypanozoon* is composed of subspecies that are morphologically indistinguishable. Morphological characteristics are therefore, of limited value in distinguishing members of this subgenus, as no consistent differences are discernible (Godfrey, 1977).

2.7.2 Immunological methods

Trypanosomes have a complex antigenic profile and elicit production of a large spectrum of antibodies in the host. Since clinical symptoms are highly variable and parasitological methods laborious and with limited sensitivity, diagnosis is aided by serological tests. Serological screening of the entire population in endemic areas has become a routine strategy in field surveys of sleeping sickness (Dumas *et al.*, 1999). The trypanosomes evolving in the patient, including the initial metacyclic forms inoculated by a tsetse fly, are coated with a layer of variable surface glycoprotein (VSG). Each trypanosome has genetic potential of expressing several hundreds of distinct VSGs, corresponding to a repertoire of as many variable antigen types (VATs) (Vickerman, 1978; Barry and Turner 1991). Serotyping of VATs is currently done by immune lysis or direct agglutination tests using cloned populations and specific antisera (Isharaza, 1990). Evidence that each clone seems to have a distinct antigenic repertoire, the expression of which is not driven by the host immune system (Vickerman, 1978), has led to the development of serological methods of detection and identification. Parasites with the same repertoire belong to the same serodeme and are assumed to be homologous (Shah-Fischer and Say, 1998). The release of soluble antigens into the blood and other tissue fluids, as a response to lysis by the host, led to the development of detection systems for the trypanosome antigen in the peripheral blood and cerebrospinal fluid (CSF) of patients (Nantulya, 1988). This technique has also been used for detection of trypanosomosis in livestock (Nantulya and Linduist 1989)

2.7.3 Identification in tsetse vector

After several futile attempts to demonstrate that tsetse flies were vectors of trypanosomes, the observation of development of *T. brucei* in *G. fuscipes* showed that a developmental cycle in the fly was necessary (Hoare, 1972). This was followed by the description of the behavior of other mammalian trypanosomes in the vector (Bruce *et al.,* 1914). These differences were subsequently exploited by Lloyd and Johnson, (1924) who used the developmental sites in the fly as a method for distinguishing trypanosomes.

The dissection method of Lloyd and Johnson (1924) is still widely used in epidemiological surveys. The development of *T. vivax* is confined to the proboscis while *T. congolense* and *T. simiae* develop in the midgut. *T. brucei* ssp complete development in the salivary glands. Immature infections found only in the midgut are difficult to identify by dissection and microscopy alone. The occurrence of mixed infections in the fly also complicates the interpretation of dissection data (Godfrey, 1966). Recent advancements in biochemical methods of parasite characterization have led to the development of a wide range of technique for identification of trypanosomes in the vector. Isoenzyme analysis (Gashumba *et al.*, 1986), recombinant DNA probes (Majiwa *et al.,* 1994), monoclonal antibody based dot-ELISA (Bosompem, 1993; Ouma *et al.*, 2000) are some of the techniques developed and applied in identification of mature and immature trypanosomes in the tsetse fly.

2.8 Biochemical characterisation

2.8.1 Molecular karyotype

Molecular karyotypes are produced by size fractionation of chromosomal DNAs by pulsed field gel electrophoresis (PFGE) (Schwartz and Canter 1984). Trypanosomes of the *T. brucei* species have over 100 mini (Gibson and Borst, 1986) and at least 11 large chromosomes, in addition to several medium sized chromosomes. In practice, karyotypes are highly variable the results are more useful for identification of individuals by fingerprinting, than characterization of populations. However, some karyotpic features have been found to be characteristic of group 1 *T. b. gambiense* minichrosomes (Kanmogne *et al.*, 1997).

2.8.2 DNA hybridization

2.8.2.1 Satellite DNA

Satellite DNAs are a common constituent of eukaryotic genomes and generally appear to be transcriptionally inactive. These sequences were first described in trypanosomes as a tandem array of a basic repeat unit in *T. brucei* and *T. cruzi* (Sloof *et al.*, 1983). Initially, satellite DNA was described as DNA that banded separately from the bulk of the genomic DNA following cesium chloride gradient centrifugation with density depending on the content of guanine and cytosine (G & C) relative to adenine and thiamine (A &T). Recently, satellite DNA has been used to describe any tandemly arranged repeated sequences irrespective of whether or not they are separable by density gradient centrifugation (Beridze, 1986). When cleaved with restriction enzyme, tandemly repeated sequences generate a characteristic

ladder of fragments that are multiples of a basic repeat unit. Satellite DNA accounts for about 12% of total genomic DNA of *T. brucei* and 9% for *T. cruzi* (Borst *et al.*, 1981; Lanar *et al.*, 1981).

One of the consequences of using satellite DNA probes for identifying trypanosomes has been the re-evaluation of the current status of taxonomy within the genus *Trypanosoma*. While five morphologically indistinguishable species and subspecies are recognized within the subgenus *Trypanozoon*, they all share the same satellite DNA sequence (Gibson *et al.*, 1988). In contrast, six groups within the subgenus *Nannomonas* can be differentiated by unique satellite DNA (Masiga *et al.*, 1992). Only three of these presently have species status.

It is of value to compare satellite DNA sequences from different species and subspecies of trypanosomes since they are analogous genetic elements. They represent about 10% of the genome of the parasite, and are located predominantly on minichromosomes (Gibson *et al.*, 1988) and may offer a clue to the phylogenetic relationships of different parasite (Masiga *et al.*, 1996).

2.8.2.2 Kinetoplast DNA

The order Kinetoplasitida is characterized by possession of an unusual mitochondrial DNA that is organized into a large network of catenated minicircles and maxicircles (Simpson and Simpson, 1980). Kinetoplast DNA (kDNA) consists of 40-50 maxicircles, which are homogeneous in base sequence. They contain genes for

ribosomal RNA and proteins necessary for mitochondrial biogenesis and are thus analogous to the mitochondrial DNA of other eukaryotes (Simpson and Simpson, 1980). Although Restriction Fragment Length Polymorphism (RFLPs) of maxicircle have been used to study diversity in *T. brucei,* it is acknowledged that they are too conserved to be used realistically to distinguish between subspecies of *T. brucei* (Gibson and Wellde, 1985).

There are 5000-10,000 minicircles per kinetoplast (Staurt and Feagin, 1992). Minicircles of cyclically transmitted trypanosomes are heterogeneous in base sequence, except for a highly homologous region of 120 bases, which appears to be conserved (Borst *et al.*, 1981). The minicircles code for small RNA molecules known as guide RNAs that contain sequence information for editing maxicircle transcripts (Pollard *et al.*, 1990).

2.8.3 Restriction Fragment Length Polymorphism (RFLP)

Restriction Fragment Length Polymorphism (RFLP) analysis requires large numbers of trypanosomes and is more costly in terms of materials and reagents. Both nuclear and kinetoplast (mitochondrial) DNA have been used (Dumas *et al.*, 1999). The purified DNA is digested by various restriction enzymes. The resulting DNA fragments are separated by gel electrophoresis and visualized with ethidium staining. The are about 20 kilo base (kb) kDNA maxicircles which are homogeneous in sequence and thus appears as one or more discrete fragments after electrophoresis, depending on the restriction enzyme used. The analysis of maxicircles from *T.*

brucei species stock (*T. b. gambiense* and *T. b. rhodosiense*), showed little variation except for 2 subgroups of *T. b. brucei* (Kiboko and Sindo) that had distinctive RFLP (Gibson and Wellde, 1985). The minicircles of *T. brucei* are heterogeneous in sequence. Linearization by a single cut produces 1-kb fragments. The extreme heterogeneity of *T. brucei* minicircles makes them less useful for identification purposes than those of *T. evansi.*

2.8.4 Isoenzyme electrophoresis

Isoenzyme analysis was developed in the 1950's and has been widely applied to characterize of trypanosomes (Godfrey and Kilgour, 1976). The technique is comparatively cheap and robust, and given a good choice of enzymes, its usefulness for characterization purposes have not been significantly superseded by DNA-based techniques (Otieno *et al.,* 1990). Isoenzyme analysis is performed on highly concentrated extracts of cytoplasmic proteins and thus requires large number of trypanosomes. Multiple molecular forms of an enzyme (isoenzymes) give rise to multiple bands on a gel if the molecules are in motility. In practice, only about a quarter of amino acid substitutions give rise to electrophoretic changes (Harris and Hopkinson, 1976). A range of different enzymes therefore, needs to be screened and the use of 10-20 enzymes, which give clear and reproducible results, is recommended (Mathieu-Daude and Tibayrenc, 1994). Given that the metabolism of insect and bloodstream forms of trypanosomes is different, the isoenzyme bands seen may differ in number, mobility or intensity depending on the life cycle used, this is not a problem for DNA-based characterization (Kilgour, 1980).

2.8.5 Polymerase Chain Reaction (PCR) based techniques

The analysis of nucleotide sequences has been revolutionized by the development of polymerase chain reaction (PCR) (Saiki *et al.*, 1988), a technique that allows the *in vitro* amplification of DNA. Polymerase Chain Reaction provides a number of techniques for detecting polymorphism that do not require the use of radioactive material and can be easily adapted to most laboratories. They also provide flexibility in the collection of materials to be amplified and fewer DNA materials are required (Innis and Gelfarnd, 1989). With African trypanosomes, PCR amplification of satellites sequence from different species has been used to detect trypanosomes both from infected blood (Moser *et al.,* 1989) and tsetse flies (Masiga *et al.*, 1992). It has been shown that a PCR signal can be obtained from DNA or trypanosome extracts diluted to less than a single genome equivalent (Moser *et al.*, 1989; Masiga *et al.*, 1992; Solano *et al.,* 1995)

2.8.5.1 Random Amplified Polymorphic DNA (RAPD)

Random Amplified Polymorphic DNA is a PCR-based technique, which uses arbitrary primers to amplify fragments from genomic DNA templates. This primer has a chance of finding its complimentary sequence, roughly every million bases in any genomic DNA sample. Thus no sequence information about target DNA is necessary and experimentation will show which primers produce suitable amplification. Individual primers yield a fingerprint consisting of 10 or so bands while use of several primers rapidly generate large volumes of characterization data for strain comparison (Waitumbi and Murphy, 1993). The RAPD technique is quick

and can be easily used for characterization of trypanosome isolates. The results are reproducible and agree with those derived from isoenzyme or RFLP studies. It allows analysis of large number of individuals at a reasonable expense and does not require prior DNA sequence information nor involve radioactivity. Polymorphisms are easily detected and many markers can be screened on a single run (Mathieu-Daude and Tibayrenc, 1994). The main disadvantage is that the target sequences amplified are unknown thus the data is not open to interpretation in terms of individual loci and alleles. In addition, contamination of trypanosome DNA with DNA from other sources must be avoided, since RAPD primers are not specific and that the method depends on dominant markers (William, 1982). The technique has been applied in identification, paternity testing, purity testing, detection of genetic diversity and relatedness (Mathieu-Daude and Tibayrenc, 1994).

2.9 Genotyping of *Trypanosoma brucei*

Genotype is the genetic make-up of an individual, including traits not expressed in the phenotype since they are determined by recessive alleles in an individual who is heterozygous at these loci (MacLean 1990). In recent years a wide variety of biochemical and molecular typing systems have been employed in the study of parasite diversity aimed at investigating the level of genetic and delineating relationships between different species and subspecies. However, such methods have failed to differentiate between two of the classically defined subspecies of *T. brucei*, the human infective *T. b. rhodesiense* causative agents of African sleeping sickness,

and the non-human infective *T. b. brucei*. This has led to the hypothesis that *T. b. rhodesiense* is a host variant of *T. b. brucei* (MacLeod *et al.*, 2000).

2.9.1 Amplified Restriction Fragment Polymorphism (AFLP)

Amplified Restriction Fragment Polymorphism (AFLP) detects DNA polymorphisms at specific restriction enzyme sites and enables these polymorphisms to be detected at multiple independent restriction sites simultaneously. It is applicable to the detection of variation in DNA of any origin and complexity. This is a technique for profiling the entire genome. Initially developed for analysis of plants (Vos *et al.*, 1995), it has now been adapted to parasites (Masiga *et al.*, 2000). The method is based on the selective amplification of sets of restriction fragments from genomic DNA, which is cut by restriction enzymes and double stranded adaptors are used to amplify the fragments. PCR primers designated to match the sequence of the adaptors are used to amplify the fragment (Vos *et al.*, 1995). DNA amplification products are electrophoresed, usually through denaturing gels to produce a profile. These profiles may be used as a tool for determining the identity of a specific DNA sample or to assess the relatedness between samples. They can also be used as the source for genetic markers to generate linkage maps or to identify molecular markers linked to phenotypic traits and or genetic loci (Weising *et al.*, 1995; Heolzel, 1998; Mueller and Wolfenbager, 1999). This technique does not require prior sequence knowledge, and the use of high stringency PCR makes it reliable and reproducible. AFLP markers are dominant where heterozygous and homozygous loci both have a band present (Vos *et al.*, 1995).

2.9.2 Hypervariable Minisatellites

Minisatellites are short tandem regions of eukaryotic genomes, many of which show high levels of allelic length variation due to differences in the number of repeat units (Jeffrey *et al.*, 1985). These loci are highly informative genetic markers and have been used extensively for individual identification in man, paternity testing (Barrett *et al.*, 1997), linkage mapping (Nakamura *et al.*, 1987) and in studying the structure of natural populations (Hoelzel, 1998). They have also been used in the analysis of parasite genomes such as the characterization of *T. brucei* (MacLeod *et al.*, 2000). However, alleles at minisatellite loci tend to vary not only in terms of the number of repeat units that they contain, but also in the precise sequence of each repeat unit. The development of minisatellite variant repeat (MVR) mapping by PCR as a digital approach to DNA typing (Jeffrey *et al.*, 1985) accesses this variation, while overcoming many of the drawbacks of minisatellite length analysis. The use of locus-specific primers to amplify minisatellite markers enables genotyping trypanosomes even when contaminated with large quantities of DNA, in addition to allowing the analysis of small quantities of DNA. Due to their high level of polymorphism, minisatellite markers are particularly important in determining variation between populations due to geographical barrier, defining mating systems and detecting heterogeneity within a sample (MacLeod *et al.*, 1999).

2.9.3 Microsatellites

Microsatellites or simple sequence repeats (SSRs) are short tandemly repeated sequence motifs consisting of repeat units of 1-6 base pair (bp) in length (Hoelzel, 1998). These microsatellites are ubiquitous among eukaryotes. Most of them occur interspersed in the genome, depending on the repeated motif and species considered. They are highly polymorphic DNA markers with discrete loci and co-dominant alleles. They mostly arise from slippage mechanisms during replication or DNA repair according to the stepwise mutation model (Biteau *et al.*, 2000). The mutation rate of SSRs and thus the variability is higher than that of isoenzyme or RFLP markers (Weber and Wong, 1993). Thus, SSR microsatellite analysis is highly suitable for studying the relationships between closely related species or within populations of the same species. They are highly variable, reliable, reproducible and give co-dominant makers, making them the makers of choice in behavioral ecology, since they allow determination of paternity and kinship. The main disadvantages are that the development cost is high and polyacrylamide gel electrophoresis (PAGE) is preferred. Microsatellites can also be used to analyze samples having only minute DNA quantities or highly degraded DNA. For example, microsatellites have been amplified from skeletal remains, museum specimens, hair roots and feaces (Hoelzel 1998). They are now becoming increasingly important in studying the structure of natural populations (Biteau *et al.*, 2000).

2.10 Serum resistance-associated (SRA) gene

The trypanosome lytic factor (TLF) is a primate specific innate defense mechanism that restricts the host range of African trypanosomes. *Trypanosoma b. rhodesiense* is resistant to the cytolytic action of TLF (Hawking, 1977). The *T. brucei* species consists of three sub-species; *T. b. rhodesiense*, *T. b. gambiense* and *T. b. brucei*. The first two sub-species are human infective forms, while the last sub-species is non-human infective. It is lysed upon exposure to cytotoxic factor in human serum. *T. b. rhodosiense* can however, occur as a serum-resistant (R) and as serum-sensitive form (S). The serum resistance associated (SRA) mRNAs which is absent in several isolates from *T. b. brucei* and *T. b. gambiense*, indicates that more than one mechanism may be involved in the phenomenon of serum resistance in the *T. b. brucei* group. Neither *T. evansi*, nor *T. equiperdum* expresses the SRA-transcript. The transcript of the resistant (R)-specific transcript is not maintained during the life cycle of *T. b. rhodesiense.* In the procyclic forms the SRA-mRNAs are no longer present (Rifkin *et al.*, 1994). Potentially then, the SRA gene holds the key to identification of *T. b. rhodesiense* (Welburn *et al.*, 2001; Gibson *et al.*, 2001). The resistance towards normal human serum is a labile character and is determined by the environment in which the parasite lives (De Greef *et al.*, 1994).

The differentiation of *T. b. rhodesiense* and *T. b. brucei* has always been difficult and the only absolute method has been the infection of human volunteers. However, the blood incubation infectivity test (BIIT) of Rickman and Robson (1970) has generally been accepted as a reasonable alternative. More recently Godfrey (1979)

and Gibson *et al.*, (1980) have reported differences in the isoenzyme patterns of various *T. brucei* stabilates. This has however, failed to differentiate positively *T. b. brucei* from *T. b. rhodesiense* (Jenning and Urquhart, 1985). The search for molecular markers that distinguish *T. b. rhodesiense* from *T. b. brucei* and *T. b. gambiense* has been unrewarding until now. This has been achieved with the identification and characterization of human serum resistance associated (SRA) gene described from *T. b. rhodesiense* (De Greef *et al.*, 1989).

CHAPTER THREE
MATERIALS AND METHODS

3.1. Study area

This project was undertaken in two administrative districts of Western Kenya, Busia and Teso. Rukada village in Busia district, and Amoni, Amase, Ongariama and Obuchun villages in Teso district, were selected on the basis of recent cases of sleeping sickness. These villages were geo-referenced at the sampling sites and their locations are shown in fig. 4. Teso is bordered by Bungoma district to the north, Kakamega district to the east and Uganda to the West. Busia lies on the shores of lake Victoria where it shares a border with Uganda and Siaya district to the east. The two districts lies between latitude 0^0 and 0^0 250 north and longitude 33^0 54 East. They cover an area of 1819 square kilometers; 137 square kilometer of this is under permanent water surface, mainly composed of lake Victoria. The altitude varies from 1130m to 1375m. The mean annual rainfall is 1500mm divided between two seasons, while annual maximum temperature range from 26^0 C to 30^0 C and minimum temperature varies from 14^0 C to 18^0 C. The areas studied are dominated by mixed farming. Farmers keep cattle, sheep, goats and pigs (Republic of Kenya, 1997-2001). Trypanosomosis is endemic in Western Kenya and the area is infested with important tsetse fly species, mainly *G. f. fuscipes* and *G. pallidipes*. The vegetation is dominated by various species of indigenous and exotic trees that belong to the tropics and small shrubs providing abundant browsing throughout the year. Various grass species are also numerous but depend on rainfall. Various seasonal and permanent rivers are also found that are important for farming activities.

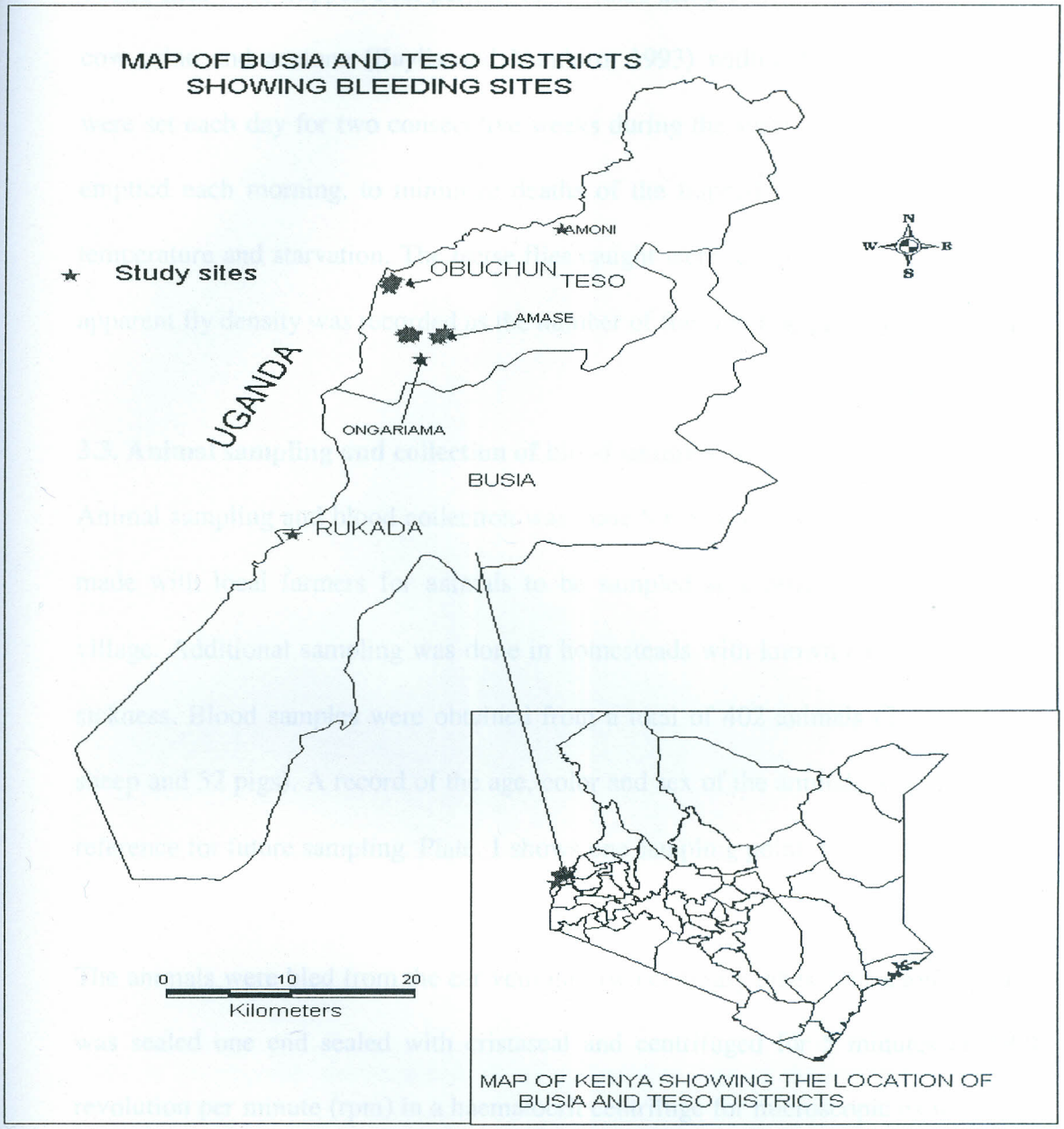

Figure 4: A map showing villages in which sampling was done. The study sites shown are the exact georefrenced positions where sampling was done.

3.2 Trapping of tsetse fly and their densities

Tsetse flies were trapped over periods of 48 hours using 6 biconical traps baited with cow urine and acetone (Baylis and Nambiro, 1993) within the study sites. Traps were set each day for two consecutive weeks during the study period. Catches were emptied each morning, to minimize deaths of the trapped tsetse flies due to high temperature and starvation. The tsetse flies caught were identified and counted. The apparent fly density was recorded as the number of flies per trap per day (FTD).

3.3. Animal sampling and collection of blood samples

Animal sampling and blood collection was done for two weeks. Arrangements were made with local farmers for animals to be sampled at a central location in each village. Additional sampling was done in homesteads with known cases of sleeping sickness. Blood samples were obtained from a total of 402 animals (255 goats, 95 sheep and 52 pigs). A record of the age, color and sex of the animals was made on a reference for future sampling. Plate. 1 shows one sampling point in Amoni village.

The animals were bled from the ear vein into two capillary tubes. One capillary tube was sealed one end sealed with cristaseal and centrifuged for 5 minutes at 9000 revolution per minute (rpm) in a haematocrit centrifuge for microscopic examination of the buffy coat and recording of Packed Cell Volume (PCV). The second capillary was used for preparation of a PCR template. All animals positive by microscopy were bled from the jugular vein and blood collected into 2 ml vials containing EDTA and transported in cool boxes to KETRI for analysis using PCR.

Plate 1: Photograph shows sheep, goats and pigs in one of the sampling point in Amoni village.

3.4 Parasitological examination of blood

The blood in the capillary tubes with one end sealed with cristaseal was centrifuged for 5 min at 9000 r.p.m in micro haematocrit centrifuge. The PCV was determined on the haematocrit reader. After spinning the capillary tube was then cut 1 mm below the buffy coat to include the top layer of red cells. The contents of the capillary tube were then expressed onto a clean slide, mixed and covered with a cover slip. At least two hundred microscope fields of the preparation were examined using dark field microscopy. The trypanosomes were identified and parasitaemia estimated according to the methods described by Adema and Makau, (1998) and Murray *et al.*, (1977).

3.5 Preparation of field samples for PCR

Templates for PCR were prepared by lysis with a non-ionic saponin detergent. This involved mixing 300 µl of each blood sample with 500 µl of saponin lysis buffer (0.15% saponin, 0.2% NaCl, 1 mM EDTA) in 600 µl capacity micro-centrifuge tubes by vortexing. This was followed by centrifugation at maximum speed for 10 minutes in a micro centrifuge (in this case eppendorf centrifuge 5415 C). The procedure was repeated three times, each time re-suspending and recovering the pellets. In the forth cycle the pellets were washed in 200 µl of 1X PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$ 10 mM Tris-Cl, pH 8.3). The recovered pellets were then suspended in 100 µl of triple distilled de-ionised water and heated for 20 minutes at 95^{o} C by a thermal cycler. Two microliters of this extract were used as template for PCR amplification.

3.6. PCR amplification

The protocol described by Masiga *et al.,* (1992) was used for amplification. The amplification was carried out in 25 µl reaction volumes. The reaction mixture contained 1x PCR buffer (10mM Tris-HCl, pH 8.3, 50mM KCl, 1.5mM $MgCl_2$), 200µM each of the four dNTPs, primers (specific for *T. congolense* Savanna, *T. congolense* Kilifi, *T. simiae, T. vivax, T. evansi* and *T. brucei* as shown in Table 1) at 1µM, 2µl DNA template and 1 unit of Taq DNA polymerase. The reaction mixture were then placed in a thermal cycler (GeneAmp^R PCR system 2700) and incubated at 94°C for 1 minute, then 30 cycles of 94°C for 30 seconds (denaturation), 60°C for 45 seconds (annealing) and 72°C for 30 seconds (extension). Fifteen microliter of each PCR product were resolved by electrophoresis (using Pharmacia Biotech electrophoretic system) in a 1.5% ethidium bromide-stained agarose gel and photographed under ultraviolet (UV) illumination. Samples that were positive for *T. brucei* were genotyped using microsatellite and analyzed for the presence of SRA gene.

3.7. PCR analysis of serum resistant gene (SRA)

All PCR amplifications were carried out in 25µl reaction volumes containing 200µM of each dNTPs, 0.2µl SRA primers (SRA-A and SRA-E) shown in Table 1, 1 unit of Amplitaq DNA polymerase, 1x PCR buffer containing (200mM $(NH_4)_2SO_4$, 750 mM Tris HCL (pH 8.8) and 0.1% Tween 20), 2 µl of the template and triple distilled water. Thirty five cycles of amplification were carried out, with denaturation at 95°C

for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 60 seconds using (GeneAmp^R PCR system 2700) (Gibson *et al.*, 2001). Twenty microlitre of each PCR product were resolved by electrophoresis (using Pharmacia Biotech electrophoretic system) in a 1.5 % ethidium bromide stained agarose gel and photographed under UV illumination.

3.8 Microsatellite PCR reaction and detection

Protocol described by Biteau *et al.*, (2000) was used. All PCR amplifications were carried out in 25µl reaction volumes containing 200µM of each dNTPs, 0.2µM of each primer (MT3033 AT, MEST19-AT/GT, MORF 2-CA, MT3033 AC/AT and M6C8 CA) shown in Table 2 and 2µl of purified genomic DNA. The reaction was carried out in the presence of 1 unit of Amplitaq DNA polymerase, 1x PCR buffer containing (200mM $(NH_4)_2SO_4$, 750 mM Tris HCL (pH 8.8) and 0.1% Tween 20). Then the samples were processed through 2 cycles of 45 seconds at 95°C, 45 seconds at 60°C and 45 seconds at 72°C, followed by 28 cycles of 45 seconds at 95°C, 45 seconds at 55°C and 45 seconds at 72°C and 5 minutes at 72°C using (GeneAmp^R PCR system 2700). The samples were resolved through 6-8% denaturing polyacrylamide gels and visualized by silver staining.

3.8.1 Separation of Microsatellite products

PCR products were separated on a 8% denaturing polyacrylamide gel. This was prepared by mixing 32ml SequaGel concentrate (Acrylamide, methylene-bisacrylamide and urea in a deionized aqueous solution), 58ml sequaGel diluent

(Urea in deionzed) and 10ml sequaGel buffer (0.89M Tris-Borate-20mM EDTA buffer ph 8.3 (10X TBE) and urea (Native Diagnostics). Forty microliter of TEMED was added to the casting solution and swirled gently to mix. Eight hundred microliters of freshly prepared 10% ammonium persulfate was added to the casting solution and the mixture set with appropriate spacers and combs. Ten microliters of the samples were loaded and electrophoresed at 150V for 4 hours with a 4% polyacrylamide stacking gel. The DNA was visualized by silver staining.

3.8.2 Silver staining

The gel glasses were removed and the gels fixed for 30 minutes in 15% ethanol and 5% acetic acid. They were then rinsed three times with water for 2 minutes each. Staining was done for 30 minutes with $AgNO_3$ (1g/L), 100µl 37% formaldehyde per 100ml. The gels were then rinsed for 20 seconds with water. Gel development was done for 15 minutes with 2.5% $NaCO_3$, 100µl 37% formaldehyde per 100ml and NaS_2O_3 2mg/l and stopped when thought to be well developed (Visual control). The development was then stopped for 10 minutes with 2% (w/v) glycine and impregnated with 5% (v/v). The gels were then stored in the cold room (Wray *et al.*, 1981).

Table 1: Oligonucleotide primers and their sequence. F/A and R/E refer to forward and reveres respectively.

Primer	Strand	Sequence 5'-3'	Size (bp)	Specificity	Reference
TCS	F R	CGAGAACGGGCACTTTGCGA GGACAAACAAATCCCGCACA	320	*T. Congolense* savannah	Masiga *et al.*, 1994
TV	F R	CCCGGCAGGTTGGCCGCCATC TCGCTACCACAGTCGCAATCG CAATCGTCGTCTCAAGG	399	*T. vivax*	Masake *et al.*, 1994
TSM	F R	CCGGTCAAAAACGCATT AGTCGCCCGGAGTCGAT	437	*T. simiae*	Masiga *et al.*, 1992
TCK	F R	GTGACCAAATTTGAAGTGAT ACTCAAAATCGTGCACCTCG	294	*T. congolense* Kilifi	Masiga *et al.*, 1992
TBR	F R	GAATATTAAACAATGCGCAG CCATTTATTAGCTTTGTTGC	164	*T. Brucei*	Moser *et al.*, 1989
SRA	A E	GACAACAACTACCTTGGCGC TACTGTTGTTGTACCGCCGC	460	*T. b. rhodesiense*	Gibson *et al.*, 2001
EVA	F R	ACATATCAACAACGACAAAG CCCTAGTATCTCCAATGAAT	150	*T. evansi*	Gibson *et al.*, 2001

Table 2: The 5 microsatellite markers analyzed and their sequence. P and M stand for plus and minus strand. These sequences are taken from Biteau *et al* (2000).

Maker	Strand	Sequence 5'-3'	Repeated sequence	Size (bp)
MORF2-CA	P M	TTTATCTCACATTACTCGGCG GCGTCGATCATGTCTACCGTAC	(CA) n	115-253
M6C8-CA	P M	CTTTCAACCGCCTTATCAGC GGCTAGTTACACTGTAGTTCTC	(CA) n	84-360
MT3033-AC/TC	P M	GAGTGACAATGGTGAAGATCG TTTTTCTTTGGTGCTTGTGAG	(AT/TC) n	119-201
MT3033-AT	P M	CTCACAAGCACCAAAG ATGGAACTTCGCAAGTGTG	(AT) n	164-178
MEST19-AT/GT	P M	TACACAAAACGTTCTCAAC GACAGAGTATACGAGAAGTG	(AT/GT) n	257-340

3.9 Data Analysis

Randomized block design was used to compare location of sampling, species of animal, sex and age of animals and the prevalence of trypanosomosis. Chi-square was used to compare differences in infection cases in the villages. Similarly differences in infection cases between the animal sexes and ages were also analyzed using chi-square (SAS 1996). Data that had replicates lower than five were analyzed using Fisher's exact (2-tail) test. Example comparison of infections cases by sex in pigs and ages in sheep, goats and pigs were assessed using Fisher's exact (2-tail) test. Least square means under the General Linear Models (GLM) was used to compare PCV, while t test was used for the comparison of mean PCV between the animals and locations (SAS, 1996).

CHAPTER FOUR

RESULTS

4.1 Tsetse fly collection

Total catches of tsetse flies and biting flies that were trapped on 4 different days are shown in table 3. In total 22 tsetse flies were caught, sixteen of which were *Glossina fuscipes fuscipes,* and 6 *G. pallidipes*. All the flies caught were non-teneral, fifteen were females and 5 were males. Of the biting flies caught, there were 18 stomoxys and 2 tabanids. The apparent tsetse fly densities recorded as flies/trap/day (FTD) for the localities are shown in table 3.

Table 3. The number and species of tsetse flies caught and their densities in the five villages. F= females and M= male.

Location	Species	Non-Teneral (M)	Non Teneral (F)	Teneral (M)	Teneral (F)	Total	FTD
Rukada	***G. f. fuscipes*** Stomoxys	2	4	0	0	6 7	0.5
Ongariama	***G. f. fuscipes*** ***G. pallidipes*** Stomoxys Tabanids	1 1	3 0	0 0	0 0	4 1 3 2	0.417
Amase	***G. f. fuscipes*** ***G. pallidipes*** Stomoxys	1 2	5 3	0 0	0 0	6 5 8	0.917
Obuchun	None	0	0	0	0	0	0
Amoni	None	0	0	0	0	0	0

4.2 Prevalence of trypanosomes by microscopy

A total of 402 animals were sampled in the 5 villages. In Teso district there were 42 goats, 37 sheep and 3 pigs sampled from Amoni. In Amase 35 goats, 8 sheep and 9 pigs were sampled, 55 goats, 7 sheep and 6 pigs were sampled in Ongariama, while in Obuchun 86 goats, 13 sheep and 13 pigs were sampled. There were 37 goats, 30 sheep and 21 pigs sampled from Rukada village of Busia district (Fig. 4). The buffy coat and heamacrotic centrifuge technique (HCT) examination of blood from the 402 animals showed 5 trypanosome infections (Table 4). The trypanosome species were *T. congolense* 1, *T. vivax* 1 and *T. brucei* 3. No mixed infections were observed. Two cases of infection were in Amase. One *T. vivax* infection was from a male sheep, while 1 case of *T. congolense* infection was detected in a female pig. One *T. brucei* infection was isolated in an adult female goat in Obuchun, one in a young female goat in Ongariama and another from a female sheep from Rukada. There were no infections recorded in Amoni.

Table 4. Number of trypanosomes identified from sheep, goats and pigs by microscopy in the five villages.

Village Species	No. of Animals	Trypanosome species		
		T. congolense	*T. vivax*	*T. brucei*
Amoni				
Goat	42	0	0	0
Sheep	37	0	0	0
Pig	3	0	0	0
Amase				
Goat	35	0	0	0
Sheep	8	0	1	0
Pig	9	1	0	0
Ongariama				
Goat	55	0	0	1
Sheep	7	0	0	0
Pig	6	0	0	0
Obuchun				
Goat	86	0	0	1
Sheep	13	0	0	0
Pig	13	0	0	0
Rukada				
Goat	37	0	0	0
Sheep	30	0	0	1
Pig	21	0	0	0
Total	**402**	**1**	**1**	**3**

4.3 Trypanosome identification by PCR

All 402 blood sampled in the 5 villages were subjected to PCR analysis for detection and identification of trypanosomes and the results are shown in table 5. PCR detected 85 infections (21.14%) out of the 402 sampled animals. From Amoni 23 out of 82 (28.05%) animals sampled were found infected. In Obuchun 31 out of 112 (27.68%), 14 out of 68 (20.6%) in Ongariama, 10 out of 52 (19.2%) in Amase and 7/88 (7.9%) in Rukada. Amoni had significantly higher infections than those from Obuchun, Ongariama, Amase and Rukada (χ^2 =10.375, df=1, P=0.001).

In Amoni 16 out of 37 sheep and 7 out of 42 goats were found infected by PCR and none of the pigs sampled from this village were found infected. From Obuchun 22 of the 86 goats, 5 out of 13 pigs and 4 out of 13 sheep sampled were found infected. In Ongariama 12 of the 55 goats and 2 out of 6 pigs sampled were infected while none of Sheep were infected. Five out of 35 goats, 4 out of 9 pigs and 1 out of 8 sheep sampled from Amase were found infected, while In Rukada 4 out of 30 sheep and 3 out of 37 goats were infected, there were no infections in pigs.

Table 5. Trypanosomes species detected by PCR amplification from the five villages.

Village Species	No of animals	*T. congolense* Savannah	*T. congolense* Kilifi	*T. simiae*	*T. vivax*	*T. brucei*
Amoni						
Goat	42	1	3	1	0	2
Sheep	37	1	6	5	0	4
Pig	3	0	0	0	0	0
Amase						
Goat	35	0	0	0	4	1
Sheep	8	0	0	0	1	0
Pig	9	1	0	0	1	2
Ongariama						
Goat	55	0	0	7	4	1
Sheep	7	0	0	0	0	0
Pig	6	0	0	1	1	0
Obuchun						
Goat	86	4	0	8	7	3
Sheep	13	0	1	0	1	2
Pig	13	0	0	0	4	1
Rukada						
Goat	37	1	1	0	0	1
Sheep	30	1	1	0	0	2
Pig	21	0	0	0	0	0
Total	**402**	**9**	**12**	**22**	**23**	**19**

4.4 Characterization of trypanosomes by PCR from five villages

T. vivax was the most prevalent species by PCR and was detected in 23 (5.7%) out of 402 sampled blood. Twelve out of 23 *T. vivax* cases were reported in Obuchun, while Amase and Ongariama recorded 6 and 5 cases respectively, none was detected in animals sampled from Amoni and Rukada. Fig. 5 shows the picture to indicate how *T. vivax* was identified by PCR.

T. simiae detected in 22 blood samples (5.5%) out of 402 was the second prevalent species. Eight out of the 22 *T. simiae* cases were reported in animals sampled from Ongariama, 8 in Obuchun and 6 in Amoni. No *T. simiae* cases were seen in animals from Amase and Rukada. Fig. 6 illustrates the picture to show how *T. simiae* was identified by PCR from the blood of sheep, goats and pigs.

Twenty-one (5.2%) out of the 402 sampled animals were infected with *T. congolense,* of which 12 (57.2%) were *T. congolense* Kilifi and 9 (42.86%) were *T. congolense* Savanna. Out of 21 *T. congolense* infections 11 were detected in animals sampled from Amoni, 5 in Obuchun, 4 in Rukada and 1in Amase. Figs. 7 and 8 show the pictures to illustrate how *T. congolense* Kilifi and *T. congolense* Savanna were identified by PCR.

T. brucei was the least common species and was detected in 19 (4.72%) out of the 402 sampled animals, 6 in Amoni and in Obuchun, while 3 in Amase and in Rukada and 1 in Ongariama. Fig. 9 shows the picture to illustrate how *T. brucei* was identified by PCR from the blood of sheep, goats and pigs. *T. evansi* is morphologically similar to other members of the subgenus *Trypanozoon.* It was identified using specific

primers EVA A and B. None of the 402 sampled animals were found infected or harboring *T. evansi* (results not shown).

A number of mixed infections were observed in this study; *T. congolense* Savannah and *T. brucei* were encountered twice. There were also a single case of the following mixed infections: *T. congolense* Savannah and *T. congolense* Kilifi, *T. congolense* Kilifi and *T. simiae T. congolense* Savannah and *T. simiae*, *T. vivax* and *T. brucei* and *T. simiae* and *T. brucei*.

Goats had the most infection cases of *T. brucei* accounting for 9 out of 19, 8 in sheep and 2 in pigs. The highest *T. simiae* infection cases were reported in goats that had 16 out of 22, while 5 in sheep and 1 in pigs. *T. congolense* cases were distributed as follows; 11 in goats, 9 in sheep and 1 in pigs. Goats had the highest number of *T. vivax* infections, accounting for 16 followed by 5 in pigs and 2 in sheep.

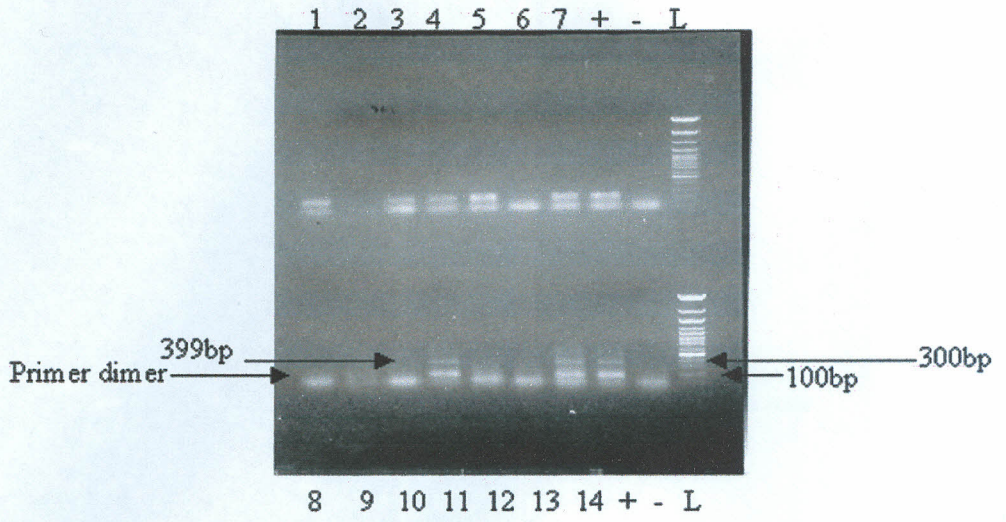

Figure 5: Ethidium bromide stained 1.5% agarose gel illustrating PCR identification of *T. vivax* using TV F and R primers. Samples 1, 3,4,5,7,11 and 12 are positive giving the expected fragment size of 399bp. The lane marked L is a 100bp size marker; + and – are positive and negative controls respectively.

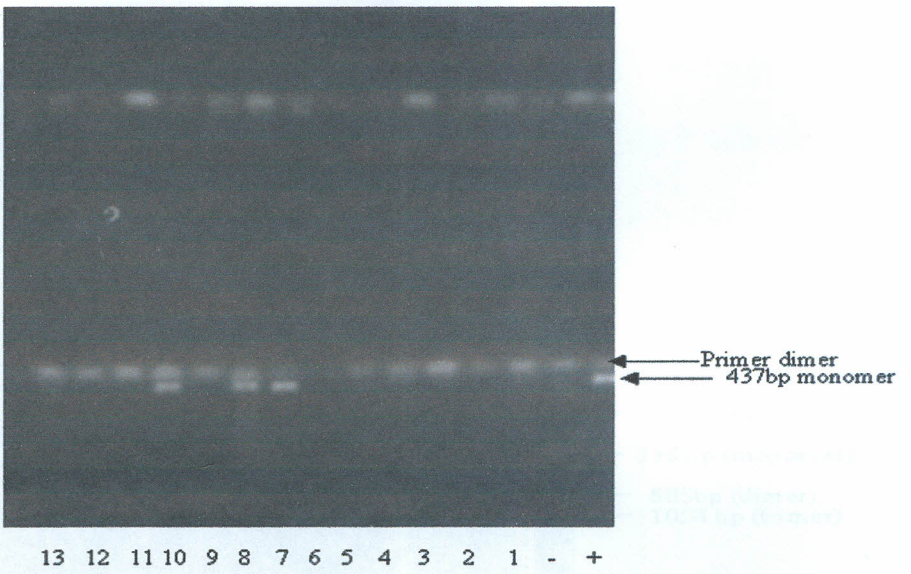

Figure 6: Ethidium bromide stained 1.5% agarose gel illustarting PCR identification of *T. simiae* using TSM F and R primers. Samples 7, 8 and 10 are positive giving the expected fragment size of 437bp. + and – are positive and negative controls respectively.

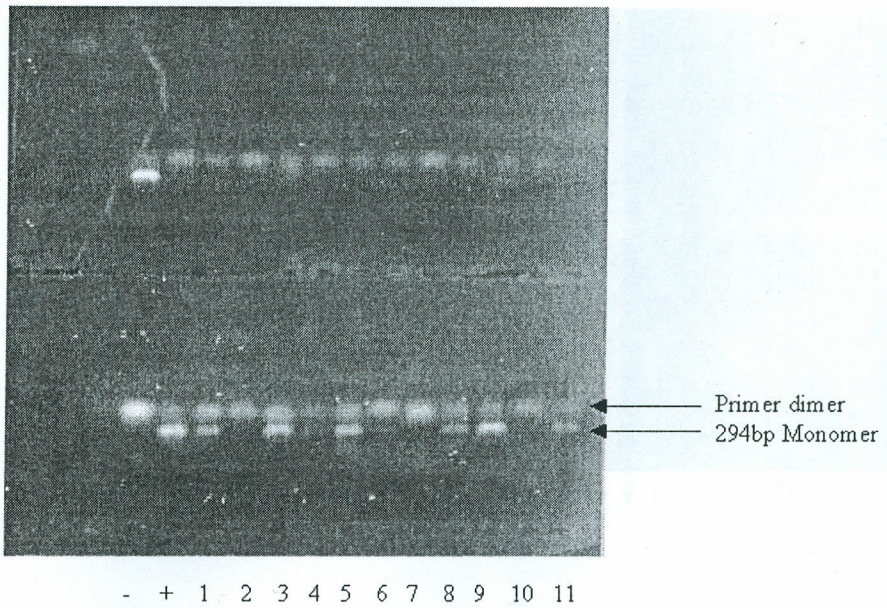

Figure 7: Ethidium bromide stained 1.5% agarose gel illustarting PCR identification of *T. congolense* Kilifi using TCK F and R primers. Samples 1, 3,5,8,9, 11 and 12 are positive giving the expected fragment size of 294bp. + and – are positive and negative controls respectively.

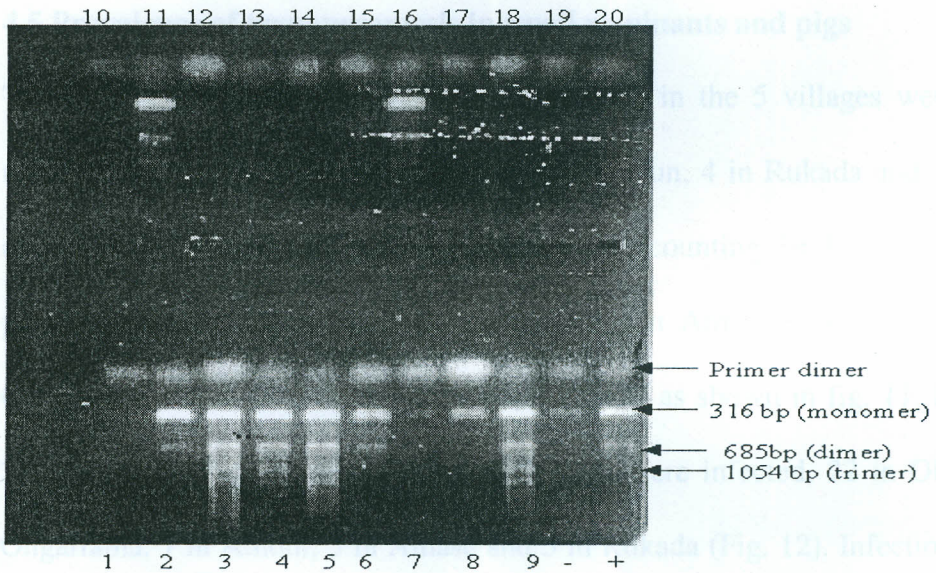

Figure 8: Ethidium bromide stained 1.5% agarose gel illustrating PCR identification of *T. congolense* Savanna using TCS F and R primers. Samples 2, 3,4,5,6, 8, 9, 11 and 16 are positive giving the 316bp product, which represents the monomer for the detected amplicon. Multiple products can be seen in same lanes, consistent with amplification from tandemly repeated sequences. + and – are positive and negative controls respectively.

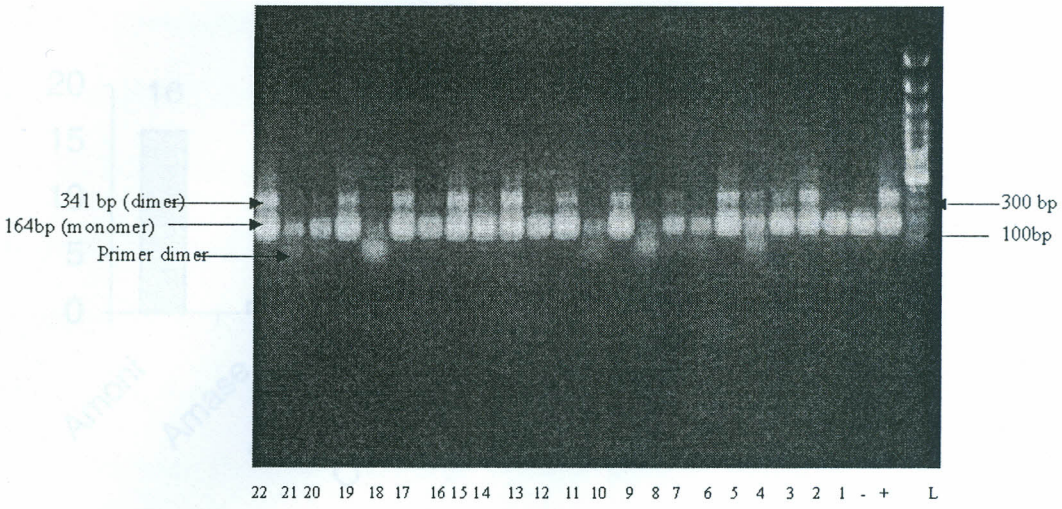

Figure 9: Ethidium bromide stained 1.5 % agarose gel illustrating PCR identification of *T. brucei* using TBR F and R primers that gives 164 bp products representing the monomer size for the detected amplicon. Multiple products can be seen in some lanes, consistent with amplification from tandemly repeated sequences. The lane marked L is a 100bp size marker, + and – are positive and negative controls respectively.

4.5 Prevalence of trypanosomosis in small ruminants and pigs

Twenty-five (26.06%) out of 96 sheep sampled in the 5 villages were infected by trypanosomes, 16 were from Amoni, 4 in Obuchun, 4 in Rukada and 1 from Amase (Fig. 10). Pigs had the second infection cases accounting for 11 (21.15%) out of 52 pigs sampled in the 5 villages. Four were from Amase, 5 in Obuchun and 2 in Ongariama, while none from Rukada and Amoni as shown in fig. 11. Forty-nine out 254 (19.19%) sampled goats in the 5 villages were infected, 22 in Obuchun, 12 in Ongariama, 7 in Amoni, 5 in Amase and 3 in Rukada (Fig. 12). Infections reported in sheep were significantly higher than those in pigs and goats (χ^2 =12.225, df=4 and P=0.016).

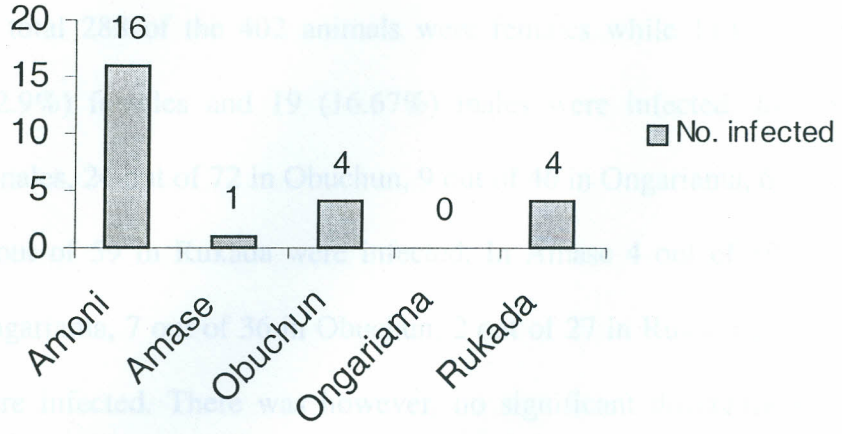

Figure 10: Shows the distribution of infections in sheep sampled from the 5 villages.

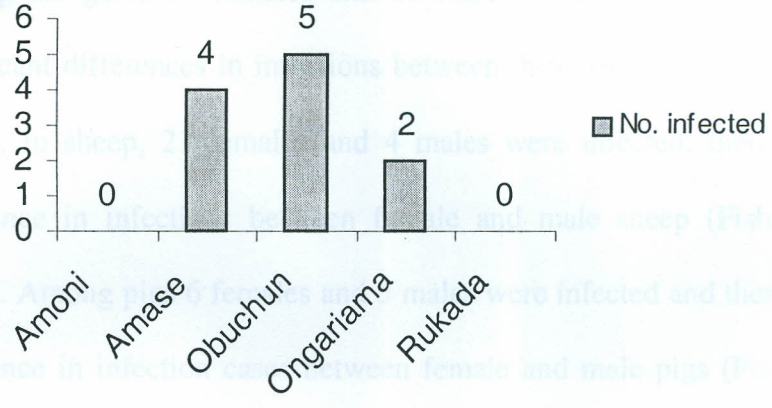

Figure 11: Shows the distribution of infections in pigs sampled from the 5 villages.

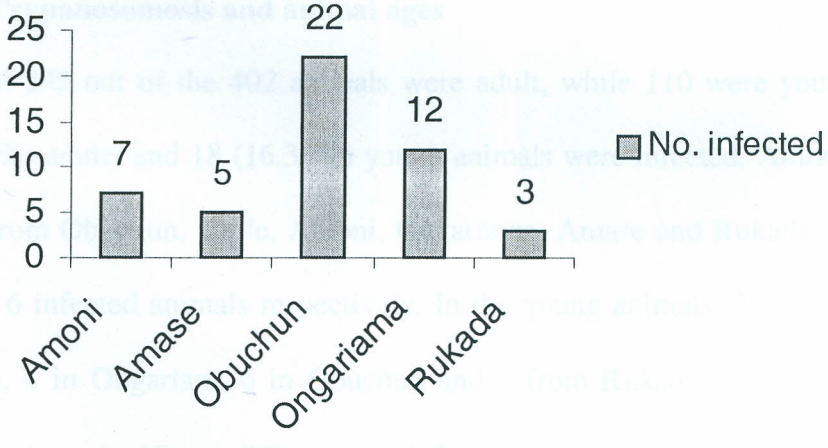

Figure 12: Shows the distribution of infections in goats sampled from the 5 villages.

4.5.1 Trypanosomosis and animal sexes

In total 288 of the 402 animals were females while 114 were males of which 66 (22.9%) females and 19 (16.67%) males were infected. In Amoni 22 out of 62 females, 24 out of 72 in Obuchun, 9 out of 46 in Ongariama, 6 out of 42 in Amase and 5 out of 59 in Rukada were infected. In Amase 4 out of 10 males, 5 out of 22 in Ongariama, 7 out of 36 in Obuchun, 2 out of 27 in Rukada and 1 out of 19 in Amoni were infected. There was however, no significant difference in infections between females and male in the 5 villages (χ^2 =4.538, df=4 and P= 0.338).

Among the goats 39 females and 10 males were infected, however there was no significant differences in infections between these two sexes (Fisher's exact test, P= 0.949). In sheep, 21 females and 4 males were infected, there was no significant difference in infections between female and male sheep (Fisher's exact test P = 0.328). Among pigs 6 females and 5 males were infected and there was no significant difference in infection cases between female and male pigs (Fisher's exact test P = 0.179).

4.5.2 Trypanosomosis and animal ages

In total 292 out of the 402 animals were adult, while 110 were young of which 67 (22.95%) adults and 18 (16.36%) young animals were infected. Among the adults 25 were from Obuchun, while, Amoni, Ongariama, Amase and Rukada reported 20, 10, 6, and 6 infected animals respectively. In the young animals, 3 from Amoni, 4 from Amase, 4 in Ongariam, 6 in Obuchun and 1 from Rukada were infected. However there was no significant difference in infections between adult and young animals (χ^2 =1.99, df=4 and P = 0.703).

Of the total 49 infected goats, 37 were adult while 12 were young ones, however there was no significant difference in infection cases between adult and young goats (Fisher's exact test P = 0.436). In total 22 out of the 25 infected sheep were adult while 3 were young ones and there was no significant difference in infection cases between these two age groups (Fisher's exact test P = 1.000). For the 11 infected pigs 8 were adult while 3 were young ones but there was no significant difference in infections cases between these two age groups (Fisher's exact test P = 0.786).

4.6 Effect of trypanosomosis on anaemia

The overall mean % PCV for goats, sheep and pigs for the 5 villages were 23.6 (SD± 7.2), 27.3 (SD± 7.3) and 27.7 (SD± 9.0) respectively (Table 6). The infected livestock generally had low PCVs compared to the non-infected ones. Mean % PCV for infected goats in the 5 villages was 22.5 (SD± 7.9), while 23.9 (SD± 7.0) for non-infected ones. Infected sheep recorded mean % PCV of 26.8 (SD± 7.5), while 28.8 (SD± 6.2) for the non-infected ones, while infected pigs had mean % PCV of 27.4 (SD± 9.4) and 29.0 (SD± 7.3) for the non-infected ones. However, comparison of the mean % PCV of the infected animals and non-infected animals from the 5 villages showed no significant difference (t-test, P=0.2581, P= 0.2128 and P= 0.5852 for goats, sheep and pigs respectively).

Table 6. Mean % PCV values for sheep, goats and pigs. SD stands for standard deviation

Overall mean % PCV (±SD)		
Goat	**Sheep**	**Pig**
23.6 (7.2)	27.3 (7.3)	27.7 (8.8)
Adult 23.8 (7.3)	Adult 27.2 (7.1)	Adult 29.3 (8.8)
Young 23.1 (7.1)	Young 27.8 (8.1)	Young 24.8 (8.9)
Female 23.6 (7.3)	Female 27.2 (6.5)	Female 27.6 (8.8)
Male 23.6 (7.0)	Male 27.6 (8.9)	Male 28.3 (9.4)

The adult goats recorded mean % PCV of 23.8 (SD± 7.3) while 23.2 (SD± 7.1) was for the young ones and the two means were not significantly different (t- test, P=1.000). For the female and male goats, the mean % PCV were 23.6 (SD± 7.3) and 23.6 (SD± 7.0) respectively which were not significantly different (t- test, P= 0.8518). In general 23 infected goats of both ages and sexes had mean % PCV of less than 15.

Among the sheep, the mean % PCV for adult was 27.2 (SD± 7.1) while 27.8 (SD± 8.1) was recorded in young ones and these two means were not significantly different (t-test, P=0.3499). For the sexes, the mean % PCV for female and male sheep were 27.2 (SD± 6.5) and 27.6 (SD± 8.9), respectively which were significantly different (t-test, P=0.0254). Five infected sheep of both ages and sexes had PCV of less than 15.

The adult pigs had mean % PCV of 29.3 (SD± 8.8), while 24.8 (SD± 8.9) was recorded in the young ones, however these two means were not significantly different (t-test, P= 0.4784). For the sexes, the mean % PCV for female and male were 27.6 (SD± 8.8) and 28.3 (SD± 9.4), respectively and the two means were not significantly different (t-test, P=0.9291). Four infected pigs of both ages and sexes had PCV of less than 15.

4.7 Detection of human infective trypanosomes (*T. b. rhodesiense)*

All the 34 isolates positive for *T. brucei spp*, on the basis of specific DNA targeting satellite DNA were subjected to evaluation for the presence of the SRA gene, 6 were positive (Fig. 13). Thus the 6 isolates (S05, G04, G13, G14, P02 and P05) were designated as *T. b. rhodesiense* while the remaining 28 isolates (S01, S02, S03, S04, S06, S07, S08, S09, S10, S11, S12, S13, S14, S15, G01, G02, G03, G05, G06, G07, G08, G09, G10, G11, G12, P01, P03 and P04) were designated *T. b. brucei* (Table 7).

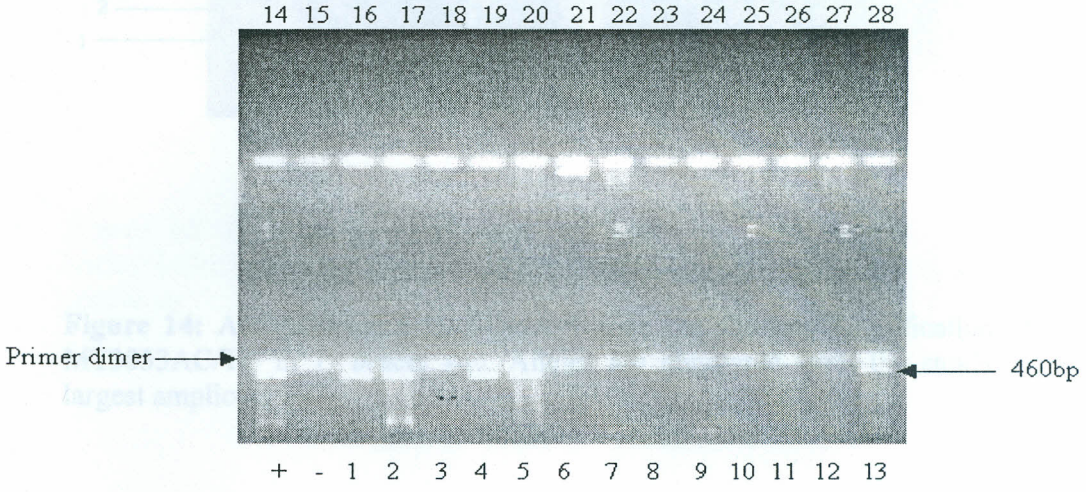

Figure 13: Ethidium bromide stained 1.5% agarose gel showing PCR identification of *T. b. rhodesiense* using SRA F and R primers. Sample 1,2,4,5, 13 and 21 are positive giving the expected fragment size of 460bp. + and – are positive and negative controls respectively.

4.8 Microsatellite analysis of *T. brucei spp.*

The microsatellite markers were used to determine the number of alleles and hence the genotypes present in 28 *T. b. brucei* (14 from sheep, 11 from goats and 3 from pigs) and 6 *T. b. rhodesiense* (1 from sheep, 3 from goats and 2 pigs). There were no stutter bands and the amplification products were scored for the different sized alleles (bands) present. The analysis of the alleles at these loci in two sub species of *T. brucei* shows that there are at least 5 distinct alleles at the locus MT3033AC/TC (Fig. 15). While 4, 6, 4 and 5 distinct alleles were observed at the MEST19-AT/GT, MORF2-CA, M6C8-CA and MT3033-AT loci, respectively (Figs. 15, 16, 17 and 18). A total of 24 distinct alleles were detected from the analysis of these 5 loci, showing high level of allelic variation.

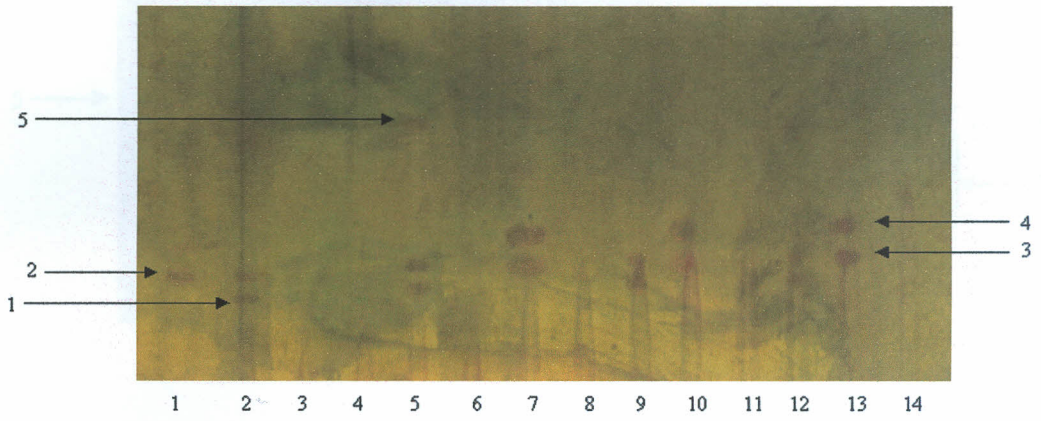

Figure 14: A denaturing 8 % polyacrylamide gel showing amplification of locus MT3033AC/TC in *T. brucei spp.* Alleles are numbered from the smallest to the largest amplicon.

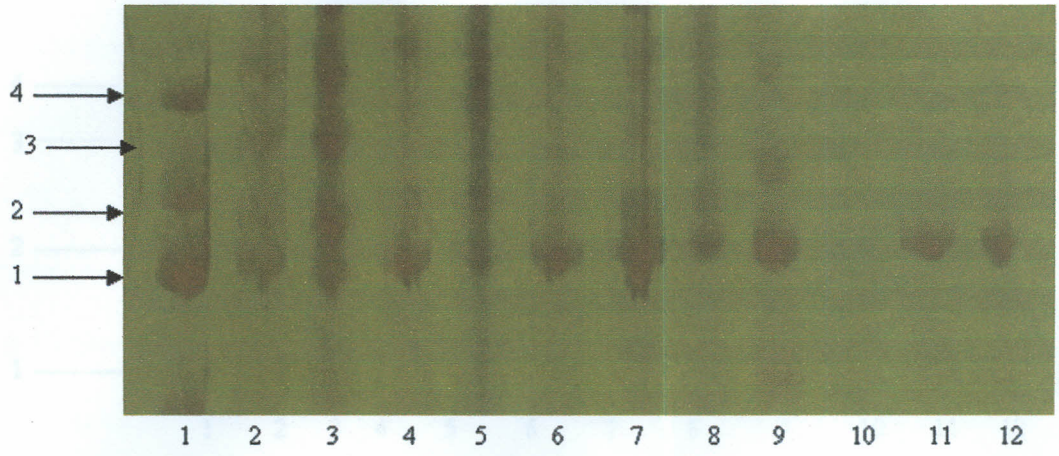

Figure 15: A denaturing 8 % polyacrylamide gel showing amplification of locus MEST19-AT/GT in *T. brucei spp.* Alleles are numbered from the smallest to the largest amplicon.

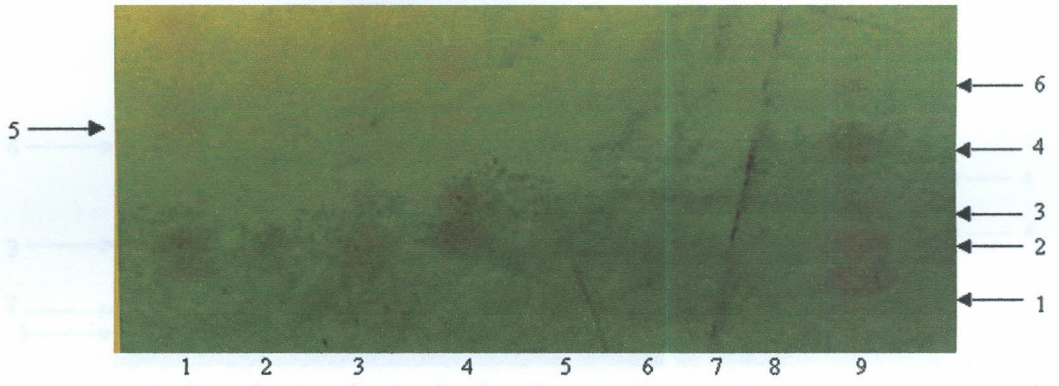

Figure 16: A denaturing 8 % polyacrylamide gel showing amplification of locus MORF2-CA in *T. brucei spp.* Alleles are numbered from the smallest to the largest amplicon.

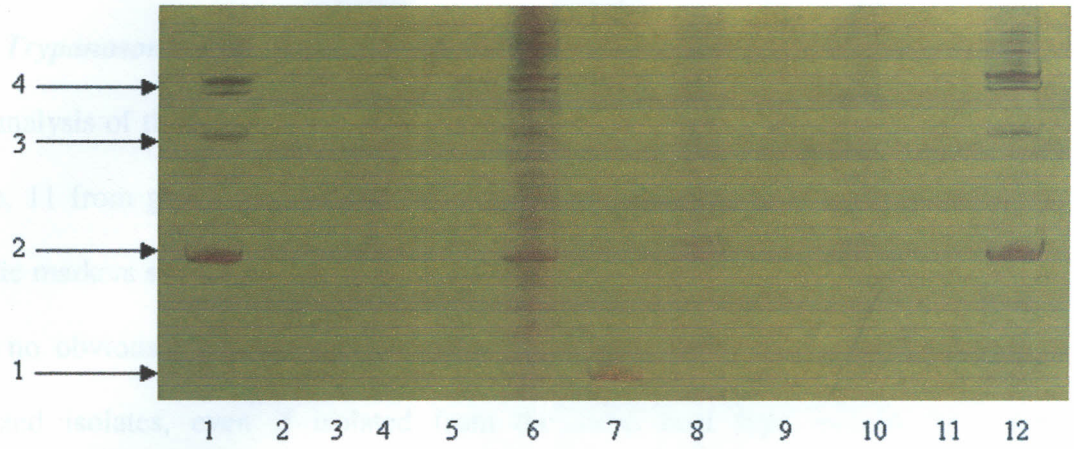

Figure 17: A denaturing 8 % polyacrylamide gel showing amplification of locus M6C8-CA in *T. brucei spp.* Alleles are numbered from the smallest to the largest amplicon.

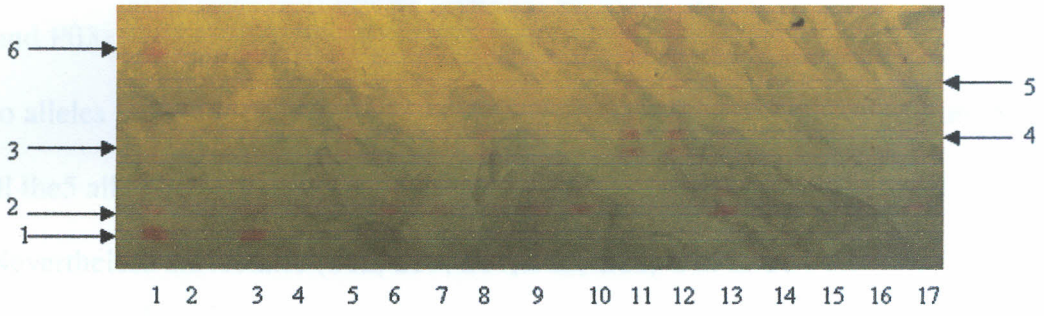

Figure 18: A denaturing 8 % polyacrylamide gel showing amplification of locus MT3033-AT in *T. brucei spp.* Alleles are numbered from the smallest to the largest amplicon.

4.8.1 *Trypanosoma brucei brucei* isolates

The analysis of the *T. b. brucei* isolates using the 5 loci showed that 14 isolates from sheep, 11 from goats and 3 from pigs were genotypically distinct. All the analyzed genetic markers showed remarkable genetic diversity within this sub-species and there were no obvious relationships between the analyzed isolates. In deed none of the analyzed isolates, even if isolated from the same host type and in the same geographical region displayed identical genotypes and no consistent pattern of allele distribution could be observed. However isolates (S03, S04, S12, S15, G06, G07, G08, G11 and P03) lacked alleles at the locus MT3033-AC/TC while isolates (S02, S03, S04, S07, S12, S15 and P03) lacked alleles at the locus MEST 19-AT/GT. Similarly isolates (S02, S03, S04, S07, S09, S11, S15, G06, G07, and G08) had no alleles at MORF 2- CA, Isolates (S01, S09, S10, S12, S14, S15, G02, G06, G07, G09, G12 and P03) lacked locus MT3033 AT while isolates S08, S11, G08, G10, and P03 had no alleles at M6C8 CA (Table 7). Isolates S01, S02, S04, S10, G06, G12 and P04 had all the5 alleles at locus M6C8 CA, while isolate P03 had only one locus MORF 2-CA. Nevertheless the isolates (S02, S09, S14, S15, G02, G03 and P04) share alleles at locus MT3033AC/TC. Isolates S06, S08, S09, S11, G03, G09, G10, G11, G12 and P04 share common alleles at locus MEST 19-AT/GT. Isolates S01, S02, S04, S10, G06, G12 and P04 have common alleles in locus M6C8-CA. Isolates S14 and S15 share alleles at locus MORF2 CA. While S06, S08, S13, G01, G03, G10, G11, P01 and P04 share alleles at locus MT3033AT. The isolates G01, G03, and P04 had at least two alleles at all the five loci analyzed (Table 7) thus comprises of more than one genotype.

4.8.2 *Trypanosoma brucei rhodesiense* isolates

Two distinct groups arise within the *T. b. rhodesiense* stocks, corresponding to different distribution of alleles. The first group comprises S05, G04 and P02, which are characterized by 3 alleles at the locus MT3033-AC/TC (Table 7). In addition, all except S05 have the locus MORF2-CA. Three isolates G13, G14, and P05 characterized by 2 distinct alleles at the locus MT3033 AC/TC and MORF 2-CA form the second group. All except G14 have alleles at the locus MT3033 AT (Table 7) Isolates G04, G13, P02, and P05 have more than two distinct alleles at all the 5 loci analyzed and are composed of more than one genotype (Table 7). Some *T. b. brucei* and *T. b. rhodesiense* stocks from different region and host shared common alleles at locus MORF2-CA, in isolates P04 and S12 *T. b. brucei* and all the *T. b. rhodesiense* stocks. Similarly, S01, S02, S04, S10, S14, G06, G07, G11, G12 and P04 have common alleles at the locus M6C8-CA found in *T. b. rhodesiense* stocks.

4.8.3 Microsatellite analysis of *T. congolense, T. vivax* and *T. simiae*

The microsatellite analysis of other species of trypanosomes revealed that these 5 loci appear to be absent in the genome of *T. congolense (*Savannah and Kilifi), *T. vivax* and *T. simiae*, (results not shown). No amplification products were evidenced when tested against purified DNAs from representative stocks.

Table 7: Shows the analysis of *T. brucei spp* using SRA and microsatellite markers. + and - are for positive and negative respectively. 1,2 etc. shows different alleles/bands observed

Stock	Host	Site	SRA	MT3033-AC/TC	MEST19-AT/GT	MORF 2 -CA	MT3033 AT	M6C8 CA
S01	Sheep	**Amoni**	-	2,3	1,2	1	-	1,2,3,4,5
S02	Sheep	**Amoni**	-	1,2	-	-	2	1,2,3,4,5
S03	Sheep	**Amoni**	-	-	-	-	2	3,4
S04	Sheep	**Amoni**	-	-	-	-	2	1,2,3,4,5
S05	Sheep	**Rukada**	+	2,3,4	2,3,4	-	1,3,4	-
S06	Sheep	**Rukada**	-	-	1,2,3	2	1,4	1,2,3,4
S07	Sheep	**Obuchun**	-	-	-	-	1,3,4	1,2
S08	Sheep	**Obuchun**	-	-	1,2,3	1,2,4	1,4	-
S09	Sheep	**Busia**	-	1,2	1,2,3	-	-	3,4,5
S10	Sheep	**Teso**	-	-	1,2	2,3,5	-	1,2,3,4,5
S11	Sheep	**Teso**	-	-	1,2,3	-	1,2,3,4	-
S12	Sheep	**Teso**	-	1	-	5,6	-	1,2,3
S13	Sheep	**Teso**	-	-	2,3	1,2,6	1,4	1,2,3,4
S14	Sheep	**Busia**	-	1,2	1,2	1,2,6	-	3,4,5
S15	Sheep	**Busia**	-	1,2,	-	-	-	3,4
G01	Goat	**Amoni**	-	1,2,5	2,3	1,2	1,4	1,3,4
G02	Goat	**Amase**	-	1,2	2,3	2,3	-	1,2,3
G03	Goat	**Obuchun**	-	1,2	1,2,3	2,3	1,4	1,2
G04	Goat	**Rukada**	+	2,3,4	2,3,4	4,5,6	1,3,4	3,4,5
G05	Goat	**Ongariama**	-	3	2,3	1,2,4	1,3,4	1,2,4
G06	Goat	**Obuchun**	-	-	2,3	-	-	1,2,3,4,5
G07	Goat	**Obuchun**	-	-	2,3	-	-	3,4,5
G08	Goat	**Obuchun**	-	-	2,3	-	1,2,3,4	-
G09	Goat	**Busia**	-	3,4	1,2,3	1,2,4	-	3,4
G10	Goat	**Busia**	-	2,3	1,2,3	2,3,5	1,4	-
G11	Goat	**Busia**	-	-	1,2,3	1,2,3	1,4	3,4,5
G12	Goat	**Busia**	-	1,2,3,5	1,2,3	1,2,3,4	-	1,2,3,4,5
G13	Goat	**Busia**	+	2,3	2,3,4	5,6	1,3,4	3,4,5
G14	Goat	**Teso**	+	2,3	2,3,4	5,6	-	3,4,5
P01	Pig	**Amase**	-	3	1,2	4,5,6	1,4	3,4
P02	Pig	**Amase**	+	2,3,4	2,3,4	4,5,6	1,3,4	3,4,5
P03	Pig	**Amase**	-	-	-	2,3	-	-
P04	Pig	**Busia**	-	1,2	1,2,3	2,3,5	1,4	1,2,3,4,5
P05	Pig	**Teso**	+	2,3	2,3,4	5,6	1,3,4	3,4,5

CHAPTER FIVE

DISCUSSION

5.1 PCR as a method of identification

The accurate identification of different species, subspecies and strains of trypanosomes has been a fundamental problem in epidemiological studies of trypanosomsosis in Africa (Mugittu *et al.*, 2001). The risk to livestock and man can only be fully recognized if the pathogenic trypanosomes are identified unequivocally (Masiga, 1994). The ideal methodology for the identification of parasites is one that is reliable, giving no false positives and identifying all true infections (Ole-Moi Yoi, 1987). In this study 21.1% infections of the 402 animals were identified by PCR based technique as opposed to 2.1% by microscopic methods. PCR detection of infections as observed, has proven to be reliable and very sensitive. Test for sensitivity has shown that 1 trypanosome or less could be identified by PCR (Masiga, 1994), compare to the routinely used microscopic techniques, which has a sensitivity of about 500 trypanosomes per milliliter of blood (WHO, 1986). An infection describes the presence of live pathogens in the host. As PCR detects DNA it is conceivable that an infection that has terminated could still be detected before DNA from dead parasite is eliminated from circulation (Mugittu *et al.*, 2001).

PCR has also shown to be very specific for the target trypanosome species. For example *T. congolense* and *T. simiea* that are members of subgenus *Nannomonas* are difficult to distinguish by microscopic methods. These two species were distinguished by specific PCR amplifications, with the amplification product of the

correct size being obtained only when the target DNA is present in the reaction mixture (Masiga, 1994).

The most extensive studies assessing the importance of trypanosomosis in sheep and goats were carried out by microscopic examinations (Giffin and Allonby, 1979a; b; Masiga *et al.*, 2002). Since parasitological methods are laborious and insensitive, diagnosis has been aided by serological tests (Asongayi *et al.*, 1990; Kayang *et al.*, 1997). Although the microscopic techniques are known to have poor sensitivity (Murray *et al.*, 1977), the use of this technique provided an important basis for investigations into the importance of trypanosomosis in small ruminants and pigs.

5.2 Trypanosomosis in sheep and goats

This study showed that sheep and goats are infected by trypanosomes. Although, it was observed that tsetse fly find sheep and goats rather unpalatable and use them only when very hungry, these livestock appear attractive to tsetse flies (Boyt 1971). The villages in which this study was done were also inhabited by large numbers of cattle and other domesticated and wild animals yet sheep and goats were infected by trypanosomes.

From this work, sheep were more infected than goats. This finding agrees with various other studies by Griffin and Allonby (1979b) who ed sheep as being more frequently affected by trypanosomosis than goats in Kenya. Makumyaviri *et al* (1989) observed more infections in sheep than in goats in Zaire. Kalu *et al* (1996) also observed more infections in sheep than in goats in Nigeria. Katunguka-

Rwakishaya (1996) observed infection rates of 26.7% in sheep than 8.8% in goats in Uganda. Osaer *et la* (1999), observed higher trypanosome prevalence in sheep than goats which was significantly higher in the moderate risk area in Gambia, Masiga *et al* (2002) observed sheep as being more prone to infection than were goats in Kenya. Griffin and Allonby (1979a) and Irungu *et al* (2002) observed high financial losses in sheep than goats and attributed this to high susceptibility of sheep to trypanosomosis than goats. In contrast to these observations, Kayanga *et al* (1996) observed high infections in goats as opposed to sheep in Ghana while Goossens *et al* (1998) observed no significant difference in infection rates between sheep and goats in Gambia.

The diverse feeding habits of goats compared to sheep could have contributed to the lower infection in goats than sheep. Behavioral attributes of goats such as skin rippling and leg thumping that may make it more difficult for tsetse flies to feed on them (Vale, 1977) could be a possible reason for the low infection cases observed in goats. Sheep and pigs have a relatively inactive lifestyle as opposed to goats, which are very agile, this makes sheep and pigs a good target for tsetse flies as a source of blood meal. Other contributory factors to the difference in infection cases could be due to coat thickness, color of the host and tsetse preference to the host could also contribute to the marked differences in infections between sheep and goats.

5.3 Trypanosomosis in pigs

The second highest infection rates by animal species after sheep were recorded in pigs (21.2%). Although all the sampled pigs were under zero grazing management,

and the population of goats and sheep were high, infections were detected in pigs. Various works assessing the prevalence of trypanosomes in pigs have made similar observations. Katunguka-Rwakishaya (1996) observed high infection rates of 32.4% in pigs as compared to 26.7% in sheep and 8.8% in goats in Uganda. Makumyaviri *et al* (1989) showed pigs as being highly infected than sheep and goats in Zaire. Asonganyi *et al* (1990) also reported significant infections in pigs in Cameroon. Hide *et la* (1994) encountered high mortality in pigs due to trypanosomosis in Uganda.

5.4 Characterization of trypanosomes in small ruminants and pigs

Trypanosoma vivax was the most prevalent species infecting sheep, goats and pigs, followed by *T. simiae, T. congolense* and *T. brucei.* Three cases of *T. b. rhodesiense* based on the presence of SRA gene were detected infecting these livestock. There were no cases of *T. evansi* reported in these livestock. These trypanosome species are also known to infect cattle and some are responsible for sleeping sickness in man. It is potentially then, that sheep, goats and pigs can act as reservoir of both Animal and Human African trypanosomosis.

Similar prevalence rates have been observed in small ruminants and pigs. Kalu *et al* (1996), observed 43% infections in cattle, sheep and goats as a result of *T. vivax*, while 25% was due to *T. congolense* and 2% due to *T. brucei* in Nigeria. Kayang *et al* (1997) observed *T. vivax* as the most predominant species infecting sheep and goats. *T. vivax* was also found to be predominant species infecting sheep and goats which was followed by *T. congolense* in Ghana (Osear *et al.*, 1999).

Other studies have found different prevalence rates. Griffin and Allonby (1979a and b) observed high *T. congolense* infection rates of 82% in sheep and goats. Makumyaviri *et al* (1989) also observed predominance of *T. congolense* infecting pigs (76%), sheep (31%) and goats (7%). *T. brucei spp* infections were second and *T. vivax* reported only on two occasions in Zaire. Fakae and Chiejina (1993) reported 50% *T. brucei*, 43% *T. congolense* and 36% *T. vivax* in Nigeria. Katunguka-Rwakishaya (1996) working in Uganda found that *T. brucei* was detected in 66.7% and 30% of all infections in goats and pigs. However, Masiga *et al* (2002) observed *T. congolense* as the most prevalent species accounting for most infections, with *T. brucei* was only encountered once in Kenya.

Trypanosoma simiae is essentially a parasite of pigs, where it causes a fulminating disease with death occurring in a few days (Hoare, 1972). Infections by this trypanosome in sheep and goats have been limitedly reported (Mackenzie *et al.*, 1975). In this study sheep, goats and pigs were found infected by *T. simiae.* No *T. evansi* infections were observed in sheep, goats and pigs. Jacquiet *et al* (1993) reported that goats could harbor *T. evansi* for more than 200 days without displaying any signs of disease. This indicates that goats could act as good reservoirs for trypanosomes.

5.5 Variation of Trypanosomosis with animal age and sex

The general comparison of infections between adult and young animals showed no significant difference. Study by Goossens *et al* (1998), observed no significant difference in infection between adult and young sheep and goats in Gambia.

Beably *et al* (1996) however, observed adult goats to be more prone to infections than were the young ones.

The higher potential productivity of the older animals, in terms of fecundity and milk yield apparently places them at a greater risk of serious trypanosomosis compared to the young animals. Helmithosis and other parasite such as theilaria are a major constraint on small ruminant and pig production (Ademosun, 1994). It was also expected that intercurrent infections, and synergistic effects of trypanosomes and other infections such as helminthes could have also exacerbated the severity of tyrpanosomsosis in adult animals. Haematocrit values decline from birth and a stable mean value occurs in early adulthood, when erythroporetic activity also declines (Jain, 1986). When trypanosome infections are superimposed on these physiological changes it may be expected that the disease will have different effects in different age groups, the adult animals being more susceptible. The low % PCV recorded in older animals compared to those of young animals or their immediate offspring that were less parasitaemic, was expected to contribute to high infections in adult than young animals.

From the study there was no significant difference in infections between female and male animals. Katunguka-Rwakishaya (1996) observed no significant difference in infection rates in both female and males of sheep and pigs in Uganda. However, a study by MacLennan (1974) showed that lactating goats had an increased prevalence of trypanosomal infections than non-lactating females and males. Similarly, female goats were more susceptible to infections than the males (Beably *et al.*, 1996) in Zambia. The significant stress created by lactation in

female animals (lactating and those which have kidded or given birth) could be a reflection in infection cases in females than in male animals. Whist stress of lactation may have some influence on the level of trypanosomal prevalence infections; it is likely that it is only a contributory factor.

5.6 Trypanosomosis and anaemia

The study shows that goats had the least mean % PCV followed by sheep and pigs. However, a comparison of mean % PCV for infected livestock and the uninfected ones showed no significant difference. There were also no significant difference in mean % PCV between the two age groups and sexes in the livestock. However, several animals were found with low mean PCV, a level 15, considered fatal. Twenty-three goats, 5 sheep and 4 pigs had PCV lower than15. It is clear that trypanosomosis due to both cattle and human infective has profound effect on the level of red blood cell.

Griffin *et al* (1981), noted high cases of aneamia in goats as a result of this disease that was primarily due to haemolysis. Initially accompanied by a marked stimulation of erythropoiesis, in chronic infections the anaemia is complicated by depressed erythopoiesis arising from iron deficiency. Munyua, 1981, observed high mortality cases in goats due to trypanosomosis, which was marked with considerable reduction in PCV of up to 16%. Goats that survived infection and had a severe anaemia, made rapid recovery after the disappearance of the parasite from the blood. The effects of trypanosomosis in small ruminants and pigs is reflected in the mean change in PCV, body weight and temperature and mortality rates (Beably *et al.*, 1996; Masiga *et al.*, 2002).

5.7 Tsetse fly density

Trapping of tsetse flies was done in mid October 2001, during the short rains. The fly catches were low, possibly due to combination of the control measures being undertaken by various organizations in the project of Farming in Tsetse Infested Areas (FITCA). The tsetse densities estimated as fly/trap/day (FTD) was highest in Amase at 0.92 and lowest in Ongariama at 0.42. Rukada recorded an FTD of 0.5. FTD. Omukuba (1995) had previously observed FTD value of 0.21 for the same area.

The role of biting flies in the transmission of trypanosomosis in Busia and Teso districts, as in other areas where tsetse transmitted trypanosomes predominates, is unknown. That *T.* vivax can be transmitted mechanically is well known, especially in view of the fact that this parasite has been maintained for many centuries in South America in the absence of tsetse flies. Their role in this area clearly warrants investigation. Tabanids and stomoxys have been associated with mechanical transmission of disease agents, many of which require transfer between host within minutes or hours (Foil *et al.*, 1987). Trapping of tsetse flies along the riverbanks accounted for the large proportion of *Glossina. f. fuscipes* , which have riverine distribution (Maudlin, 1989). The observation of the presence of *G. pallidepes* concurs with the early made observations of the now increasing number of this fly that dwells in savanna in the study areas.

5.8 Genetic relationship. *T. b. brucei* and *T. b. rhodesiense.*

Specific PCR at the five-microsatellite loci revealed a high degree of size polymorphism among *T. brucei spp.* Contrary to Biteau *et al* (2000) who used agarose gel electrophoresis to resolve the product of these 5 loci, resolution under denaturing conditions were undertaken which generated far greater levels of polymorphism. These markers have revealed far greater level of genetic variation than those observed with minisatellite (MacLeod *et al.,* 2000), isoenzyme electrophoresis (Gibson *et al.,* 1980; Godfrey *et al.,* 1990) and RFLP in ribosomal DNAs (Hide *et al.,* 1990; 1994) on similar analyses. Microsatellite analysis is therefore, a suitable technique for genotyping *T. brucei* isolates on large scale.

The microsatellite analysis of the *T. b. brucei* and *T. b. rhodesiense* has shown tha genetic diversity exists among them. The *T. b. rhodesiense* from the 4 villages of Teso districts share more common alleles at the 5 loci, which are distinct from the *T. b. rhodesiense* isolated from Busia district (Rukada village). Characterization data support this idea that a degree of micro heterogeneity in *T. b. rhodesiense* within and between different foci occurs (Dumas *et al.,* 1999). The *T. b. rhodesiense* stocks from different regions Botswana and Zambia have been shown to be quite distinct from those in Uganda and neighboring areas of Kenya, both by isoenzyme electrophoresis (Godfrey *et al.,* 1990) and by RFLP (Hide *et al.,* 1994). The *T. b. brucei* stocks were however, more heterogeneous in this study than *T. b. rhodesiense*. Each the *T. b. brucei* isolates although they share one or two alleles at the 5 loci, showed distinct allelic distribution in all the 5 loci analyzed in agreement with the conclusions of Hide *et al* (1994).

Microsatellite analysis does not only allow the unequivocal identification of alleles, but the relationship between alleles can be deduced from the region of homology. *T. b. brucei* and *T. b. rhodesiense* are shown to share common alleles at MORF 2-CA and M6C8 loci which may have impotant implication in gene flow, suggesting that genetic exchange and hence traits of importance such as human infective occurs between the human infective and non-human infective stocks. The fact that there is a great range of different *T. b. brucei* allelic variation compared to *T. b. rhodesiense*, suggest that the human infective isolates are a subset of the more heterogeneous *T. b. brucei* isolates, in agreement with the conclusion of Hide *et al* (1994) and MacLeod *et al* (1999).

Microsatellite analysis were also able to detect those isolates which contain more than one genotype of trypanosomes by virtue of these isolates containing more than two alleles at each locus. In principle, this could suggest that the trypanosomes were polypoid (a condition where a cell or organism has more than it's normal number of sets of chromosomes), however, this is unlikely as this has only been reported in the progeny from laboratory crosses and genotyping of single trypanosomes from field isolates only shows diploid patterns. Using these markers we have found evidence that genetically distinct trypanosomes co-exist in the blood stream of a significant proportion of small ruminants and pigs. Similar diversity of genotypes in animals was observed by Hide *et al.*, 1998, In contrast, only 49% mixed genotypes were observed in tsetse flies using minisatellite analysis (MacLeod *et al.,* 1999), while 9.6% mixed genotypes in tsetse flies were reported by isoenzyme data (Godfrey *et al.,* 1977). Low levels of mixed genotypes by isoenzyme, RFLP, RAPD and PFGE analyses Komba *et al.,* 1997).

Microsatellite mapping technique, which is able to identify allele unequivocally are especially suitable for distinguishing T. *b. brucei* from *T. b. rhodesiense* based on characteristic banding profiles that are unique to each sub species. This analysis has clear;y demonstrated that. *T. b. brucei* and *T. b. rhodesiense* are distinct, in support of the findings of MacLeod *et al* (2000). Similarly this technique has shown significant degree of genetic exchange between the *T. b. brucei* and *T. b. rhodesiense*. If this is the case, it has great implications in the spread and inheritance of traits of medical and economic importance such as serum resistant associated gene.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

Molecular methods of identification and detection of infection has proven sensitive and very reliable than parasitological methods. However, the cost of operation of such methods and the technical knowledge required is high than microscopy and hence the disease management will still rely heavily on parasitological detection of the parasite.

A marked difference in infection cases has been demonstrated in Western Kenya. From this work it however cannot be ascertained whether these differences in were genetic in origin, or due to other factors such as tsetse preference, difference in grazing habits, coat thickness, color of the coat or due to animal age or sex. Such possible causes of variation in infection should be investigated further and be considered in selection programs for upgrading local breeds.

Trypanosoma vivax was the most prevalent species followed by *T. simiae, T. congolense* and *T. brucei* . The variation in prevalence and the possible explanations for such variations should be investigated.

In this study, small ruminants and pigs were observed to harbor and were infected by trypanosomes that are a threat to human and cattle. Their role as reservoirs of trypanosomes in the area has been underestimated. Future disease management programs will need to pay more attention and proper care for controlling the infection in these animals.

Variation of infection cases among the five villages was observed. The factors responsible for such variation should be clearly investigated to check the spread of the disease to other regions and countries that are not affected by the disease.

The vector *Glossina pallidipes* that is restricted within the savanna regions and is the true vector of trypanosomes responsible for AAT and HAT is on the increase in Western Kenya. The actual role of this species together with other tsetse fly and other biting flies in the disease epidemiology is not clearly understood should be monitored periodically. Similarly the role of other biting flies *Stomoxys* and *Tabanids* spp in the transmission of trypanosomes in Western Kenya as in other areas beyond tsetse fly belt clearly warrants investigation.

The analysis of genetic relationship between *T. b. brucei* and *T. b. rhodesiense* showed significant degree of gene flow between these two subspecies of *T. brucei.* Small ruminants and pigs were observed to harbor a mixture of genotypes of *T. brucei*. Cloned isolates should be used for genetic analysis to checked if the variation in the 5-microsatellite loci observed were due to mixed genotype or due to polypoidy. Similarly, other genetic tools that are co-dominant and are suitable for genetic analysis should confirm the population genetic structure obtained by microsatellite.

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