USE OF cELISA IN VERIFICATION OF DRUG LEVELS IN THE SERUM FOLLOWING TREATMENT BY VARIOUS PERSONNEL IN LAMU DISTRICT

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DECLARATION

I hereby declare that this thesis is my original work and that it has not been presented to any other university or institution for the award of a degree.

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We confirm that the work reported in this thesis was carried out by the candidate under our supervision.

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DEDICATION

This work is dedicated to my children JARED DIELS ASHIEMBI and LACINA LEAH AGUMBI for their perseverance at very tender age when I was carrying out this work, my late father JARED ASHIEMBI for what he instilled in me, my mother GRACE ASWANI, brothers and sisters; the ASHIEMBIS for their encouragement and inspiration and my husband EDWARD ANGOTE.

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ACRONYMS AND ABBREVIATIONS

AAT	Animal Africa Trypanosomosis
AHAs	Animal Health Assistants
cELISA	Competitive Enzyme-linked Immunosorbent Assay
CF	Complement Fixation
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleic triphosphates
FAO	Food and Agriculture Organization
FMD	Foot and Mouth Disease
FTD	Fly per Trap per Day
G	Glossina
GC	Gas Chromatography
GDP	Gross Domestic Production
HPLC	High Pressure Liquid Chromatography
ITS	Internal transcribed spacer
Km^2	Kilometer squared
MS	Mass Spectrometry
OD	Optical density
PAAT	Programme of African Animal Trypanosomosis
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline tween 20
PCR	Polymerase chain reaction
PCV	Packed Cell Volume
QCs	Quality Controls
RFLP	Restriction fragment length polymorphism

SIT	Sterile Insect Techniques
SPP	Species
T.	*Trypanosoma*
TLC	Thin Layer Chromatography
TMB	Tetramethyl benzidine
Vet	Veterinary

ABSTRACT

Animal trypanosomosis is a serious constraint to animal production in many parts of Africa. Economic losses in Kenya are estimated at Kshs.700million annually from livestock deaths. The cost of importation of trypanocidal drugs and pesticides amounts to KShs.300 million and 60 million respectively. Control of this disease is mainly by use of chemoprophylactic and chemotherapeutic drugs including diminazene aceturate, isometamidium and homidium bromide. Farmers have been experiencing treatment failure mainly attributable to improper use of trypanocidal drugs, development of resistance and poor quality of drugs. This study was carried out to determine the role of trypanocidal drug use in treatment failure in cattle in five sites within Lamu District. Cattle were randomly selected and marked for identification from five selected sites of Lamu. Blood was collected from the ear vein using heparinised capillary tubes for parasitological examination of trypanosomes and (PCV) determination. The presence of trypanosomes was determined using the micro-haematocrit centrifugation and buffy coat/dark ground techniques. Blood collected from the jugular vein was used to determine the serum drug level by competitive enzyme-linked immunosorbent assay (cELISA). The type of trypanocidal drug used, personnel administering the drug, the time when the cattle were treated last were recorded from the farmers. The cELISA for determination of drug levels in the serum after extraction in 0.1M borax at pH 9.7 was carried out for the three drugs (diminazene, homidium and isometamedium). The analysis of serum prepared from blood samples collected from the five sites in Lamu district showed an overall treatment failure of 12.9%. This study has demonstrated that drug levels can be used to evaluate treatment failure and drug use which can contribute to efficacy of trypanocidal drug. The study has also established that the three types of trypanocidal drugs are being used in the area, both the veterinary personnel and farmers administer the drug and the drugs were administered intra-muscularly. There was a small percentage (20%) of treatment due to drug resistance, there were also few cases of mis-diagnosis. Treatment failure was largely due to under-dosing. Overall, livestock keepers under dosed 135 animals as compared to 52 by Veterinary personnel. There was a big percentage (81.2%) of drug levels below therapeutic level. The study has demonstrated further that cELISA method can be used in the verification of trypanocidal drug use as a cause of treatment failure.

CHAPTER ONE: INTRODUCTION

1.1 Background

Cattle trypanosomosis is widespread in Kenya and occurs both within and outside tsetse belts. It is estimated that 25% of Kenya, comprising 7 out of 8 administrative provinces and 60% of the productive, well watered rangelands are tsetse infested (Chemuliti *et al.,* 2005 ; Otindo, 2005). Lamu District, in Coast Province of Kenya, covers a strip of northeastern coastal mainland and the islands of Pate, Manda and Lamu. Smaller islands include Kiwayu, which lies in the Kiunga Marine National Reserve, and Manda Toto. Lamu District has an area of 6,166.7 sq Kilometers and population of 84,146 as lamu district annual progress report 2008/09 financial year (http://www.aridland.go.ke/semi_annual_reports/semi_arid_08-09/lamu_08-09.pdf). The district lies between latitude 1'40' and 2'30' south and longitudes 40'15' and 40'38' east. The district is divided into seven administrative divisions: Amu (Lamu Island), Kiunga, Faza, Kizingitini, Mpeketoni, Hindi and Witu. There are extensive mangrove forests and savannah grasslands in the area which are ideal habitats for tsetse flies. (http://www.enotes.com/topic/lamu-district,kenya.aspx, http://lamu-district.co.tv, http://wikien4.appspot.com/wiki/lamu-district).

A cross-sectional and a partial longitudinal surveys were conducted by Mdachi *et al.,* (2008 and 2006b) to determine the prevalence of trypanosomosis, tsetse

challenge and the effectiveness of control strategies including trypanocidal drugs as control measures for trypanosomosis in Lamu District. From his study, there was 78.8% relapse infections observed in cattle in the district. The prevalence of relapse infection increased significantly with infection rate in cattle. There were also cases of trypanocidal treatments that were considered to have failed to effect cure.

1.1.1 Economic importance of trypanosomosis

Trypanosomosis reduces meat and milk production in cattle by at least 50% (Swallow, 1999). It also limits opportunities for farmers in terms of crop and livestock production: there is less efficient nutrient cycling, less access to animal traction, lower income from milk and meat sales and less access to liquid capital. The economic losses in cattle production alone are between US $1 and 1.2 billion annually while losses, in terms of agriculture GDP in Africa, amounts to about US $4.75 billion per year (Aksoy, 2003; Cleveland, 2007). Economic losses from AAT related livestock deaths in Kenya are estimated at Kshs.700 million annually. The cost of importation of trypanocidal drugs and pesticides amounts to Kshs.300 million and 60 million, respectively (Otindo, 2005).

1.1.2 Treatment failure

Cases of treatment failure by use of the three trypanocidal drugs could be attributed to reduced sensitivity of trypanosomes (drug resistance) and / or improper drug use by farmers. Earlier study has demonstrated that despite

presence of drug resistant trypanosomes in Lamu District, therapeutic and prophylactic drugs are still efficacious consequently, by using quality drugs correctly, trypanosomosis can be controlled effectively (Mdachi *et al.,* 2006b). Some factors relating to the farmer and the parasite have been identified as contributing to treatment failure (Mdachi *et al.,* 2006b).

1.1.3 Trypanocidal drug used in Lamu

Many farmers indicated they had used trypanocidal drugs which included diminazene aceturate, isometamidium and homidium bromide for chemotherapy and chemoprophylaxis (Swallow, 1999; Machila *et al.,* 2003; Mdachi *et al.,* 2006). Chemotherapy remains the principal control method of both animal and human trypanosomosis (Kagira and Maina, 2007). Despite the widespread use of trypanocidal drugs and the other reported control technologies, trypanosomosis continues to be a major constraint to livestock production in Lamu district. Three types of trypanocidal drugs have been reported to have been used in the control of trypanosomosis in Lamu district; homidium bromide chloride/bromide (Ethidium^{R} Chloride, Novidium^{R}, Ethidium^{R} bromide), isometamidium chloride (Somarin^{R}, Trypamidium^{R}) and diminazene aceturate aceturate (Berenil^{R}, Ganaseg^{R}, Trypazen^{R}, Veriben^{R}).

1.1.4 Mode and personnel administering the drugs

The treatment of trypanosomosis is carried out by the farmers themselves and veterinary personnel. Farmers increasingly treat their own cattle but often do not accurately diagnose diseases (Machila *et al.,* 2003), mainly to avoid the cost of involving veterinary personnel, following the introduction of cost sharing of services earlier provided free by the Government. Furthermore the privatization of veterinary services in Africa means that farmers rather than veterinary personnel now commonly give drugs (Maudlin, 2003) to avoid paying for the charges.

The mode of drug administration used to treat the animals by both farmers and veterinary personnel is intra-muscular as recommended (Mdachi *et al.,* 1995). This is either on the neck muscles or on the quarters. However, treatment failure is in the increase. Sub-therapeutic blood drug levels attained following intra-mascular administration of various trypanocides by farmers and vet personnel might be the basis of treatment failure. cELISA has been used to determine whether therapeutic trypanocidal drug levels are attained following intra-mascular administration of trypanocides by both vet personnel and farmers and to associate this with treatment failure in Lamu district. The drugs are recommended to be used at the dose rate of 3.5 mg/kg for diminazene aceturate products (Fairclough, 1963; Uilenberg, 1998; Murilla *et al.,* 2003), 1mg/kg for homidium bromide products (Leach and Roberts, 1981; Uilenberg, 1998; Murilla *et al.,* 1999) and 0.5-1.0 mg/kg for isometamidium products (Kinabo and McKellar, 1990). It is not known

whether treatment failure experienced in this area is due to underdosing or drug resistance. The results will determine whether the treatment failure is due to underdosing or drug resistance.

1.2 Problem statement and justification

1.2.1 Problem statement

Trypanosomosis is a major disease affecting livestock at the Kenyan coast (Thorpe *et al.,* 1993). The control of trypanosomosis in cattle is mainly by use of only three drugs; diminazene aceturate, isometamidium chloride and homidium bromide. Disease diagnosis and treatment is often left to cattle keepers and extension workers who lack any formal clinical knowledge or diagnostic ability (Eisler *et al.,* 2004). In the absence of effective diagnosis, preventive measures and treatment are either used incorrectly or inappropriately (Eisler *et al.,* 2004). The aim of this study was to determine whether therapeutic trypanocidal drug levels are attained following administration of trypanocides by both vet personnel and farmers by using cELISA.

1.2.2 Justification

The problem of trypanocidal treatment failure has been a common occurrence in coastal region (Eisler *et al.,* 2004; Mdachi *et al.,* 2006). This has resulted in increased cost of treatment and losses of animal through death leading to loss of income. Trypanocidal drugs remain the only widely available control method

affordable by farmers (Maudlin, 2003). Over 90% of farmers use trypanocidal drugs to control trypanosomosis (Swallow, 1999; Machila *et al.,* 2003; Mdachi *et al.,* 2006). Treatment failure has been recorded within this region (Mdachi *et al.,* 2006b). Few studies have been done in evaluating the use of the three trypanocidal drugs in the area. In this region, trypanocides are administered by trained veterinary professionals as well as untrained farmers. There is a possibility that the administered dosages do not deliver effective drug concentrations in the serum for effective treatment of the disease. There is no documented evidence to show the concentration of drugs in the serum following treatment of animals by farmers and by the veterinary personnel in the region.

1.3 Research question

Can cELISA be used to determine the association between trypanocidal administration and treatment failure in Lamu district?

1.4 Hypotheses

The hypotheses of this study were;

1. Drug levels can be used to evaluate and detect drug failure in control of trypanosomosis.
2. The administration of trypanocides to cattle by farmers and their neighbors deliver sub-therapeutic levels of drugs and lead to treatment failure.
3. cELISA method can be used in the verification of trypanocidal drug use as a cause of treatment failure.

1.5 Objectives

1.5.1 Main objective

Determine whether cELISA can be used to establish the role of trypanocidal drug use in treatment failure in cattle within Lamu District.

1.5.2 Specific objectives

Specific objectives were;

1. To establish the types of trypanocidal drugs used, the mode and the personnel administering them in five sites of Lamu District.
2. To establish the rate of treatment failure in cattle.
3. To establish the drug concentration achieved following administration of trypanocidal drugs in cattle by farmers and veterinary personnel.

1.6 Significance and anticipated output

The aim of this project will establish if improper use of trypanocidal drugs is one of the causes of treatment failure in Lamu district. The results would be useful to policy makers in developing strategies to reduce the misuse of the drugs and assist in developing a strategy to educate smallholder farmers and extension workers of trypanocidal drug use practices, and to assess their impact.

CHAPTER TWO: LITERATURE REVIEW

2.1 Animal trypanosomosis

Animal trypanosomosis is a vector borne disease caused by protozoan parasites of the genus *Trypanosoma* (T) (Finelle, 1983). There are different species of *Trypanosoma* found in African domesticated animals (Table 2.1). *T.congolense* is mostly found in sub-Saharan Africa and it causes trypanosomosis in cattle and other domestic animals including wild animals (Uilenberg, 1998). Other species include; *T. vivax* and *T. brucei* which causes trypanosomosis in cattle, antelope, horses and camel, *T. evansi* which causes trypanosomosis in camels, horses and deers and *T. equiperdum* which causes trypanosomosis in horses, donkeys) (Desquesnes, 2004).

Table 2.1: Species of trypanosomes occurring in various animal, their reservoir hosts and laboratory animals that can be infected.

Trypanosome species	Domestic animals affected	Reservoir hosts	Laboratory animals
T. congolense	Cattle, camels*, horses, dogs, sheep, goats, pigs	Several groups of wild mammals	Rats, mice, guinea pigs, rabbits
T. simiae	Pigs	Wart hog, bush pig	Rabbits, monkeys
T. godfreyi	Pigs	Wart hog	None susceptible
T. vivax	Cattle, sheep, goats, domestic buffalo, horses	Several groups of wild mammals	Usually none susceptible
T. uniforme	Cattle, sheep, goats	Various wild ruminants	None susceptible
T. brucei brucei	Horses, camels*, dogs, sheep, goats, cattle, pigs	Several groups of wild mammals	Rats, mice, guinea pigs, rabbits
T. brucei gambiense, T. brucei rhodesiense	Human sleeping sickness; affect domestic animals as *T. brucei brucei***	Several groups of wild mammals (particularly *T. brucei rhodesiense*)	As for *T. brucei brucei* (after initial adaptation where *T. brucei gambiense* is concerned)
T. evansi	Camels, horses, dogs, domestic buffalo, cattle	Several wild mammals in Latin America	As for *T. brucei brucei*
T. equiperdum	Horses, donkeys, mules	None known	As for *T. brucei brucei* (after initial adaptation)
T. theileri and *T. ingens* (subgenus *Megatrypanum*)	Cattle, domestic buffalo*** (not pathogenic)	Various wild ruminants	None

*Camels are highly susceptible to *T. congolense* and to *T. brucei*, but do not usually penetrate into tsetse country.

**In particular, the behavior of *T. b. rhodesiense* in domestic animals is

quite similar that of *T. b. brucei,* whereas *T. b. gambiense* is on the
average more chronic (as in humans).

*** Of the two only *T. theileri* was reported from domestic buffalo (Uilenberg, 1998).

2.1.2 Distribution of tsetse flies and animal trypanosomosis

Trypanosomosis occurs mostly in Sub-Saharan Africa north of Kalahari Desert where the vector; tsetse flies is endemic (Nordeen and Schneider, 2006). Tsetse flies occur in 36 countries (Ng'ayo *et al.*, 2005) and a total of about 10 million Km^2 in sub Sahara Africa (Samdi *et al.,* 2010; Connor, 1991). The number of cattle in Africa is assessed to be 160 million heads; however, the distribution is very uneven among various regions (Finelle, 1983). The distribution of Animal Africa Trypanosomosis (AAT), (Figure 2.1) is restricted between latitude 14°N and 29°S of African continent where the tsetse vector occurs (Nordeen and Schneider, 2006; Samdi *et al.,* 2010). It is estimated that 25% of the Kenya, comprising 7 out of 8 administrative provinces and 60% of the productive, well watered rangelands are tsetse infested (Chemuliti *et al.*, 2005; Otindo, 2005). Of approximately 7-10 million Km^2 of land that are infested by tsetse fly only 20 million cattle are raised. Under different circumstances, this land could support more than 140million cattle and increase meat production by 1.5million tones (Nordeen and Schneider, 2006).

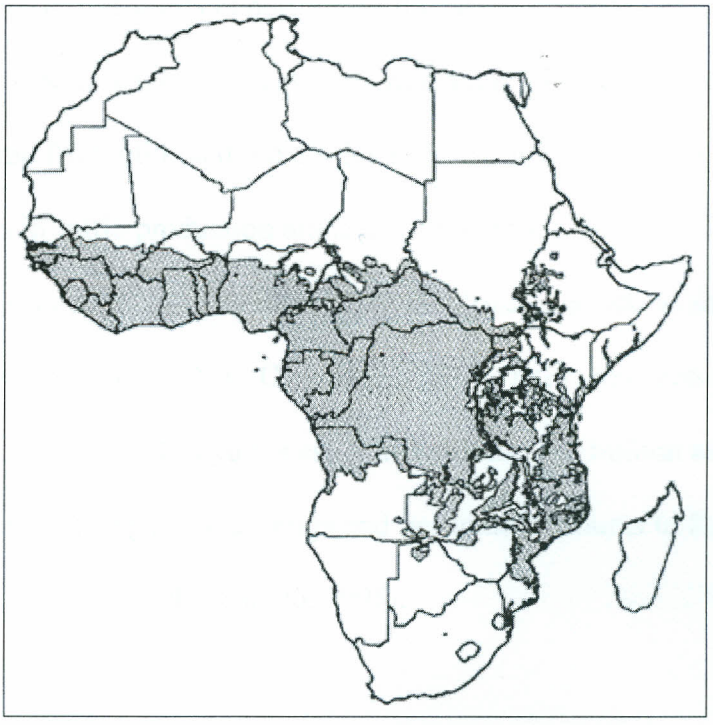


Figure 2.1 Distribution of tsetse flies on the African continent.
IAEA bulletin-vol 20, no.3; by Willem Takken and Michael Weiss (Uilenberg, 1998).

Key: Shaded areas indicate the tsetse fly infested areas.

2.1.3 Economic importance of trypanosomosis

About 50 million cattle and 20 million small ruminants are considered to be at risk of tsetse-transmitted trypanosomosis (Nordeen and Schneider, 2006) while 35 million doses of trypanocides are administered to 45 to 60 million cattle at risk of trypanososmosis (Kristjanson *et al.,* 1999; Sones, 2001). About 3 million cattle die of AAT annually in Sub-Saharan Africa (Connor, 1991; Cleveland, 2007). In tsetse-infested areas, trypanosomosis reduces meat and milk production by at least

50% (Swallow, 1999). It also limits opportunities for farmers in terms of crop and livestock production: there is less efficient nutrient cycling, less access to animal traction, lower income from milk and meat sales and less access to liquid capital. The economic losses in cattle production alone are between US $1 and 1.2 billion annually while losses, in terms of agriculture GDP in Africa, amounts to about US $4.75 billion per year (Aksoy, 2003; Cleveland, 2007). Economic losses from AAT related livestock deaths in Kenya are estimated at Kshs.700 million annually. The cost of importation of trypanocidal drugs and pesticides amounts to Kshs.300 million and 60 million, respectively (Otindo, 2005).

2.2 Transmission and life cycle of trypanosomes

2.2.1 Transmission by Tsetse flies

Tsetse flies are large biting flies that inhabit much of mid-continental Africa between the Sahara and the Kalahari deserts. They live by feeding on the blood of vertebrate animals and are primary biological vectors of Trypanosomes, which cause human sleeping sickness and animal trypanosomosis, also known as nagana. The tsetse flies are dipteric flies of the genus *Glossina*. Biologists have identified 23 different species of tsetse flies (*Glossina, G*) which all but three (*G. palpalis, G. morsitans* and *G. pallidipes*) transmit trypanosomal infection to mammals (Nordeen and Schneider, 2006). Both male and female flies feed on blood and are biological vectors of the parasite. The major pathogenic trypanosomes in Kenya

are *T. congolense* and *T. vivax* in cattle , *T. simiae* in pigs, and *T. evansi* in camels and horses (Otindo, 2005).

The life cycle of trypanosomes have a mammalian and a tsetse cycle (Figure 2.2). During a blood meal on the mammalian host, an infected tsetse fly injects metacyclic trypomastigotes into skin tissue. The parasites enter the lymphatic system and pass into the bloodstream. Inside the host, they transform into bloodstream trypomastigotes, are carried to other sites throughout the body, reach other blood fluids (e.g., lymph, spinal fluid), and continue the replication by binary fission. The entire life cycle of African Trypanosomes is represented by extracellular stages. The tsetse fly becomes infected with bloodstream trypomastigotes when taking a blood meal on an infected mammalian host. In the fly's midgut, the parasites transform into procyclic trypomastigotes, multiply by binary fission, leave the midgut, and transform into epimastigotes. The epimastigotes reach the fly's salivary glands and continue multiplication by binary fission. The cycle in the fly takes approximately 3 weeks. Humans are the main reservoir for *Trypanosoma brucei gambiense*, but this species can also be found in animals. Wild game animals are the main reservoir of *T. b. rhodesiense* (http://dpd.cdc.gov/dpdx/html/Trypanosomiasis Africa.htm; Murakami).

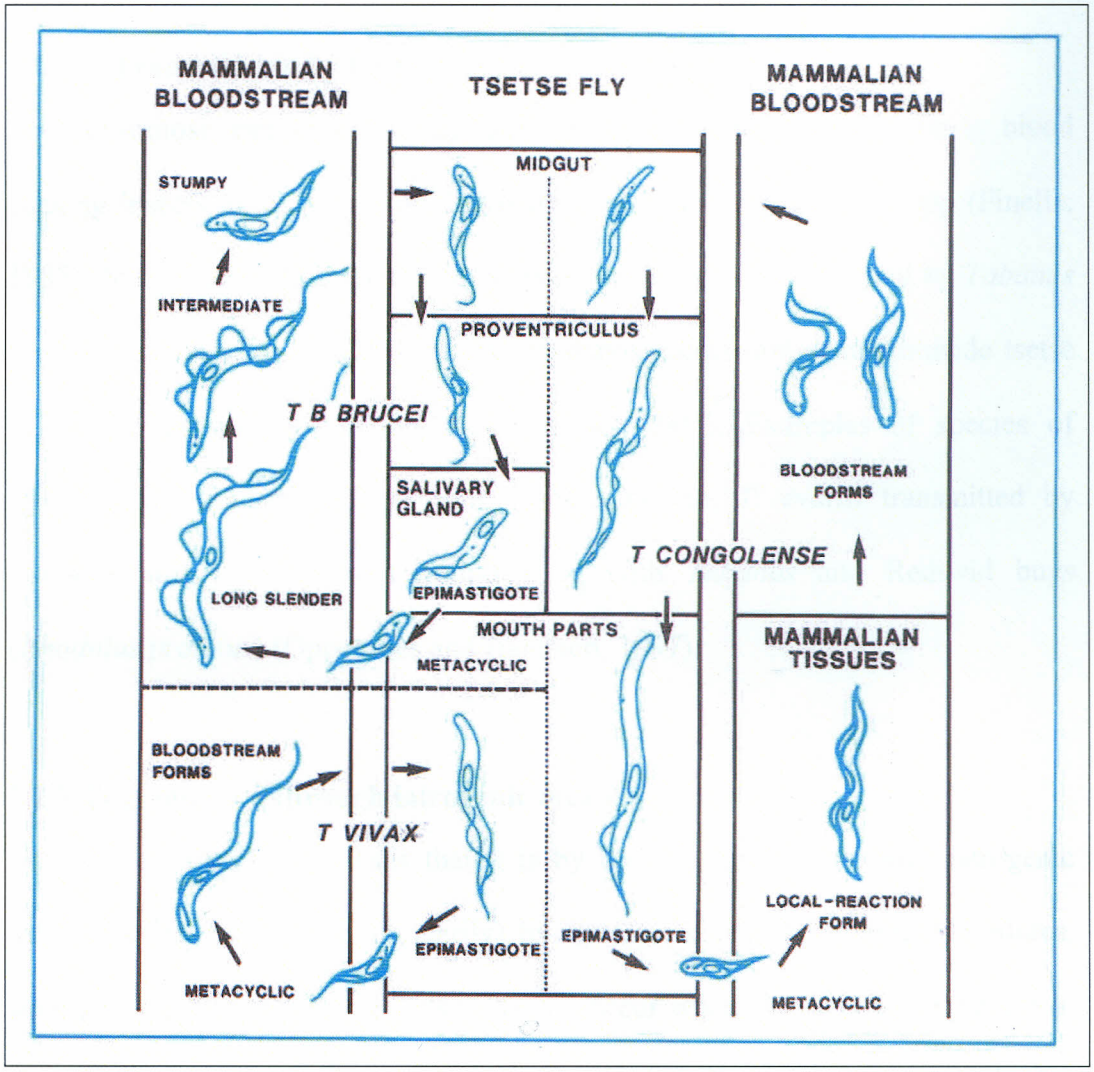


Figure 2.2. Life cycle of *T b brucei, T congolense* and *T vivax.*

Heavy outlines indicate parasite forms with surface coats consisting of variable glycoprotein antigens. Light outlines indicate uncoated forms which are not infective to mammals. T b brucei *develops in the tsetse midgut, proventriculus and salivary glands, where metacyclic forms occur which are infective to mammals.* T congolense *develops in the tsetse midgut, proventriculus and mouth parts, where infective metacyclic forms are produced. T vivax develops in the tsetse mouthparts* (ILRAD, 5th Annual Report, 1981).

2.2.2 Transmission by biting flies

Trypanosomosis can also be transmitted mechanically that is non cyclic by blood sucking insects as flies such as *Tabanus* (horseflies) and *Stomoxys* spp (Finelle, 1983). In Southeast Asia trypanosomosis is mechanically transmitted by *Tabanus* (Irwins and Jefferies, 2003). In Kenya trypanosomosis that occurs outside tsetse belts is transmitted by biting flies (Otindo, 2005). Examples of species of trypanosomes transmitted by biting insects include; *T. evansi* transmitted by *Tabanus* and *T. equinum* transmitted by both *Tabanus* and Reduvid bugs (*Rhodnius prolixus*) (Opperdoes and Hannaert, 1997).

2.2.3 Transmission through iatrogenic means

Iatrogenic transmission means that it is by the (veterinary) operator. Iatrogenic infections are induced (involuntarily) by operator using unhygienic procedures, such as contaminated instruments. This can occur when using the same needle or surgical instrument on more than one animal, at sufficiently short intervals that blood on the needle or instrument does not dry. It is not an uncommon occurrence when animals are vaccinated or treated by injection, or when blood is collected from several animals in a row, without changing or disinfecting needles or pins. It may also occur when several animals are subjected at short intervals to a surgical

intervention (dehorning, castration) without properly disinfecting instruments (Uilenberg, 1998).

During foot and mouth disease (FMD) vaccination campaigns it was found that use of one needle for several animals could also contribute to the spread of *T. vivax* (Davila and Silva, 2000).

2.3 Diagnosis of trypanosomosis

Diagnosis refers to methods for detecting infection, either by identifying the parasites themselves or by interpretation based on the results of immunological tests (Luckins, 1998). A disease may be diagnosed on the basis of the clinical signs and symptoms (anaemia, weight loss, abortion, possibly death) but none of these are pathognomonic (Sinshaw *et al.,* 2006; Molyneux, 2002; Ooijen, 1993; Nantulya, 1990), by demonstration of the causative organism or by reactions to diagnostic tests (Luckins, 1998). There are a number of techniques available for the diagnosis of animal trypanosomosis (Ooijen, 1993).The type of diagnostic test used in the detection of infections caused by the animal trypanosomiases will vary according to the epidemiological characteristics of the disease and the strategy for control (Molyneux, 2002). Diagnostic procedures vary not only according to the tools available, but often even more to what one wishes to know (Sinshaw *et al.,* 2006).

2.3.1 Clinical diagnosis

The clinical signs of disease caused by these organisms vary according to the trypanosome species, the virulence of the particular isolate and the species of host infected. Clinical signs are so varied and the ecological conditions under which trypanosomosis occur so diverse that, in terms of identifying animals with active infections, clinical diagnosis is too imprecise a procedure to use as a basis for the control of trypanosomosis, and other means of diagnosis must be employed (Luckins, 1998). The clinical manifestation of trypanosomosis in animals is influenced by the host as well as the trypanosome species and "strain". In general, the disease is characterised by severe anaemia, weight loss, reduced productivity, infertility and abortion, with death occurring in some animals during the acute phase of the disease. Owing to these varied clinical manifestations, diagnosis of trypanosomosis cannot be based on clinical signs alone. Laboratory confirmation of the diagnosis is an absolute necessity (Nantulya, 1990). The clinical disease in cattle varies considerably in severity and duration and the signs are often considered to be either acute or chronic (Ooijen, 1993).

A disease may be diagnosed on the basis of the clinical signs and symptoms (anaemia, weight loss, abortion, possibly death) but none of these are pathognomonic (Nantulya, 1990; Ooijen, 1993; Molyneux, 2002; Sinshaw *et al.*, 2006). Although clinical signs may be important in some situations, they are not

considered specific enough for accurate assessment of the parasites to which a herd may be exposed (Sinshaw *et al.,* 2006).

2.3.2 Parasitological diagnosis

The easiest technique for detection of trypanosomes in peripheral blood is by direct microscopic examination of blood, either by the wet film method to detect motile trypanosomes or, as stained thick and thin smears, when parasites are identified on the basis of their morphology by light microscopy. Examination of wet blood films is quick and the method is suitable for screening large numbers of animals. This method, however, is insensitive as half of the infected animals may be missed (Nantulya, 1990).

The sensitivity of direct microscopic examination can be improved through concentration of the parasites by centrifugation. When unclotted blood is spun in a haematocrit centrifuge, the trypanosomes are concentrated at the buffy coat. Examination of the buffy coat is thus more sensitive than examination of blood films. Initially used for the detection of avian trypanosomes, this technique has gained wide application through various modifications. The microhaematocrit centrifugation technique is particularly useful in that the status of anaemia in the test animals can be assessed at the same time. This technique, however, requires the use of electricity which limits its application in the field ((Nantulya, 1990).

Blood from suspect animals can also be inoculated into susceptible laboratory animals, usually mice or rats. This technique is more sensitive than direct microscopic examination of the blood sample. Animal inoculation has the added advantage that trypanosome isolates can be collected for other studies in the laboratory. This, however, is not a practical technique because diagnosis is not immediate. In addition, the cost of maintaining the animals makes the method prohibitively expensive for routine diagnosis, especially in the field. Furthermore, some trypanosome isolates, notably East African *T. vivax, T. simiae* and, to a lesser extent, *T. congolense,* do not infect laboratory rodents (Nantulya, 1990). It can thus be seen that despite several improvements in the techniques for trypanosome detection, a high proportion of infections still go undetected as the majority of infections are chronic and often aparasitaemic. Also, the intermittent parasitaemia arising from the phenomenon of antigenic variation may preclude detection of the parasites even in acute infections. Moreover, some of the techniques are not practical enough to be applied in the field (Nantulya, 1990).

2.3.3 Immunological diagnosis

Several techniques have been developed for immunodiagnosis of trypanosomosis. Most of these are based on the detection of immune responses of the animal to the infection (Nantulya, 1990).

2.3.3.1 Antibody detection tests

As early as 1899, it was shown that sera from trypanosome-infected animals caused lysis of trypanosomes. Trypanosome lysis was later shown to be both complement- and antibody-dependent. This was the basis for the development of the complement fixation (CF) test for the diagnosis of trypanosomosis. The main handicap of the CF test is the difficulty encountered in preparation and standardization of the antigen for use in the test. Moreover, the test itself is difficult to perform; it is cumbersome and requires supplies of sheep red blood cells, complement, a centrifuge and a refrigerator. Thus, it is frequently unsuitable as a routine diagnostic tool. The next generation of tools for immunodiagnosis of trypanosomosis emanated from the observation that sera from trypanosome-infected animals were gelatinized. However, the tests were not specific for any one disease. Moreover, later investigators failed to reproduce, let alone correlate, the results of these test with patent parasitaemia in subsequent studies. Major improvements in the specificity and sensitivity of immunodiagnostic techniques were recorded through the introduction of the indirect haemagglutination test. The basic problems with this technique were that the antigens used were not well-defined and as a result, the test was difficult to standardize with regard to sensitivity and specificity. Moreover, coupling of the antigen onto the red blood cells can be irregular; the cells may be unstable on storage and nonspecific agglutination can be encountered due to heterophile antibodies (Nantyula, 1990).

One of the most significant improvements in trypanosomiasis serodiagnosis was the introduction of the indirect immunofluorescent antibody test, but there are two major problems associated with this method of antigen preparation. First, preparation of large numbers of blood smears to provide a standardized antigen preparation is cumbersome and the slides require ultra-low temperatures for storage and transportation. Second, the antigens so prepared provide substantial non-specific reactions (Nantyula, 1990). The introduction of enzyme immunoassays was a major breakthrough in the field of immunodiagnosis. A major limitation of cELISA in its application as a routine diagnostic test is the nature of the antigens used in the assay. The antigen is usually a crude trypanosome lysate, the quality of which is ill-defined. This makes the test difficult to standardize with regard to specificity and sensitivity. The persistent disadvantage of trypanosomiasis serodiagnosis has always been the lack of well-defined, standardized antigens (Nantyula, 1990).

All the serological tests described above measure host antibody responses to antigens of the infecting trypanosomes, specifically (i.e. using trypanosome antigens) or non-specifically (i.e. measurement of immunoglobulin rise). Antibody detection systems, however, can provide only a presumptive diagnosis as they do not differentiate between current and past infections. Therefore, a positive antibody detection test does not necessarily form a good basis for the decision to treat the animal. This is because anti-trypanosomal antibody levels may persist for

several months following successful trypanocidal therapy or spontaneous self-cure. They are thus more useful as epidemiological tools rather than as diagnostic procedures. For this reason, attention has continued to be focused on the development of tests that can differentiate between exposure and a current infection (Nantyula, 1990).

2.3.3.2 Antigen detection test

An alternative approach to antibody detection would involve the use of assays to detect trypanosome-specific antigen in the blood of infected animals as a means of diagnosis. This polyclonal antibody system has, however, been found to have low specificity because cross-reactions occur with non-targeted trypanosome species and possibly with other parasitic diseases (Nantyula, 1990). There is a small proportion of animals in which antigens cannot be detected despite the patent parasitaemia. These results have also been observed under experimental conditions where very early in infection, high parasitaemia may not be associated with the presence of antigen in serum. Since the basis for this test is that trypanosomes have to be destroyed to release the antigens in circulation, it is conceivable that the test will not be positive until after a critical number of trypanosomes have been destroyed by the immune response of the host. Second, there is a high proportion of animals without parasitaemia which test positive for antigens. Considering that the control animals from trypanosomiasis-free areas do not test positive for antigen, the cases detected by this test may represent true infections that cannot be

diagnosed by parasitological techniques. The most effective diagnostic strategy, therefore, will be to combine antigen-trapping cELISA with one of the more sensitive standard trypanosome detection methods (Nantulya, 1990).

2.3.4 Deoxyribonucleic acid (DNA) based techniques

The diagnosis of trypanosomes has been improved since the 1980s by DNA-based techniques. DNA diagnosis is either based on hydridization profiles of parasite DNA with

DNA probes or polymerase chain reaction (PCR) technology (http://www.diss.fu-berlin.de/diss/servlets/MCRFileNodeServlet/FUDISS_derivate_000000002052/06_litre.pdf;jsessio). Currently, several PCR based diagnostic assays have been developed to improve the detection of pathogenic trypanosomes (Thumbi *et al.,* 2008). Reliable DNA based methodologies to determine prevalence of trypanosome species in domestic livestock have been available for over 10 years (Picozzi *et al.,* 2002). Despite that, they are rarely used to generate baseline data for control operations for this disease in the field (Picozzi *et al.,* 2002). Rather such operations tend to rely on data which can be generated using low technology methods such as direct observation of parasites by microscopy. Diagnosis by PCR on buffy coat preparations on Whatman^{R} FTA^{R} matrices is most sensitive (Picozzi *et al.,* 2002).

A number of important epidemiological issues have been addressed through the application of PCR and DNA probe technology to the problem of animal trypanosomoiasis. These applications have clearly demonstrated the potential for wide-scale application of DNA-based diagnostics in the epidemiology of trypanosomoiasis (Mauldin *et al.,* 2004). A single repetitive DNA probe can identify all members of subgenus *Trypanosoma* as well as non-tsetse-transmitted trypanosomes (Gibson, 2002). Use of this battery DNA probes to identify the trypanosomes carried out by tsetse flies in the field has yielded some surprises about the accuracy (or inaccuracy) of previous identification methods (Gibson, 2002). An unexpectedly high prevalence of mixed infections has been found in all the field studies carried out so far. The large number of infections that remain unidentified by the available probes suggests the existence of other, as yet, unknown trypanosome species.

A DNA-probe is a known DNA sequence which can be obtained by cloning or by PCR with labeled nucleotides (enzymes or isotopes). DNA probing entails exposing a denatured DNA sample fixed on nitro-cellulose to a labeled DNA-probe under specific salt and temperature conditions. If the complementary DNA sequence is present in the sample, the probes will bind to it and remain on the nitro-cellulose where they can be visualized (Desquesnes and Dàvila, 2002). Probes have been developed for the main pathogenic trypanosomes (Masiga and Gibson, 1990), but the sensitivity of this technique is limited to 100 parasites

(Masiga *et al.*, 1992), which is not sufficient for trypanosome detection in mouthparts of the vectors or in host blood when the parasitaemia is low.

The PCR is an *in vitro* technique which allows the amplification of a specific DNA region that lies between two regions of a known DNA sequence. The technique exploits a thermostable enzyme, *Taq* polymerase, which synthesizes a new strand of DNA by copying an original DNA template (Mullis *et al.*, 1986). PCR was originally developed to amplify sequences of interest and to increase the sensitivity of detection using DNA-probes (Saiki *et al.*, 1986). PCR amplification of DNA is achieved by using oligonucleotide primers. These are short, single-stranded DNA molecules which are complementary to the ends of a defined sequence of DNA template. The primers are extended on a single-stranded denatured DNA template by a DNA polymerase, in the presence of deoxynucleoside triphosphates (dNTPs) under suitable reaction conditions. This results in the synthesis of a new strand complementary to the template strands. Following 30 – 40 cycles, the DNA will have been amplified several million times, and the resulting PCR product can be visualized on an agarose or polyacrylamide gel, after staining with ethidium bromide and exposing it under ultraviolet light.

The specific size of the PCR product is evaluated by simultaneous migration of molecular size markers and a positive control. A negative control is run together to evidence any DNA contamination. The actual sequence amplified can be analyzed

by sequencing. Generally, once the specificity of the primers has been established, the size of the PCR product is sufficiently characteristic for diagnostic purposes (Newton and Graham, 1997; Desquesnes and Dàvila, 2002). The use of quantitative PCR techniques has been shown to be of potential value for other types of parasitic infections in domestic animals and gives indication of level of parasite burden whereas convectional PCR simply indicate the presence or absence of parasite DNA (Mauldin *et al.,* 2004).

2.3.4.1 Diagnostic targets of PCR

PCR diagnosis aims to identify the parasite at the species level, which can be done using various targets. The preferred targets are those which are present in a high copy number in the genome of trypanosomatids; the more copies of the target, the greater the chances of amplifying it by PCR. Single copy genes are more difficult to amplify (MacLeod *et al.,* 1997) and are rarely targeted since low parasitaemia is a characteristic of trypanosome infection and the sensitivity would be too low (Desquesnes and Dàvila, 2002). Mini-chromosomes of the nuclear DNA contain satellite DNA which has been the most favoured target in the development of species-specific primers able to detect very small amounts of parasite DNA. Such primers were developed for the main pathogenic

trypanosomes: *T. vivax* (Masiga *et al.,* 1992 and *T. evansi* (Artama *et al.,* 1992). A single animal may contain a diversity of *Trypanosoma* species, therefore, PCR diagnosis carried out will require several PCR reactions; for example, in cattle, up

to five reactions per sample may be required. Research is now focusing on a diagnosis based on the amplification of the internal transcribed spacer-1 (ITS-1) of ribosomal DNA which presents the advantages of being a multi-copy locus (100–200), having a small size (300–800 bp), which varies from one taxon to another but is conserved in size in a given taxon. This may lead to the development of a multi-species-specific diagnostic protocol using a single PCR (Desquesnes and Dàvila, 2002).

This technique is limited in that, the sensitivity of the primers is lower than that of satellite DNA primers, due to partial homology of the sequences and their limited repetitiveness. DNA satellite sequences are repeated 10, 000-20,000 times as compared to ITS-1 sequences of only 100-200. A single polymerase chain reaction (PCR) - restriction fragment length polymorphism (RFLP) assay can be used to characterise all important bovine trypanosome species (Geysen *et al.,* 2003). Restriction enzyme analysis using *Msp*1 and *Eco*571 gave a clear destinction between *T. congolense*, *T. brucei*, *T. vivax* and *T. Theileri* (Delespaux *et al.,* 2003). Several subgroups within the *T. congolense* group could be distinguished using this method, although no difference could be detected between the species belonging to the subgenus *Trypanozoon* (Delespaux *et al.,* 2003).

2.3.4.2 Specificity and sensitivity of PCR diagnosis and typing

Several levels of specificity have been achieved from sub-genus to species, sub-species and even types. Random priming of trypanosome DNA has even allowed "isolate specific" identification. PCR has allowed an increase in the specificity of diagnosis in vectors such as tsetse flies. Direct determination of the trypanosome species-type in the vector or host is likely to provide a more reliable estimate of parasite prevalence since no selection is exerted on the ability of the parasite to grow either in culture or in animals (Majiwa *et al.,* 1993). Many natural trypanosome infections of either tsetse or livestock are due to more than one trypanosome species (Nyeko *et al.,* 1990). Precise evaluation of such infections by PCR requires that the oligonucleotide primers for PCR retain their specificities under standard assay conditions. Species-specific DNA probes have been shown to detect simultaneous infection of cattle with *T. vivax*, *T. b. brucei*, and *T. congolense* when conventional methods revealed only single infections (Nyeko *et al.,* 1990).

PCR coupled with DNA probe hybridisation could prove to be a highly sensitive tool for the diagnosis and assessment of the therapeutic efficacy and disease progress especially in chronic trypanosomosis (Clausen *et al.,* 1999). Individual hosts often harbour more than one parasite clone. It was previously thought that certain *Nannomonas* species were limited to particular ecological niches but it has been proved that this is not the case, as revealed when these probes are used in

epidemiological studies (McNamara *et al.,* 1995). *T. (N.) congolense* comprises morphologically identical but genotypically heterogeneous trypanosomes that express different phenotypes in terms of vector and host specificities, and disease symptoms (Godfrey, 1982; Young and Godfrey, 1983; Majiwa *et al.,* 1986). Four genotypic groups of *T. (N.) congolense* have been recognised. They are designated Savannah-type *T. (N.) congolense*, West African forest/riverine-type *T. (N.) congolense*, Kilifi-type *T. (N.) congolense* and the Tsavo-type *T. (N.) congolense* (Young and Godfrey, 1983; Majiwa *et al.,* 1985; Majiwa *et al.,* 1993). Repetitive, tandomly arranged DNA sequences and oligonucleotide primers, specific for each of these types, for use in polymerase chain reaction (PCR) amplification, have been described (Majiwa *et al.,* 1985; Kimmel *et al.,* 1987; Gibson, *et al.,* 1988; Dickin and Gibson, 1989; Majiwa *et al.,* 1993).

PCR provides a highly specific diagnosis of *Trypanosoma* infections in animals. The most used primer sets for trypanosomes have never failed in terms of their specificity (Desquesnes and Dàvila, 2002). However, species-specific primers have not yet been described for some trypanosome species such as *T. equiperdum*. Although it is possible to distinguish *T. b. rhodesiense* from *T. b. brucei* using three mini-satelite markers (McLeod *et al.,* 2000) or using serum-resistance-associated gene (Welburn *et al.,* 2001), couples of sub-species-specific primers are not yet available (Desquesnes and Dàvila, 2002). PCR allows the detection of a single specific sequence of DNA; consequently, a single parasite (0.1 pg of DNA)

or even fewer can be detected when using satellite DNA (Desquesnes and Dàvila, 2002). This very sensitivity also brings a very high risk of false positive results since a very small amount of other biological materials can contaminate the sample. The sensitivity threshold of the trypanosome detection by PCR generally ranges from 1 to 20 parasite/ml of blood, depending on the technique used. Below this level of parasitaemia, PCR cannot detect the infection, the volume of the samples processed and the concentration need to be increase through the parasitological techniques to increase the sensitivity. There is still a need to design primers based on repetitive sequences for a sensitive detection and specific identification of *T. b. brucei*, *T. b. rhodesiense* and *T. b. gambiense* for epidemiological studies in vectors, domestic and wild reservoirs of human sleeping sickness (Desquesnes and Dàvila, 2002).

DNA-probe technique is laborious and time consuming (Desquesnes and Dàvila, 2002), in recent years the development of PCR alone has been mainly devoted to diagnosis without the need of probes. Limited use of PCR has been made for diagnosis of human and animal trypanosomoiasis, due to its high cost (Gibson, 2002). The rate of adoption of diagnostic DNA technology by laboratories in developing countries appears to be limited not only by cost but also by widespread perception that is highly complex. This diagnostic techniques do have the potential to function efficiently even in hands of modestly trained technical staff. It

can totally automate, thereby minimizing steps involved in sample handling and decreasing the possibility of contamination (Maudlin *et al.,* 2004).

Diagnosis of trypanosomosis is notoriously difficult, not only are there no specific clinical signs but the intermittent and usually low parasitemias make detection of trypanosomes difficult (Connor, 1991; Eisler *et al.*, 1998; Njiru *et al.,* 2002; Ouma *et al.,* 2007). The clinical manifestation of trypanosomosis in animals is influenced by the host as well as the trypanosome species and "strain" (Nantulya, 1990). Animals which survive often remain infected for several months or years, exhibiting a low level of fluctuating parasitaemia which serves as a reservoir for the disease. Occasionally, however, the infected animals may undergo spontaneous recovery. Owing to these varied clinical manifestations, diagnosis of trypanosomosis cannot be based on clinical signs alone (Nantulya, 1990). The clinical signs of disease caused by these organisms vary according to the trypanosome species, the virulence of the particular isolate and the species of host infected (Luckins *et al.*, 1994).

Disease diagnosis and treatment is often left to cattle keepers and extension workers who lack any formal clinical knowledge or diagnostic ability, leading to preventive measures and treatments being used incorrectly or inappropriately (Eisler *et al.,* 2004). Important consideration in the diagnosis of trypanosomosis are that, firstly, the number of detectable parasites is not necessarily directly

related to the severity of the disease and secondly, in trypanosomosis-enzootic areas the disease is a herd problem (Connor, 1991). In tsetse-infested areas clinical signs of trypanosomosis are well recognized (Connor, 1991). Diagnosis is important to monitor the incidence of infection in rural level, assists in management decisions on large farms and is essential for disease surveillance and monitoring control programmes at district and provincial levels (Connor, 1991). The clinical signs are so varied and the ecological conditions under which trypanosomosis occur so diverse that, in terms of identifying animals with active infections, clinical diagnosis is too imprecise a procedure to use as a basis for the control of trypanosomosis (Luckins, 1998). Thus, mis-diagnosis which leads to treatment failure.

2.4 Control, prevention and treatment

Many techniques have been used to reduce tsetse populations with earlier crude methods being replaced in more recent times by methods which are cheaper, more directed, and ecologically better considered. These include: bush and game clearing, live bait techniques, traps and targets, insecticides and pesticides (Uilenberg, 1998; Kuzoe and Schofield, 2005). These methods are crude, costly, require commitment and most of them are more of an environmental problem than a benefit (Kuzoe and Schofield, 2005). Chemoprophylactic and chemotherapeutic trypanocidal drugs is another way of controlling trypanosomosis and is a common practice by the Kenyan pastoralists (Otindo, 2005). There are three different

compounds commonly used and have been in the market for over 40 years; diminazene aceturate (Berenil^{R}), isometamidium (Samorin^{R}, Trypamidium^{R}) and homidium bromide (Novidium^{R}, Ethidium^{R}) (Geerts *et al.*, 2001). It is estimated that about 35 million doses of trypanocidal drugs are currently used in Africa per year (Geerts and Holmes, 1998). Pharmacokinetics of trypanocides is incompletely understood, but the rate of excretion of the different compounds is known to affect their activity. Diminazene aceturate is rapidly excreted and therefore used for therapeutic purposes, while isometamidium is excreted slowly and is effective as a prophylactic compound. Homidium bromide is excreted more slowly than diminazene aceturate but more rapidly than Isometamidium and thus has limited prophylactic activity (Connor, 1991; Murilla 1999).

2.5 Causes of treatment failure

Cases of treatment failure have been widely reported and are on the increase (Geerts and Holmes, 1998). There are different causes of drug failure including; under dosing, poor quality drugs and drug resistance. Under dosing is due to underestimation of body weight, over dilution of trypanocide and incorrect injection techniques (Connor, 1991; Eisler *et al.,* 1996; Pecoul *et al.*, 1999). Poor quality of drugs is due to counterfeits, poor manufacturing and drug decomposition; inaccessibility due to high cost or withdrawal from the market (Shakoor *et al.*, 1997). Although trypanocidal drugs remain the principal method of animal trypanosomosis control in most African countries, there is a growing concern that their future effectiveness may be severely curtailed by widespread

drug resistance (Geerts and Holmes, 1998). The number of case reports on drug resistance is increasing but there is a lack of reliable data at the regional or national level on the true prevalence and impact of drug resistance (Geerts and Holmes, 1998).

Drug resistance is the ability of a parasite to survive, what was previously determined to be lethal concentrations of a toxic drug. It occurs when trypanosomes are in contact with a sub-curative dose of trypanocide or when the administration is insufficient to ensure the destruction of the parasite (Shakoor *et al.,* 1997). The increasing frequency of drug resistance has been attributed to combination of microbial characteristics, selective pressures of anti-microbial use and societal and technologic changes that enhance the transmission of drug resistance organisms (Cohen, 1992). So far, resistance to one or more of the three trypanocides used in cattle has been reported in at least thirteen countries in Sub-Saharan Africa (Geerts and Holmes, 1998). This is probably an underestimation of the true situation, as in several countries, surveys for resistances have not yet been carried out. Little is known on mechanisms of drug resistance, and much of what is known has been based on studies on bacteria. Possible mechanisms of drug resistance in parasites include; conversion of the drug sensitive site, increased efflux or decreased influx, alternative pathway to by-pass inhibited reaction, increased production of drug sensitive enzymes, increase in the amount of an enzyme substrate; i.e. to compete with drug, decreased requirement for product of

inhibited reaction and failure to activate the drug (Sutherland and Holmes, 1993; Mulugeta *et al.* 1997). Drug resistance may be associated with: prolonged under-dosing due to underestimation of body weights, failure to calculate adequate dosage, deliberate under-dosing, poor preparation and administration, high incidence of trypanosomosis and erratic treatments with prophylactics Connor, 1991; Peregrine *et al.*, 1997; Delespaux and Harry, 2007;). Drug failure can be determined by sensitivity tests, determining the quality of the drugs and drug levels in circulation of treated animal (Sinyangwe *et al.,* 2004).

2.6 Methods of drug serum level determination

The determination of drug levels in circulation of treated animal can be carried out by use of different analytical methods such as High Pressure Liquid Chromatography (HPLC), Gas Chromatography (GS), Mass Spectrophotometry (MS), Thin Layer Chromatography (TLC) and Enzyme Linked Immunoassay (ELISA) (Pincus and Abraham, chapter 23).

2.6.1 High Pressure Liquid Chromatography (HPLC)

HPLC has a high resolution, results are gotten in minutes, and it is sensitive: can measure molecules in ng to fg, has reproducibility of ±1%, high accuracy and automation. However, the method is too costly, the equipment is complex and it has low sensitivity for some compounds. There is irreversible adsorption of

compounds which cannot be detected (http://www.ehow.com/list_5911530_disadvantages_advantages_hplc.html).

2.6.2 Gas Chromatography/Mass Spectrophotometry (GC/MS)

These are the most specific and sensitive screening techniques in identification of drugs and drug metabolites; it can quantify drug concentration in a fluid sample and there is no cross reactivity. However, this technique is very expensive thus used primarily for the confirmation of preliminary test. The facility and the individual who conduct the testing procedure and interprets the results may have an effect on the actual accuracy of test (http://www.sge.com/support/training/fast-gc-analysis/advantages-/-disadvantages-of-fast-gc).

2.6.3 Thin Layer Chromatography (TLC)

This method is not sensitive, selective nor effective in determination of drug levels in circulation of treated animal. TLC is only qualitative exhibit cross-reactivity and cannot test low level concentration of drugs or drug metabolites (http://chemwiki.ucdavis.edu/VV Lab_Techniques/Thin_Layer_Chromatography).

2.6.4 Competitive Enzyme Linked Immunoassay (cELISA)

Competitive enzyme linked immunoassay method for trypanocides has been developed and optimized for detection of drug levels in treated animals (Eisler *et al.,* 1993; Murilla *et al.,* 1999; Karanja *et al.*, 2002). Competitive enzyme-linked

immunosorbent assay (cELISA) is a biochemical technique used mainly in immunology to detect and quantify the presence of an antigen or antibody in a sample (http://www.answers.com/topic/elisa). It is widely used in research, diagnosis and testing (Martz, 2003). It has many advantages over other methods which include; rapid process, easily standardized kits, highly sensitive, accurate, fewer working steps, reduced pipeting or dilution errors, more precise, more specific, cheap, it has been automated to perform large numbers of tests therefore large numbers of sera can be tested within a relative short time (Decker, 2006).The only major disadvantage of cELISA is that each kit is specific for a particular antibody or antigen and if more than one species are involved then series of cELISA tests need to be done.

There are different types of cELISA method; direct, indirect, sandwich, and competitive cELISA (http://www.piercent.com//proteomics/browse.Cfm?fIdID). They have both advantages and disadvantages. cELISA is advantageous over the others in that it detects small molecules for instance drug molecules thus used when the antigen is small and has only one epitope (http://en.wikipedia.org/wiki/elisa). It also requires minimal sample processing. Studies and surveys on use of trypanocidal drugs have been carried out worldwide; Tanzania, Uganda, Zimbabwe, Burkina Faso, Zambia, South America, South East Asia. In Kenya, studies have been done in various parts; Busia (Machila *et al.*, 2003), Kilifi (Paling *et al.*, 1987), Lambwe Valley (Turner, 1989), Tana River

(Catley *et al.*, 2003). Extensive studies have been done in Kwale district (Mugunieri *et al.*, 2000; Machila *et al.*, 2003; Mdachi *et al.*, 2003; Bett *et al.*, 2004; Ohaga *et al.*, 2007;).

CHAPTER THREE: MATERIALS AND METHODS

3.1 Experimental design

Cattle were randomly selected and marked for identification from five selected sites of Lamu. Blood was collected from the ear vein using heparinised capillary tubes for parasitological examination of trypanosomes and packed cell volume (PCV) determination. The presence of trypanosomes was determined by using the microhaematocrit centrifugation and buffy coat/dark ground techniques. Blood collected from the jugular vein using a plain vacutainer tube with a 19 gauge needle was aliquoted into 2-ml storage tubes, stored at -20°C and used to determine the serum drug level by cELISA.

The serum samples were collected in 1996 and 1997. The areas where the samples were collected were identified, type of trypanocidal drug used, personnel administering the drug and the time when the cattle were treated last recorded. Out of the 500 animals identified for sample collection, 301 serum samples were collected from the animal identified to be treated with trypanocidal drug. Only 223 samples that were available at the time of analysis were analyzed.

Farmers were interviewed through a questionnaire (Appendix 1).This was to find out the mode of administration, the dosage, personnel administering the drug,

types of trypanocidal drug used, source of trypanocidal drugs and the last date the animal was treated. The animals that were not treated with trypanocidal drug were excluded from this study. The dilution used in the analysis of the serum samples which were collected in 1996 and 1997 was determined by doing the titration of antisera and conjugate.

3.1.1 Parasitological examination and PCV

Blood from a marginal ear vein, punctured using a sterile lancet (Unilet^{R} JBMSL Ltd. Hamilton, Scotland) or a needle was drawn into a heparinised capillary tube. The capillary tube was filled to about 5mm from the top of the tube. It was then sealed on one end with Cristaseal (labpak Ltd., Coventry, England). The tube was placed in the microhaematocrit centrifuge (Hawksley, Lancing, West Sussex, England) and spun for 10minutes at 12,000g. The packed cell volumes were determined as ratio of red cell to plasma volumes using PCV reader (Hawksley). For trypanosomes detection, blood was prepared as described and after centrifugation, capillary tube was cut just below the buffy coat with a diamond pencil (Merck, Lutterworth, England). The buffy coat under dark-background illumination according to Murray *et al.* (1977). Parasitaemia was quantified by counting the number of parasites in 50 fields using a tally counter (Fisher Scientific).

3.2 Experimental animals

The animal species of focus was mainly cattle. In areas inhabited by pastoralist, the cattle breed was mainly local Orma zebu, whereas in Uziwa and Baharini were mixtures of local zebus, crosses of the local cattle with exotics crosses and a few exotic dairy cattle. The cattle selected for sampling were those that the individual farmers regarded as sick, having trypanosomosis. Five sites were selected and 100 cattle were sampled from each site.

3.3 Determination of serum drug concentration by c-ELISA

Serum drug concentrations of the samples were determined as in appendix 2.

3.4 Data analysis

Parasitological, treatment history and PCV data were entered into Microsoft excess database. Treatment failure rate was calculated as a percentage of the number relapse infection over the number of animals treated. Descriptive statistics of PCV was carried out using Microsoft excel. Statistical data analysis on infection rate, serum drug level concentration and rate of treatment failure were performed using Statview for windows Computer Programme version 5.0.1 (SAS Institute Inc.).

3.5 Study site

Five sites; Kilimani, Uziwa, Baharini, Didewaride and Moa were selected from three administrative divisions (Figure 3.1) based on farmer interviews regarding

disease prevalence and usage of tsetse and trypanosomosis control technologies. Kilimani, Didewaride and Moa are typically pastoralist areas inhabited by the Orma herdsmen and their families. Uziwa and Baharini are largely areas of permanent settlement, where most of the bushes have been cleared to give way to crop farming. In latter areas, farmers are mainly Bantu who have settled in the area since the early '70s practising mixed farming. The farmers grow crops and keep livestock. The two sites were selected based on the high disease challenge in comparison to other sites and the fact that the inhabitants there are pastoralists who solely depend on livestock for their up keep.

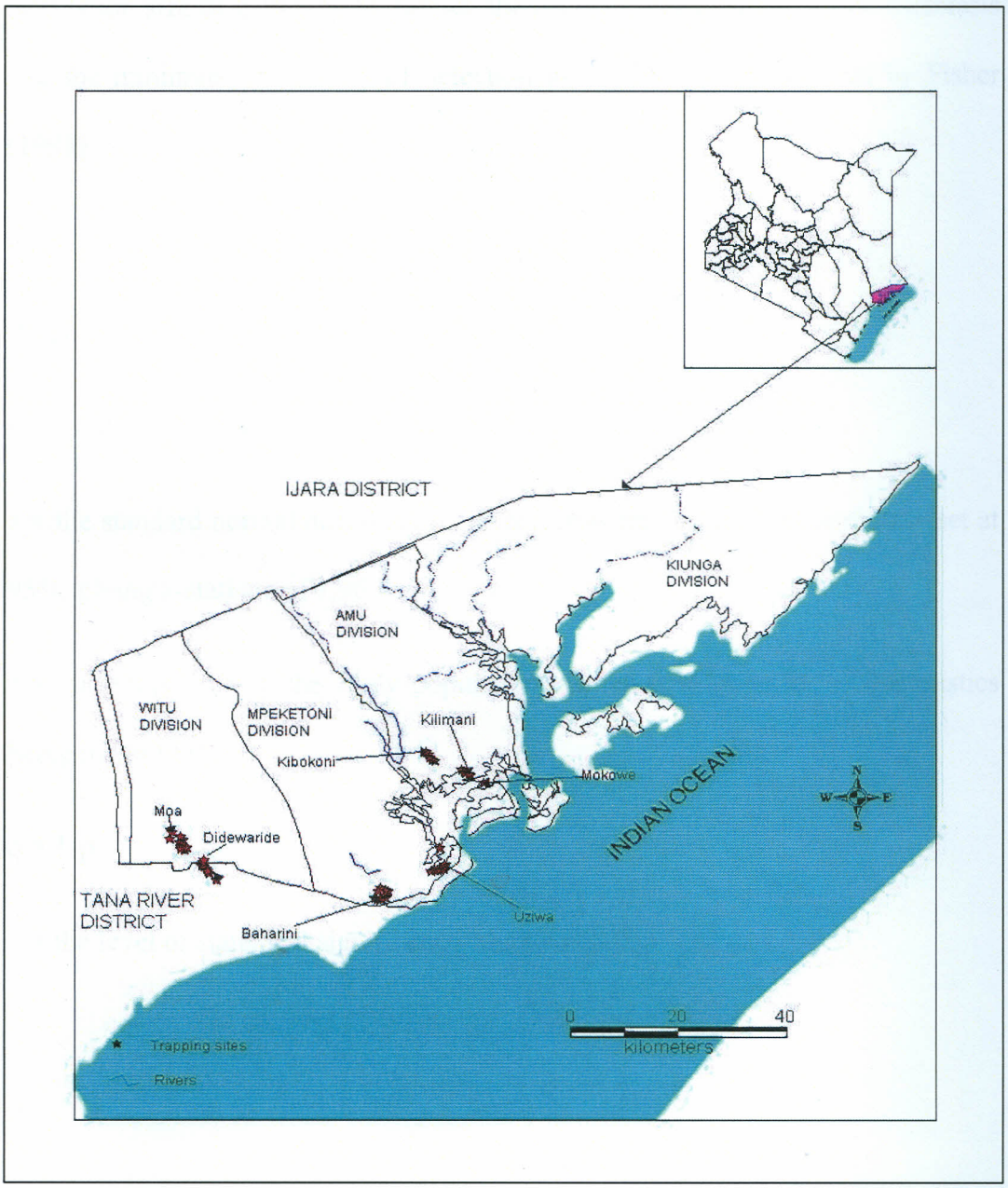


Figure 3.1: Map of Lamu district showing the administrative divisions and cattle sampling sites.

(Mdachi *et al.*, 2006).

3.6 Sample size

The sample size of 100 animals per site was determined as a function of population size of cattle in the district, the average prevalence of trypanosomiasis and the minimum probability of detection using the formula as used by Fisher (1998).

$n = z^2 pq/ d^2$

Where

n = the desired sample size

z = the standard normal deviate at required confidence level. This is usually set at 95%, giving z-statistic of 1.96

p = the proportion in the study population estimated to have the characteristics being measured

q = 1-p

d = the level of statistical significance was set at 0.05.

CHAPTER FOUR: RESULTS

4.1 Trypanocidal drug use

4.1.1 Animals treated

The animals had been treated following clinical diagnosis by the respective groups. Out of 500 animals sampled from the five sites, 301 (60.2%) had been treated with trypanocidal drugs. Of the animals treated, the least number, 27 (9%) of the animals treated was from Didewaride while the largest number, 109(36.2%) was from Kilimani (Figure 4.1).

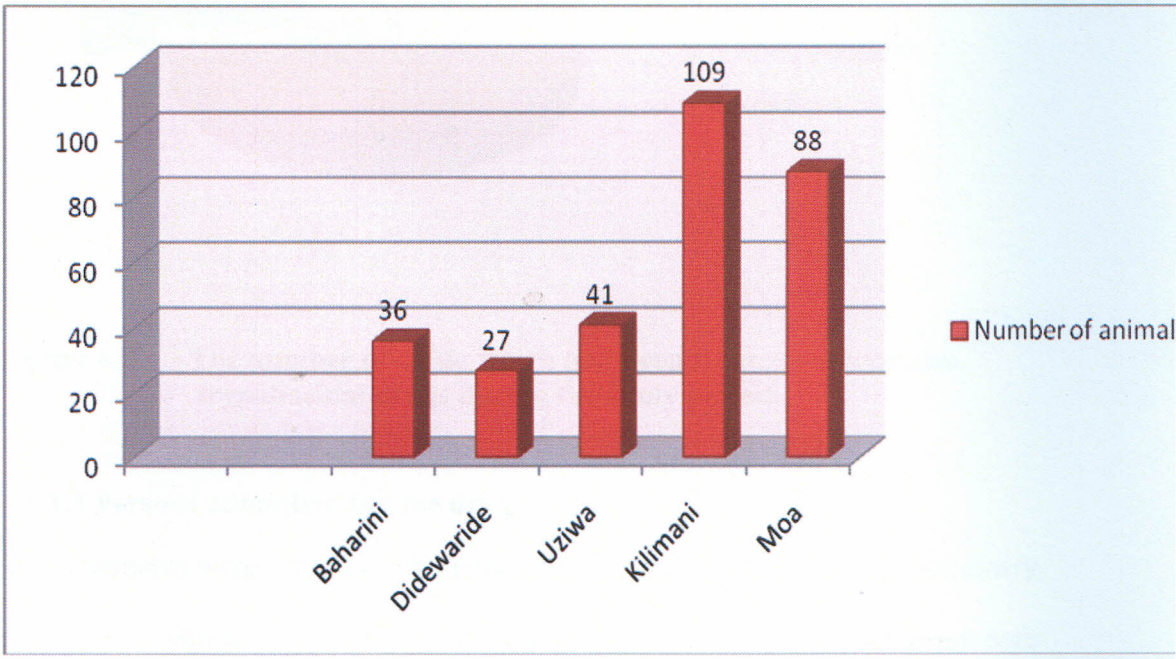


Figure 4.1 The number of animals treated with trypanocidal drugs in Baharini, Didewaride, Uziwa, Kilimani and Moa in Lamu District.

4.1.1.2 Types of trypanocidal drugs used

Three types of trypanocidal drugs are being used in the control of trypanosomosis in Lamu District; homidium bromide chloride/bromide, isometamidium chloride and diminazene aceturate. Overall 219 (72.8%) of the animals had been treated with diminazene aceturate, 62 (20.6%) had been treated with homidium bromide and 20 (6.6%) with isometamidium chloride during the study period (Figure 4.2).

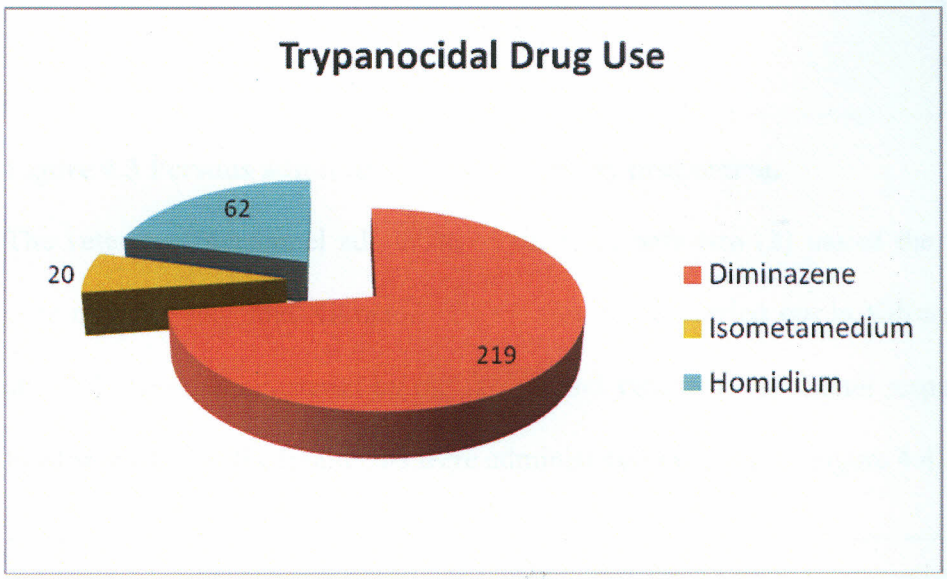


Figure 4.2 The number of cattle which had been treated with various trypanocidal drugs during the study period.

4.1.1.3 Persons administering the drug

The treatments were carried out by either the farmers themselves or the veterinary personnel. Treatment was carried out mainly by farmers with a higher percentage of 81.1% while 18.9% were veterinary personnel (Figure 4.3).

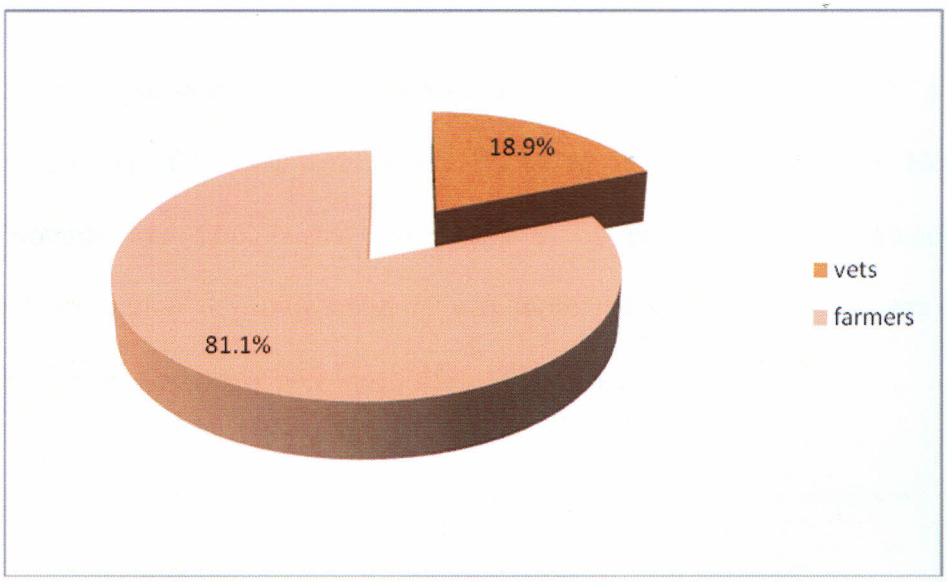


Figure 4.3 Persons administering the drugs by proportion.

The veterinary personnel administered drugs in only two (2) out of the five (5) sites namely; Moa and Kilimani. Of the treatments carried out in Kilimani 56% and 44% were administered by the famers and veterinary personnel respectively. In Moa, 89.8% of the treatments were administered by farmers (Figure 4.4).

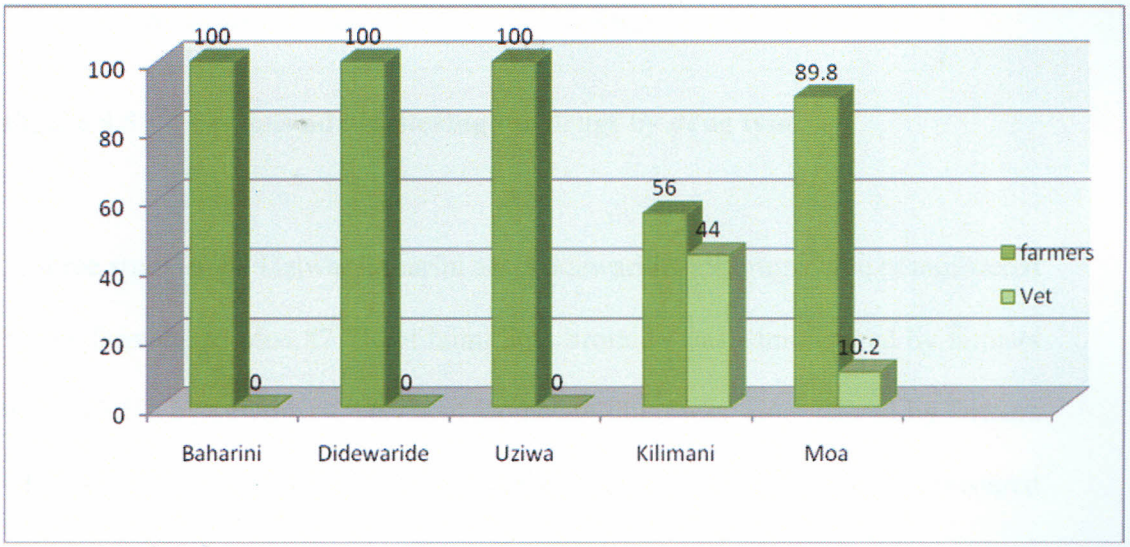


Figure 4.4 Persons administering the drugs in each of the study sites.

Diminazene aceturate was mostly administered by farmers with a higher proportion of 98.6%, with only 1.4% being administered by vets. Homidium bromide was administered more (72.6%) by the veterinary personnel. The administration of isometamidium was more fairly distributed between farmers (55%) and veterinary personnel (45%) (Figure 4.5).

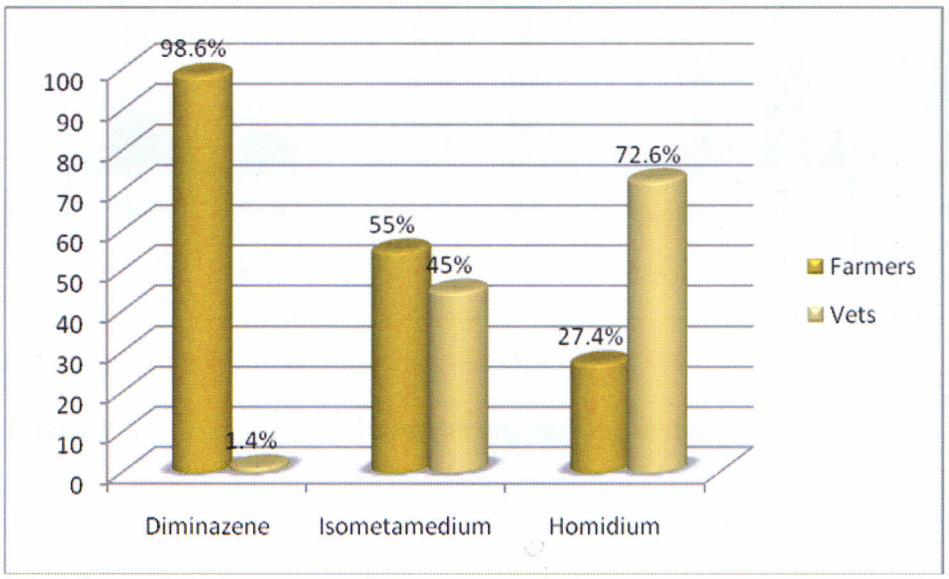
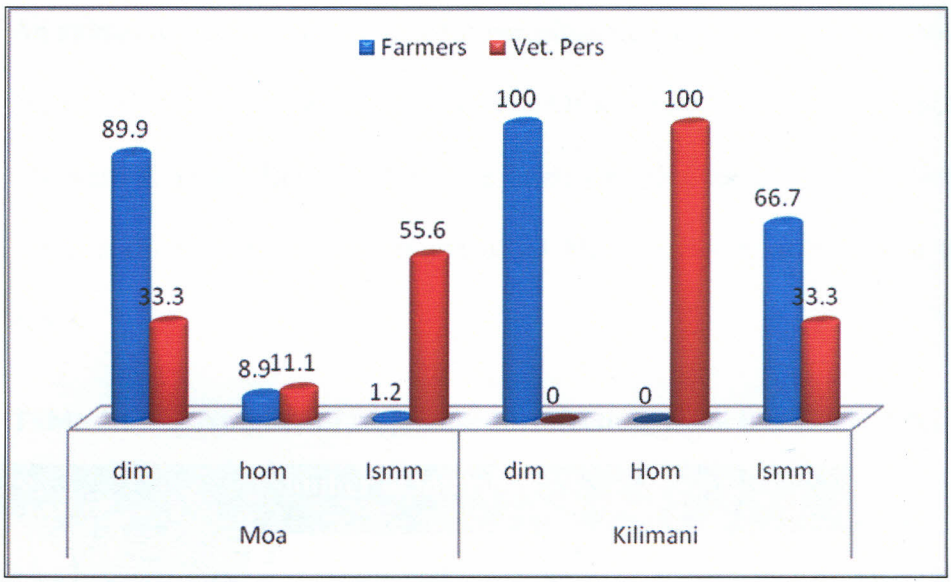


Figure 4.5 Persons administering the drugs by drug type.

In three study sites; Uziwa, Baharini and Didewaride, all drugs were administered by the farmers. In Moa 87.5% of homidium bromide was administered by farmers while 12.5% by veterinary, 16.7% of isometamidium was administered by farmers while 83.3% by veterinary and 95.9% of diminazene aceturate was administered by farmers while 4.1% by veterinary. In Kilimani 66.7% of isometamidium was

administered by farmers while 33.3% by veterinary, homidium bromide and diminazene aceturate were administered by veterinary and farmers, respectively (Figure 4.6).



Key

Hom = Homidium bromide; Dim = Diminazene aceturate; Ismm=Isometamidium

Figure 4.6 Personnel administering various drugs per site.

4.1.1.4 Mode of administration

Both farmers and veterinary personnel administered the drugs through the intra-muscular route. This was either on the neck muscles or on the hind legs. The dosage used by the farmers could not be established, however most of the farmers indicated that they used 10-15mg/kg of diminazene aceturate, 10mg/Kg of homidium bromide and 5-8 mg/Kg of isometamidium solutions. These drugs are available in powder or tablet forms. The farmers dissolved them in water before administration and the reconstitution into solution was done in an arbitrary

manner. The veterinary personnel indicated that they administered homidium bromide at the dose rate of 1mg/kg but the weight of the animals was estimated.

4.1.2 Trypanosome infection rate

An animal was considered infected if parasitaemia was demonstrated at the time of sampling. The infection rate was 10.4%. Of these 96 % were *T. congolense* and 4% were *T. vivax*. Between sites, the infection rates varied from a minimum of 3.9% in Didewaride to a maximum of 53.9% in Kilimani as shown in table 4.1 below.

Table 4.1 Number of trypanosome infection in the five Study Sites

Site	Number of infection	Infection rate (%)
Baharini	3	5.77%
Didewaride	2	3.85%
Kilimani	28	53.85%
Moa	11	21.15%
Uziwa	8	15.38%

4.1.3 Treatment failure

Treatment failure was regarded as any animal found with trypanosomes infection 21 days after treatment with diminazene aceturate, 30 days with homidium bromide or 90 days with isometamidium. Overall, 12.9% of the treatments with isometamidium, homidium bromide or diminazene aceturate did not effect cure.

There was treatment failure rate of 24.6% in animals treated by veterinary personnel and 8.9% in those treated by farmers (Figure 4.7).

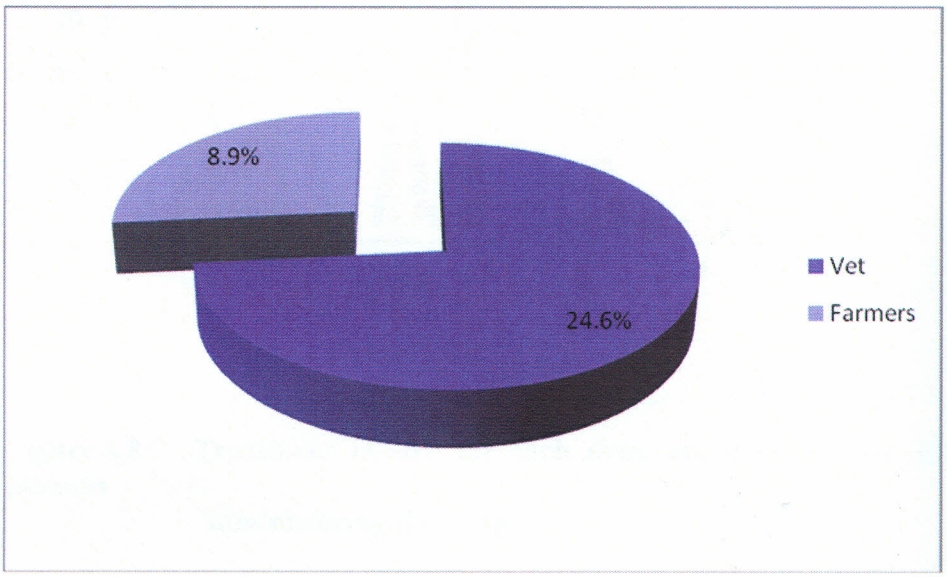


Figure 4.7 Overall treatment failure (%) according to the group of persons.

The treatment failure rate when using homidium bromide was 17.6% in animals treated by farmers and 26.7% in those treated by veterinary personnel, while treatment failure rate when using isometamidium was 18.2% by farmers and 22.2% by veterinary personnel. When using diminazene aceturate products, treatment failure rate was 7.8% by farmers and none by veterinary personnel (Figure 4.8).

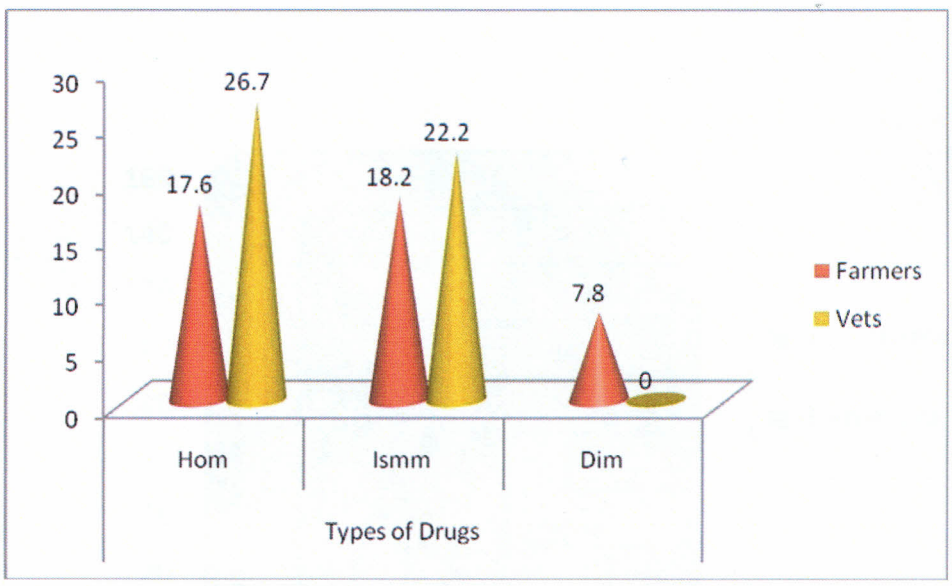


Figure 4.8 Treatment failure for each drug according to the groups of persons administering the drug.

Hom = Homidium chloride/bromide
Ismm = Isometamidium
Dim = Diminazene aceturate

4.1.4 Serum trypanocidal drug analysis

4.1.4.1 Samples analyzed by drug cELISA

The serum drug concentrations of the three trypanocidal drugs; homidium bromide, diminazene aceturate and isometamidium were determined. A maximum of 223 (74.1 %) out of the 301 samples collected from animals that had been treated by either the farmers or the veterinary personnel were analyzed, 78 (25.9%) of the samples were not analyzed. Out of the samples analyzed, 141 (63.2%) were analyzed for diminazene aceturate, whereas 62 (27.8%) and 20 (9.0%) were analyzed for homidium bromide and isometamidium respectively (Figure 4.9).

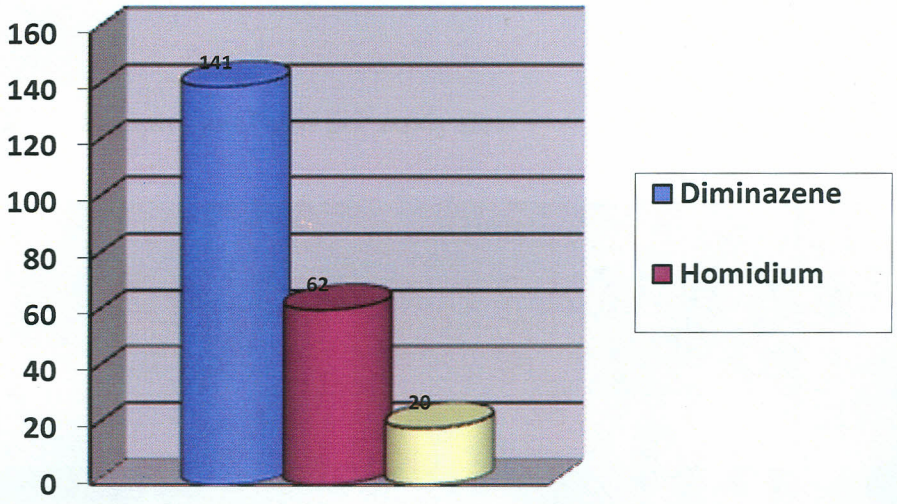


Figure 4.9 Number of serum samples analysed for the three types of trypanocidal drugs (Diminazene, Homidium and Isometamedium).

Out of 33 samples analyzed from Uziwa, 28 (84.8%) were analyzed for diminazene aceturate, 4 (12.2%) for homidium bromide and 1 (3.0%) for isometamidium. Out of 37 samples analyzed from Baharini, 31 (83.8%) were analyzed for diminazeneaceturate, 5 (13.5%) for homidium bromide and 1 (2.7%) for isometamidium. Out of 27 samples analyzed from Didewaride, 26 (96%) were analyzed for diminazene aceturate and 1 (4%) for homidium bromide. Out of 47 samples analyzed from Moa, 33 (70.2%) were analyzed for diminazene aceturate, 8 (17.0%) for homidium bromide and 6 (12.8%) for isometamidium. Out of 79 samples analyzed from Kilimani, 23 (29.1%) were analyzed for diminazene

aceturate, 44 (55.7%) for homidium bromide and 12 (15.2%) for isometamidium as represented in Table 4.2 below.

Table 4.2 Samples analyzed per study site

Sites	Types of Drugs			Total
	Diminazene aceturate	Homidium bromide	Isometamidium	
Baharini	31	5	1	37
Kilimani	23	44	12	79
Didewaride	26	1	0	27
Moa	33	8	6	47
Uziwa	28	4	1	33

4.1.4.2 Drug concentrations in serum

Diminazene aceturate levels in samples analyzed varied from undetectable levels to a maximum of 4200 ng/ml with a mean concentration of 220.8 ± 537.6 ng/ml. Homidium bromide drug levels varied from undetectable levels to a maximum of 14.5 ng/ml with a mean of 0.5 ± 1.86 ng/ml, whereas isometamidium drug levels varied from a minimum of 0.02 ng/ml to a maximum of 29 ng/ml with a mean of 2.47 ± 6.46 ng/ml.

From the samples analyzed, 81.2% had drug levels below therapeutic levels while the rest of the samples (19.8%) had drug concentrations above therapeutic levels of either of the drugs. Drug concentrations of correct therapeutic levels for diminazene aceturate, homidium bromide and isometamidium were 16.3%, 11.3% and 50% of the samples analyzed for the respective drugs (Figures 4.10, 4.11 and 4.12). Of the 23 samples that had therapeutic levels of diminazene aceturate, 87.0% were from cattle that had been treated less than 15 days prior to sample collection. Whereas 3 were from cattle that had been treated 21 and 30 days prior to sample collection. Of the 118 samples that had drug concentrations below the therapeutic levels of diminazene aceturate, 64.4% were from cattle that had been treated less than 15 days prior to sample collection Whereas 40 (35.6%) samples were from cattle that had been treated between 30 to 120 days prior to sample collection.

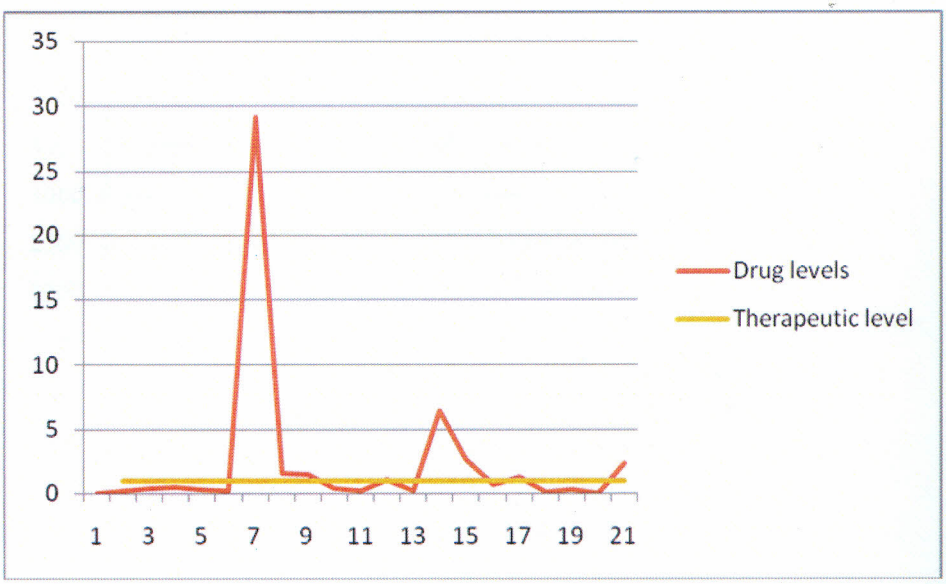


Figure 4.10 Drug concentrations of correct therapeutic levels for isometamedium.

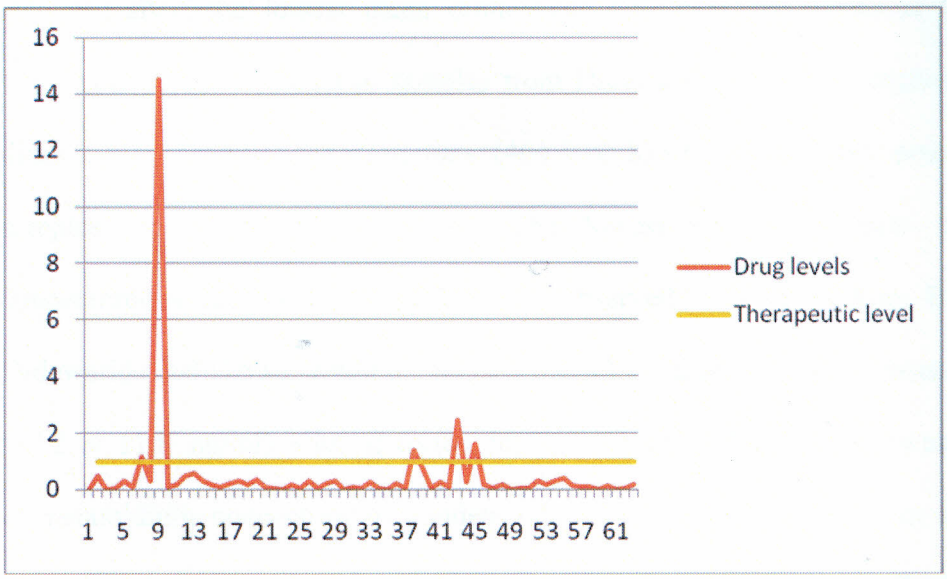


Figure 4.11 Drug concentrations of correct therapeutic levels for homidium.

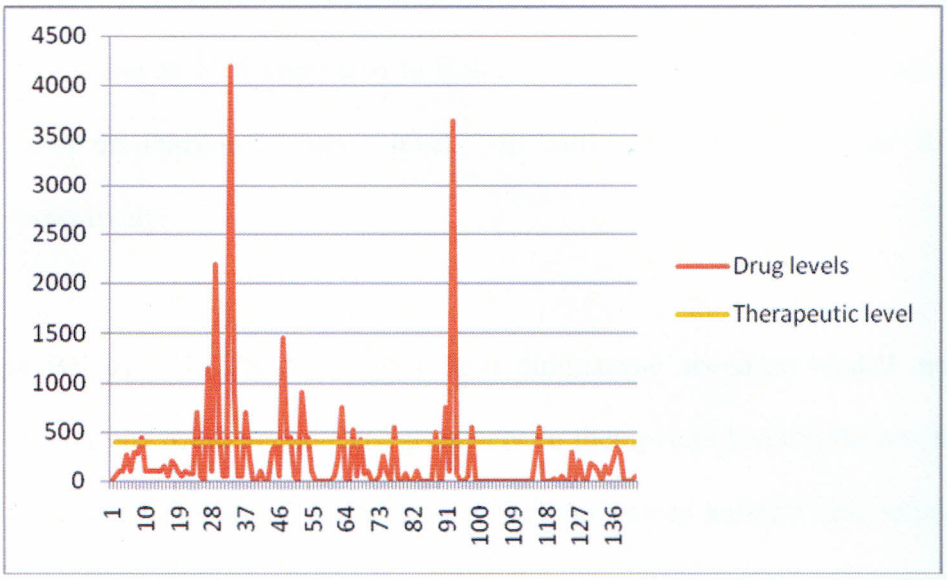


Figure 4.12 Drug concentrations of correct therapeutic levels for diminazene.

Between sites, the lowest mean diminazene aceturate concentrations (81.1 ± 109.1ng/ml) were detected in samples from Didewaride, while the highest mean diminazene aceturate concentrations (466.7 ± 833.8 ng/ml) were detected in samples from Moa (Table 4.4). The lowest mean homidium bromide concentrations (undetectable levels) were observed in samples from Baharini, Didewaride and Uziwa, while the highest mean homidium bromide concentrations (5.12 ± 11.8 ng/ml) were detected in samples from Moa. The lowest mean isometamidium concentrations (undetectable levels) were observed in samples from Baharini, while the highest mean isometamidium concentrations (2.12 ± 5.01 ng/ml) were detected in samples from Moa (Table 4.3). There was no correlation between mean drug levels in each site with the corresponding trypanosomosis prevalence. It was expected that animals in study sites with higher drug levels

would have lower prevalence of infections. However this was not the case. In Moa where mean diminazene aceturate levels in cattle were highest, disease prevalence was higher at 11% compared to Baharini (3%) and Didewaride (2%) which had mean diminazene aceturate levels in cattle of 215.8 ng/ml and 81.1 ng/ml, respectively.

In Baharini, 16.2% of samples from diminazene aceturate treated animals had diminazene aceturate concentrations above therapeutic levels whereas all samples from homidium bromide and isometamidium treated animals had respective drug concentrations below therapeutic levels. In Didewaride, all samples (from homidium bromide and isometamidium treated animals) had respective drug concentrations below therapeutic levels. In Kilimani, 8.7%, 11.4% and 58.3% of samples from diminazene aceturate, homidium bromide and isometamidium treated animals respectively had respective drug concentrations above therapeutic levels. In Moa, 36.4%, 25.0% and 33.3% of samples from aceturate, homidium bromide and isometamidium treated animals, respectively had drug concentrations above therapeutic levels.

Table 4.3 Descriptive analysis of homidium bromide, diminazene aceturate and isometamidium concentration in cattle in each site in Lamu District

Sites	Drug Type	Number of samples	Mean conc (ng/ml)	Minimum Conc.	Maximum Conc.	Standard Deviation
Baharini	Dim	30	215.83	0.00	3650.00	680.34
	Hom	5	0.20	0.07	0.40	0.15
	Ismm	1	0.02	0.02	0.02	
Didewaride	Dim	26	81.10	0.00	350.00	109.01
	Hom	1	0.16	0.16	0.16	
Kilimani	Dim	22	140.34	50.00	450.00	99.40
	Hom	44	0.29	0.00	2.43	0.46
	Ismm	12	1.36	0.09	6.44	1.77
Moa	Dim	33	466.67	0.00	4200.00	833.82
	Hom	8	2.12	0.01	14.52	5.02
	Ismm	6	5.12	0.15	29.20	11.80
Uziwa	Dim	28	119.95	0.00	750.00	199.86
	Hom	4	0.05	0.01	0.14	0.06
	Ismm	1	2.39	2.39	2.39	

Dim = Diminazene aceturate;
Hom = Homidium chloride/bromide;
Ismm= Isometamidium chloride.

In Uziwa, 14.3% of samples from diminazene aceturate treated animals had diminazene aceturate concentrations above therapeutic levels whereas all samples from homidium bromide treated animals had homidium bromide concentrations below therapeutic levels and all samples from isometamidium treated animals had isometamidium concentrations above therapeutic levels.

4.1.5 Evaluation of causes of treatment failure

Two causes of treatment failure were recorded in this study; sensitivity of infecting trypanosomes to drugs and under dosing. Trypanosome infections detected in cattle which had either diminazene aceturate serum concentrations above 800 ng/ml or homidium bromide and isometamidium concentrations above 1ng/ml respectively were considered to be resistant to the drug. Under dosing was regarded as serum concentration that were below expected values as per pharmacokinetics following treatment of cattle at the recommended doses of 3.5 mg/kg for diminazene aceturate products, 1 mg/kg for homidium bromide products and 0.5 mg/kg for isometamidium products according to pharmacokinetic of the appropriate drugs; within 21 days for diminazene aceturate, 30 days for homidium bromide and 90 days for isometamidium.

4.1.5.1 Drug resistance

Out of the 15 cases of homidium bromide treatment failure analyzed, 3 (20%) were due to resistance of trypanosomes to the above therapeutic circulating levels of the drug. All cases of diminazene aceturate and isometamidium treatment failure that were analyzed for drug levels had trypanosomes when drug concentrations were below therapeutic levels.

4.1.5.2 Under dosing

Overall, of the animals treated 83.9% were under dosed, 4.5% were over dosed with only 11.6% having correct dose with the respective drugs (Figure 4.13).

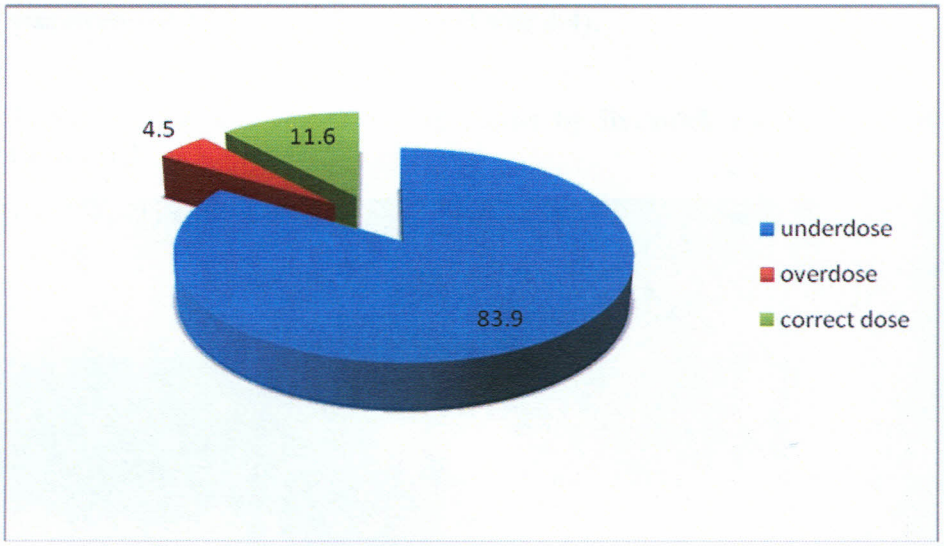


Figure 4.13 Overall drug dose levels as determined from sera.

Of the animals treated with diminazene aceturate 81.6% were under dosed and 5% were over dosed. Whereas 93.6% and 70.0% of the animals treated with homidium bromide and isometamidium respectively were under dosed while 15% treated with isometamidium were over dosed and no animals treated with homidium bromide were over dosed.

Of the animals treated by the livestock keepers, under dosing of diminazene aceturate, homidium bromide and isometamidium was 81.6% (115), 82.4% (14) and 54.6% (6) respectively while the same drugs were overdosed at the rate of 5%

(7) and 19% (2) respectively, except homidium bromide. Whereas, of the animals treated by the veterinary personnel, under dosing rates of homidium bromide and isometamidium were 97.8% (44) and 88.9% (8) respectively and isometamedium was overdosed at rate of 11.1% (1) (Table 4.4).

Table 4.4 Various drugs dosing status by livestock keepers and veterinary personnel

Who treated the Animals	Drug Type	Dosing Type	Number of Animals	Rate of Dosing
Livestock Keepers	Diminazene	Correct-dose	19	13.48%
		Over-dose	7	4.96%
		Under-dose	115	81.56%
	Homidium	Correct-dose	3	17.65%
		Under-dose	14	82.35%
	Isometamidium	Correct-dose	3	27.27%
		Over-dose	2	18.18%
		Under-dose	6	54.55%

Veterinary Personnel	Homidium	Correct-dose	1	2.225
		Under-dose	44	97.78%
	Isometamidium	Over-dose	1	11.11%
		Under-dose	8	88.89%

The drugs under dosing rates in Baharini, Didewaride and Uziwa were 67.6% (25), 100% (27) and 72.7% (24) respectively while overdosing was at the rates of 5.4% (2) and 3.0% (1) except in Didewaride. The drug administration in these sites was carried out by farmers. In Moa, drug under dosing rates by farmers and veterinary personnel were 80.5% (33) and 83.3% (5) respectively and over dosing rates were 9.8% (4) and 16.7% (1) respectively. Whereas in Kilimani, under dosing rates by farmers and veterinary personnel were 83.9% (26) and 97.9% (47) respectively and over dosing rate by farmers was 6.5% (2) with no cases of under dosing by veterinary personnel (Table 4.5).

Table 4.5 Number of animals under dosed or over dosed with the appropriate drug by livestock keepers and veterinary personnel at each study site

Personnel Treating the Animals	Site	Dosing Type	Number of Animals	Rate of Dosing (%)
Livestock Keepers	Baharini	Under-dose	25	67.6%
		Overdose	2	5.4%
	Didewaride	Under-dose	27	100%
		Overdose		
	Kilimani	Under-dose	26	83.9%
		Overdose	2	6.5%
	Moa	Under-dose	33	80.5%
		Overdose	4	9.8%
	Uziwa	Under-dose	24	72.2%
		Overdose	1	3.0%
Veterinary Personnel	Kilimani	Under-dose	47	97.9%
		Overdose		

	Moa	Under-dose	5	83.3%
		Overdose	1	16.7%

4.1.6 Cases of trypanosomosis incorrect-diagnosis

Incorrect diagnosis of trypanosomosis was considered as animals that were diagnosed as having trypanosomosis by farmers and Veterinary Personnel and were treated, but the serum drug levels were below therapeutic levels and the animals were not infected 7 days post-treatment. Overall, of the 77 cattle treated for trypanosomosis a week prior to sampling 62.3% were wrongly diagnosed. Of the 75 animals treated for trypanosomosis by livestock keepers a week prior to sampling 62.7% were wrongly diagnosed, whereas 50% of the animals treated by Veterinary personnel were wrongly diagnosed. Between study sites, cases of mis diagnosis varied from a minimum of 48.1% at Moa to a maximum of 100% at Didewaride (Table 4.6).

Table 4.6 Cases of mis-diagnosis of trypanosomosis in each study site in Lamu District

Site	Number of cases of mis-diagnosis
Baharini	2
Didewaride	16
Kilimani	14
Moa	13
Uziwa	3

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

The high proportion of animals treated within four months prior to this study is a reflection of the trypanocidal drug use in Lamu District and the importance of trypanosomosis in animal health within Lamu District. An earlier survey in the district observed that 93% of livestock keepers perceived trypanosomosis to be the most important diseases affecting livestock (Mdachi *et al.,* 2006). However, the apparent trypanosomosis prevalence derived from the number of animals perceived to have acquired trypanosomosis within six months of the survey over the total number of cattle in a house hold was observed to be 61%. In the current study, the proportion of animals treated 4 months prior to sample collection would then be an accurate indication of the trypanosomosis prevalence in Lamu district. The significant variation in the proportion of animals treated between sites is a reflection of the significant variation of the disease challenge within the district, confirming the findings by Omukuba *et al.* (2000). This is also in agreement with observations of epidemiological studies carried out in this district where trypanosomosis prevalence was observed to vary from 1.9% to 22% (Paling *et al.*, 1987; Dowler *et al.*, 1989).

The three drugs that were used by either the farmers or veterinary personnel are the recommended drugs for the treatment of trypanosomosis or nagana in cattle (Murilla *et al.,* 2003). This is an indication of awareness of the disease that is endemic at the coastal region. Earlier studies showed that 79.7% of the livestock keepers at the coast ranked trypanosomosis as the most important livestock disease and were able to identify at least 3 symptoms related to the disease (Mdachi *et al.,* 2008). Most of sub-Saharan Africa, bovine trypanosomosis is primarily controlled by use of trypanocidal drugs (Holmes *et al.,* 2004). Trypanocidal drugs are the first drugs tried on cattle when they develop (any) symptoms of the disease (Geerts *et al.,* 2001). These drugs have been on the market for over 40 years and several generic forms of them from a wide range of companies have become available on the African market (Holmes *et al.,* 2004).

Isometamidium is principally used as a prophylactic drug and can provide up to 6 months protection against trypanosomosis. Whilst homidium bromide has limited prophylactic properties, it is primarily used as a therapeutic agent. Although it has been shown that diminazene aceturate provides also a short term protection of 2 to 3 weeks, it is mainly used for therapeutic purposes. Farmers in this area preferred the use of diminazene aceturate with 219 animals being treated with diminazene aceturate. This may be mainly because of their affordability and ready availability. However, some of the diminazene aceturate available were not registered for use

in Kenya (Mugunieri and Murilla, 2003). Other studies show that majority of farmers use diminazene aceturate (Bossche *et al.,* 2000).

There were more farmers than veterinary officers treating the animals in Lamu District. This is an indication that farmers in the District prefer to treat their own animals despite the fact that they may not be knowledgeable enough to administer the drug correctly as has been observed earlier (Mugunieri and Murilla, 2003; Ohaga *et al.,* 2007). In many countries unskilled persons are allowed to administer drugs (FAO, 1998). This may be because following changes in government policies and introduction of cost sharing of services earlier provided free by the Government, it has become expensive for the livestock keepers to pay for both the drugs and services. As such they would rather use the services of their neighbours and peers who may be a little more knowledgeable. The use of veterinary personnel in the two sites is not a reflection of the preference of the livestock keepers in these sites to use professional services but rather a choice of well wishers. The US marines in one of their routine visits to the coastal region during this period had offered to provide veterinary drugs to the District Veterinary Office to be used for free treatment of farmers' animals.

Intra muscular administration of the three trypanocidal drugs that were used is the recommended route of administration (Mdachi *et al.*, 1995). The drugs are recommended to be used at the dose rate of 3.5 mg/kg for diminazene aceturate

products (Fairclough, 1963; Murilla *et al.,* 2001), 1mg/kg for homidium bromide products (Leach and Roberts, 1981; Murilla *et al.,* 1999) and 0.5-1.0 mg/kg for isometamidium (Kinabo and McKellar, 1990).

There was low overall trypanosome infection rate of 10.4%. Out of 223 animals treated for trypanosomosis, 48 animals were found not to be suffering from trypanosomosis. Other studies in Lamu District have shown that 15% of cattle that farmers identified as having trypanosomosis had anaplasmosis while 2.3% had theileriosis and 1.2% had babesiosis (Mdachi *et al.,* 2006b). This could be viewed as indicative of the level of knowledge on trypanosomosis symptoms and the ability of the farmers to make differential disease diagnosis bearing in mind that there are no confirmations in the laboratory. Farmers usually treat the animals based on clinical signs and symptoms observed. The low infection rate may also be reflective of the high trypanocidal drug usage rate by farmers as indicated by the proportion of the cattle (78%) that had been treated prior to sampling thereby reducing effective transmission of the disease leading to the very low incidence (new infections) of trypanosomosis (2.2%) observed. Trypanosome infection rates in cattle varied significantly ($p<0.0001$) between sites and appeared to correspond to the tsetse challenge as indicated by the fly catches per site reported earlier (Mdachi *et al.*, 2006b).

Treatment failure is normally due to several factors that include; a) under dosing, b) using the wrong route of drug administration, c) using the wrong drug for the particular disease usually due to mis-diagnosis, d) using a fake drug and e) reduced sensitivity of trypanosomes to drugs (drug resistance) (Connor, 1991; Mdachi, 1999). The significantly ($p<0.01$) higher failure rate obtained for animals treated by Veterinary personnel is surprising. Using our criteria for drug failure, the few trypanosomosis infection observed may be an indication that the treatments by livestock keepers were effective leading to the significantly lower drug failure rate observed. Higher rate of under dosing found in this study agrees with a study carried out by Yahannes *et al.* (2010) in Ethiopia; under dosing appear to be a common practice. Another report by FAO indicated that under dosing occurs very frequently (FAO, 1998). Farmers have the tendency to under estimate the weight of their animals when they have to treat them. In addition, as the drugs are relatively expensive there is a temptation to over dilute the drug and hence under dose (FAO, 1998). Given the fact that in many countries unskilled persons are allowed to administer drugs, errors easily occur in calculating the correct doses for the treatment of the animals (FAO, 1998).

Studies in the southern coastal area of Kwale showed that livestock keepers' mis diagnosed 50% of the clinical cases observed (Machila *et al.*, 2003). Trypanocides were mainly used to treat clinically sick animals (not necessarily infected with trypanosomes, Bossche *et al.* (2000) and they are often the first drugs tried by

farmers when their cattle develop (any) signs of the disease (Geerts *et al.,* 2001). In this study vets used the prescribed doses of 1mg/kg of homidium while farmers used higher doses of 10-15mg/kg of diminazene aceturate, 10mg/kg of homidium and 0.5-8mg/kg of isometamidium. Other studies carried out in Kwale District of the coast province showed that despite administering the drug correctly, veterinary personnel (AHAs) used lower doses than the farmers (Mdachi *et al.,* 2003b). The veterinary personnel would normally use the drugs at the prescribed doses which may no longer be effective at the coast (Dolan *et al.,* 1990; Mdachi, 1999) due to development of resistance. Whereas the livestock keepers due to on farm experience and concern for their animals would reconstitute more drugs with the same amount of water thereby administer a high dose, which would be more effective in treating infection of reduced sensitivity (Machila *et al.,* 2003).

There was a significant association between treatment failure rate and trypanosome prevalence. The treatment failure rate increased significantly ($p<0.001$) with increase in the disease prevalence. Kilimani, the study site with the highest disease prevalence had the highest rate of treatment failure. This is in agreement with findings from earlier studies in Galana ranch, Kilifi District (Dolan *et al.,* 1990; Mdachi 1999) and Kwale District (Mdachi, 1999). These authors found increase of cases of diminazene, homidium bromide and isometamidium treatment failure during periods of high prevalence of trypanosomosis and high tsetse challenge. It is postulated that trypanosome infections affect the disposition

of trypanocidal drugs and increase the elimination rate of the drug from systemic circulation leading to lower blood drug levels. This drug elimination rate increases with increase in blood trypanosome burden (Eisler *et al.*, 1994).

It might be possible that the prevalence of other diseases like anaplasmosis, theileriosis and babesiosis are very low as compared to trypanosomosis, due to lower number of animals mis-diagnosed in this study. However, this improper drug practice could be contributing to drug failure and development of resistance in the region (Mdachi *et al.*, 2006). Studies carried out earlier in Kwale and Busia districts reported between 50% and 64% cases of incorrect diagnosis (Machila *et al.*, 2003). Trypanocidal drugs are often the first drugs tried by farmers when their cattle develop (any) symptoms of the disease (Geerts *et al.*, 2001). And also, trypanocides are frequently used in the absence of diagnosis or used to treat conditions for which they are not effective (Holmes *et al.*, 2004). Trypanocides were mainly used to treat clinically sick animals (not necessarily infected with trypanosomes (Bossche *et al.*, 2000). Further work will be required to develop diagnostic methodologies that are more 'user friendly' for livestock keepers in order to improve their basis for treatment decisions. This would reduce on the cost (inappropriate treatment of cattle) by the already impoverished livestock keepers.

There was a higher rate of under dosing (83.9%). Overall livestock keepers under dosed 135 animals as compared to 52 by Veterinary personnel. Unfortunately,

under dosing occurs very frequently (FAO, 1998). The dosage used by the farmers in this study was above the recommended dose for all the three drugs while vets used the recommended dose. The sub-therapeutic levels found in this study could be due to under-estimation of animal weight, over-dilution of the drugs, wrong amount of drug might have been used when making up the injectable solution, and drugs might have not been completely dissolved before use as also observed in the report of FAO, 1998. Under dosing could be contributing to drug failure and development of resistance in the region. Under dosing is one of the major causes of resistance development. Sub-therapeutic drug concentrations exert a strong selective pressure for the emergence of resistant clones that pre-exist in the trypanosome population (FAO, 1998). Farmers have a tendency to under-estimate the weight of their animals when they have to treat them (FAO, 1998). In this study vet personnel have also been found to be under dosing and this may be mainly due to under estimation of the animal's weight. Given the fact that in many countries unskilled persons are allowed to administer drugs, errors easily occur in calculating the correct doses for the treatment of the animals (FAO, 1998). In addition, as the drugs are relatively expensive there is a temptation to over dilute the drug and hence under dose. Apart from this, under dosing of trypanocidal drugs appeared a common practice in the areas surveyed (Yohannes *et al.,* 2010).

There was no correlation between mean drug levels in each site with the corresponding trypanosomosis prevalence. It was expected that animals in the

study sites with higher drug levels should have lower prevalence of infections. However this was not the case. In Moa where mean diminazene aceturate drug levels in cattle were highest (466.7 ng/ml) had a higher disease prevalence(11%) compared to Baharini with disease prevalence of 3% and Didewaride 2% which had mean diminazene aceturate levels in cattle of 215.8 ng/ml and 81.1 ng/ml respectively. This could be an indication of presence of resistant infections and /or inappropriate use of trypanocidal drugs by livestock keepers that has previously been observed in Kwale (Mdachi, 1999; Mugunieri and Murilla, 2003) and Busia district (Machila *et al.,* 2003).

Isometamidium and homidium bromide serum concentrations above 0.5ng/ml and diminazene aceturate concentration above 400 ng/ml were considered therapeutic to a sensitive trypanosome infection (Eisler *et al.,* 1996; Mdachi, 1999). Despite the fact that farmers used higher dosage and vet personnel used the recommended dose, the drug levels found in the serum in most cases for both three drugs were below therapeutic levels. It was only in very few cases where therapeutic levels were attained. The sub-therapeutic levels in the serum is a contributing factor of treatment failure in the area.

From the results obtained, there is a low percentage of drug resistance (20%). This was observed in cattle treated with homidium bromide (the parasitological examination had shown presence of trypanosomes in these samples while the drug

levels of the same samples were above therapeutic levels). Both diminazene aceturate and isometamedium for the samples which had trypanosomes, the drug levels were below therapeutic levels. This is an indication that majority of cases of drug failure in Lamu District may be due to inadequate drug concentrations in the blood to effect cure. This could be attributed to improper drug use.

5.2 Conclusion

It is confirmed that;

- Treatment failure in Lamu District is associated with under dosing whereby therapeutic drug levels are not attained following treatment by farmers and vets.
- cELISA can detect treatment failure associated with under dosing and can also detect drug resistance where treatment failure is associated with therapeutic drug levels in blood.

5.3 Recommendation

This study was a cross-sectional one and in order to fully understand the factors associated with treatment failure which includes sub-therapeutic levels of drugs in the serum and under dosing, I recommend a longitudinal study in the area.

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APPENDICES

Appendix 1: Questionnaire

Name……..

Area; Moa ☐ Uziwa ☐ Baharini ☐

Kilimani ☐ Didewaride ☐

Type of drug used; Isometamedium Homidium

Diminazene Others specify……

Source of drug; Veterinary shop……

Hawkers……

Others specify……

Last treatment date……

Mode of administration……

Personnel treated; Self ☐ Veterinary officer

Appendix 2: Preparation of Reagents

Preparation of washing buffer

For one litre 980ml of distilled water was measured using a measuring cylinder, 0.5ml of tween 20 and 20ml of PBS ×10 added using micropipette and pipette then shaken well.

Preparation of PBST

For one litre 900ml of triple distilled water was measured, 0.5ml tween 20 and 100ml of PBS ×10 added and then well shaken. This was refrigerated at 4-8°C.

Preparation of coating buffer

Solution A; Sodium bicarbonate (Na_2C0_3) - 8.4g was dissolved in one liter of double distilled water and 100ml removed from it.

Solution B; sodium carbonate (Na_2C0_3) - 2.65g was dissolved in 250ml of double distilled water. Then adjust pH of $NaHC0_3$ using Na_2C0_3 to pH 9.2.

Preparation of PBS ×10

40g of NaCl, 7.4g Na_2HPO_4 and 2.15g KH_2PO_4 were weighed and dissolved in 500ml of triple distilled water.

Preparation of 1M orthophosphoric acid

Given purity and density of the orthophosphoric acid, the molarity is calculated using the formula: $m_1v_1=m_2v_2$ formula was used.

Preparation of TMB

Mix solution A and solution B in ratio of 1:1.

Preparation of control serum

Blood was collected from jugular vein of untreated bovine. It was then put in centrifuging tubes then centrifuged for 10 minutes at 4000rpm using Centrifuge, eppendort 5416 Germany. Supernatant was then decanted into a cleaned plastic tube and was put in freezer at -20°C until use.

Preparation of quality controls (QCs)

QC0 was prepared by taking blank serum prepared as in 3.5. QC1(10ng/ml) was prepared by pipetting 10ul of the prepared standard in a tube labeled 1 and diluting it in 1ml blank serum and QC2 was prepared by pipetting 20ul from tube labeled 1 and diluting it in 1ml blank serum.

Preparation of standards

Standards ranging from 500ng/ml to 0.025ng/ml was prepared by weighing 0.05± 0.0003g (50mg) of either drug (isometamidium, homidium bromide or diminazene aceturate) into 100ml volumetric flask , dissolved and topped up to the mark with distilled water. 1/10 dilution was made by pippeting 1ml from the above dilution into 9ml of distilled water. 2ml plastic tubes labeled 1-10 were prepared and 500ul of blank serum was pipetted twice in each tube. 500ul of blank serum was pipetted thrice in a separate tube labeled x. 45ul was discarded from tube x and replaced with 1/10 dilution drug. 500ul was then pipetted from it to tube labeled one, this was mixed by vortexing using Padded vortex, then 500ul pipetted from it to tube labeled two the same procedure was repeated until the 10^{th} tube (first tube contained 500ng/ml while the 10^{th} contained 0.025ng/ml).

Evaluation of standards

Plate was coated with dilution of samples as in cELISA procedure. Conjugate was prepared the following day with the dilution of the sample. 225ul of the conjugate was pipetted and mixed with 25ul of the standard; 100ul of the above was pipetted in the washed plate incubated for 1 hour and 30 minutes on varishaker incubator TMB added and after ten minutes of incubation the reaction was stopped with adding 100ul of 1M orthophosphoric acid (stopping solution) then OD read at 450nm. The curve was then drawn. (Tubes 2 and 3 were excluded in development of the curve as their readings were almost the same).

Antisera and enzyme conjugate titration

Antisera titration ranging from 1/100 to 1/204800 and conjugate ranging from 1/1000 to 1/128000 was carried out to determine the competition for binding sites between free and enzyme-conjugate.

Determination of assay accuracy

Was determined by making QC samples with QC0 being blank serum, QC1 spiked with 5ng/ml drug and QC2 with 10ng/ml drug. This was run together with standards for every analysis of samples.

***c*ELISA**

100ul of antisera which was diluted in coating buffer (3.4.3) was pipetted into wells of microtitre plate (96 wells), incubated at 4°C-8°C overnight in the fridge then put in the freezer at -20°C the following day. Plate was then thawed and washed five times using washing buffer (3.4.1). 100ul of standard, QCs and samples diluted in conjugate in PBST (3.4.2) was added in each well, plate was then incubated for 1hour 30minutes at 37°C in Varishaker Incubator then washed as above removing unbound compounds. 100ul of TMB (3.4.6) was added and incubated for 10 minutes in Varishaker Incubator, the reaction was then stopped by adding 100ul of 1M orthophosphoric acid (3.4.5). Absorbance (OD) was then

measured at 450nm using cELISA. Concentrations of analytes were read directly from calibration curve generated from standard curve.