TRANSMISIBILITY AND VIRULENCE STUDY OF DRUG SENSITIVE AND RESISTANT *Trypanosoma congolense* IN MICE

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DECLARATION AND RECOMMENDATION

Declaration

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

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Recommendation

This thesis has been submitted for examination as per university regulations with our approval as supervisors

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DEDICATION

I dedicate this thesis to my wife, Lyllie Sangwa Fatuma, my children Mellyssa Cheusi, Merveille Binti, Chancelle Anjelani, Mechrist Muloye, Benedicte Furaha for supporting me during the long and difficult time away from them and to my other family members for being there for me and giving me moral support that enabled me to undertake this degree course.

In memory of my Parents Louis Mutangala Muloye, Binti Ngongo and younger sister Michou Mutangala who passed away while I was carrying out this research and will never have time to see the fruits of this work. May God rest their souls in eternal peace.

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ABSTRACT

To enhance our understanding of epidemiology of *Trypanosoma congolense* infection and to mitigate the development of drug resistance in the field, this study sought to assess whether there is any correlation between sensitivity and/or resistance to drug and virulence, and also to establish the relative efficiency of transmission of drug sensitive and resistant *T. congolense* by *Glossina pallidipes* in mice. The other aim was to assess possible molecular changes in *T. congolense* (savannah) before, during and after transmission. Three groups of 10 mice each were used for virulence study and were infected with either the drug sensitive or drug resistant *T. congolense*, respectively. The results showed that the drug sensitive *T. congolense* in mice had a shorter mean pre-patent period of mean of 11dpi; the mice started to die early from 11dpi, thereafter the infection became chronic. The drug resistant *T. congolense* showed a mean pre-patent period of 15dpi and mice started to die at 23dpi after which the infection became acute until all animals died by 38dpi. Parasitaemia profiles of the two isolates in mice were significantly different. The mean values for weight and PCV were not significantly different between the two isolates, however the values of the two isolates when compared to the control PCV showed significant drop for drug resistant and no significant difference compare to drug sensitive isolate, furthermore body weight for the two isolates were significantly different compared to the control in both cases. The drug sensitive trypanosome had a 42.4% infection rate in *Glossina pallidipes* compared to 39.6% for drug resistant isolate. Using the polymerase chain reaction -restriction fragment length polymorphism (PCR-RFLP) there were no detectable molecular differences between the two strains but within each strain some molecular changes occurred after passage in both flies and mice. The PCR picked more positive cases than microscopy.

Results indicate that there are differences in the pathogenesis of drug resistant and susceptible trypanosomes in the host which could responsible in production of various disease expression observed in the field. At the same time the drug resistant phenotype of the parasite is maintained drug cryopreservation and transmission of the trypanosomes in the flies and host thus posing a great challenge in the control of the trypanosomes using chemotherapy.

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LIST OF ABREVIATIONS

AAT	African animal trypanosomosis
CSF	Cerebro-spinal fluid
DA	Diminazene aceturate
dpi	Day post infection
EDTA	Ethylene diamine tetra acetate
EtBr	Ethidium bromide
GARP	Glutamate and alanine rich protein
ILRI	International Livestock Research Institute
ISM	Isometamidium chloride
KARI	Kenya Agricultural Research Institute
TRC	Trypanosomiasis Research Centre
KtDNA	Kinetoplast DNA
MT	Metacyclic trypomastigote
PCR	Polymerase chain reaction
PCV	Packed cell volume
PSG	Phosphate saline glucose
PT	Procyclic trypomastigote
SSA	Sub-Saharan Africa
VSG	Variant surface glycoprotein

CHAPTER ONE

INTRODUCTION

1.1 Background

African trypanosomosis is an infectious disease of humans and animals of similar etiology and epidemiology. The causative agents of the disease are protozoan parasites of the genus *Trypanosoma* that live and multiply extracellularly in blood and tissue fluids of their mammalian hosts and are transmitted by the bite of infected tsetse flies (*Glossina* sp.) (Dietmar, 2008). The distribution of trypanosomosis in Africa corresponds to the range of tsetse flies and comprises currently an area of 8 million km^2 between latitude 14 degrees North and 20 degrees South (Molyneux *et al.,* 1996). Throughout history, African trypanosomosis has severely repressed the economic and cultural development of the entire continent (Dietmar, 2008).

African animal trypanosomosis (AAT), nagana or surra is a disease caused by either *Trypanosoma congolense*, *Trypanosoma vivax* or *Trypanosoma brucei spp*. In wild animals, these parasites cause relatively mild infections while in domestic animals they cause a severe, often fatal disease. All domestic animals can be affected by nagana and the symptoms are fever, restlessness, emaciation, hair loss and discharge from the eyes, edema, anemia and paralysis (Dietmar, 2008). As the illness progresses the animals weaken more and eventually become unfit for work, hence the name of the disease "N'gana" which is a Zulu word that means "powerless/useless" (Dietmar, 2008).

Because of nagana, stock farming is very difficult within the tsetse belt. Treatment and prevention of animal trypanosomosis relies essentially on three drugs, namely diminazene aceturate, homidium chloride (Novidium®) or bromide (Ethidium®) and isometamidium chloride (Samorin® Trypamidium®) (Anene *et al.,* 2001). However, almost all of these trypanocides are gradually losing their efficacy due to drug resistance (Schrevel *et al.,* 1996). Experimental studies have demonstrated the occurrence of drug resistant trypanosomes to both diminazene (Moloo *et al.,* 1990) and isometamidium (Schonefeld *et al*., 1987; Clausen *et al*., 1992). This has been confirmed by studies carried out in Ghibe valley, Ethiopia (Codjia *et al*., 1993; Peregrine, 1994).

In addition, there is evidence for the development of multiple drug resistance in Burkina Faso (Sones *et al*., 1988; Clausen *et al*., 1992) and Ethiopia (Codjia *et al*., 1993; Mulugeta *et al*., 1997). Previous studies have shown the prevalence of drug-resistant trypanosomes in cattle herds of Ethiopia. Afewerk, (1998) reported that *T. congolense* field isolates from the Metekel region, expressed resistance to both isometamidium chloride and diminazene aceturate when parasites were passaged in mice. Adem (1998) identified a population of trypanosomes in north Omo zone, which expressed resistance to both isometamidium chloride and diminazene aceturate. Afewerk *et al* (2000) confirmed multiple drug resistance in cloned *T. congolense.* A lot of work has been done at KARI-TRC to determine the prevalence of phenotypes of drug sensitive and resistant *T. congolense,* with KETRI 3913 being sensitive and KETRI 3805 being shown to be resistant to multiple treatments.

Type II restriction enzymes which are most useful in molecular biology recognize a specific DNA sequence, usually four, five or six nucleotides in length and cleave the DNA within this restriction site. This is a method for the investigation of alteration of genes or DNA due to mutations. When a DNA sample has been restricted with a given enzyme it must be cut into a precise mixture of fragments that will form a reproducible pattern of bands when subjected to agarose gel electrophoresis. The differences in the reproducible patterns in DNA from various organisms are known as restriction fragment length polymorphisms (RFLPs) and result from differences in positions of restriction sites between DNA species (Keith, 2000).

The analysis of RFLPs takes advantage of base sequence variation in DNA sequences, revealing the loss or gain of restriction enzymes sites, or on the absence of target sequences altogether.

Polymorphisms in trypanosome DNA can be examined using a set of restriction enzymes and DNA probes and within the genus *Trypanosoma*, this methodology has been used most extensively to determine relationships between *T. brucei* subspecies (Masiga *et al*., 1992).

1.2 Statement of the Problem

Trypanosoma congolense savannah is one of the most important species of the livestock pathogenic trypanosomes. It is important to note that the most effective control measure involves the use of trypanocidal drugs. However the main constraint to this approach is that this parasite has rapidly developed resistance to multiple drug treatments in the field complicating the use of the few drugs available for treatment. The mechanisms of development of drug resistance are however poorly understood.. Furthermore, the study investigated the relationship between the relative degrees of virulence and drug sensitivity and/or resistance among *T. congolense* savannah isolates.

1.3 Objectives

1.3.1 Main objective

To determine cyclic transmissibility, virulence and molecular changes between drugs-sensitive and resistant *T. congolense* before, during and after transmission in both mice and tsetse flies.

1.3.2 Specific objectives

1. To determine the identity of *T. congolense* subtypes in storage at KARI-TRC.

2. To assess whether there is any correlation between sensitivity and/or resistance to drug and virulence.

3. To establish the efficiency of transmission of drug sensitive and resistant *Trypanosoma congolense* by *Glossina pallidipes* in mice.

4. To assess possible molecular changes of *T. congolense* savannah before, during and after transmission using PCR and restriction fragment length polymorphism.

1.4 Hypotheses

1. There is no difference in subtypes of *T. congolense* isolates stored in KARI-TRC cryobank.

2. There is no specific correlation between virulence and drug sensitivity and/or resistance of *Trypanosoma congolense* savannah.

3. There are no differences in transmissibility by *Glossina pallidipes* between drug sensitive and drug resistant *T. congolense*.

4. There are no molecular changes during transmission of *T. congolense* savannah through mice and tsetse flies.

1.5 Justification

Trypanosomosis depresses every aspect of production including reduced fertility, poor young stock growth, low milk yields, poor carcass quality, lack of stamina and strength in affected animals and high mortality rates. However, the pathogenicity of the different species of trypanosomes in the different species of livestock varies and within a trypanosome species there is a range of virulence. Trypanosomosis is a debilitating disease of both man and livestock. Tsetse flies infest about eleven million square kilometers, about half of the arable land of Africa. In this area, about 45 million cattle and 55 million people are at risk of the disease. Estimated total losses due to trypanosomosis range from USD 1.3 to 5 billion depending on the methodology used, assumptions made and type of loss estimated. In countries with large areas affected by trypanosomosis, total agricultural production is reduced by 2-10%. Several technologies exist for the control of both trypanosomosis and tsetse flies. However, each technology has its advantages and disadvantages. Although the use of trypanocidal drugs is the main method for trypanosomosis control, it is threatened by increasing cases of drug resistance. Indeed, it is unknown if there is any association between drug resistant phenotype and specific *T. congolense* genotypes or between certain *T. congolense* phenotypes and transmissibility by tsetse flies. The proposed study seeks to investigate the relationship, if any, between drug sensitive and resistant phenotype versus transmissibility by tsetse and virulence.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

Trypanosoma congolense is a species of trypanosomes that is the major pathogen responsible for the disease nagana in cattle and other animals including sheep, pigs, goats, horses and camels. This parasite is spread by the tsetse fly vector and in its mammalian host, *Trypanosoma congolense* only lives in blood vessels and causes anemia (Mugera and Kiptoon, 1979).

African animal trypanosomosis (AAT) is a serious disease of livestock in many parts of the tropics and sub-tropics. AAT is considered a major constraint to livestock productivity in Sub-Saharan Africa (SSA) (Leak *et al.*, 1993) and has profound effects on rural development over vast areas (Holmes, 1997). It is estimated that tsetse flies are distributed over approximately 11 million km^2 of Africa (Jordan, 1996), which is about 37% of the continent (FAO/WHO/OIE, 1982). This area has average fertility and in size it exceeds the total area of the United States of America. Out of the estimated 172 million head of cattle, approximately 44.7 million are at risk of trypanosomosis (Gilbert and Vance, 2001). The economic losses attributed to AAT are due to decreased meat and milk production as a result of mortality, morbidity and infertility. When the impact on crop productivity due to reduced animal draught power and manure is considered, the true economic losses could be much higher (Budd, 1999). The main trypanosome species causing trypanosomosis in East Africa include *T. congolense, T. vivax, T. brucei* and *T. evansi*. All except *T. evansi* that affects camels are cyclically transmitted by tsetse flies. In East Africa the most important tsetse flies are *G. pallidipes, G. longipennis, G. swynnertoni, G. brevipalpis* and *G. morsitans*, whereas biting flies include Stomoxys and Tabanus which mechanically transmit *T. vivax* (Dwinger and Hall, 2000). In Eastern Africa, *T. congolense* is the commonest species of trypanosome causing nagana in cattle while *T. vivax* is the next in importance (Mugera and Kiptoon, 1979). Virulence of the disease depends on species of the trypanosomes and the host, in addition to the frequency of exposure to infected tsetse bites (Connor *et al.*, 1989).

2.2 Pathogenesis and Clinical Signs

The pathogenesis of trypanosomosis depends on species of trypanosomes, host and the nutritional status of the host. On infection, *Trypanosoma congolense* enters capillaries in which they live their entire life. They cause their effects almost entirely by blocking capillaries and causing anemia (Mugera *et al*., 1979). Cattle infected with *T. congolense* show a rise in temperature 5 to 10 days after exposure. The fever is usually intermittent and animals under stress such as work oxen develop the disease faster than other animals and might take up to several weeks before any symptoms are seen (Murray *et al.*, 1982). The clinical symptoms of the disease include dullness, anorexia, lethargy, lacrimation and photophobia. Later the animal loses body condition; the eyes appear sunken with a starring coat and lymphadenopathy that may be confused with that caused by East Coast fever (Mugera *et al*., 1979). In the last stages of the disease anemia is evident and the eyes become opaque with the animal becoming extremely emaciated and weak. Some animals may show edematous swellings of the throat, dewlap and ventral abdomen. *T. congolense* infection causes more severe disease in adult cattle and when left untreated, death of infected animals occurs in 2 to 4 months post infection.

2.3 Diagnosis of Trypanosomosis

2.3.1 Difficulties of diagnosis

Diagnosis for trypanosomosis is unreliable and difficult to carryout. Not only are there no specific clinical signs, but the intermittent and usually low parasitaemia make detection of the trypanosomes difficult (Connor *et al.*, 1989). Furthermore, infection is not synonymous with disease with many subclinically affected animals living in a delicate balance with potentially pathogenic trypanosomes. An element of clinical judgment, therefore, enters into the diagnosis of trypanosomosis. Comparisons of the different diagnostic methods have been made by several authors (Nantulya, 1990) and detailed descriptions of parasitological methods of diagnosis are available (Boyt, 1984).

Important considerations in the diagnosis of trypanosomosis are that the number of detectable parasites is not necessarily directly related to the severity of the disease. In addition in trypanosomosis-enzootic areas the disease is a herd problem. In most tsetse infected areas

clinical signs of trypanosomosis are well recognized with farmers and veterinary personnel commonly resorting to treatment of sick animals and thereafter using response to therapy to provide retrospective diagnosis (Connor *et al.*, 1989). In these areas, a history of the presence of tsetse and the use of trypanocidal drugs when considered with presenting clinical signs are sufficient to make a tentative diagnosis. However, the presence of concurrent disease may mask trypanosomosis and complicate the clinical picture (Connor *et al.*, 1989). Thus, the only way to confirm a diagnosis in clinically affected animals is to demonstrate and identify the parasites in body fluids.

2.3.2 Direct diagnostic methods

For diagnosis purposes the body fluid most commonly examined is blood, either capillary blood from the tail tip, or venous blood from the jugular or from the ear (Connor *et al.*, 1989). Lymph, aspirated from a punctured superficial lymph node, usually the pre-scapular gland, provides useful supplementary diagnostic material. Whereas in the diagnosis of human sleeping sickness, cerebrospinal fluid (CSF) is routinely examined for the presence of trypanosomes (Simarro *et al.*, 2008), this is exceptional in veterinary medicine. Fresh blood is examined microscopically, as a wet preparation with a low objective lens. For routine diagnosis dry thick and thin blood smears are usually made. The smears are stained with Giemsa stain and examined under an oil immersion lens. The most sensitive direct method to detect *Trypanosoma congolense* and *T. vivax* infections is by examination of wet preparations of the microhaematocrit buffy coat, under phase contrast illumination (Connor *et al*., 1989).

A method of polymerase chain reaction (PCR) has been introduced into diagnostic tests for detection of African trypanosomes infection in human and animals as well as tsetse flies (Masiga *et al*., 1992). Specific repetitive nuclear DNA sequences can be amplified for *T. vivax, T. simiae* and each of the three *T. congolense* subgroups namely Savannah, forest and Kenya coast (Kilifi) (Moser *et al*., 1989; Artama *et al.*, 1992; Masiga *et al*., 1992). A common primer set is also available for detection of the three *T. brucei* subspecies namely *T. b. brucei, T. b. gambiense* and *T. b. rhodesiense* (Moser *et al*., 1989).

2.3.3 Indirect diagnostic methods

Because of the difficulty of detecting trypanosomes in the blood due largely to the phenomenon of antigenic variation, numerous indirect tests have been applied for the diagnosis of trypanosomosis. Although a range of serological tests have been applied to the diagnosis of animal trypanosomosis, a major problem arises from the lack of specificity of antigens which have been used (Connor *et al*., 1989). Even when anti-trypanosomal antibodies are detected they do not distinguish between current and past infection, and cross reactions occur between some trypanosome species (Magona *et al*., 2004). The card agglutination test (CATT) adopted to assist diagnosis of West African human sleeping sickness relies on the presence of anti-trypanosomal antibody to agglutinate intact, stained and preserved trypanosomes (Connor *et al*., 1989).

2.4 Drugs in Use Against Trypanosomosis

Trypanosomes are covered by a glycoprotein coat that is encoded by up to a thousand genes that are antigenically distinct, thus making the parasites able to engage in an immuno-protective process of antigenic variation (Borst and Fairlamb, 1998). As a result prospects for vaccine development are poor. Drugs are the preferred available choice of tackling this problem although all of them have serious side effects and resistance is increasing (Mulugeta *et al.*, 1997). Drug resistance in animal pathogenic trypanosomes including *T. congolense*, *T. vivax* and *T. evansi* to the major drugs such as diminazene, isometamidium, homidium, suramin and quinapyramine is well established (Anene *et al.*, 2001). Similarly multiple-drug resistance has been described for *T. congolense* (Afewerk *et al*., 2000). The difference in sensitivity was in a range of more than 120 fold and for quinapyramine more than 160 fold (El Rayah *et al*., 1999). These large differences in sensitivity clearly indicate the existence of drug resistant pathogenic trypanosomes in the field (Brun *et al*., 2001). However the chemotherapy of animal trypanosomosis has been comprehensively reviewed during the last two decades (Leach and Roberts, 1981; Holmes and Scott, 1982; Tacher, 1982; Peregrine *et al*, 1994). Over this period the number of compounds marketed for trypanosomosis has dwindled as some compounds have been withdrawn because of the emergence of drug resistance. There are no new trypanocides on the horizon, neither is there a prospect for a vaccine. The high cost of vector control operations and inadequate methods to protect areas reclaimed from tsetse infestation have led to a

widespread dependence on trypanocides, of which only four compounds are now available. Diminazene, homidium and isometamidium are used mainly in cattle, goats and sheep (Anene *et al.*, 2001). The fourth compound, quinapyramine was withdrawn from the market in 1977 because of the emergence of widespread resistance among trypanosomes in cattle (Magona *et al.*, 2004). It was however reintroduced in 1985 mainly to treat *T. evansi* infections in camels and horses. It is also useful in the treatment of African trypanosomosis in horses and pigs (Connor *et al*., 1989). Although suramin, the oldest trypanocide still marketed is used to treat early cases of human sleeping sickness as well as *T. evansi* infections in camels, it is ineffective against the other species of trypanosomes (Connor *et al*., 1989). Similarly, the other trypanocides also differ in their activity against the different species of parasites. Diminazene is active against *T. congolense* and *T. vivax* at a dosage rate of 3.5mgks^{-1}, but must be used at 7.0mgkg^{-1} or more to remove *T. brucei* infections. The phenanthridinium related compounds homidium and isometamidium are active against *T. congolense* and *T. vivax* but less so against the *T. brucei* group (Connor *et al.*, 1989).

2.5 Mechanisms for Evading Immune Responses

African trypanosomes are protozoan parasites living in the blood stream of the mammalian host. Different species of African trypanosomes cause different patterns of disease (Mulligan and Potts, 2006) but they all share the characteristic of being covered by a dense layer of variant surface glycoproteins (VSG) (Cross, 1990; Gerald *et al.*, 1996). *Trypanosoma congolense* is the most pathogenic species infecting livestock. Mice are also susceptible to *T. congolense* infections (Shi *et al*., 2007). There is evidence indicating that resistance and/or susceptibility to *T. congolense* infections in mice is controlled by at least five different quantitative trait loci on chromosomes 17, 5 and 1 (Kemp *et al.*, 1997; Iraqi *et al.*, 2000). The relevant genes and their products are unknown. Control of parasitaemia is due to antibodies specific for VSG (Jackson *et al.*, 1978; Bussler *et al.*, 1998). Most of the initial antibodies specific for the VSG of *T. congolense* or *T. brucei* are of the immunoglobulin M (IgM) class and are produced in a T cell independent fashion (Campbell *et al.*, 1978; Shi *et al.*, 2007).

2.6 Mechanisms of Drug Action

The pharmacokinetic of trypanocides is incompletely understood, but the rate of excretion of the different compounds is known to affect their activity (Kinabo and Bogan, 1988). Diminazene, which is rapidly excreted, is used only for its therapeutic effect, whereas isometamidium is slowly excreted and is the most effective prophylactic compound currently available (Kinabo and Bogan, 1988). Homidium is excreted more slowly than diminazene, but more rapidly than isometamidium and thus has limited prophylactic activity. Whilst slow excretion is advantageous for prophylaxis, it is disadvantageous because of the drug residue effect produced and thus the trypanocides have recently been scrutinized by the joint FAO/WHO Expert Committee on Food Additives (WHO, 1989).

Although diminazene is rapidly excreted it has been shown to have a prophylactic effect, against intravenous challenge with *T. congolense* for a period of 12 days (Wellde and Chumo, 1983). Isometamidium, which is widely considered to be the drug of choice in the prevention of bovine trypanosomosis, is administered by deep intramuscular injection. The usual sequel to the correct injection of isometamidium is that an encapsulated lesion forms within the muscle, from which the drug is slowly released to give prolonged protection from infection by trypanosomes. The duration of protection appears to be largely dose related and thus the higher the dosage rate, the longer the period of protection (Peregrine *et al.*, 1988).

Homidium is mainly used for its therapeutic effect, but it does have prophylactic activity for several weeks (Dolan *et al.,* 1990). It's also administered by deep intramuscular injection but is less irritating than isometamidium.

Quinapyramine sulphate is soluble in water and is administered by subcutaneous injection to treat *T. evansi* and *T. brucei* infections (Connor *et al*., 1989). The prosalt formulation is a combination of the soluble dimethyl sulphate and the insoluble chloride salt of quinapyramine. After subcutaneous injection of the prosalt suspension, the soluble salt exerts a therapeutic effect, whilst the chloride provides a depot from which the trypanocide is slowly absorbed and therefore providing prophylaxis (Connor *et al*., 1989).

2.7 Drug Resistance

When the use of trypanocides does not produce the expected cure or protection, there is a tendency to assume that drug resistant has arisen (Williamson, 1979; Leach and Roberts, 1981). Whilst this may be true, there are many other reasons which contribute to drug treatment failure. Only after carefully investigating the practical points of drug administration and eliminating as causes of failure, is it valid to investigate the likelihood of there being true drug resistance.

Before trypanocides can be said to have failed, there must be parasitological evidence that trypanocidal treatment either has not removed an established infection or has not provided protection against infection or re-infection (Rowlands *et al.*, 1990). Thus, parasitological monitoring is a prerequisite to drug usage. From an analysis of properly kept records of routine monitoring is it possible to determine whether administration of a therapeutic dose of a trypanocide cures an infected animal. A recent report from Ethiopia has demonstrated the value of a field appraisal to determine the efficacy of trypanocidal drugs in an area where trypanocide failure occurred (Rowlands *et al.*, 1990).

Drug resistance to trypanocides is a well known phenomenon (Williamson, 1979; Leach and Roberts, 1981). The introduction of every new trypanocide has been followed by the emergence of resistance to it, and in the cases of homidium, quinapyramine and prothidium has led to their withdrawal from the market (Connor *et al*., 1989). Resistance seems to develop in a stepwise manner with trypanosomes resistant to a low dose of a trypanocide being removed by a higher dose of the same compound (Connor *et al*., 1989). The problem is that, because of the narrow therapeutic indices of the trypanocides, there is only limited scope to overcome resistance by increasing the dosage.

2.8 Mechanisms of Drug Resistance

The P2 aminopurine transporter, encoded by TbAT1 in African trypanosomes in the *Trypanosoma brucei* group, carries melaminophenyl arsenical and diamidine drugs into these parasites. Loss of this transporter contributes to drug resistance. Analyses have been made on a *Trypanosoma congolense* contig coding named TcoATI, a putative P2-like nucleoside transporter. The sequence included a start and stop codon and presents a high similarity with the

gene TbAT1 of *T. brucei* with the smallest sum probability of 2.8e−136. To investigate a possible link between point mutations and diminazene aceturate (DA) resistance in mice, the TcoAT1 putative genes was identified. Studies of drug resistance in kinetoplastids have indicated that decreased levels of drug accumulation are responsible for tolerance to a number of trypanocides as is the case in arsenical compounds. Activity of the adenine-sensitive adenosine transporter termed the P2 subtype is absent from populations induced to be melarsen-resistant in the laboratory (Carter and Fairlamb, 1993). The diamidine group of trypanocides also seems to enter the trypanosome by means of the P2 adenosine transporter subtype, which is consistent with reports of cross-resistance to arsenical compounds**.**

The resistance mechanism thus seems to be specific for the homidium moiety, not the m-amidinophenyl-azo-amine functional group common to Samorin and Berenil. Furthermore, populations of *T. congolense* seem to express only inosine-sensitive (i.e. P1 type) adenosine transport; uptake of isometamidium is not inhibited by this compound (Wilkes *et al*., 1997). Thus in contrast with the diamidines, adenosine transport systems do not seem to be involved in the transport of phenanthridines.

Mulugeta *et al* (1997) described the accumulation of isometamidium chloride in *T. congolense* and showed mitochondrial electrical potential to be a major determinant of the process. Furthermore, *T. congolense* populations with different sensitivities to the drug were shown to exhibit consistent relationships between their level of resistance, rate of drug accumulation and intrinsic mitochondrial potential. He proposed that modulation of mitochondrial potential was a mechanism responsible for resistance to isometamidium in this organism. This represents a novel mechanism of drug resistance in naturally occurring populations of protozoan parasites

CHAPTER THREE

MATERIALS AND METHODS

3.1 Selection and Characterization of *T. congolense* Stabilates

3.1.1 Parasite selection and DNA extraction

Two *T. congolense* savannah stabilates that are known to be either drug sensitive or resistant were randomly selected from KARI-TRC trypanosome bank. Those samples were collected from the field then characterized phenotypically. The trypanosome preparation was made using the edge of the slide and ends with a filmbrited tail where the red cells are one layer thick. The film was air-dried and then fixed using methanol followed by staining using Giemsa stain. The film was then examined for identification of the parasites using immersion oil at x100 magnification. *T. congolense* was recognized by it polymorphic morphology, the absence of free flagellum, the presence of slightly developed undulating membrane, marginal or central sub-terminal kinetoplast and posterior end that are either rounded or flat.

T. congolense that is drug sensitive or drug resistant was obtained by taking 50µl of EDTA blood containing stabilates that had been thawed and which was lysed by mixing with 10 volumes (500µl) of lysis buffer (0.2% NaCl, 0.15% saponin and 1mM EDTA). After centrifugation for 10 minutes at 12,000 rpm in an eppendorf centrifuge, the supernatant was discarded and the pellet washed in lysis buffer. The pellet was rewashed in PCR buffer (50mM KCl, 1.5mM $MgCl_2$, 10mM Tris-HCl, pH 8.4) and re-suspended in 50µl of Tris EDTA (TE) buffer after which the sample was boiled in water for 20 minutes. Two µl of the material was then used for each PCR reaction.

3.1.2 Subtypes characterization using PCR

Three primer pairs were used for PCR analysis; TCS1 and TCS2; TCK1 and TCK2 and TCF1 and TCF2 as described by Masiga *et al* (1992). PCR analysis was performed in 20µl reaction volumes containing PCR buffer (200µM dNTPs, 2.5µM primer, 1.5µM $MgCl_2$) (Promega USA), 0.1 units of Tag polymerase (Applied Biotechnologies) and 10-20ng genomic DNA obtained after extraction as described in section 3.1.1. PCR reactions were then carried out

in a PE 2700 thermal cycler (Perkin Elmer) using the profile of 98^{o}C for 1 minute, then 98^{o}C for 30 seconds and 60^{o}C for 45 seconds and thereafter an extension at 72^{o}C for 45 seconds repeated 30 times. Thereafter a final extension at 72^{o}C for 4 minutes was carried out. PCR products were size-separated by electrophoresis on a 2% agarose gel, stained with ethidium bromide and visualized under ultraviolet illumination (Masiga *et al*., 1992). The gels were examined for the presence of distinct band to confirm the subtypes present as either savannah, kilifi or forest *T. congolense* subtypes.

3.1.3 Experimental animals

Animals were obtained from KARI-TRC small animals breeding unit after ethical approval from TRC Institutional animal care and use committee (IACUC). They were classified either as donors or experimental.

The experimental design for all the studies undertaken is as summarized in table 1.

Table 1: Experimental design for the transmissibility and virulence studies of drug sensitive and resistant *Trypanosoma congolense* in mice

Study Type/No of animals required			
Animals/Infection type	Validation/sensitivity testing	Virulence in mice	Transmission through tsetse flies and mice
Donors			
Sensitive	2	2	10
Resistant	2	2	10
Experimental (infected, treated)			
Sensitive	12	10	100
Resistant	12	10	100
Infected Controls (Infected, not treated)			
Sensitive	3	-	-
Resistant	3	-	-
Non-infected controls	-	10	10

3.1.3a Donor mice

A total of twenty-eight donor Swiss white mice aged 6-8 weeks and weighing 20-30g were used for validation/sensitivity, virulence and transmissibility studies. Animals were obtained from KARI-TRC small animal breeding unit. They were maintained on a diet of commercial mice pellets (mice pellets®, Unga Ltd, Nairobi, Kenya) and water provided *ad libitum.* The mice were immunosuppressed using cyclosphosphamide at 300mg/kg intra-peritoneally for 3 consecutive days before infection to evade immune response. This was followed by infection with either drug sensitive or drug resistant *T. congolense* stabilates from KARI-TRC trypanosome bank. The mice were examined for parasitaemia development as the infection progressed. A drop of tail blood was collected daily on a clean slide and covered with a

cover slip, examined under microscope at magnification of x40 and the parasitaemia score correlated to a score sheet as outlined by Herbert and Lumsden (1976). At the peak of parasitaemia, the mice were euthanized by placing them in an environment of concentrated carbon dioxide (CO_2) and bled from the heart. The blood was collected into a tube containing EDTA and trypanosomes quantified using the improved Neubauer chamber. An inoculum dose of 0.2ml (1 $x10^5$ trypanosomes) was prepared using phosphate saline glucose (PSG) pH 8.0 for infecting experimental mice as described in sections 3.3 and 3.4.

3.1.3b Experimental mice

A total of two hundred and seventy Swiss white mice aged 6-8 weeks and weighing 20-30g was used for sensitivity testing, virulence and transmissibility studies. Animals were obtained from small animal breeding unit at KARI-TRC. They were maintained on a diet of commercial mice pellets (mice pellets®, Unga Ltd, Nairobi, Kenya) and water provided *ad libitum.* Experimental procedures followed were as described under relevant sections namely section 3.3 and 3.4. Drug sensitivity study was conducted on two selected *T. congolense* isolates from KARI-TRC trypanosome bank. The isolates were known to be sensitive or resistant to isometamidium chloride and diminazene aceturate.

3.1.4 Multiplication of trypanosomes and preparation of inoculum

Four adult donor mice described under section 3.1.3a were used. The two selected stabilates were either drug sensitive or drug resistant *T. congolense.* The stabilates were inoculated into two donor mice each for multiplication then the infected mice were kept two per cage with sawdust as bedding material. The mice were examined for parasitaemia development as the infection progressed. The inoculum for infection of experimental mice was prepared as described under section 3.1.3a.

3.2 Experimental Procedure for the Drug Sensitivity Study

Thirty experimental mice as described under section 3.1.3b were used. They were numbered using cupric acid and kept six per cage with sawdust as bedding material. Pre-infection data for both weights and packed cell volume (PCV) was collected every two days for a period of two weeks prior to infection. The thirty mice were divided into five (I-V) experimental

groups of six mice each. Groups I and II were infected with the selected drug sensitive *T. congolense* (KETRI 3913) and treated with either isometamidium (1mg/kg) or diminazene (20mg/kg) twenty hours after infection. Groups III and IV were infected with the selected drug resistant *T. congolense* (KETRI 3805) and treated with either isometamidium (1mg/kg) or diminazene (20mg/kg) at twenty hours after infection as summarized in table 2. The fifth group (group V) served as infected untreated controls and was treated with distilled water. Each mouse in the four groups (group I–IV) was weighed on a flat pan balance prior to administration of trypanocidal drug. For the treated groups, the mice were monitored for trypanosomes as described under section 3.1.3a every two days for a period of sixty days post-treatment

Table 2: Experimental design for the validation of drug sensitive and resistant *Trypanosoma congolense* in mice

Trypanosome isolates	**Drug**	**Dose**	**Number of mice**
Selected drug sensitive *T. congolense*	Isometamidium	1mg/kg	6
	Diminazene	20mg/kg	6
Controls infected, not treated	Distilled water	0.2ml	3
Selected drug resistant *T. congolense*	Isometamidium	1mg/kg	6
	Diminazene	20mg/kg	6
Controls infected, not treated	Distilled water	0.2ml	3

3.3 Study to Determine Virulence of Drug Sensitive and Resistant *T. congolense* in Mice

This study was undertaken to determine how fast the parasites of either drug sensitive or drug resistant nature establish and how they multiply causing infection in mice.

3.3.1 Multiplication of trypanosomes and preparation of inoculum

Trypanosomes were multiplied using the procedure as described under section 3.1.3a.

3.3.2 Experimental procedure for virulence study

Thirty mice were used. They were numbered using cupric acid and kept ten per cage with sawdust as bedding material. The animals were divided into three experimental groups of ten mice each (group I-III). The first two groups (group I-II) formed the infected groups. Ten mice of the group I were infected with the selected drug sensitive *T. congolense* and another ten (group II) were infected with the selected drug resistant *T. congolense.* Group III consisting of 10 mice were used as non-infected control. The infected mice were monitored daily for sixty days post-infection. The parameters that were determined included parasitaemia level, pre-patent period, body weight, packed cell volume and survival period.

3.3.2a Parasitaemia and pre-patent period

Mice were examined as described under section *3.1.3a*. The animals were examined for the parasitaemia development as the infection progressed and pre-patent period determined.

3.3.2b Packed cell volume and body weight

Body weight for each mouse was measured daily using analytical balance. Thereafter packed cell volume was determined as outlined by Naessens *et al* (2005). Infected blood was collected from the tail vein daily using heparinized capillary tubes sealed with plasticin at one end. The sealed capillaries were then centrifuged in a haematocrit centrifuge at 10,000 revolutions per minutes (RPM) for 5 minutes and thereafter PCV read using the haematocrit reader.

3.3.2c Survival period

The infected mice were examined daily and death time for each animal recorded. The data were used to calculate the mean survival period.

3.4 Study to Assess Transmission of *Trypanosomes congolense* Through Tsetse and Mice

3.4.1 Trypanosomes for infection

Two *Trypanosoma congolense* stabilates were obtained from KARI-TRC cryobank for use. The selected isolates of *T. congolense,* which were known to be drug sensitive or drug resistant to isometamidium and diminazene were propagated in Swiss white mice.

3.4.2 Donor mice

Twenty donor mice as described under section 3.1.3a were used. The two selected stabilates, drug sensitive and drug resistant *T. congolense* were inoculated into ten donor mice each for multiplication. The infected mice were kept ten per cage with sawdust as bedding material. They were examined for parasitaemia as described in section 3.1.3a.

3.4.3 Transmission procedure in tsetse flies using infected mice

The donor mice were monitored for parasitaemia daily using the tail blood as described under section 3.1.3a. At peak parasitaemia, 100 teneral flies starved for two days and placed in groups of 20 per cage, were infected by feeding them on the trypanosome infected donor mice. The infected flies were thereafter maintained in the insectary by *in vitro* feeding for 35 days from infection to maturity. The infected flies were then starved for two days and then separated into individual fly tubes. These flies were probed onto probing slides placed on the fly feeding mat at 37 ^{0}C. Each of the infected and probed fly was fed individually on individual clean mice to test their infection transmission ability. The flies were returned to the insectary and dissected 24 hours after individual feeding on mice for microscopical examination of trypanosomes in the gut, salivary glands and proboscis. The same organs were thereafter preserved in eppendorf tubes for molecular analysis where the positive organs were analyzed for polymerase chain reaction-restriction fragment length polymorphism analysis (PCR-RFLP) as described in section 3.4.4. A control group of 100 clean teneral flies were fed on clean mice and underwent the same procedure as described for the infected group.

3.4.3a Probed slides

These probed slides were stained using the Giemsa stain and thereafter microscopically examined at x100 objective using oil immersion to determine the presence of mature trypanosomes and thus infection.

3.4.3b Fly dissection

Thereafter the flies were euthanized using chloroform. The dead flies were then placed on slides and dissected using the dissecting microscope to collect organs for molecular analysis.

3.4.4 Molecular analysis

Molecular analysis was performed using normal PCR followed by RFLP methods as described in sections 3.4.4a and 3.4.4b.

3.5 DNA extraction, polymerase Chain Reaction and Restriction Fragment Length Polymorphism Analysis (PCR-RFLP)

DNA extraction and PCR were performed as described in subsections 3.1.1 and 3.1.2, respectively.

The PCR products at various stages of transmission using subspecies specific primers and fusion enzyme as DNA polymerase were subjected to restriction enzymes, Mbo II, Bam HI, Hind III, Hae III and HinfI. Briefly, a mixture of 5µl DNA, 2µl enzyme, 2µl of 10 times buffer and 11µl of distilled water served as Master Mix for one reaction and was used for each of the restriction enzymes. The mixture was incubated at 37^{o}C for one hour after which it was subjected to agarose gel electrophoresis. The RFLP was performed using samples from three sources namely; blood of donor mice after infecting tsetse flies, tsetse flies with mature infection in the salivary glands and blood from positive mice infected using infected tsetse flies. Five microlitres of the PCR products were mixed with 1 x restriction buffers to make a total volume of 20µl. On completion of incubation, the whole 20µl was loaded into an agarose gel containing ethidium bromide in 1x TAE buffer. At the end of the run, the gels were photographed both with a digital camera and UVITEC images. PCR was performed from two sample sources for diagnosis purpose namely, all organs from the infected flies and blood from mice infected using tsetse flies

monitored for development of parasitaemia for 30 days. At the same time uninfected mice were also euthanized and bled to act as negative controls for PCR analysis.

3.6 Data Analysis

The data were entered in Microsoft Excel (version 2007) and analyzed by student's t-test and analysis of variance (ANOVA). The first and fourth hypotheses were assessed by visual inspection of presence/absence of DNA bands among isolates and between parasite infection stage/host using specific primers and restriction enzymes respectively. Chi-square analyses were used to assess the second and third hypotheses by the correlation between sensitivity and/or resistance to drug and virulence and comparing the rate of transmission between the two isolates. Analysis of mean parasitaemia, PCV and body weights by the student's t-test, which compared the differences between mean values for measured parameters of the control mice and those of the infected group for virulence. Significance was considered at p value <0.05. The analysis of survival was carried out employing Kaplan-Meier method for determination of survival distribution function on StatView (SAS Institute, Version 5.0.1). Rank tests of homogeneity were used to determine the effect of treatment on early (during early phase of infection) and larger (during late phase of infection) survival times respectively. . In all analyses, a level of $p < 0.05$ was considered statistically significant.

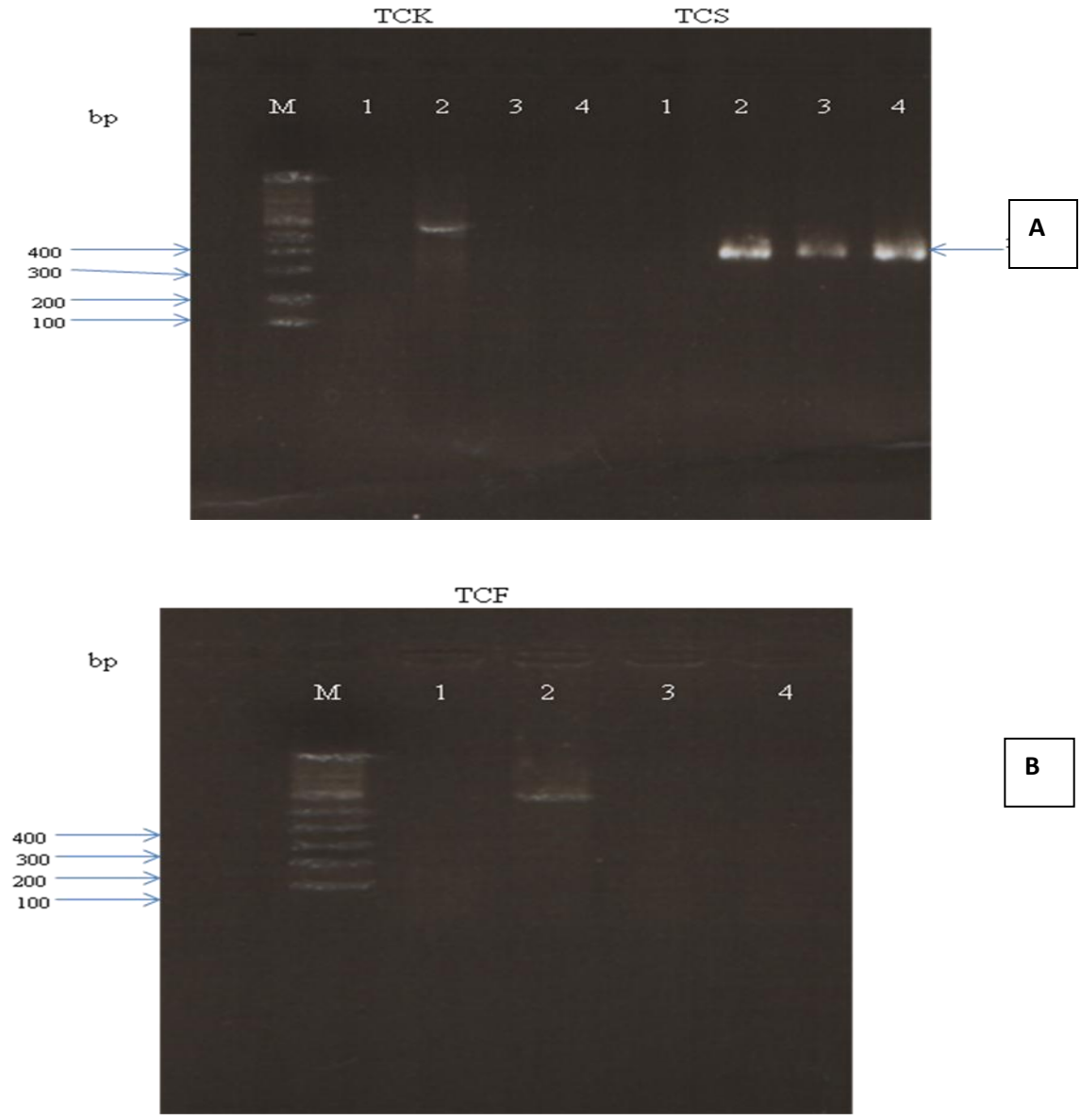
CHAPTER FOUR

RESULTS

4.1 Subtypes characterization

Results obtained following amplification of PCR products from the two isolates, drug sensitive and drug resistant trypanosomes, are shown in figure 1. Results indicated the negative controls did not show any band(s) while there was a strong DNA band of size 369bp in drug sensitive, drug resistant and the positive control where the savannah specific primers were used, confirming the presence of *T. congolense savannah*.

All samples of drug sensitive, drug resistant *T. congolense* and negative control subjected to PCR using TCK1 and 2 and TCF1 and 2 did not yield any bands indicating absence of the Kilifi and forest subtypes. No mixed infections were observed in the samples analyzed.



Figures 1 A and B: 2% agarose gel electrophoresis following amplification of *Trypanosoma congolense* sub-species and visualized through ethidium bromide staining. Lanes 1 and 2 are negative (distilled water) and positive controls (*T. congolense savannah*), respectively while lanes 3 and 4 are drug sensitive (KETRI 3913) and drug resistant (KETRI 3805) respectively from KARI-TRC cryobank. M is DNA molecular weight marker (100bp ladder).

TCK, TCS and TCF are *T. Congolense* Kilifi, *T. congolense* savannah and *T. congolense* forest primers , respectively

4.2 Drug Sensitivity/Validation Study

Results on drug sensitivity test in mice are summarized in table 3. When the mice were treated at 24 hours post-infection with either isometamidium at 1mg/kg body weight or diminazene at 20mg/kg body weight none of them developed any parasites in circulation and remained parasitaemia negative over the experimental period of 60 days post treatment giving 100% cure and thus confirming that this isolate were sensitive to the two drugs used.

In the case of drug resistant isolate, five of the six mice treated at 24 hours post-infection with isometamidium at 1mg/kg body weight developed parasitaemia over the experimental period of 60 days post treatment indicating approximately 80% were not cured. All the mice treated with diminazene at 20mg/kg body weight developed parasitaemia over the experimental period of 60 days post treatment. This indicated 100% were not cured.

One mouse infected with the drug resistant trypanosome and treated with isometamidium did not develop parasites in circulation at any time during the experimental period. These results confirmed that this isolate was resistant to the two drugs used.

Table 3: Cure rate in mice infected with either drug sensitive or resistant *Trypanosoma congolense* isolates and treated with either isometamidium at 1mg/kg or diminazene at 20mg/kg body weight

Trypanosome isolates	**Drug**	**Dose**	**No. of mice cured /No. exposed**	**% Cure rate**
KETRI 3913 (Drug sensitive)	Isometamidium	1mg/kg	6/6	100
	Diminazene	20mg/kg	6/6	100
KETRI 3805 (Drug resistant)	Isometamidium	1mg/kg	1/6	16.7
	Diminazene	20mg/kg	0/6	0

4.3 Determination of Virulence of Drug Sensitive and Resistant *T. congolense* in Mice

4.3.1 Pre-patent period, survival time and parasitaemia progression of Swiss white mice infected mice

Results of mean (range) pre-patent period and survival time of Swiss white mice infected with either drug sensitive or drug resistant isolate are shown in figure 2 and table 4. Mice infected with drug sensitive isolate had a mean pre-patent period (PP) of 11 days post infection (dpi) with a range of between 7 to 21 dpi. One mouse became positive on day 7, 4 mice on day 9, 3 mice on day 10, 1 mouse on day 14 and the remaining 1 mouse became positive on day 21 post-infection. While mice infected with drug resistant isolate had a longer mean PP of 15 days with a range of 9 to 17 dpi. In the drug resistant infected mice, one mouse became positive on day 9, 4 mice on day 13, 1 mouse on day 15, 3 mice on day 17 and the 1 mouse on day 21 post-infection. The results indicate that the mean pre-patent period was shorter (11 dpi) in mice infected with drug sensitive isolate when compared to a similar group of mice infected with drug

resistant isolate (15 dpi). The drug sensitive group of mice had a mean survival period of 35 days post infection. The first mouse in the drug sensitive group died on day 11, 2 mice on day 14, one mouse each died on days 16, 25, 43, 49, 56, 58. However one mouse in the group survived up to 60 dpi when the experiment was terminated.

In contrast mice infected with the drug resistant trypanosome had a mean survival period of 30 dpi. Three mice in the drug resistant group died on 23 dpi, one on 27 dpi, three on 32 dpi, one mouse each on days 35, 37 and 38.

Table 4: Mean pre-patent period and survival time of Swiss white mice infected with drug sensitive (n=10) and drug resistant (n=10) *T. congolense* isolates

Isolates	**Mean pre-patent period (days)**	**Mean survival time (days)**
Drug sensitive isolate (KETRI 3913)	11 (range 7-21)	35 (range 11-60)
Drug resistant isolate (KETRI 3805)	15 (range 9-17)	30 (range 23-38)

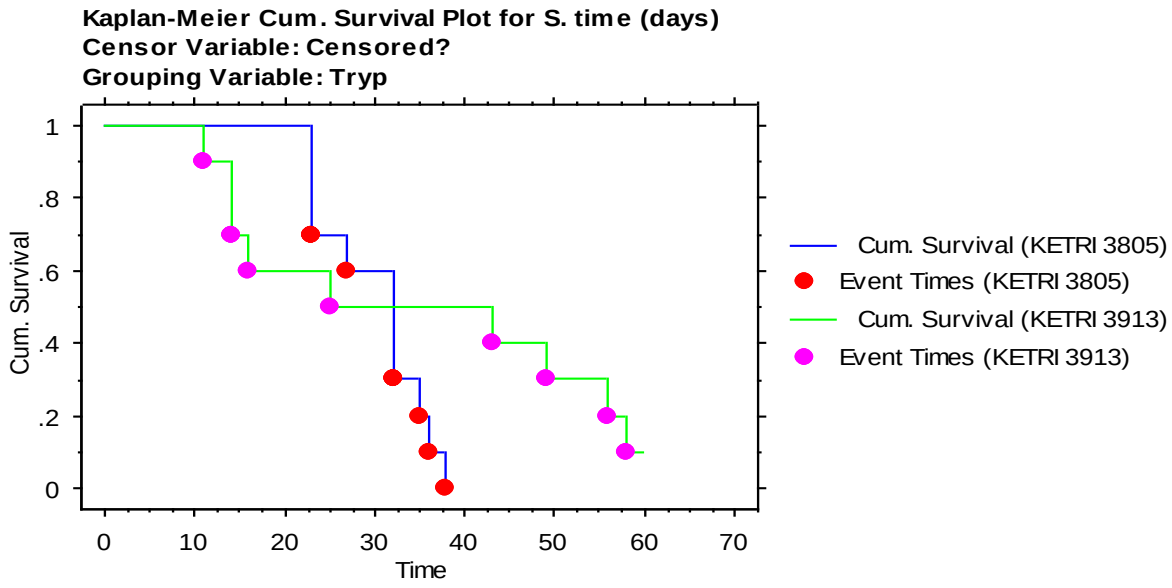


Figure 2: Cumulative survival (days) of Swiss white mice infected with either drug sensitive or drug resistant *T. congolense*

The mean parasitaemia progression over observation period of 60 days post-infection is presented in table 5 and figure 3. Several waves of parasitaemia were observed. The drug sensitive isolate showed the first peak of parasitaemia of $1x10^7$ parasites/ml blood occurring at 14 days post-infection (dpi) and declined to $1x10^6$ parasites/ml blood at 17 dpi. The parasitaemia increased gradually reaching the second peak of $1x10^8$ parasites/ml blood 28 dpi. However, a slight decline was observed between days 25 and 38 with a third peak parasitaemia of $1x10^8$ parasites/ml blood being observed from 45 dpi and remained relatively at same level until the end of experiment when all the mice had died.

The group of mice infected with the drug resistant isolate showed a rapid increase in parasitaemia levels to reach the first peak of $1x10^{8.4}$ parasites/ml blood 23 dpi which then decreased to $1x10^{7.8}$ parasites/ml blood on 30 dpi. The second and third peaks of parasitaemia of $1x10^{8.4}$ parasites/ml blood and $1x10^9$ parasites/ml occurred on days 33 and 36 post infection, respectively at which point all the mice had died. Results of the parasitaemia progression were significantly different between the two isolates $p<0.05$.

Table 5: Showing the mean ±SE parasitaemia progression (log) of mice groups infected with either drug sensitive or drug resistant *T. congolense* savannah at different periods of the experiment

Trypanosome isolates **Infection period**	**Drug sensitive** **(KETRI 3913)** **No of parasite/ml blood (log)**	**Drug resistant** **(KETRI 3805)** **No of parasite/ml blood (log)**
Pre infection	0	0
14 dpi	*6.77±0.65	*3.56±0.61
21 dpi	*7±0.39	*8.19±0.18
35 dpi	*7.62±0.30	*8.55±0.32

*$p<0.05$= significant

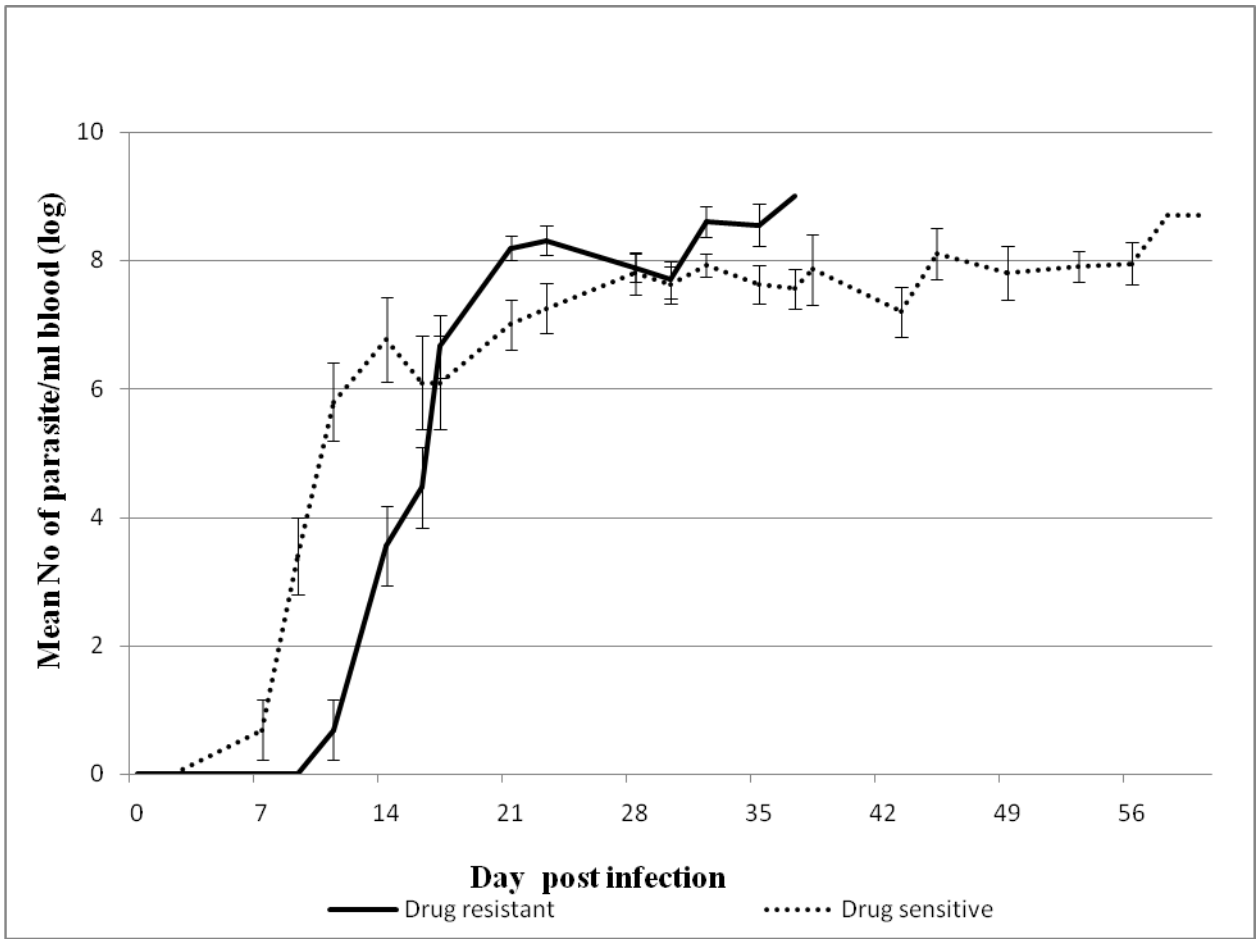


Figure 3: Parasitaemia profiles of mice infected with either drug sensitive or drug resistant *Trypanosoma congolense* savannah

4.3.2 Packed cell volume in mice infected with drug sensitive and drug resistant *T. congolense*

The packed cell volume (PCV) values of the infected and control mice are presented in figure 4 with a summary of the mean trends in table 6. The mean PCV for the uninfected mice were maintained around 51% throughout the experimental period of 60 days. However, PCV levels for both groups of infected mice showed steady decline from 17 dpi a trend that continued throughout the period of infection. The decline in the mean PCV levels of animals infected with drug resistant isolates produced a significantly faster decline in PCV level compared to those infected with the drug sensitive trypanosomes ($p<0.05$). By 35 dpi the PCV levels for drug resistant and drug sensitive trypanosome infected mice were significantly different and had

reached levels of 24.5±1.57 and 37.8±1.35, respectively ($p<0.05$). Similar trends in decline of PCV levels for the infected mice were maintained throughout the infection period.

Table 6: The mean (±SE) Packed cell volume of mice infected with trypanosomes at different periods of the experiment

Infection period	**Group drug sensitive (KETRI 3913) (%)**	**Group drug resistant (KETRI 3805) (%)**	**Control (%)**
Pre infection	50.5±0.42	49.6±1.28	48±0.96
21 dpi	39.3±1.15	*32.2±0.74	*52.9±0.44
35 dpi	37.8±1.35	*24.5±1.57	*47.9±0.64
56 dpi	*26.5±1.57	-	*54.2±0.45

*$p<0.05$= significant

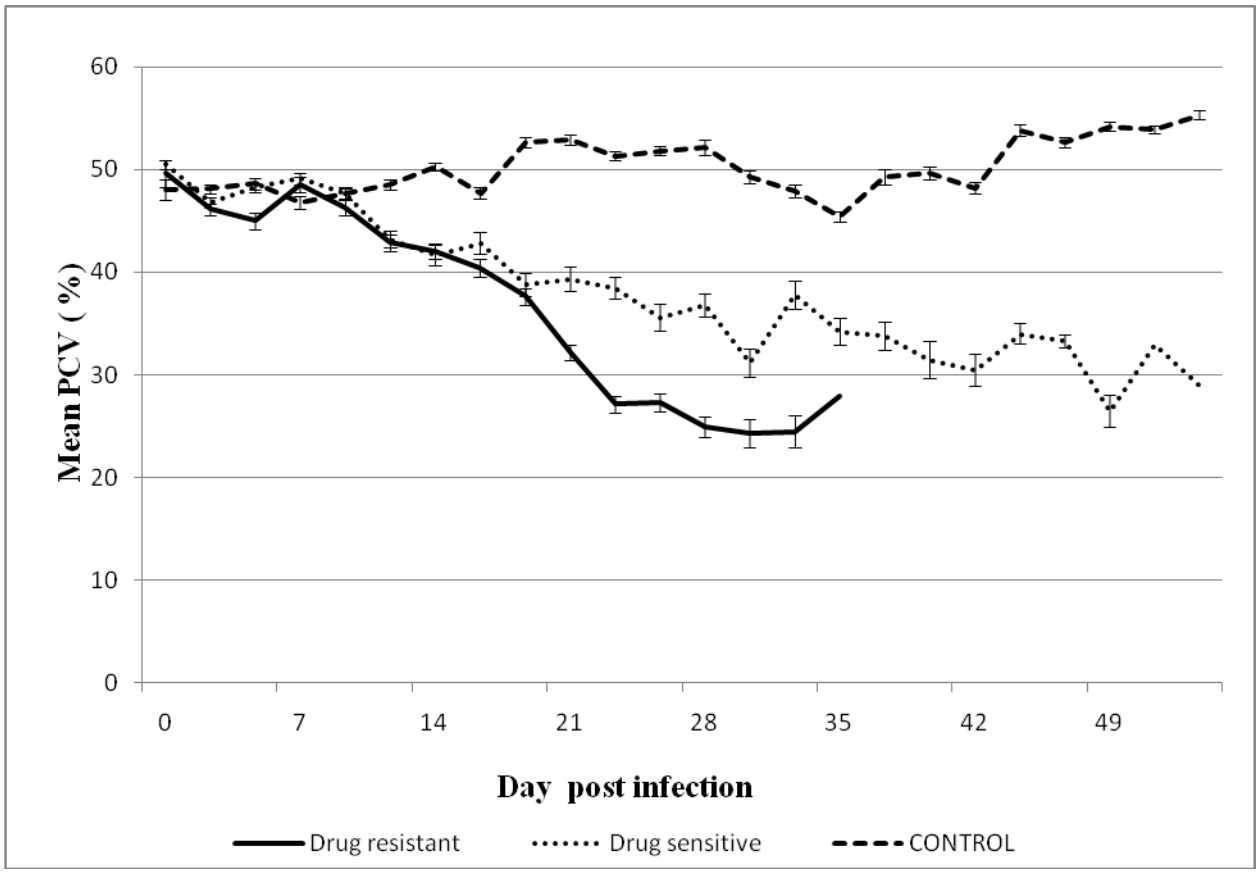


Figure 4: Changes in packed cell volume of Swiss white mice infected with either drug sensitive or drug resistant *T. congolense* isolates and the control group

4.3.3 Body weight

The changes in body weight between the infected and uninfected mice are presented in figure 5 with the summary of mean body weight trends as in table 7. The body weight of the uninfected mice remained within a narrow range of 23.4±0.52g and 24.8±0.51g during the pre-infection and up to 21 dpi, respectively. Thereafter the uninfected mice showed a gradual but consistent increase in body weight to reach mean weights of 26.7±0.54g at 60 dpi when the experiment was terminated. The mean body weight of the infected groups of mice increased in a similar manner during the initial period of infection. However, those infected with drug resistant trypanosome stabilate showed a drastic decline in body weight from 28 dpi till all the mice had

died by 35 dpi. On the other hand those mice infected with drug sensitive trypanosome stabilate, showed a similar drastic decline in the mean body weight but this occurred later on, from 35 dpi. Statistical comparison of the infected and control groups indicate that there were no significant differences between infected and control groups and also between the infected groups ($p>0.05$).

Table 7: Showing the mean ±SE body weight (g) of mice infected with drug sensitive and resistant *T. congolense* at different periods following infection

Infection period	**Drug sensitive (KETRI 3913)**	**Drug resistant (KETRI 3805)**	**Control**
Pre infection	24.4±1.06	25.4±0.26	23.5±0.88
7 dpi	25.7±0.57	24.6±0.31	23.4±0.52
14 dpi	23.71±0.73	25.7±0.28	24.3±0.49
28 dpi	26±0.86	26.83±0.56	25.6±0.55
35 dpi	27±0.88	25±1.45	24.6±0.55
56 dpi	22±1.18	-	26.7±0.54

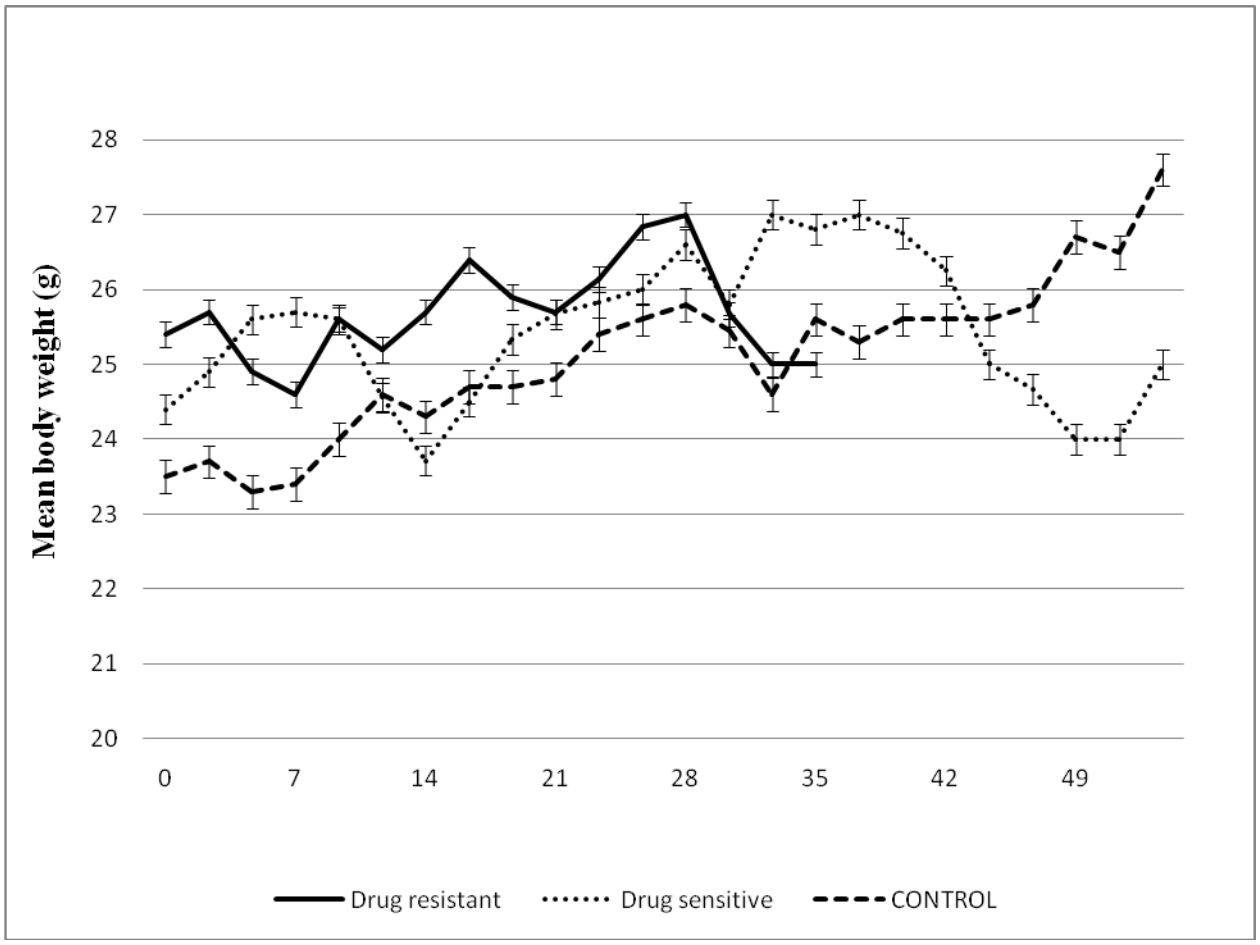


Figure 5: Body weight changes in mice infected with either drug sensitive or resistant strains of *T. congolense* and the control group

4.4 Transmission of *Trypanosoma congolense* Through Tsetse and Mice

4.4.1 Feeding of clean *Glossina pallidipes* on donor mice infected with either drug sensitive or drug resistant *T. congolense* and un-infected controls

The results for the fly and mice transmission experiments are summarized in table 8. One hundred tsetse flies (*Glossina pallidipes*) were fed on 5 immuno-suppressed mice infected with drug sensitive isolate and thereafter maintained in the insectary by *in vitro* feeding for 35 days for infection to mature. By day 35 only 59 out of the 100 flies had survived (59%). The 59 flies were then probed of which 39 were successfully probed (66.1%). Following microscopic examination only one fly out of 39 was found to be trypanosome positive (2.56%). All the 59

surviving flies were each individually fed on one clean mouse. Only 52 flies successfully fed with 7 flies failing to feed.

Thereafter all the 59 flies were dissected and samples from midgut and mouthparts were microscopically examined for the presence of trypanosomes. The results from dissection did not show any positive fly infections in both midgut and mouth parts. For each of the 59 flies, the mouthparts and midguts were pooled and examined for trypanosomes using PCR method of which 13 were detected positive for trypanosomes (22.03%).

Following the feeding of flies on mice, the animals were monitored for the development of parasitaemia for a period of 30 days post feeding. Results showed that none of the animals were microscopically positive during the experimental period of 30 days post feeding. Thereafter all the negative mice were bled from the heart and blood samples collected for examination using PCR method of which 25 were positive for trypanosomes (42.37%).

Similarly one hundred *Glossina pallidipes* tsetse flies were fed on 5 immunosuppressed mice infected with drug resistant isolate and thereafter maintained in the insectary by *in vitro* feeding for 35 days for the infection to mature. After the 35 days, only 48 out of the 100 flies had survived (48%). The 48 flies were then probed of which only 29 flies were successfully probed (60.41%). On microscopic examination of the probing slides only three out of 29 were found to have trypanosomes.

The 48 surviving flies were then individually fed on single clean mice. Only 40 of the flies successfully managed to feed while 8 failed to feed. Thereafter all the 48 flies were dissected and samples from midgut and mouthparts were microscopically examined for presence of trypanosomes. The results showed that two flies had trypanosomes in both midgut and mouthparts while one had trypanosomes in mouthparts only (6.25%).

For each of the 48 flies dissected, mouthparts and midguts were pooled and examined for trypanosomes using PCR. Five of the 48 flies (10.4%) were positive for trypanosomes using the PCR method.

A control group of 51 clean teneral flies fed on clean mice underwent the same procedure as described for the infected group. None of the clean fly samples were found positive for trypanosomes by both microscopy and PCR techniques.

Table 8: Survival of *G. pallidipes* and the infection rates due to drug sensitive and resistant *T. congolense* in the flies

T. congolense isolate	Flies fed on Infected Donor Mice	% flies survived at 35 days after feeding on donor mice and subsequently allowed to feed on experimental (clean) mice	Infected Flies		
			Microscopy+	PCR+	Total
Drug sensitive	100	59	1, probing 0, dissection	13	14
Drug resistant	100	48	3, probing 3, dissection	5	11
Negative control	100	51	0	0	0

Table 9: Infection rates in mice due to transmission of drug sensitive and resistant *T. congolense* by *Glossina pallidipes*

T. congolense isolate	No. of mice on which flies were allowed to feed	Infected mice		
		Microscopy	PCR	Total (%)
Drug sensitive	59	0	25	25 (42.4)
Drug resistant	48	1	18	19 (39.6)
Negative control	10	0	0	0 (0)

4.4.2Transmissibility of trypanosomes to mice using *Glossina pallidipes*

The results showed that a higher proportion of flies were able to pick and transmit the drug sensitive trypanosomes than the resistant (Table 9)

4.4.3 Determination of variability in parasites passaged through flies and mice using PCR-Restriction Fragment Length Polymorphism (PCR-RFLP)

Normal PCR conditions (see 3.1.1 above) were followed to amplify DNA from the two isolates namely; drug sensitive or drug resistant *T. congolense* isolate samples from donor mice infected intra-peritonealy using a syringe, tsetse flies fed on the infected mice, and finally from mice which were fed upon by the infected tsetse flies.

DNA was extracted from *T. congolense* during the transmission phases by *G. pallidipes* vector. The DNA was amplified by PCR using *T. congolense* savannah specific primers and amplicons digested by HinfI, HaeIII, HindII, BamHI and and MboII restriction endonucleases. The digest products were resolved in 2 % agarose gels stained with ethidium bromide. Figure 6 shows the results of PCR-RFLP.

Brighter bands were detected on lanes 5 and 6, indicating higher concentration of trypanosome DNA from donor mice. In general, there were minor changes in the gel phenotypes depending on the restriction enzyme used.

Using Mbo II as restriction enzyme the result showed that samples from donor mice infected intra-peritonealy using a syringe gave two major bands (200bp and ~370bp) with the ~370bp being the major one. The trypanosome DNA from the flies gave only one band (~370bp). There was no digestion of samples from mice infected through infected tsetse bites. This enzyme did not show any difference between the drug sensitive and drug resistant *T. congolense* but showed marked changes in the cutting sites in the gene in different hosts.

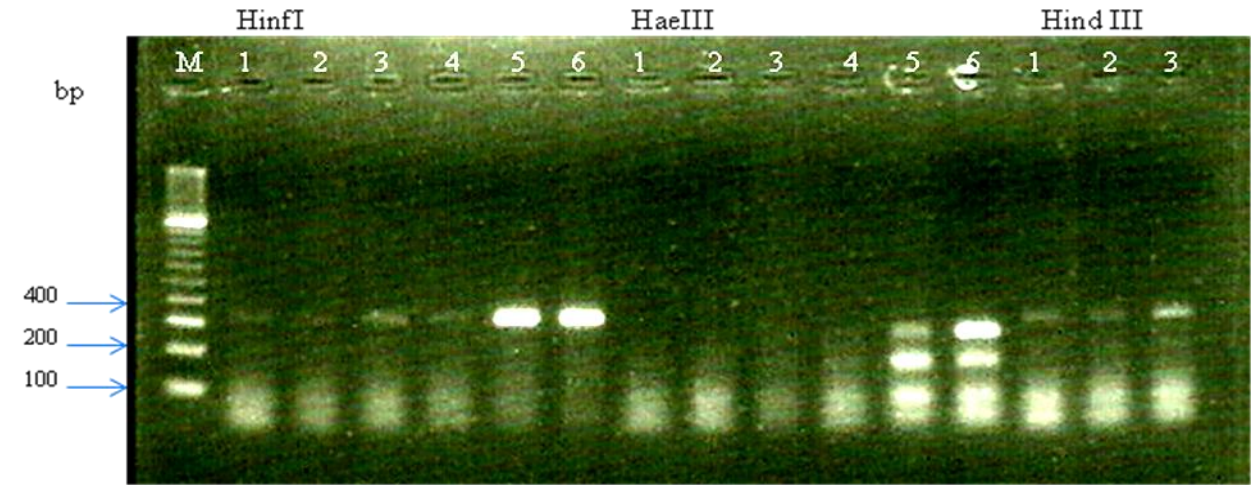
The results of Bam HI as restriction enzyme were almost synonymous with that of Mbo II but here samples from mice infected through infected tsetse bites showed a band (~370bp).

Digestion with Hind III revealed two bands in donor mice infected intra-peritonealy using a syringe (200bp and ~370bp). Samples from flies were similar to those ones from mice infected through infected tsetse bite (100bp and ~370bp). Samples from donor infected intra-peritonealy

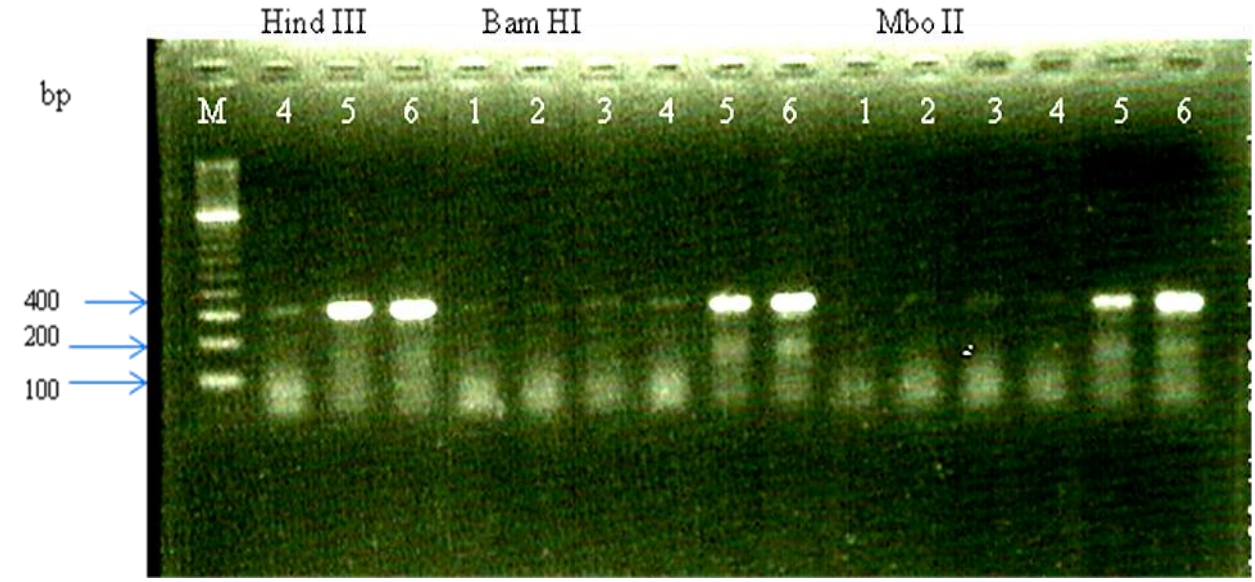
using a syringe were different from the ones from infected flies and mice infected through infected tsetse bite.

Using Hae III as restriction enzyme samples from donor mice infected intra-peritonealy using a syringe gave three major bands (100bp, 200bp and ~370bp) with the major bands being those of 200bp and ~370bp. The trypanosome DNA from flies yielded two bands (100bp and 200bp). The mice infected through infected tsetse bite gave only one band (100bp). This enzyme did not show any difference between the drug sensitive and drug resistant *T. congolense* but showed marked changes in the cutting sites in gene in the different hosts.

Using Hinf as restriction enzyme, the results of digestion with this enzyme were all synonymous in samples from donor mice infected intra-peritonealy using a syringe, flies and mice infected through infected tsetse bite (100bp and ~370bp). Furthermore, this enzyme did not show any difference between the drug sensitive and drug resistant *T. congolense*. Results are shown in figure 6 A and B



A



B

Figure 6 A and B: A 2% agarose gel electrophoresis following amplification of *Trypanosoma congolense* drug sensitive and drug resistant using different restriction enzymes (HinfI, Hae III, Hind III Bam HI and Mbo II). Numbers denote the following origins of *T. congolense* DNA samples; 1-2 = infected mice by fly bites; 3-4 = flies infected by feeding on donor mice and 5-6 = donor mice infected intra-peritonealy using a syringe. All even and odd numbered samples denote drug sensitive and resistant *T. congolense*, respectively.

CHAPTER FIVE

DISCUSSION

The results of this study demonstrated that the drug sensitive isolates (KETRI 3913) and drug resistant (KETRI 3805) held at KARI-TRC cryo-bank have retained their viability and characteristic of being drug sensitive and resistant, respectively to multiple drugs as previously described (Murilla *et al.,* 2002). These results show that lengthy cryopreservation doesn't affect drug sensitivity/resistance phenotype.

PCR amplification of DNA using specific primers (TCS1 and 2; TCK1 and 2 and TCF1 and 2) followed by analysis on 2% agarose gel electrophoresis showed DNA bands of only TCS for both sensitive and resistant parasites which means that those two isolates are pure *Trypanosoma congolense* of the subtypes savannah. In the field, it has been recorded that three subtypes of *Trypanosoma congolense* namely; *T. congolense* savannah, *T. congolense* Kilifi and *T. congolense* forest exist. Indeed various combinations of mixed infections can occur in the tsetse fly (Majiwa and Otieno, 1990) and in cattle hosts (Nyeko *et al*, (1990). Current results indicate that *T. congolense* savannah is the most frequent subtype. Similar findings have also been reported by other studies (Ouma *et al*., 2000; Mugittu *et al.,* 2001).

The results of the present study also showed rapid development of parasitaemia in mice infected with drug sensitive *T. congolense*, with a pre-patent period of between 7 and 21 dpi and a mean of 11days whereas the drug resistant isolate had a pre-patent period range of 9 to 17 dpi with a mean of 15 days. Whereas infection developed faster in mice infected with the drug sensitive isolate, 50% of the experimental animals developed a chronic infection and survived for periods ranging from 43 to over 60 dpi. In contrast, mice infected with resistant strain developed the infection later with mortalities recorded between 23 and 38 dpi. Thus all animals were dead by day 38 post-infection. Mean survival period for the mice was 35 and 30 dpi for drug sensitive and resistant isolates, respectively. In studies reported by Masumu *et al*. (2006a), 31 genetically different *T congolense* belonging to the Savanna sub-group and isolated from cattle in 11 sites in eastern Zambia were assessed and their virulence compared in mice. Strains were categorized as extremely virulent with pre-patent period of 2.3±0.3 and survival of 5-9 days; moderately virulent with pre-patent period of 3.6±1.6 and survival of 10-30 days; and low virulence with

pre-patent period of 3.5±1.6 and survival of more than 30 days. From the results of the present study, the two *T. congolense* isolates could be characterized as moderate and low virulence for resistant and sensitive, respectively. However, the pre-patent periods did not concur with those reported by Masumu *et al.*, (2006a) since in the current study the PP was longer. However, the results were closer to those reported by Opara and Fagbemi (2010) of 6-10 days. The differences could result from geographical distribution of the various isolates and also the fact that the parasites from previous studies were not screened for drug sensitivity/resistance in order to determine whether there is any association between virulence and sensitivity/resistance to drugs. These results have demonstrated marked differences in virulence in *T. congolense* isolates of the Savanna sub-group collected from more than one geographical region. In addition the virulence could be related to the host source of the isolates. Indeed in their study Murilla *et al*., (2002) used strains from Zambia isolated from cattle whereas those used in the present study were from isolated from the lion (KETRI 3913) and cow (KETRI 3805).

One of the effects of *T. congolense* is the destruction of red blood cell and thus anaemia (Ngure *et al,* 2008) due to nitric oxide inhibition of the bone marrow (Mabbot and Sterberge, 1994) and/or by oxidative destruction of RBC membranes by free radicles (Tabel *et al,* 2000). Studies by Masumu *et al*., (2006a) indicated that the rate of decline in PCV was faster in the extremely virulent strains. The decline in PCV was less steep in mice infected with moderate and low virulent strains. These findings concur with the observations from the current study which showed that the resistant isolate, which may be characterized as moderately virulent showed faster decline in PCV than the sensitive strain of low virulence. The significant fall in PCV values in mice after infection suggests that mice developed anaemia after they were challenged with *T. congolense*. This is agreement with previous studies (Anosa, 1977; Grootenhuis *et al*., 1990; Trail *et al*., 1992; and Opara and Fagbemi, 2010).

Body weights of mice infected with the two isolates in the present study were significantly different compared to the uninfected control ($p<0.05$). This is consistent with the pathogenic nature of the trypanosomes used to infect the mice with the parasites causing a wasting away of the diseased animal due to inability to feed due to loss of appetite and the production of the cytokine tumour necrosis factor alpha (TNF-α) (Beutler and Cerami, 1988).

Production of TNF-α results in a defect in lipid metabolism thus causing catabolism of fat in the body leading to cachexia (Beutler, 1988).

Following infection of *G. pallidipes* (tsetse flies) by feeding on infected mice, only one out of 59 (1.69%) flies in the drug sensitive group was detected positive by microscopy compared to 3 out of 48 (6.26%) in the resistant group. Using PCR detection, these values were 25 positive giving a transmission rate of 42.4% and 19 positive transmission rate of 39.6% for sensitive and resistant strains, respectively. The results showed that PCR technique was more sensitive and detected more positives than microscopy. The proportion of flies with infections due to sensitive *T. congolense* isolate was significantly lower than that due to resistant isolate, using the microscopy technique; however, these results were in reverse when PCR was applied as the detection method. Thus, only results from microscopy detection concurred with findings of Masumu *et al*. (2006b); probably if the investigators had applied the PCR technique, the results would have been different as demonstrated by the current study.

Trypanosomes undergo molecular changes (polymorphism) in various hosts and this can clearly be demonstrated using the PCR-RFLP technique. Results of this study established that there were no differences at molecular level between the drug sensitive and resistant, using PCR-RFLP technique. However, differences were detected in gel phenotypes within each isolate as it was passaged through the tsetse flies and mice, indicating that changes do occur during passage through various hosts. Although all samples analysed by PCR-RFLP yielded multiple bands following digestion with HaeIII, BamHI, and MboII, samples obtained from mice infected i/p showed greater number of bands. This may seem to suggest that there are genes most likely of VSG genes type which are expressed by the bloodstream parasites that are not expressed in the tsetse fly. However, it is unclear why parasites obtained from mice infected by tsetse flies did not yield all bands exhibited by samples from mice infected i/p. Perhaps certain genes are silenced by cyclic transmission of parasites through the tsetse fly. In this study, it was further observed that lanes 5-6 representing samples from mice infected i/p by syringe yielded brighter bands than other samples. This can be attributed to the route of infection such as i/p that resulted into higher parasitaemia. It seems syringe infection resulted in greater initial parasite inoculum than cyclic transmission of parasites by tsetse flies.

The outcome of the above experiments on transmissibility of *T. congolense* isolates shows clear differences, based on virulence, the sensitive isolate being less virulent than the resistant in mice. This observation confirms the theory for evolution and maintenance of virulence in parasite population and may explain the persistence of virulent trypanosome populations in a susceptible host population. More studies need to be done with a larger sample size to determine the adaptability of various strains as they are passaged through tsetse flies and mice.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

This study concludes that the drug resistant trypanosome population is more virulent than the drug sensitive in mice, and that drug sensitivity/resistance was found to influence transmissibility of *T. congolense* parasites. The more virulent trypanosome population also reduced survival period of the animals. Using the five restriction enzymes (HinfI, HaeIII, HindIII, BamHI and MboII) there were no differences at molecular level between the drug sensitive and resistant using PCR-RFLP. Differences were detected in gel phenotypes within each isolate as it was passaged through the tsetse flies and mice i.e. changes do occur during passage through various hosts.

6.2 Recommendations

(i) A large-scale study is required on the subtypes characterization of the drug sensitive and resistant isolates in order to assess impact on virulence of passaging parasites several times in both mice and flies. This will generate useful data and information to confirm the stability of virulence in *T. congolense*. Information will also be useful in developing strategies to mitigate resistance in the field and reduce losses due to trypanosomosis.

(ii) It is recommended that for better resolution, sequencing should be done in the future.

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