Factors influencing the spread and selection of drug resistance in Human African Trypanosomiasis

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Abstract

A growing problem with drug resistance in Human African Trypanosomiasis has necessitated the implementation of screening programmes to monitor for its spread. This thesis describes the study of several factors that can influence the selection and propagation of drug resistance in *T. brucei*.

Human African Trypanosomiasis (HAT) is caused by *T. brucei gambiense* and *T. brucei rhodesiense*. The few drugs used for the treatment of the disease are either toxic, cause severe side effects or suffer from parasite resistance. The *T. brucei* P2 transporter, which is encoded by the gene *TbAT1*, mediates uptake of melaminophenyl arsenicals and diamidines. Reduced P2 uptake is associated with drug resistance. A number of point mutations found in a laboratory derived melarsoprol resistant *T. brucei* stock (STIB 777R) allowed development of a PCR/RFLP based molecular method to identify resistance alleles. By 1999, 20-30% of patients treated in Omugo, NW Uganda were failing to respond to melarsoprol. PCR/RFLP analysis indicated that mutant alleles accounted for 58.5% of those in circulation. Melarsoprol was withdrawn in 2001 and by 2003 mutant *TbAT1* alleles accounted for only 14% of those in circulation in NW Uganda. The current study aimed to determine the incidence of the PCR/*Sfa NI TbAT1* mutant alleles in 2006, some five years after melarsoprol had been withdrawn as first-line treatment. Successful molecular analysis of 91 of 132 (68.9%) *T. b. gambiense* field isolates from Omugo and Moyo in NW Uganda indicated the presence of only *TbAT1* wild type alleles. Mutant alleles thus appear to have disappeared. This may be the result of parasite fitness cost following the withdrawal of melarsoprol as a stage II first-line drug from Omugo health centre, Arua, since 2001. This apparent instability of *TbAT1* mutants in the field may be exploited for rational or alternating use of melarsoprol and eflornithine (DFMO) to ensure a longer life for eflornithine, delaying the onset of resistance.

Insight into the overall population structure of the *T. b. gambiense* from Omugo, Arua (N=54) and Moyo (N=17) was obtained using mini/microsatellite marker analysis. Genetic diversity was observed to be more intra than inter regional. Multilocus genotype data analysis revealed the Omugo, Arua, population was genetically distinct from the Moyo population (Nei's genetic distance=0.176). The evidence indicated surprisingly little genetic exchange with an excess in homozygosity (Fis >0) and alleles in linkage disequilibrium (P<0.05) within the Omugo, trypanosome population. This excess in homozygosity may be due to population sub-structuring, trypanosome inbreeding, or

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migration of patients. The latter is likely occurring from the neighbouring *T. b. gambiense* endemic disease focus in Southern Sudan. The findings suggested that the *T. b. gambiense* from Arua is not panmictic, clonal or epidemic but there is some level of genetic exchange.

The possibility that *T. b. gambiense* can infect animals raises the prospect that wild or domestic animals may act as a reservoir and that a veterinary link to *gambiense* Human African Trypanosomiasis exists. Treatment of animals for babesiosis and trypanosomes with diminazene, uptake of which is mediated through TbAT1/P2 could select for P2-defective drug resistant trypanosomes, thereby threatening control of the human disease as well. Species detection by PCR for animal and human trypanosomes in dog isolates (N=190) from the tsetse fly endemic Jos Plataeu, Nigeria did not reveal *T. b. gambiense*, but multiple infections with *T. brucei* (95%), *T. vivax* (89%), and subspecies *T. congolense* forest (54%) and savannah (50%) were detected. The dogs were also infected with other parasites, including *Babesia canis* (22%) and *Hepatozoon canis* (16%). Multiple infections can make correct diagnosis difficult and the infections are likely to be missed by the less sensitive microscopy method.

The trypanocidal action of the diamidine group of trypanocides, diminazene, pentamidine and furamidine (DB75) are principally mediated through the TbAT1/P2. In addition, pentamidine is taken up by two additional *T. brucei* transporters called High Affinity Pentamidine Transporter (HAPT1) and the Low Affinity Pentamidine Transporter (LAPT1). DB75 also has a secondary unknown route. Loss of TbAT1/P2 leads to significant resistance to DB75 and diminazene but not pentamidine. Identification of other markers of resistance is necessary to determine if other routes of drug entry do exist apart from P2 and whether these can be exploited for the delivery of new trypanocides into the trypanosomes. Adaptation of the *T. brucei tbat1* knock-out cell line to higher concentrations of diminazene by *in vitro* selection for resistance led to loss of HAPT1. The resultant phenotype was similar to the previously characterised pentamidine resistant clone B48, but more resistant to diminazene and DB75. The adapted line was still capable of accumulating 1 μM radiolabelled diminazene suggesting both HAPT1 and LAPT1 as possible routes for diminazene uptake. Adaptation of the *T. brucei tbat1* knock-out cell line to a high concentration of DB75 over the same 6 months period did not lead to increased resistance.

Overall the project has confirmed an important role for *tbat1/P2* in development of resistance to melarsoprol in the field. Importantly, it appears that removal of the selection pressure of melarsoprol leads to a loss of *tbat1* alleles associated with resistance in a

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population of trypanosomes capable of genetic exchange in NW Uganda. Although evidence for a dog reservoir for *T. b. gambiense* in Nigeria was lacking in this study, a risk of selecting resistance in animals must remain high on any list of consideration. I have further shown that the diamidine drug, diminazene, used in veterinary medicine also appears to enter *T. brucei* via the HAPT1 transporter, as well as the P2 transporter. Loss of HAPT1 through selection with diminazene leads to high level pentamidine resistance, which could indicate a further risk in selection of human infectious trypanosomes also resistant to drugs like pentamidine.

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Dedication

This thesis is dedicated to my loving husband John Fred Kazibwe

and

Our children Jotham Semuyaba and Joanne Nabulime

To the true knowledge of God

Through it all, the Lord stood with me and strengthened me 2 Timothy 4:17

Declaration

This thesis and the results presented in it are entirely my own work except where explicitly stated in the text.

Anne Juliet Nalunkuma Kazibwe

Abbreviations

BLAST	Basic Local Alignment Search Tool
bp	Base Pair
BSA	Bovine Serum Albumin
CATT	Card Agglutination Test for Trypanosomiasis
CO_2	Carbon dioxide
dATP	Deoxyadenosine Triphosphate
dTTP	Deoxythymidine Triphosphate
dGTP	Deoxyguanosine Triphosphate
dCTP	Deoxycytidine Triphosphate
dNTP	Deoxynucleotide Triphosphate
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
DTT	1,4-dithio-DL-threitol
E. coli	*Escherichia coli*
EDTA	Ethylenediamine tetraacetic acid
ENT	Equilibrative nucleoside transporter
g	Gram
GP	Glandular puncture
HCl	Hydrochloric acid
HCT	Haematocrit centrifugation test
IC_{50}	50% Inhibitory Concentration
IPTG	Isopropyl-β-D-thiogalactopyranoside
Kb	Kilo base
KCl	Potassium Chloride
kD	Kilo Dalton
K_m	Michaelis Menten constant
Ki	Inhibition constant
KO	Knock Out
L	Litre
LP	Lumbar Puncture
LB	Luria-Bertani
M	Molar

Abbreviations

mg	Milligram
$MgCl_2$	Magnesium Chloride
ml	millilitre
N_2	Nitrogen
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
nM	Nanomolar
O_2	Oxygen
OD	Optical Density
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
RBC	Red Blood Cell
PCV	Packed cell volume
RNA	Ribonucleic Acid
rRNA	ribosomal Ribonucleic Acid
SDS	Sodium Dodecyl Sulfate
SOC	Super Optimal broth-Catabolite repression
TAE	Tris-acetate EDTA
TbAT1/P2	*Trypanosoma brucei* adenosine transporter 1
TBE	Tris-borate EDTA
TE	Tris-EDTA
T_m	Melting point temperature
TRIS	2-amino-2-hydroxymethyl-1, 3-propanediol
UV	Ultra Violet
Vmax	Maximum velocity
X-Gal	5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside
WHO	World Health Organisation
μCi	Micro Curie
μg	Microgram
μl	Microlitre
μM	Micromolar
α	Alpha
β	Beta
γ	Gamma

Chapter 1

1 Introduction

1.1 Human African Trypanosomiasis

Human African trypanosomiasis (HAT) or sleeping sickness is a deadly disease that occurs in 36 countries in sub-Saharan Africa. It is one more public health problem alongside other diseases including malaria, human immune deficiency virus, filariasis and tuberculosis with a major impact on social and economic development. It is endemic in several countries, which include Uganda, Angola, the Democratic Republic of Congo (DRC), Sudan, the Central African Republic (CAR), and the Republic of Congo. HAT is caused by a group of parasitic haemato-protozoa called trypanosomes, most of which are solely transmitted by the tsetse flies of the genus Glossina. There are three subspecies of *Trypanosoma brucei* (*T. b.*) namely *T. b. brucei*, *T. b. rhodesiense* and *T. b. gambiense*. Only two of these infect humans: *T. b. rhodesiense* and *T. b. gambiense*. *T. b. gambiense*, which causes the chronic form of the disease, is seen in West and Central Africa and *T. b. rhodesiense*, which causes the acute form of the disease, is found in East and Southern Africa (Figure 1.1). The West and Central African form of the disease is transmitted by the riverine species (*G. palpalis*, *G. fuscipes* and *G. tachinoides*) tsetse flies, which prefer a humid and dense riverine habitat while the East and South African form is transmitted by the savannah species (*G. morsitans, G. pallidipes*, and *G. swynnertoni*), which prefer dry, open woodland, and savannah habitat. An exception is the East African *T. b. rhodesiense* form, which is transmitted by the riverine species *G. fuscipes* (Smith *et al*, 1998;Waiswa *et al*, 2006). The disease has an early stage which later progresses into a late stage and is fatal if left untreated. The chronic form of the disease due to *T. b. gambiense* is responsible for more than 90 % of all HAT patient cases (Barrett, 2006).

An equivalent disease transmitted by tsetse flies in animals is referred to as nagana and is caused by *T. b. brucei*, *T. congolense* (sub-genus Nannomonas) and *T. vivax* (sub-genus Dutonella). While wild and domestic animals do act as reservoirs of infection for *T. b. rhodesiense*, the importance for humans of the *T. b. gambiense* infection in wild and domestic animals is still unclear although there is some evidence of animals acting as host reservoirs of this parasite (Gibson *et al*, 1978;Nkinin *et al*, 2002;Herder *et al*, 2002;Simo *et al*, 2008a).

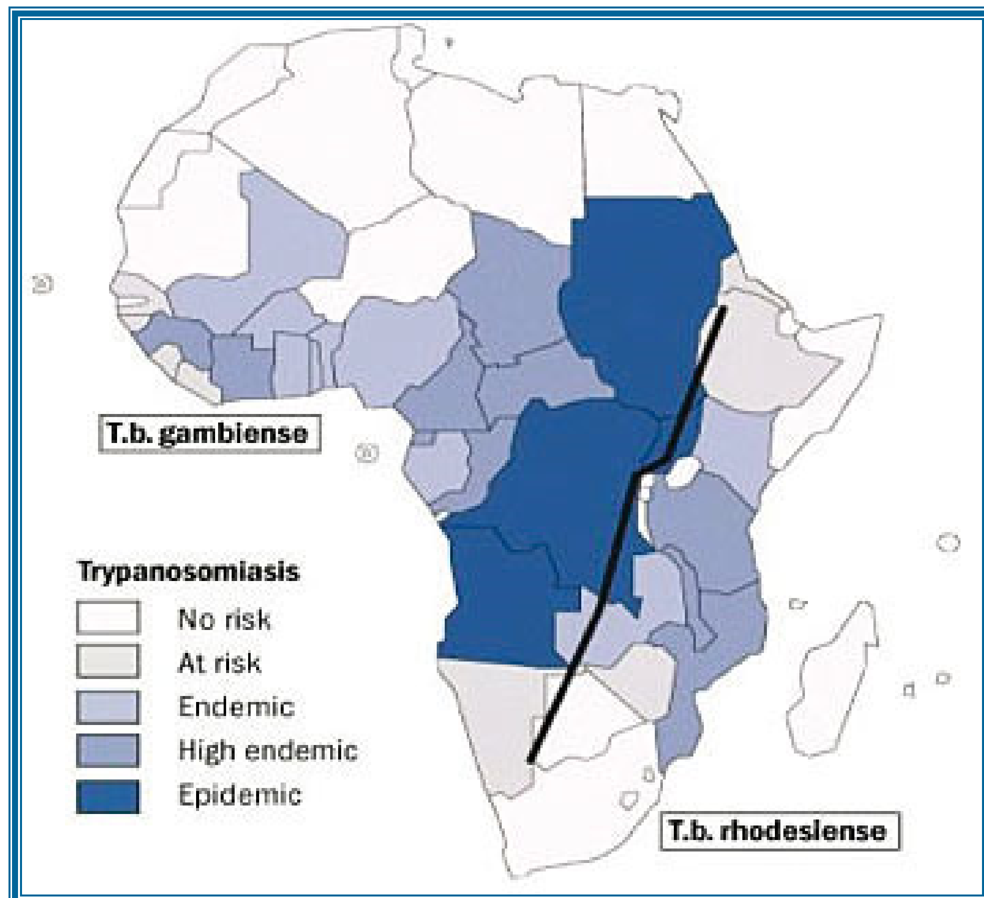


Figure 1.1 Map showing the distribution of *T. b. gambiense* and *T. b. rhodesiense* in Sub-Saharan Africa (World Health Organisation, 2001).
Melarsoprol treatment failures are confined to a belt stretching from Angola, through Congo, NW Uganda and S.Sudan (Matovu *et al*, 2001).

Chemotherapy has been widely used for many decades as one of the control strategies in addition to vector control. Treatment of the disease is still carried out using the few available old drugs, which can cause adverse side effects, suffer from parasite resistance and require a parenteral mode of administration hence making effectiveness of treatment and efforts to prevent the disease very difficult. Pentamidine and suramin are effective for treatment of early stage *T. b. gambiense* and *T. b. rhodesiense* respectively while melarsoprol is used for treatment of both *T. b. gambiense* and *T. b. rhodesiense* late stage disease. Melarsoprol is known to be toxic, causing a fatal reactive encephalopathy in 5-10 % of the treated patients (Pepin *et al*, 1995;Barrett, 2000). Eflornithine (DFMO), the most recently introduced drug, is only effective against *T. b. gambiense* and used for late stage West African sleeping sickness. Nifurtimox, although not yet registered, is another promising drug when used in combination with eflornithine (Priotto *et al*, 2006;Checchi, 2007). Melarsoprol with eflornithine and melarsoprol with nifurtimox are other

combinations for treatment of late stage disease although the latter combination is more promising following recent drug trials (Bisser *et al*, 2007).

Since drug treatment is the major control strategy used, there is an urgent need to develop rapid laboratory tools for the diagnosis and detection of any early signs of developing drug resistance in the field. This is one of the highest priorities in HAT research. Until recently, there has been no molecular marker for the detection of melarsoprol resistance in HAT. However, mutations in the *Trypanosoma brucei* adenosine transporter 1 gene that encodes the P2 activity (TbAT1/P2) that are linked to melarsoprol treatment failure (Mäser *et al*, 1999), have been identified in a limited number of relapsed patient isolates (Matovu *et al*, 2001b;Nerima *et al*, 2007). Decreased drug uptake due to loss of the TbAT1/P2 transporter, or mutations in *TbAT1*, is implicated in the development of arsenical and diamidine resistance (Carter & Fairlamb, 1993;Barrett *et al*, 1995;Ross & Barns, 1996;Mäser *et al*, 1999;de Koning & Jarvis, 1999;de Koning *et al*, 2000;Matovu *et al*, 2001b;Witola *et al*, 2004;Stewart *et al*, 2005;Delespaux *et al*, 2006).

1.1.1 Clinical manifestation

Both *T. b. gambiense* and *T. b. rhodesiense* are known to infect and cause clinical disease in humans. Both types of parasites cause early and late stage disease, which if not treated leads to death. Transmission to a human occurs from the bite of an infected tsetse fly (Cattand & de Raadt, 1991;Pentreath, 1995). *T. b. rhodesiense* causes the acute form of the disease that can lead to death with in a matter of weeks to 6-8 months (Odiit *et al*, 1997). The observed difference in the rate of progression of the disease has been attributed to geographic variation (MacLean *et al*, 2004). The disease due to *T. b. gambiense,* which causes the chronic form, manifests with few specific symptoms over a period of several months to years (≥ 2 years) (Taelman *et al*, 1987). During the early stage of the disease when the parasites multiply by binary fission in the bloodstream and the lymphatic system, the patient develops a primary chancre at the site of the tsetse fly bite (Moore *et al*, 2002). The parasites then invade all organs of the body including the heart. Swelling of the cervical nodes (Winterbottom’s sign), cyclical fever patterns and headaches are some of the symptoms of the early stage disease. The late stage or meningo-encephalitic stage occurs after the parasites cross the blood-brain barrier and get into the central nervous system. This invasion leads to inflammation of the brain tissues (and visceral organs) as well as the infiltration of the spinal nerve tissue triggering psychological changes in the

brain. The patient deteriorates rapidly as a result of alterations of the mental state, sensory disorders, coordination problems, changes in the sleep/wake cycle, organ and immunological malfunctions, loss of weight which eventually lead to irreversible coma and death (World Health Organisation, 2001;Barrett *et al*, 2003).

1.1.2 Historical

The parasite that causes sleeping sickness (the human form of the disease) or nagana (the animal form of the disease) is transmitted by the tsetse fly vector. Sleeping sickness was reported by the descriptions given by Arab merchants and slave traders. But little was known of the disease until the end of the 19th century (1896) and early 20th century (1906) when serious epidemics of nagana and sleeping sickness were reported in the Congo basin and Uganda. *T. b. gambiense* was discovered by Forde in 1902 and this was followed by the identification of its mode of transmission and its life cycle in the tsetse fly by Bruce in 1903 (Cattand *et al*, 2001;Cox, 2004). *T. b. rhodesiense* was first identified by Stephens and Fantham (1910) in Zambia (Fevre *et al*, 2006a). It was at that time that the two parasites were confirmed as the cause of sleeping sickness. The second epidemics, which occurred between 1920 and 1945, were in Angola, Cameroon, Sudan, Liberia, Sierrra Leone and Guinea leaving millions of people dead (Duggan, 1970). By the early 1960s, the disease was successfully brought under control to a level of almost being eliminated in most foci; an example being Uganda (World Health Organisation, 2001). However, the disease resurged in the 1970s resulting again into serious epidemics in the 1990s (Figure 1.2). This was blamed on the collapse of the health systems as result of national neglect, insufficient budgets, and unending armed conflicts in the most affected regions which made it impossible for screening and vector control activities to continue (Barrett, 1999). With the assistance of various organisations, the current situation is now different (section 1.3).

Recent reports have predicted an overlap between the geographical distributions of the two diseases in Uganda (Enyaru *et al*, 1999;Hutchinson *et al*, 2003) but there are no reports showing evidence that this has happened yet. Furthermore, the areas that were once endemic for *T. b. gambiense* are not affected by *T. b. rhodesiense* and *vice versa* indicating that the endemic foci of the two diseases have hardly changed (Fevre *et al*, 2005).

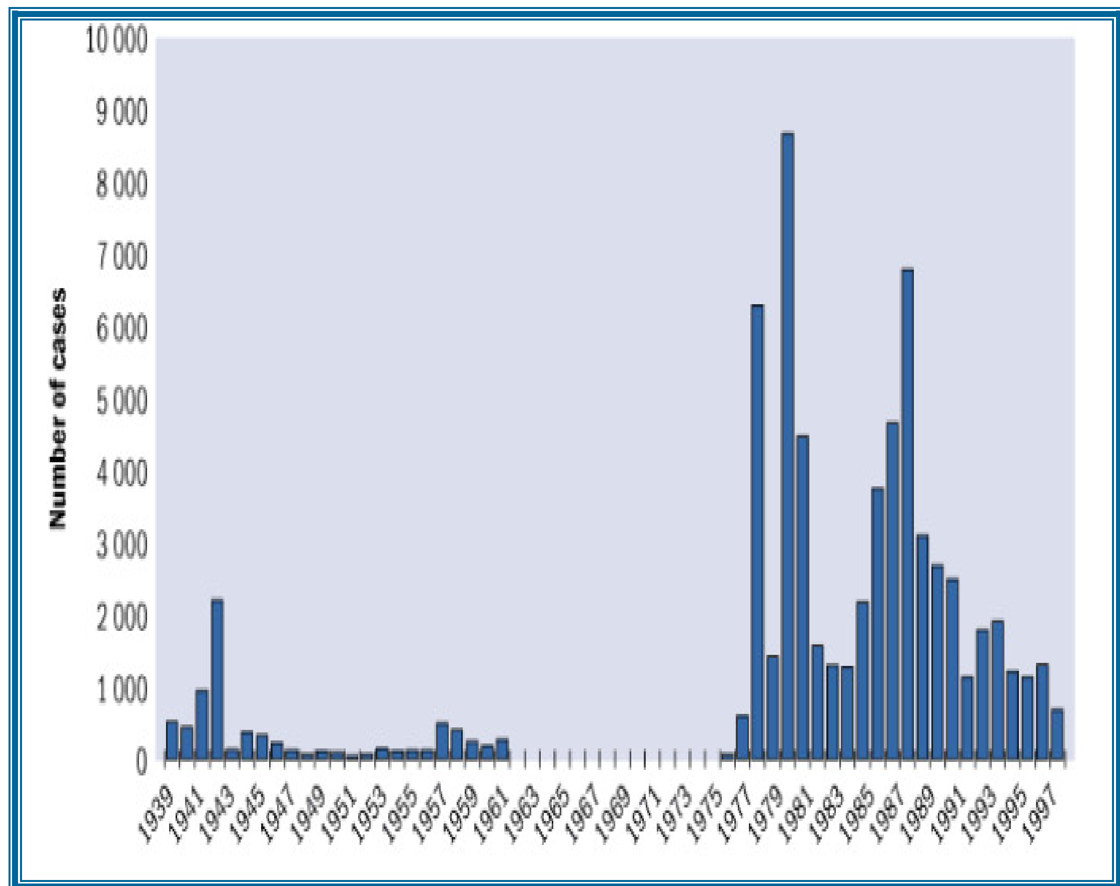


Figure 1.2 Annual trends in reported number of cases of African Trypanosomiasis in Uganda between 1939 and 1998.
(WHO Report on Global Surveillance of Epidemic-prone Infectious Diseases, 2001).

1.1.3 Scale of the problem

By the end of the twentieth century, the deadly disease was estimated to affect approximately 0.5 million people annually with 1.59 M disability adjusted life years (DALYs) leaving 60 million people at risk (Barrett, 1999;World Health Organisation, 2001), although the number of afflicted people is considered an under-estimate as a result of low detection rates (Fevre *et al*, 2006a). Current reports indicate a decline in disease incidence with an estimate of about 70,000 people infected annually (World Health Organisation, 2006;Barrett, 2006). Most of the reported cases occurred in regions of high endemicity (> 500 cases per year) namely; Angola, the Democratic Republic of Congo, southern Sudan, Uganda and Central African Republic. The globally declining trend is considered a positive step towards the control of the disease. This is thought possibly to be a result of the renewed efforts put in by various international organisations, academic institutions, pharmaceutical companies, and non-governmental organisations working

together with the national control programmes and health authorities of the affected countries. For some regions of low-level endemicity (<50 cases/year) like Chad, Ivory Coast, Gabon and Cameroon it was noted that when the control activities are relaxed, a resurgence of the disease is observed (World Health Organisation, 2001;Cattand *et al*, 2001). This is therefore an indication that increased surveillance has to continue in all foci to be able to successfully control the disease.

The disease caused by *T .congolense* and *T. vivax*, which is also devastating in livestock, leads to massive reduction in production amounting to US $1-1.2 billion seriously affecting livestock keepers socially as well as having a huge impact on the rural economy as a whole (Shaw, 2004).

In spite of all the continued efforts, which include screening, diagnosis, treatment and vector control, it is not yet possible to completely eliminate the disease both in humans and animals. This is thought to be due to several factors. The presence of an animal reservoir for example in West Africa has been thought to play an important role in the resurgence of the disease (Gibson *et al*, 1978;Zillman *et al*, 1984;Nkinin *et al*, 2002;Herder *et al*, 2002;Fevre *et al*, 2005;Fevre *et al*, 2006c;Simo *et al*, 2006;Njiokou *et al*, 2006). Also human /animal movements to and from an endemic region possibly increases the chance of introducing the disease to a disease free region as in the case of Uganda (Fevre *et al*, 2001;Fevre *et al*, 2006c;United Nations Report, 2004). Another factor of major importance to consider is the existing problem of emerging parasite strains that are resistant to the available drugs used for treating both humans and animals (Kalu, 1995;Mäser *et al*, 1999;Matovu *et al*, 2001c;Geerts *et al*, 2001;Anene *et al*, 2001).

1.2 Animal Trypanosomiasis as a reservoir

T. b. rhodesiense is a zoonotic parasite, which infects both humans and domestic animals. Cattle are mostly affected but also pigs, goats and sheep are known to be infected and these serve as reservoirs of this parasite (Gibson & Gashumba, 1983;Welburn *et al*, 2001a;Waiswa *et al*, 2003;Njiru *et al*, 2004;Fevre *et al*, 2005). Dogs are also reported to act as host reservoirs of this parasite (Gibson & Gashumba, 1983). Apart from causing acute disease ,*T. b. rhodesiense* can also cause mild disease in humans as reported for Malawi (MacLean *et al*, 2004). Therefore both humans and animals can act as host reservoirs of this parasite since they are implicated in the human disease transmission. *T. b.*

gambiense is known to cause the chronic form of the disease in humans who are major reservoir

Because of the endemic nature of the disease, it has been speculated that there could be an animal link to *gambiense* Human African Trypanosomiasis. The possibility that *T. b. gambiense* can infect animals suggests that wild or domestic animals may have a role to play. This has been hypothesized in an attempt to explain the continued endemic situation and recurrent epidemics in high and low-level endemic foci in most of sub-Saharan Africa in spite of animal-tsetse-human transmission generally being considered to be of low importance. The human infective *T. b. gambiense* parasite is transmitted to animals by the same tsetse fly vector, *Glossina palpalis*. With the previous and new advancements in molecular methods using molecular markers specific for each trypanosome species it is possible to differentiate between *T. brucei* species infecting humans, animals and tsetse flies (Moser *et al*, 1989;Masiga *et al*, 1992;MacLeod *et al*, 1999;Biteau *et al*, 2000;Welburn *et al*, 2001a;Gibson *et al*, 2002;Radwanska *et al*, 2002a;Radwanska *et al*, 2002b;Herder *et al*, 2002;Njiru *et al*, 2004). Studies done to identify the different trypanosome species that infect animals do implicate animals as a possible reservoir of *T. b. gambiense* (Gibson *et al*, 1978;Mehlitz *et al*, 1982;Zillman *et al*, 1984;Nkinin *et al*, 2002;Herder *et al*, 2002;Simo *et al*, 2006;Njiokou *et al*, 2006). These studies have revealed that domestic animals, mostly pigs, dogs and wild animal species (rodents, ungulates, primates, carnivores, and reptiles), harbour *T. b. gambiense*. It was also revealed that animals belonging to different species were also infected with other animal trypanosome species which include *T. b. brucei*, *T. congolense*, *T. vivax* and *T. simiae* (Herder *et al*, 2002;Simo *et al*, 2006). In a more recent study performed in Central Africa involving the use of Amplified Fragment Length Polymorphism (AFLP), which is a more sensitive molecular method, it was revealed that pigs were infected with a *T. b. gambiense* genotype similar to the one that affects man in the same and other different foci (Simo *et al*, 2008a). This finding further strengthens and supports the earlier hypothesis that domestic animals can act as host reservoirs of *T. b. gambiense* and therefore play a role in the maintenance and circulation of this parasite in the human population (Simo *et al*, 2008a).

1.3 Control Programmes and their role

The control and management of HAT has been made at least possible by the inputs of national programmes, national governments, non-governmental organisations, the World

Health Organisation (WHO) and international donors. This was initiated by the WHO which encouraged and supported endemic countries and those prone to epidemics (WHO report, 1998) to set up National Sleeping Sickness Control Programmes (NSSCP) to help in the surveillance and control of the disease (Cattand *et al*, 2001). Uganda, Kenya, Tanzania, Angola, the Democratic Republic of Congo, Ivory Coast, Chad, Cameroon, Central African Republic, Gabon, and Sudan are some of the countries, which have established control programmes. Other countries including Ghana, Liberia, Sierra Leone and Nigeria, although among the affected, still lack national surveillance programmes. However, Nigeria is currently seeking support from the WHO to ensure a surveillance programme is set up. The main aim of these programmes is to eliminate the disease. HAT is commonly referred to as a disease of the poor and the most marginalised communities in sub-Saharan Africa, which are ravaged with wars, insecurity, social instability, and weak health systems. The role of the control programmes is to ensure that the people at high risk, mostly those from rural areas not easily accessible and lacking adequate health services, are reached and treated. Well trained technical and health personnel, reagents, screening and diagnostic tools and drugs are some of the requirements. The control programmes also ensure reinforced monitoring, which involves mobile teams for active case detection, and established health centres or hospitals for passive case detection http://www.who.int/trypanosomiasis_african/resources/afro_tryps_strategy.pdf. Control of the disease is also ensured through networks that bring together affected countries annually for exchange of information hence leading to the setting of new strategies for the future. An example is the East African Network for Trypanosomiasis (EANETT), which was set up eight years ago http://www.eanett.org/whatis/ and includes Uganda, Kenya, Tanzania, Sudan, Ethiopia and Zambia. Malawi has just recently come on board.

Control programmes require sufficient financial and human resources to be able to carry out such activities successfully. In most of the affected countries where the disease re-emerged in the late 1970s this was next to impossible because of inadequate budgets and the lack of political will to recognise the disease as of public health importance (Barrett, 1999). Various international organisations stepped in to try and alleviate the problem; The United Nations Children's Fund, United Nations Development Programme, World Bank and World Health Organisation Tropical Diseases Research (UNICEF-UNDP-World Bank-WHO/TDR), research institutions (e.g. Bill & Mellinda Gates Foundation, Drugs for Neglected Diseases Initiative (DND*i*), Foundation for Innovative New Diagnostics (FIND), Swiss Tropical Institute-Basel (STI), University of North Carolina, Institute of

Tropical Medicine- Antwerp (ITMA), University of Glasgow, University of Dundee), pharmaceutical companies (Sanofi-Aventis, Bayer (World Health Organisation, 2006;Barrett, 2006). Non-governmental organisations like Médecins Sans Frontières (MSF), an international humanitarian aid organisation that provides emergency medical assistance to people at risk in affected countries, have also played an important role regarding control of the disease.

1.4 Drugs (Human and Veterinary)

Chemotherapy is one of the major strategies used for the control of the disease in both man and animals. The drugs used for the treatment of disease due to *T. b. gambiense* and *T. b. rhodesiense* differ because of the differences in their clinical manifestations. In the case of humans, treatment relies on a few drugs which have been in use for more than 50 years namely; pentamidine, suramin and melarsoprol (MelB). Eflornithine (DFMO), which is the most recently (1990) licensed drug, is used not only for the treatment of melarsoprol refractory late stage *T. b. gambiense* but it is also used increasingly as first-line therapy. Nifurtimox, which was developed in the 1960s for the treatment of Chagas' disease, is another drug used on a compassionate basis in combination with eflornithine although it is not yet licensed for treatment of sleeping sickness (Priotto *et al*, 2006;Priotto *et al*, 2007;Checchi, 2007). Its use still requires further investigations. Other combinations of melarsoprol and eflornithine or nifurtimox have also been used (Bisser *et al*, 2007). DB75 (2,5-bis(4-amidinophenyl)furan) or furamidine a diamidine whose prodrug DB289 (the O-methyl amidoxime) derivative (pafuramidine) is a promising oral drug currently undergoing phase III clinical trials for use against early stage sleeping sickness (Jannin & Cattand, 2004).

Diminazene aceturate (Berenil), Isometamidium chloride (Trypamidium), and Homidium salt (Ethidium), cymelarsan and suramin are the drugs on which the treatment of African animal trypanosomiasis relies.

1.4.1 Drug resistance

It is of concern that the few available drugs relied on for the treatment of both human and animal African trypanosomiasis have been threatened by the emergence of parasite strains that are resistant to the drugs. In the case of malaria, infection with chloroquine-resistant or

sulphadoxine-pyrimethamine-resistant *Plasmodium falciparum* (*P. falciparum*) is reported to confer immunity with age and exposure (Djimde *et al*, 2001;Omar *et al*, 2001). However, infection with drug resistant trypanosomes can occur after treatment for *T. b. gambiense* resulting in clinical disease although an *in vivo* study among adults in Zaire indicates evidence for immunity following infection with *T. b. gambiense* (Khonde *et al*, 1995).

Drug resistance is defined by (Schnitzer & Grunberg, 1957) as ‘the heritable, temporary or permanent loss of the initial sensitivity of the population of micro organisms against the active substance’. Drug resistance in *T. brucei* therefore may not necessarily be indefinite (Matovu *et al*, 2001c). Resistance may arise as a result of the parasites surviving in high drug concentrations, not tolerable to the host, or the parasites may be naturally resistant to the drug not as a result of previous drug exposure but due to other factors; for example, drug pharmacokinetics, host, vector or parasite-related (Matovu *et al*, 2001c). An example is *T. b. rhodesiense,* which is naturally refractory to eflornithine and therefore cannot be treated with this drug. This was first observed by Iten and others (1995) using *T. b. gambiense* isolates from West Africa, which after 10 days of exposure were found to be susceptible *in vitro* at 16.3 ± 7.8 µg/ml (the level of eflornithine concentration in CSF during treatment), while *T. b. rhodesiense* isolates from S.E Uganda were found to be inhibited only at the higher eflornithine concentrations of 25-100 µg/ml. *T. b. gambiense* isolates from NW Uganda displayed minimum inhibitory concentrations (MIC) ranging between 1.13-6.3 µg/ml while the *T. b. rhodesiense* isolates from SE Uganda had higher MIC values between 25-50 µg/ml, further confirming their natural refractoriness to eflornithine (Matovu *et al*, 2001a).

For reasons other than drug resistant parasite strains, treatment failure can also occur due to a complete lack of clinical response. These may include non-compliance with the duration of the dosing regimen, drug interactions, poor drug metabolism, misdiagnosis and re-infection (Burri *et al*, 1993;Geerts *et al*, 2001). In such cases exposure of parasites to sub-optimal drug levels may enhance the development of true drug resistance. Following the high incidence of melarsoprol treatment failures in NW Uganda (Legros *et al*, 1999) that are associated with melarsoprol resistance (Matovu *et al*, 2001b), *in vitro* and pharmacokinetic analyses were performed on *T. b. gambiense* isolates collected from the successfully treated and failed patients (Brun *et al*, 2001;Burri & Keiser, 2001). Results from the analyses ruled out drug resistance and instead suggested other underlying factors

such as host or parasite-related responsible for treatment failure since no differences were observed between the melarsoprol sensitivities, plasma and CSF drug concentrations of the two patient groups (Brun *et al*, 2001;Burri & Keiser, 2001).

Melarsoprol

Melarsoprol is an organic arsenical that was introduced by Friedham in 1949 is nonspecies-specific, cheap, and still widely used as first-line therapy for late stage human sleeping sickness. It is the drug of choice for both *T. b .rhodesiense* and *T. b. gambiense* late stage disease (Van Nieuwenhove, 1992). The drug is capable of crossing the blood brain barrier during a central nervous system infection. However; it is toxic and induces severe side effects. It is reported that 10% of patients treated with melarsoprol suffer an acute encephalopathy, which may result in paralysis, permanent brain damage or death in 5% of the treated cases (Pepin *et al*, 1995;World Health Organisation, 1998;Barrett, 2000). The predominant active metabolite of melarsoprol in the body is melarsen oxide (MelOx) (Keiser & Burri, 2000). The mechanism of action is still not clear although it is reported that it inhibits enzymes of the glycolytic pathway (Flynn & Bowman, 1974;Schaftingen *et al*, 1987) 6-phosphogluconate dehydrogenase (Hanau *et al*, 1996), and also forms a stable complex with trypanothione (MelT), which inhibits trypanothione reductase (Fairlamb, 1989;Cunningham *et al*, 1994). Laboratory induction of cymelarsen (an equivalent of melarsoprol) resistance in *T. brucei* was achieved by repeated selection *in vivo* in mice, suggesting another mechanism that was transport mediated. Cross resistance was also observed with the diamidine group compounds, which implicated involvement of the same adenosine transporter system (Fairlamb *et al*, 1992;Carter & Fairlamb, 1993).

In endemic areas of Uganda, Southern Sudan, Angola and DR Congo, the effectiveness of treatment and efforts to prevent sleeping sickness have been threatened by the increasing incidence of melarsoprol treatment failures (between 15-30%) (Legros *et al*, 1999;Moore & Richer, 2001;Stanghellini & Josenando, 2001;Burri & Keiser, 2001;Brun *et al*, 2001) associated with the problem of developing drug resistance (Matovu *et al*, 2001b). The high number of melarsoprol refractory cases led to a change in treatment policy, which resulted in the complete withdrawal of the arsenical and its replacement with eflornithine as first-line therapy for late stage *T. b. gambiense* in Southern Sudan, North Western Uganda (Omugo) in 2001, and recently in Angola (Chappuis *et al*, 2005;Priotto *et al*, 2006).

Pentamidine

Pentamidine, an aromatic diamidine, was introduced in the 1940s and has remained one of the principal drugs used for treatment of early stage *T. b. gambiense* sleeping sickness (Pepin & Milord, 1994). Nephrotoxicity and diabetes mellitus are among the adverse effects caused by the use of pentamidine. Diamidines are thought to act directly against the parasites, independently of their physiological action against the host. At one time it was considered that hypo-glycaemia might influence activity but their mode of action is not clear. They are reported to interact with cellular anions by binding tightly to the highly intercalated network of circular DNA molecules that comprise the kinetoplast and nucleus hence inhibiting nucleic acid replication (Shapiro, 1993). A laboratory derived *T. brucei* pentamidine resistant line generated *in vivo* and *in vitro* with an intact adenosine transport system was found to be less virulent with no cross resistance to other diamidine group compounds *in vivo* but was 2-fold resistant to melarsoprol. This suggested another mechanism of transport other than P2 or possibly a non-transport related mechanism (Berger *et al*, 1995). Very few cases of pentamidine refractory *T. b. gambiense* have been reported indicating that pentamidine resistance in the field is a rare occurrence (Pepin & Khonde, 1996). The few isolated cases are thought to be due to improper diagnosis of what would supposedly have been late stage cases (Nok, 2003). A *T. brucei* line selected *in vitro* for high level pentamidine resistance but lacking the adenosine transport system was less virulent and 15-fold cross resistant to melarsoprol thereby implicating loss of another transporter system (HAPT1) as another mechanism in addition to loss of TbAT1/P2 for development of high level pentamidine and melarsoprol resistance (Bridges *et al*, 2007).

Suramin

Suramin is a polysulphonated, symmetrical naphthalene derivative that was introduced in 1922. Since it cannot cross the blood brain barrier because of its large size and strong negative charge, it is only effective in the treatment of early stage *T. b. rhodesiense* and *T. b. gambiense* human disease (Hawking, 1940). However, it is considered the drug of choice for the treatment of early stage *T. b. rhodesiense* sleeping sickness in East Africa (World Health Organisation, 1995) because it is more effective than pentamidine (Fevre *et al*, 2006a). While pentamidine is the drug of choice for *T. b. gambiense* (Pepin & Milord, 1994) since high treatment failure rates (30%) were reported in the 1950s, following therapy with suramin http://www.who.int/tdr/publications/publications/pdf/aftryp_swg.pdf. Adrenal failure is one of the adverse effects following prolonged treatment with suramin (Feuillan *et al*, 1987). Its mode of action is not clear although earlier studies showed that

suramin might interfere with trypanosome metabolism by inhibiting several enzymes (Misset & Opperdoes, 1987;Wierenga *et al*, 1987). Later studies have suggested its mode of action is linked to the binding of low density lipoproteins (Vansterkenburg *et al*, 1993). Reports on suramin resistance in HAT are very rare. Like pentamidine, treatment failures are attributed to misdiagnosis of late stage cases (Burri *et al*, 2004). However, expression of resistance to suramin in bloodstream form *T. brucei* incubated 24 hours in axenic *in vitro* cultures has been demonstrated in a laboratory derived suramin induced line (Scott *et al*, 1996). Resistance to suramin in animal trypanosomiasis has also been reported for *T. evansi* from China (Zhou *et al*, 2004). But the drug has been shown to successfully treat the first known *T. evansi* infection that occurred in a human in India (Joshi *et al*, 2006).

Eflornithine or α-Diflouromethyl ornithine (DFMO)

Eflornithine (DFMO) is an ornithine analogue that was developed as an anti-cancer agent (Wickware, 2002). It was introduced over a decade ago (Nightingale, 1991). Eflornithine was confirmed effective against human trypanosomes in animal models (mice) and humans as well as having an anti-neuroinflammatory role in animal models and humans (Burri & Brun, 2002). It is only effective in the treatment of both early and late stage *T. b. gambiense* human sleeping sickness (Burri & Brun, 2003). Eflornithine has the ability to cross the blood-brain barrier and is currently licensed for the treatment of melarsoprol refractory cases but it is also becoming widely used as a first line treatment. However, its mode of administration, which requires a hospital setting, and high cost limit its use although in the last few years it has been provided free to the World Health Organisation by Sanofi-Aventis (Barrett, 2006). Eflornithine is the only drug whose mode of action is well understood. It irreversibly inhibits *ornithine decarboxylase* (ODC) an essential enzyme in polyamine biosynthesis (i.e. formation of putrescine from ornithine), critical to cell division and which offers protection against oxidant stress in the parasites (Bacchi *et al*, 1980). Trypanocidal activity was shown to be abolished when eflornithine was administered together with putrescine confirming its inhibition of ODC (Bacchi *et al*, 1980).

Unlike *T. b. gambiense*, *T. b. rhodesiense* cannot be treated using eflornithine. In an *in vitro* study done using *T. b. rhodesiense* field isolates, it was demonstrated that these parasites are naturally refractory to the drug (Iten *et al*, 1995). In a follow-on study, the *T. b. rhodesiense* parasites were observed to have a higher ODC turnover with the enzyme having a shorter-half life ($t_{1/2}$~4 hours) compared to the *T. b. gambiense* parasite ODC

turnover ($t_{1/2}$~19 hours). The fast rate at which the *T. b. rhodesiense* enzyme is replaced explains their natural refractoriness (Iten *et al*, 1997). There were no reports of eflornithine resistance or cross-resistance with other drugs (World Health Organisation, 2001), but some instances of eflornithine failure have appeared which suggest eflornithine resistance is a possibility (Dr. Simmaro, WHO, personal communication, Dr. Truc, OCEAC, personal communication, Dr. Matovu, MUK, personal communication). *In vitro* selection for resistance to eflornithine by passage in increased drug concentration has been demonstrated in *T. brucei* procyclics which were found to have a 7-fold decrease in drug uptake and increased levels of ornithine (Phillips & Wang, 1987;Bellofatto *et al*, 1987). In Leishmania, which also belong to the kinetoplastid group, this increased ODC activity was also suggested as another mechanism as observed in a *Leishmania tarentolae* line selected for resistance to arsenite as (Légaré *et al*, 1997). However, *in vitro* selection for resistance to eflornithine by passage in increased drug concentration has also been demonstrated in *T. brucei* blood stream forms (Barrett *et al*, unpublished).

Nifurtimox

Nifurtimox is mainly used for the treatment of Chagas' disease which is caused by *T. cruzi* in South and Central America (Rodrigues & de Castro, 2002). Its mechanism if action is linked to the generation of free radicals following the bioreduction of the nitro group, which is considered identical in both T. cruzi and *T. brucei* (Delespaux & De Koning, 2007). It has been successfully used in the treatment of melarsoprol-refractory late stage *T. b. gambiense* patients but it lacks the TbAT1/P2 transporter recognition motif suggesting its mode of uptake is not P2 related and neither is it transported by HAPT1 (Delespaux & De Koning, 2007). Although unlicensed, it is another potential drug with the hope of being used in combination with other drugs for the treatment of human African trypanosomiasis. This is part of the effort to improve on the efficacy and protection against resistance to the drugs used for late stage therapy. There are currently no new drugs in development with the exception of the novel diamidine DB75 whose orally active prodrug DB289 is still undergoing phase III clinical trials. However, its inability to cross the blood-brain barrier implies that, if successfully developed, it will be licensed for the treatment of early stage disease (Jannin & Cattand, 2004). Clinical trials which involved using nifurtimox in combination with eflornithine have indicated promising results (Priotto *et al*, 2006;Priotto *et al*, 2007;Checchi, 2007).

Diminazene aceturate (Berenil)

Diminazene, an aromatic diamidine, is widely used for treatment of the three common animal trypanosome species (*T. b. brucei, T. vivax and T. congolense*) but the development of resistance to this drug is now a serious problem in different regions of sub Saharan Africa for example Burkina Faso, Nigeria, and Ethiopia, (Clausen *et al*, 1992;Kalu, 1995;Mulugeta *et al*, 1997;Afewerk *et al*, 2000;Anene *et al*, 2001;Geerts *et al*, 2001;Anene *et al*, 2006). This has complicated the control of the disease. Berenil has been used in experimental studies to successfully treat humans although it is not licensed (Delespaux & De Koning, 2007). The drug is taken up through the same P2 transporter system like for the arsenicals and other diamidine compounds whose loss leads to resistance. However, it is speculated that treatment of animals acting as reservoirs of the human infective *T. b. gambiense* with this less effective trypanocide could lead to selection of drug resistant human *T. b. gambiense*. This is likely to result in cross resistance to human P2 trypanocides as reported for a *T. brucei* berenil resistant isolate from a dog that was cross resistant to pentamidine (Anene *et al*, 2006). The drug is also used for treatment of other animal parasites e.g. babesiosis, which might make this route a possible way of selecting P2 drug resistant parasites if the infected animals are treated for one disease instead of another. The idea of considering licensing of diminazene for human use is of great concern because of the loss of effectiveness. A variety of generic brands of diminazene aceturate are available which contain lower levels of the stated active component diminazene (Atsriku *et al*, 2002). This may also contribute to the drug being less effective although this was found not be responsible for resistance in a *T. brucei* berenil resistant isolate from a dog in Nigeria (Anene *et al*, 2006).

Isometamidium Chloride (Trypamidium or Samorin)

Introduced in 1961 (Berg *et al*, 1961), isometamidium chloride is a phenanthridine-aromatic amidine (Leach & Roberts, 1981), a complex of part of the diminazene molecule and homidium. Isometamidium was first used in 1963 (Delespaux & De Koning, 2007). It is used widely both as a prophylactic and therapeutic treatment for all the three principal African animal trypanosomes, especially in cattle and small ruminants. However, it has also been threatened by resistance in Somalia, Nigeria, Kenya, Ethiopia and Burkina Faso (Schönefeld *et al*, 1987;Kaggwa *et al*, 1988;Clausen *et al*, 1992;Gray *et al*, 1993;Codjia *et al*, 1993;Kalu, 1995;Mulugeta *et al*, 1997;Afewerk *et al*, 2000).

Homidium bromide/ homidium chloride(Ethidium)

Homidium, a phenanthridine, is another drug that has both chemophylactic and therapeutic effects like isometamidium chloride. Ethidium was first used in *in vitro* experiments as a dye (tryparsamide) which lost its trypanocidal activity when incubated with susceptible *T. b. rhodesiense* trypanosomes but this activity was not lost on incubation with resistant trypanosomes, which suggested that there was less affinity for the drug target or changes in the transport system (Yorke *et al*, 1931;Hawking, 1936). The drug is mostly used for *T. vivax* and *T. congolense* animal infections. Resistance is associated with diminazene aceturate and isometamidium and has been reported in animal isolates from West and East Africa tested either *in vivo* or *in vitro* (Codjia *et al*, 1993;Mulugeta *et al*, 1997;Olila *et al*, 2002).

1.4.2 Possible mechanisms of drug resistance in T. brucei

The mechanism by which parasites can acquire resistance to drugs depends to a great extent on the drug's mode of action. Paul Ehrlich, in 1907, was the first person to undertake experiments to understand the mechanism of drug resistance in *T. brucei* species (Maser *et al*, 2003). He formulated the 'chemoreceptor' hypothesis, which states that drugs act via specific receptors and that drug resistance is caused by a reduction of affinity of the respective target or reduction of net drug uptake due to loss of a transporter. This was demonstrated using tryparsamide (the first clinically effective arsenical for HAT) containing medium which lost its trypanocidal activity when incubated with susceptible *T. b. rhodesiense* trypanosomes but this activity was not lost on incubation with resistant trypanosomes, which suggested that there was less affinity for the drug target or changes in the transport system (Yorke *et al*, 1931;Hawking, 1936). Later on lower accumulation levels of diamidines 4',6-diamidino-2-phenyl-indole (DAPI) and Hoechst 33342, the phenanthridine ethidium bromide, and the acridine acriflavine were demonstrated in both the blood stream melarsoprol resistant *T. b. brucei* and *T. b. rhodesiense* clones that were cross resistant to pentamidine and berenil *in vivo* when compared to the sensitive *T. brucei* clones (Frommel & Balber, 1987). However, accumulation of DAPI was shown to be the same in both sensitive and the resistant clones following treatment with the detergent Triton-X which suggested alterations in the surface membrane were responsible for low drug accumulation observed in the drug resistant parasites (Frommel & Balber, 1987). The mechanism, which involves the *T. brucei* adenosine transporter 1 responsible for P2 activity, the high affinity pentamidine transporter (HAPT1) and the low affinity

pentamidine transporter (LAPT1), will be discussed in detail later in section 1.6. However, resistance in *T. brucei* procyclics selected for resistance to eflornithine *in vitro* has been associated with alterations in a biochemical target other than a membrane target. This was because of the accumulation of more of the polyamine ornithine than the drug as observed in the resistant parasites in comparison to the drug sensitive parasites (Phillips & Wang, 1987).

Increased drug export by the multidrug–resistance associated protein that belongs to the ATP-binding cassette (ABC) subfamily C transporters is another mechanism that may be linked to the development of drug resistance in *T. brucei* (Shahi *et al*, 2002;Luscher *et al*, 2006). These proteins are able to pump out of the cytosol products formed between drugs and complexes of trypanothione, which is a glutathione-spermidine conjugate, and melarsen oxide (Maser *et al*, 2003). In *T. brucei*, Shahi and others (2002) characterised two of these proteins and were able to demonstrate that over expression of the protein TbMRPA resulted in reduced susceptibility to melarsoprol/ melarsen oxide by 10-fold *in vitro*. Over expression of two of the enzymes involved in synthesis of glutathione and hence trypanothione (ornithine decarboxylase and gamma-glutamyl-cysteine) resulted in a 2-4 fold reduction in susceptibility to melarsoprol (Shahi *et al*, 2002). To assess the effect of reduction of the production of TbMRPA could have on melarsoprol susceptibility, RNA interference was used to reduce its over expression in patient isolates, which were then tested *in vivo* in mice (Alibu *et al*, 2006). The isolates were found to be hypersensitive to melarsoprol and attempting to increase the levels of trypanothione so that the trypanosomes could over express TbMRPA turned out to be ineffective *in vivo* which suggested that lack of a TbMRPA expression mechanism *in vivo* alone may not lead to melarsoprol resistance.

Alterations in TbAT1 or loss of P2 activity and over-expression of TbMRPA were pinpointed as possible mechanisms leading to melarsoprol resistance. Based on those observations a study was undertaken to assess the melarsoprol sensitivities in *T. brucei in vitro* using *T. b. brucei* bloodstream form trypanosomes lacking TbAT1/P2 activity and at the same time over expressing TbMRPA. Such lines were found to be 6-fold less susceptible to melarsoprol when compared to the *T. brucei* line lacking only P2 activity (Luscher *et al*, 2006). This finding suggests that more than one mechanism can potentially play a role in the development of resistance to melarsoprol, which is in agreement with the loss of both TbAT1/P2 and HAPT1 (Bridges *et al*, 2007) and the hypersensitivity observed

in the in trypanosomes shown to lack over expression of TbMRPA *in vivo* (Alibu *et al*, 2006).

Hypotheses are still being considered to further understand the mechanisms underlying the development of drug resistance in *T. brucei*. One hypothesis is of a veterinary link to human disease (see section 1.2) and the continuous treatment of animals with the trypanocide diminazene aceturate leading to resistance problems (Geerts *et al*, 2001), which may contribute to the selection of resistant trypanosomes that can infect man (Barrett, 2001). Diminazene resistance in the field is a problem in some regions of sub Saharan Africa for example Burkina Faso Nigeria, and Ethiopia, (Clausen *et al*, 1992;Kalu, 1995;Anene *et al*, 2001;Afewerk *et al*, 2000). Diamidine resistance in *T. brucei* is associated with loss of P2 function in *T. brucei* (section 1.4.2). Uptake of diminazene has been shown to principally depend on the P2 transporters (Matovu *et al*, 2003;de Koning *et al*, 2004).

A diminazene (berenil) resistant *T. b. brucei* isolate from a dog in Nigeria used to infect albino rats which were treated with berenil (7 mg/kg) and pentamidine (4 mg/kg) and was confirmed to be resistant to berenil (relapse rates 83-100%) and cross resistant to pentamidine (relapse rate 50%) (Anene *et al*, 2006). This possibly indicated that other transporters such as HAPT1, LAPT1 were also missing from these trypanosomes. The problem of animals acting as reservoirs of human-infective trypanosomes has led to speculation that this might enhance the selection of P2 resistant trypanosomes in the field hence worsening the drug resistance situation for the few drugs which are in use (Geerts *et al*, 2001). It is also speculated that through this mechanism faster development of resistance may occur for the promising new diamidine compound DB75 (pro drug DB289) whose uptake also occurs through P2 transport although an unknown secondary route is also involved in the uptake of this drug (Lanteri *et al*, 2006) and loss of P2 alone gives only low levels of resistance *in vivo*.

1.5 Human African Trypanosomiasis in Uganda

Uganda is the only country in East Africa affected by both the *T. b. gambiense* and *T. b. rhodesiense* forms of disease, although both species have also been seen in Angola, which is located in Southern Africa. North-west Uganda suffers epidemics due to *T. b. gambiense* while south-east Uganda suffers epidemics due to *T. b. rhodesiense.* The two regions are

separated by a vast terrain, mostly plateau with a rim of mountains, where no documented evidence of sleeping sickness has emerged. The number of reported cases has been shown to be rising since the early 1980s (Figure 1.2). Human trypanosomiasis seems to be restricted to particular foci and occurs during epidemics but the animal form of the disease is endemic and spreads over wide areas.

1.5.1 T. b. rhodesiense in S.E. Uganda

T. b. rhodesiense which causes acute disease and serious epidemics in SE. Uganda constitutes a major public health problem, which affects both man and livestock hence having a huge impact on rural development and the general health of the rural poor. The disease was first reported from the Busoga region in 1898 (Picozzi *et al*, 2005). The disease, which is endemic in 12 districts, is fatal if not treated. It is a zoonotic disease with evidence showing that cattle are the primary reservoir following the recent out-break in Soroti (Welburn *et al*, 2001a;Fevre *et al*, 2001). The continuous movement of infected animals is another way that can lead to the spread of the disease in to new areas infested with tsetse flies. Alarming reports of a possible overlap (Figure 1.3) between the traditional SE Uganda focus and foci further North have appeared (Enyaru *et al*, 1999;Hutchinson *et al*, 2003;Picozzi *et al*, 2005). Moreover the serological test used for screening *T. b. gambiense* infections does not work for *T. b. rhodesiense* and parasitological diagnosis by microscopy cannot distinguish between *T. b. rhodesiense* and *T. b. brucei*. Clinical symptoms and the PCR technique, using the serum resistance associated (*SRA*) and the *T. b. gambiense* specific glycoprotein (*TgsGP*) as markers specific for *T. b. rhodesiense* and *T. b. gambiense* are used to define the two species (Welburn *et al*, 2001a;Gibson *et al*, 2002;Radwanska *et al*, 2002b;Radwanska *et al*, 2002a). The Soroti outbreak was later on linked to the uncontrolled movement of animals (Fevre *et al*, 2005;Fevre *et al*, 2006c) as the hospitalised patients from the SE Uganda were confirmed as infected with *T. b. rhodesiense* and patients from NW Uganda were confirmed as infected with *T. b. gambiense* indicating no overlap (Welburn *et al*, 2006).

1.5.2 T. b. gambiense in N.W. Uganda

T. b. gambiense, which causes chronic disease that recently resurged in NW. Uganda, is a public health problem with a prevalence estimated at 1% (Matovu *et al*, 2001a). The disease was first recognised in this part of the country in 1902 (Picozzi *et al*, 2005).

Humans are the major source of the *T. b. gambiense* parasite. The HAT outbreak in south Sudan (Moore & Richer, 2001) coupled with movements of displaced refugees as a result of civil unrest (Hovil & Bagenda, 2003;United Nations Report, 2004) is implicated in the spread of the disease to other districts in NW Uganda (Picozzi *et al*, 2005) (Figure 1.3).

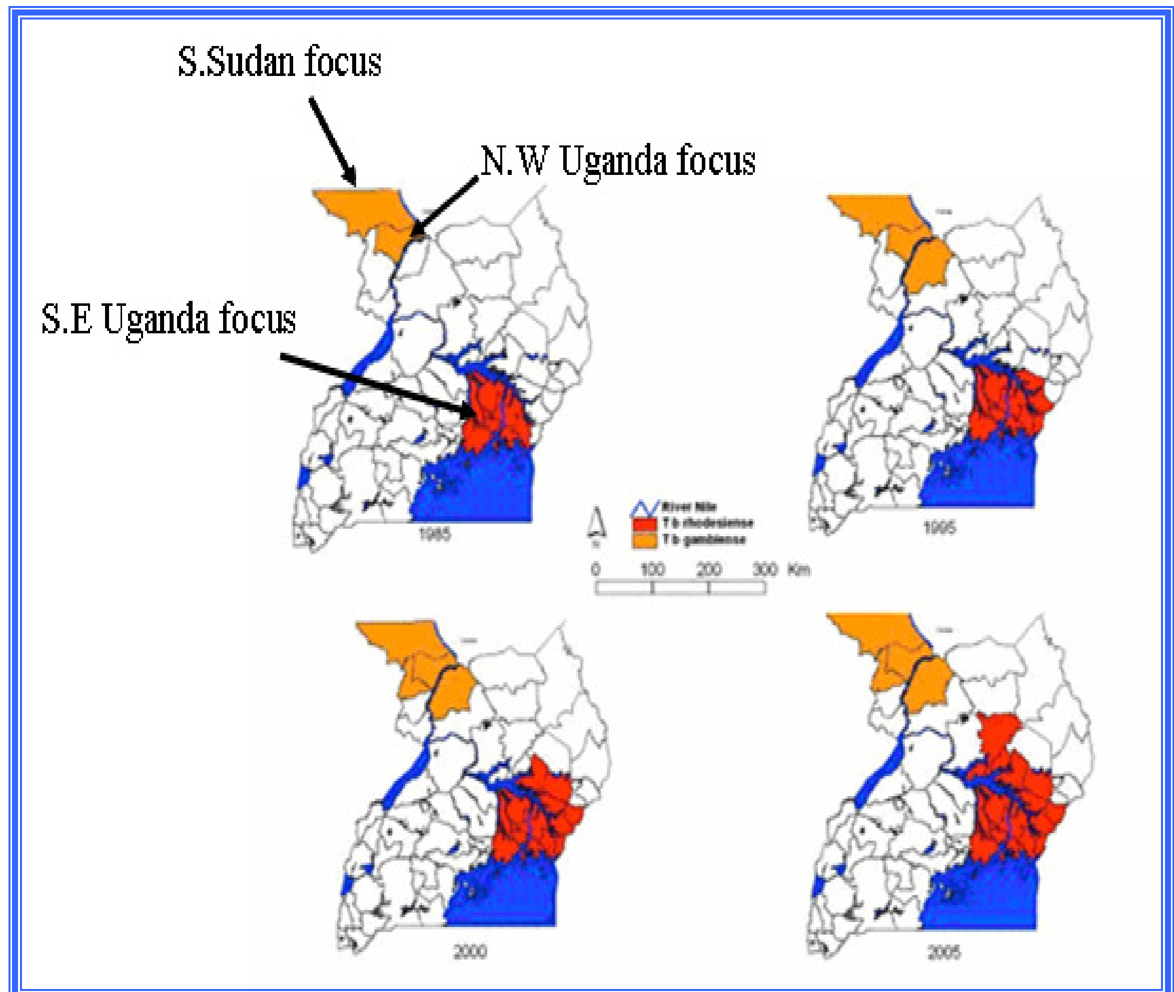


Figure 1.3 Maps showing the convergence of *T. b. rhodesiense* and *T. b. gambiense* in Uganda.
Distribution of *rhodesiense* (red) and *gambiense* (orange) (Picozzi *et al*, 2005).

1.5.3 Local policies

One of the policies applied to the control of Trypanosomiasis in Uganda is the mass screening of people at risk followed by proper diagnosis of sleeping sickness patients, before monitored treatment with the appropriate drug, administered at specific health care facilities. As a result of surveillance, reports of high melarsoprol failure rates associated with drug resistance in Arua, NW Uganda (Legros *et al*, 1999;Matovu *et al*, 2001b) led to a change in treatment policy in 2001 for Omugo Health Centre with melarsoprol being replaced with DFMO as first-line treatment for late stage disease.

Livestock farmers, on the other hand, acquire drugs easily through decentralised and private veterinary services, for example agroveterinary suppliers, local pharmacists and the informal sector. This has affected the livestock management practices of many farmers who misuse the drugs without proper diagnosis and this can lead to development of drug resistance (Geerts *et al*, 2001). A central government funded programme is currently responsible for the treatment of cattle with Trypanosomiasis (DFID Animal Health Programme, 2003). As a result of the reports of the spread of the disease (Picozzi *et al*, 2005) that was linked to the uncontrolled movement of animals in Soroti (Fevre *et al*, 2005;Fevre *et al*, 2006c) a new policy was implemented to reduce the risk of spreading the parasites to disease free areas (Wendo, 2002). Cattle transported from one district to another as part of the restocking programme receive a single dose of trypanocide as prophylaxis at the point of sale. It is a rule that before an animal permit to leave the market is issued, traders and livestock farmers must purchase as part of the overall market fee the injectable drug, which is administered by the district veterinary officials.

1.5.4 Drugs

Treatment of both stages of human disease differs in the *T. b. gambiense* North west foci and *T. b. rhodesiense* South east foci in Uganda. Pentamidine, which is given intramuscularly at a dosage of 4 mg/kg body weight/day for 10 days, is used as the first-line therapy for early stage *T. b. gambiense.* However, it is reportedly less effective against early stage *T. b. rhodesiense* which is treated using 5 injections of suramin at a dosage of 20 mg/kg body weight (a maximum of 1g/ injection) at intervals of 5-7 days (World Health Organisation, 1998). Late stage disease, in Moyo, NW Uganda and SE Uganda, is treated with melarsoprol at a dosage of between 1.2 -3.6 mg/kg body weight with 4 daily injections in 3 series each separated by 7 days. Currently, the 10 day course at a dosage of 2.2 mg/kg body weight/day for 10 days (Burri *et al*, 2000) is undergoing a trial in Kaberamaido (Dr. E. Matovu personal communication). Eflornithine which is administered intravenously at 100 mg/kg body weight every 6 hours with 4 daily infusions for 14 days is used for late stage *T. b. gambiense* disease at Omugo, NW Uganda. The Nifurtimox and eflornithine combination is undergoing a trial at Omugo and Moyo in NW Uganda for treatment of late stage disease (Dr. E. Matovu, personal communication).

Treatment of animal disease in Uganda is carried out using diminazene aceturate and isometamidium chloride depending on availability. Homidium, cymelarsan and suramin,

which are other animal trypanocides, are not used. Diminazene aceturate, which is curative, is used at a single dosage of 3.5 mg/Kg body weight for *T. b. rhodesiense* infection and 7 mg/Kg body weight for *T. b. brucei* infections. The two parasites, which are indistinguishable by microscopy, are instead distinguished using clinical symptoms and the PCR technique using the SRA marker. Isometamidium, which is curative, offers more benefit to the farmers and is used at 0.5 to 1 mg/Kg body weight. Resistance to both diminazene and isometamidium in Uganda is not considered a problem and even then treatment using both drugs as a sanative pair is effective with the hope of prolonging the effective life of the drugs. This is considered because cross-resistance is thought to be a rare phenomenon (Nerima *et al*, 2007). Of concern were reports of PCR */Sfa NI TbAT1/P2* mutant alleles associated with isometamidium resistance in *T. brucei* animal isolates from Somalia, which isolates were also resistant to diminazene (Afework *et al*, 2006). However there is sceptism that the observed *TbAT1/P2* mutant alleles were due to isometamidium resistance rather than diminazene (Nerima *et al*, 2007), which is believed to be entirely depend on P2 transport although there are indications that diminazene may have a second route of entry into the parasite other than the P2 transporter (De Koning, 2008).

1.5.5 Control situation

The main strategy for the control of both human and animal trypanosomiasis in Uganda is by detection and treatment. This is currently achieved by surveillance, which is both active and passive. Active case detection and mass screening of people at risk is carried out by mobile teams, which involve personnel from various national programmes as explained later (see section1.5.6). Most of the human early stage cases are detected by active case finding while passive surveillance (patients reporting on their own at health units) is the commonest mode of detection for the late stage cases. Detection is by screening using serological and molecular methods while microscopy is used to confirm the parasitological status of the confirmed cases. However, the Card Agglutination Test for Trypanosomiasis (CATT) (Magnus *et al*, 1978) used for screening of *T. b. gambiense* is not applicable for *T. b. rhodesiense* and distinguishing between these parasites is carried as described earlier (section 1.5.1). Patients are staged as either early or late stage as will be described later in Chapter 3. Treatment is then carried out with the most appropriate drug (see section 1.5.4). This control strategy of screening has also targeted individuals who may be serologically positive but parasitological negative for *T. b. gambiense* as these may act as a reservoir of the disease, which is chronic for several months or years.

In the case of animals, block treatment is commonly used because of the low sensitivities of diagnostic tests. Therefore, detection of parasites resulting from any single infection leads to the treatment of the entire herd. This may target animals that could act as reservoirs of the human infective *T. b. rhodesiense*.

Vector control is another strategy and this needs sustainability to achieve its effect on trypanosomiasis control (http://www.afro.who.int/ddc/publications/rc55_trypanosomiasis_strategy.pdf). In the past this relied on clearing of tsetse breeding grounds and resting places like bushes. Aerial spraying of insecticides which, although successful in completely eliminating tsetse in the Delta region of Botswana (Kgori *et al*, 2006), is expensive and of environmental concern for Uganda. Cost-effective methods using insecticide treated or baited traps and community participation are the current approaches used in the control of trypanosomiasis. Mass trapping of tsetse flies using attractant or deltamethrin-treated baited pyramidal traps, and cattle derived odor baited traps have since contributed to a decline in the tsetse densities (Katunguka-Rwakishaya & Kabagambe, 1996;Magona *et al*, 2005).

1.5.6 National programmes

Activities involving the control of the disease (vector and sleeping sickness) are carried out in an integrated way between different bodies. The Ministry of Agriculture Animal Industry and Fisheries (MAAIF) embraces Livestock Health and Entomology and this body employs the Veterinary Officers, Central Government Officers, District Entomologists and Tsetse Catchers involved in Trypanosomiasis control. The National Sleeping Sickness Control Programme (NSSCP), which is under the Ministry of Health employs the District Sleeping Sickness Officers and Sleeping Sickness Assistants. Finally, the Livestock Health Research Institute (LIRI) now called the National Livestock Health Research Institute (NALIRI), which is under the National Agricultural Research Organisation (NARO) employs Research Officers and Laboratory Technicians.

The NSSCP has the duty of carrying out control activities in the *T. b. rhodesiense* region of SE Uganda. Similar activities in the *T. b. gambiense* region of North and NW Uganda also involve NSSCP in collaboration with a French non governmental organisation, Medecins Sans Frontiers (MSF). All these different organisations involved in the control of the

disease report to the Coordinating Office for Control of Trypanosomiasis in Uganda (COCTU), which is responsible for advising on policy.

Efforts by these national programmes to coordinate and control the disease are also supported by WHO and the international community. The Ugandan government pays counterpart funding, e.g. payment of salaries to control staff.

1.5.7 Drug resistance problem

The occurrence of drug resistance in human sleeping sickness in Uganda threatens the control of the disease, which depends on a few old drugs. Melarsoprol, the cheapest and most affordable drug used for treatment of human late stage disease, has been replaced by eflornithine at Omugo Health Centre in Arua as a result of high treatment failure rates (30%) associated with drug resistance (Legros *et al*, 1999;Matovu *et al*, 2001b;Matovu *et al*, 2001a). A current concern, based on findings from an earlier study, is that eflornithine may not be effective in treatment of immune suppressed patients since it is trypanostatic and not trypanocidal (Pepin *et al*, 1992). Moreover, HIV-AIDS is prevalent in North Uganda where the health system is struggling to recover after a long period of insurgency by the Lord's Resistance Army http://www.avert.org/aidsuganda.htm. Another concern is that resistance may develop rapidly if eflornithine is overused as a monotherapy. Combination therapies have been suggested to preserve efficacy and the slow development of resistance to the few drugs in use. Drug trials to assess the efficacy of the different combination therapies in Uganda have been undertaken and the treatment outcome results of some have been assessed as effective while others may require further investigations. The combination of melarsoprol and nifurtimox showed high relapse rates (>10%) with increased toxicity in patients presenting at Omugo Health Centre (Priotto *et al*, 2006). The combination of melarsoprol and eflornithine also had adverse effects while the combination of eflornithine and nifurtimox gave good efficacy with no registered relapses in patients presenting at both Omugo Health Centre and Yumbe Hospital (Priotto *et al*, 2006;Checchi, 2007). The use of these promising combinations will have to wait for licensing.
Pentamidine resistance is not considered a serious problem. The few isolated cases are associated with misdiagnosis and inappropriate treatment.

Resistance to the two commonly used animal trypanocides diminazene aceturate and isometamidium chloride has not been considered as a problem in Uganda (Dr. E. Matovu ,personal communication,).

1.6 Overview of the *TbAT1/P2* transporter and arsenical/ diamidine resistance

Parasitic protozoa such as *T. brucei* and *Leishmania* cannot synthesize purines and pyrimidines de novo so they entirely rely on membrane transporters to obtain these nutrients from their hosts (Marr *et al*, 1978;Fish *et al*, 1982). One key purine transporter is the characterised *T. brucei* adenosine transporter 1 (TbAT1) responsible for P2 activity (Mäser *et al*, 1999). *TbAT1* is a single copy gene encoding a protein of 463 amino acids with 11 transmembrane domains predicted. It is 30% identical at the amino acid level to *Leishmania donovani* nucleoside transporter *LdNT1* (Vasudevan *et al*, 1998). The trypanosomatid transporters belong to the equilibrative nucleoside transporter (ENT) family (Mäser *et al*, 1999). Alterations in *TbAT1,* or loss of the P2 transporter which is known to mediate the cellular uptake of adenosine, adenine, melarsoprol or its analogues and diamidines across the plasma membrane, is associated with the development of arsenical and diamidine resistance (de Koning, 2001a;Maser *et al*, 2003;Delespaux & De Koning, 2007). Carter and Fairlamb (1993) were the first to perform a study that showed that a *T. b. brucei* melarsen-resistant clone derived from a wild-type parental clone lacked the P2 transporter and was 67-fold less sensitive to melarsoprol *in vitro* when compared to the parental clone. Further more, it was demonstrated, using melarsen resistant *T. brucei* bloodstream forms, that the P2 purine transport system was implicated in the reduced import of the diamidine, pentamidine, leading to the suggestion of its involvement in pentamidine resistance (Carter *et al*, 1995). The P2 adenosine transport system was also found to be present in *T. equiperdum* whereby the P2 transporter of a berenil resistant clone also cross resistant to melarsoprol, had reduced activity and possibly decreased affinity for adenosine (the main P2 substrate) in comparison to the drug sensitive clone (Barrett *et al*, 1995). Recent work has shown that *TbAT1* is no longer transcribed in the resistant cells and the residual adenine sensitive adenosine transport is due to another transporter protein (Barrett *et al*, unpublished). Other *in vitro* studies also revealed that alterations in P2 transport activity are associated with diminished drug uptake giving rise to drug resistance in *T. brucei* group organisms (Ross & Barns, 1996;Scott *et al*, 1996;Matovu *et al*, 2003;Witola *et al*, 2004;Bridges *et al*, 2007).

Molecular characterisation of the gene encoding the *T. brucei* adenosine transporter (TbAT1), (Mäser *et al*, 1999) has played a major role in further understanding the mechanisms underlying drug resistance in *T. brucei*. Identification of the *TbAT1/P2* gene was undertaken by expression in the yeast *Saccharomyces cerevisiae*. The gene was found to encode an adenine sensitive adenosine transporter P2, whose alteration due to mutations resulted in melaminophenyl arsenical resistance *in vitro* and *in vivo* (Mäser *et al*, 1999;Matovu *et al*, 2001b). Mäser and others (1999) also demonstrated that the *TbAT1* gene cloned from a melarsen-resistant clone (STIB 777R) had ten nucleotide differences in its cloned *TbAT1* Open Reading Frame (ORF) when compared to the *TbAT1* of the melarsen-sensitive clone (STIB 777S). Trypanosomes are diploid in nature; therefore they possess a copy of this gene on each of the two chromosomes. Sequencing suggested the possibility of STIB 777S being homozygous for the sensitive allele. The *TbAT1* gene cloned from the melarsen-resistant clone (STIB 777R) was reintroduced into yeast and was shown to be unable to stimulate the uptake of exogenous adenosine and did not confer susceptibility to melarsen-oxide, confirming its lack of P2 activity. In this same study a PCR/ RFLP (Restriction fragment based polymorphism) method using the *Sfa NI* enzyme was shown to differentiate between the *T. brucei* melarsen sensitive (STIB 777S) and melarsen resistant clone (STIB 777R) by displaying different allele patterns, 566 bp & 111 bp for the wild type *TbAT1* and 435 bp and 242 bp for the mutated *TbAT1* (Figure 1.4). The method was also used to demonstrate the presence of a mixed banding pattern in patient samples which were either infected with trypanosomes homozygous for both the sensitive and resistant *TbAT1* alleles or heterozygous for the sensitive or resistant *TbAT1* alleles (Matovu *et al*, 2001b). Of the 10 mutations identified in the melarsen resistant clone, one led to an amino acid change (Ala^{178}→Thr (A178T) as a result of a base change G to A at position 532 which eliminated the *Sfa NI* site present in the wild type *TbAT1*. Another mutation led to an amino acid change Asn^{286}→Ser (A286S), as a result of a base change A to G at position 857 leading to a new *Sfa NI* site 325 bp further downstream (Figure 1.5). The PCR/RFLP method can differentiate between a single infection due to a trypanosome that is *TbAT1* homozygous or hemizygous for wild type allele ($TbAT1^{s/s}$ or $TbAT1^{-/s}$) and a single infection due to a trypanosome that is *TbAT1* homozygous or hemizygous for the mutant allele ($TbAT1^{r/r}$ or $TbAT1^{-/r}$). However, the method cannot be used to differentiate between a mixed infection due to both ($TbAT1^{s/s}$and $TbAT1^{r/r}$) and a single infection due to a trypanosome that is *TbAT1* heterozygous for the wild type or mutant alleles ($TbAT1^{s/r}$) in case such gene information is needed. This is one of the drawbacks of the method. Such phenotypes can be determined by cloning. A more recent

report from a field study performed in NW Uganda, shows the use of an allele specific PCR approach based on primers targeting the *TbAT1* PCR/*Sfa NI* 677 bp fragment and are specific only for the wild type *TbAT1* and mutant *TbAT1* (Nerima *et al*, 2007). This new method involves using a PCR universal reverse primer (Sfa-mut) in the same PCR assay with two forward primers one specific for the *TbAT1* wild type allele (Sfa-s) and another specific for the *TbAT1* mutant allele (Sfa-as) for detection of melarsoprol *TbAT1* mutants. However, the results correlate with those of the PCR/*Sfa NI* approach although the new method is faster and cheaper in terms of cost (Nerima *et al*, 2007).

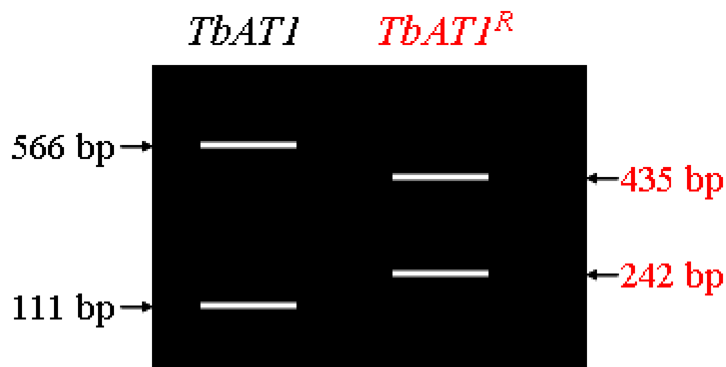


Figure 1.4 Schematic of the PCR/*SfaNI* of the wild type (Black) and mutant (red) *TbAT1*.
The schematic of the agarose gel shows the different *TbAT1* PCR/RFLP band size patterns.
***TbAT1*, wild type *T. brucei* adenosine transporter**
***TbAT1*^{R}, mutant *T. brucei* adenosine transporter.**

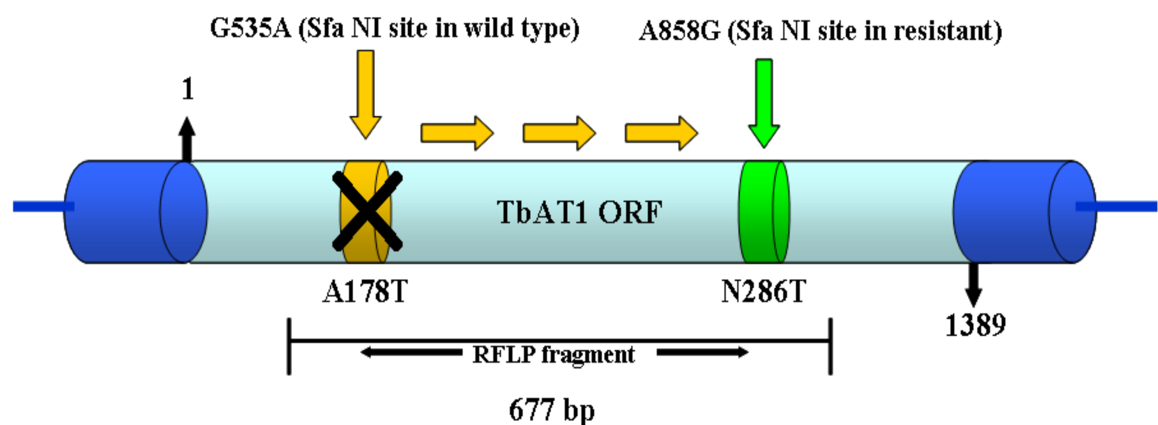


Figure 1.5 Schematic of the *TbAT1* ORF and the positions of the *SfaNI* sites
The schematic shows the nucleotide positions of the *Sfa NI* sites and the targeted *TbAT1* RFLP fragment. It also depicts the deletion of *Sfa NI* site in the *TbAT1* wild type *T. brucei* and creation of the new site 323 bp downstream in the *TbAT1* of the drug resistant *T. brucei.* ORF, open reading frame. RFLP, Restriction fragment length polymorphism.

Results from these and other studies linking the loss of P2 uptake or its loss to diamidine and arsenical resistance, led to the suggestion that loss of P2 activity may easily result in cross resistance to both classes of drugs (Barrett & Fairlamb, 1999;de Koning *et al*, 2000;de Koning, 2001b;de Koning *et al*, 2004). This was supported to some extent in a study in which the TbAT1/P2 gene was deleted by gene knock-out leading to reduced susceptibility of *T. brucei* to melaminophenyl arsenicals and diamidines, although only a 2-

3 fold resistance was observed for melarsoprol and pentamidine suggesting other altered routes of drug uptake might lead to higher level of resistance to other diamidines (Matovu *et al*, 2003). This finding was later related to a kinetic study in which it was demonstrated that, unlike the transport of diminazene which is almost entirely accumulated through P2 (de Koning *et al*, 2004), pentamidine uptake also involves at least two additional carriers termed HAPT1 and LAPT1 (de Koning & Jarvis, 2001;de Koning, 2001b). Neither HAPT1 nor LAPT1 have yet been characterised at the molecular level. Development of pentamidine resistance may therefore not occur easily because of the presence of multiple transporters. However, in a very recent study it was demonstrated using bloodstream *TbAT1* null mutants selected for resistance to pentamidine *in vitro* that loss of HAPT1 had occurred and this resulted in high levels of resistance (130-fold) to pentamidine when compared to the wild type strain 427 (Bridges *et al*, 2007). Cross resistance in the selected line to melarsoprol was increased (15-fold) while no further increase was observed for diminazene aceturate leading to the conclusion that loss of HAPT1 can occur due to pentamidine selection provided *T. brucei* already lacks the TbAT1/P2 which may lead to high arsenical resistance (Bridges *et al*, 2007). The observed increase in resistance to melarsoprol in the pentamidine resistant line KO-B48 compared to 2 to 3-fold in the *TbAT1* knock-out was an indication that HAPT1 may also be a second route of melarsoprol uptake and, for pentamidine resistance to occur; more than one transporter needs to be altered.

The animal trypanocide isometamidium has also been proposed to accumulate to some extent through the P2 transporter since it inhibited uptake of adenosine, a P2 substrate in *T. b. brucei* (Mäser *et al*, 1999). There are recent reports linking P2 changes in isometamidium resistant *T. congolense* although it is likely that isometamidium enters through other routes associated with changes in the mitochondrial membrane potential (Wilkes *et al*, 1997).

1.6.1 The North Western Uganda (Omugo) situation

Arua district, Omugo is one of the regions in NW Uganda that has been affected by the *T. b. gambiense* strains thought to be resistant or less susceptible to melarsoprol. During the time when melarsoprol treatment failures were becoming common with a prevalence as high as 30% (Legros *et al*, 1999), a field study to analyse isolates collected from 22 newly infected and 43 relapsed patients was performed by Matovu and others (2001b). They used

the PCR/RFLP based method (Mäser *et al*, 1999) to investigate whether an association between mutations in *TbAT1/P2* and treatment failure as reported in the laboratory (Mäser *et al*, 1999) was evident. They demonstrated that 38 patient isolates (59%) possessed the mutated *TbAT1* pattern and 30 of these isolates were from relapsed patients. The remaining 13 isolates from relapsed patients possessed a wild type *TbAT1*. Sequencing of *TbAT1* from the isolates revealed the presence of nine mutations, which were similar to those found by Mäser and others (1999), in *TbAT1* from the *T. brucei* melarsen resistant clone STIB 777R. Six of these mutations were expressed at the amino acid level although one mutation (ΔF316) was only found in the field isolates, but not in the laboratory derived STIB 777R. The three remaining mutations (C21T, T144C and C471T) were silent. STIB 777R was found to possess two additional mutations; L380P causing an amino acid change at position 1139 and a silent mutation (C501T), which were both absent in the field isolates K003 (a *T. b. gambiense* stock from a relapse patient from Angola) and STIB 871 (a multidrug resistant field isolate).

It was concluded from this study that mutations in *TbAT1/P2* may play a role in melarsoprol treatment failures and possibly not true resistance but the presence of wild type *TbAT1* alleles in isolates from relapse patients suggested *TbAT1* may not be the only gene responsible (Matovu *et al*, 2001b) or that multiple strains were present in individual patients. It is also a possibility that the wild type *TbAT1* alleles picked in these patient isolates were simply picked up from treatment failure due to inadequate drug treatment. However, these isolates were not tested *in vitro* for arsenical sensitivity. Eflornithine is the only other drug registered for the treatment of *T. b. gambiense* late stage disease, its use is limited because it is expensive and difficult to administer. More research was undertaken to understand how the melarsoprol-resistant parasites would respond to treatment with eflornithine. Matovu and others (2001b) performed a study using isolates collected from this region during the same period. The *T. b. gambiense* field isolates propagated in Mastomys rats and later adapted to culture medium were analysed in comparison to reference *T. b. gambiense* isolates from Ivory Coast and *T. b. rhodesiense* isolates from SE Uganda for their sensitivities to melarsoprol and DFMO using the long term feeder layer viability assay (Kaminsky *et al*, 1989). The eflornithine minimum inhibitory concentration (MIC) values were between 1.13-6.3 µg/ml indicating the NW Uganda isolates were sensitive but the melarsoprol MIC values of several isolates were higher (0.036 µg/ml and 0.072 µg/ml) than those of the isolates from Ivory Coast (0.001 µ/mg-0.018µ/mg). This indicated that the susceptibility of the NW Uganda isolates was reduced (Matovu *et al*,

2001a). A combination of these findings led to a change in treatment policy at Omugo Health Centre with melarsoprol being replaced with eflornithine in 2001.

In Moyo district, NW Uganda (another region affected by the disease) melarsoprol is still used as the treatment for late stage cases as the melarsoprol relapse situation is not as frequent as in Arua district. However, surveillance in this region is on-going and the most recently released report on a study using isolates collected from Moyo between 2003-2004 which were analysed by the PCR/*Sfa NI* RFLP based method (Mäser *et al*, 1999;Matovu *et al*, 2001b) indicated that 10 of 73 isolates possessed both wild type and mutated *TbAT1* alleles. A more sensitive allele specific PCR method identified only mutated *TbAT1* in their samples (Nerima *et al*, 2007).

Recently in South Sudan, Ibba centre, were melarsoprol was withdrawn in 2001, because of high treatment failure rates (Moore & Richer, 2001), analysis of isolates from *T. b. gambiense* sleeping sickness patients (18) collected in 2003 was carried out using the same PCR/RFLP method and sequencing. The results indicated the presence of *TbAT1* wild type alleles only and no evidence for the resistant mutations. The isolates were tested *in vitro* and they were found to be sensitive to melarsoprol leading to the suggestion that melarsoprol resistant alleles were never there or melarsoprol resistant mutations can not survive without drug pressure (Maina *et al*, 2007).

1.6.2 Drug resistance in South Eastern Uganda

Melarsoprol is still relied on as an effective trypanocide for the treatment of human late stage *T. b. rhodesiense*. There are increasing number of reports of resistance to veterinary trypanocides, including diminazene and isometamidium (Schönefeld *et al*, 1987;Kaggwa *et al*, 1988;Clausen *et al*, 1992;Kalu, 1995;Mulugeta *et al*, 1997;Afewerk *et al*, 2000;Geerts *et al*, 2001), in various sub-Saharan African countries and it is surprising that resistance to these drugs, which have been in use for 40 years, has not yet become a major problem in SE Uganda although reports indicate the potential exists (Matovu *et al*, 1997;Enyaru *et al*, 1998;Waiswa *et al*, 2003). With animals acting as reservoirs of the human infective *T. b. rhodesiense* parasite (see section 1.2), it is of concern that parasites transmitted by tsetse flies to both animals and humans may be resistant to diminazene, isometamidium and homidium (Barrett, 2001). Resistance to the drugs where the mechanism of resistance

involves the loss of the P2 transporter would have clear implications on possible treatment failure in humans using drugs that also depend on P2 for uptake.

Block treatment of animals following identification of a clinical case in a herd is commonly used in Uganda. Mass chemoprophylactic treatment of the animal reservoir with sub curative doses of drug may also result in selection of parasites with reduced susceptibility to these drugs. An *in vitro* study was performed by Matovu and others (1997) to investigate the susceptibility to melarsoprol, diminazene and isometamidium of *T. b. rhodesiense* isolates collected from sleeping sickness patients and animal reservoirs, which indicated all of the isolates were sensitive to melarsoprol but one isolate from man was found to be less sensitive to both diminazene (>100 ng/ml) and isometamidium (0.78 ng/ml) when compared to 0.8-6.3 ng/ml for diminazene and 0.01-0.20 ng/ml for isometamidium used to clear the rest of isolates from man and animals. This difference could have been due to man being infected with different parasite strains having varying responses to the drugs. An *in vivo* study by Enyaru and others (1998) to treat mice and cattle infected with the diminazene and isometamidium resistant *T. b. rhodesiense* stock from man and a *T. b. brucei* isolate that was susceptible to both drugs, revealed that 7 mg/kg of diminazene cleared both parasite infections in cattle and the *T. b. brucei* infection in mice but 14 mg/kg of diminazene could not clear the infection in all of the *T. b. rhodesiense* infected mice. This observed difference indicated possible pharmacokinetic differences between the mice and cattle or differences in the parasite strains. However, 2.0 mg/kg of isometamidium were able to clear the *T. b. brucei* infection in mice as well as both parasite infections in cattle but the same dosage failed to cure 33% of infected mice which suggested that the reduced susceptibility of this *T. b. rhodesiense* isolate *in vitro* and *in vivo* in mice could be a signal that resistance to animal trypanocides may develop with time in this region (Enyaru *et al*, 1998). Alternatively, to investigate the usefulness of treating the animal reservoir with diminazene and isometamidium, Waiswa and others (2003) used pigs infected with parasites from the *T. brucei* group and the findings showed that treatment with a 7.0 mg/kg body weight of diminazene resulted in relapses which were not observed if a dosage of 14.0 mg/kg body weight was used but a 1.0 mg/kg body weight of isometamidium registered no relapses. A similar study was done by Olila and others (2002) using *T. brucei* or *T. vivax* stabilates originally isolated from cattle in Mukono, SE Uganda (1995-1996) to determine their susceptibility to the three trypanocides diminazene, isometamidium and homidium after inoculation in cattle, goats and mice. All of the isolates were highly sensitive to both diminazene (3.5 mg/kg body weight) and

isometamidium (0.5 mg/kg body weight) when compared to reference stocks, but some isolates in goats were less susceptible to homidium (1.0 mg/kg body weight). The same trend was observed in the mice indicating that homidium susceptibility was reduced. However, homidium is not used in Uganda.

Preserving the efficacy of these drugs and to avoid the development of resistance is of great importance. Stringent vector control activities to reduce transmission and a reduction in mass treatment of animals would reduce drug pressure and may be the best options to achieving this. This would reduce the risk of the selection of resistance in humans and animals as this could complicate successful treatment of humans.

1.7 Human African Trypanosomiasis situation in other parts of Africa

West and Central African countries, Angola, the Democratic Republic of Congo (DRC), and Central African Republic (CAR) are among the regions that have suffered serious trypanosomiasis epidemics like Uganda (World Health Organisation, 2001). All of these countries which face the problem of drug resistance tend to fall within the same belt as shown in Figure 1.1. Several common factors have been linked to the disease in these parts of Africa as will be highlighted later. It is of interest to see how the Uganda *T. b. gambiense* trypanosome population structure compares with that of the chosen countries listed below. The population structures have earlier been proposed for *T. brucei* as panmictic (Tait, 1980), clonal (Tibayrenc *et al*, 1990) or epidemic (Maynard-Smith *et al*, 1993). Various methods e.g. isoenzyme analysis (Gibson *et al*, 1978;Tait *et al*, 1984), restriction fragment polymorphism (RFLP),(Hide *et al*, 1994) random amplification of polymorphic DNA (RAPD) (Stevens & Tibayrenc, 1995) and mini/ microsatellites (MacLeod *et al*, 1999;MacLeod *et al*, 2000;MacLean *et al*, 2007;Morrison *et al*, 2008) have been used for determining population structures in *T. brucei*. Availability of drug resistance candidate microsatellite markers (MacLeod *et al*, 2005b) even highlights the possibility of using such markers for identifying drug resistance which is a problem in these regions.

1.7.1 Cameroon

Cameroon is one of the countries in West Africa highly endemic for the Gambian form of the disease. The disease was thought to have been eliminated in the sixties but resurged in the early 1990's resulting in a persistent epidemic in the late 1990s. Campo, which borders Equatorial Guinea, is one of the human foci of the disease. Although the focus is still active, the disease is not as prevalent (0.2-0.5%) and the outbreak was linked to pigs in the area which were found to be carrying the parasite (Penchenier *et al*, 1999). Although an earlier study to screen domestic animals for *T. b. gambiense* in the Fontem region, another sleeping sickness focus, indicated an absence of *T. b. gambiense* (Asonganyi *et al*, 1990), recent reports show evidence of domestic and wild animals acting as reservoirs of the human infective parasite in this region (Herder *et al*, 2002;Simo *et al*, 2006;Njiokou *et al*, 2006). This may possibly be a way of maintaining the parasite in the population leading to the persistent epidemics.

1.7.2 Central African Republic

The disease, due to *T. b. gambiense*, is highly endemic in Central African Republic. The most recent outbreak occurred in the late 1990s but with no established national control programme, the control of the disease was complicated (Simarro *et al*, 2001). Animals are implicated as possible reservoirs for the disease since the epidemiology shows that human to fly contact, in comparison to pig-fly contact, varies depending on the fly species which should be of importance in prevention of the disease (Gouteux *et al*, 1993;Simo *et al*, 2008b).

1.7.3 The Democratic Republic of Congo

Located in West-Central Africa, the Democratic Republic of Congo experienced a resurgence of the disease due to *T. b. gambiense* in the mid 1980s, which resulted in epidemic levels by the mid 1990s. This was attributed to a breakdown of the surveillance and control activities (Ekwanzala *et al*, 1996;Smith *et al*, 1998). The situation has improved recently with control activities in place (Nieuwenhove *et al*, 2001) although treatment failures with melarsoprol, the first line drug, still poses a challenge. Melarsoprol relapses are treated with eflornithine as the alternative. Trials to assess the efficacy of combination therapies in treating late stage disease have been undertaken. A combination

of melarsoprol and eflornithine showed high relapse rates (Mpia & Pépin, 2002) while melarsoprol and nifurtimox proved more efficacious with low or no relapses (World Health Organisation, 2002). However, melarsoprol is still used and a randomised trial to assess its efficacy as a monotherapy at the standard regimen and at an improved 10 day short course regimen (Burri *et al*, 2000) in comparison to eflornithine monotherapy indicated that melarsoprol was less efficacious than eflornithine (Balasegaram *et al*, 2006). In spite of these findings the treatment policy has not yet been changed and melarsoprol remains the first choice for treatment in this country.

1.7.4 Congo/Brazaville

Congo is another country highly endemic for the disease, which is due to *T. b. gambiense*. The disease resurged in this country in the early 1980s with the prevalence of the disease increasing up to the early 1990s. Melarsoprol relapses are common and a combination therapy involving nifurtimox-eflornithine has been shown to be better than eflornithine alone (Priotto *et al*, 2007). Results from a similar randomised trial using melarsoprol and nifurtimox as monotherapy and as combination therapy have revealed that the 10-day short course melarsoprol and nifurtimox combination as more effective than the standard melarsoprol regimen (Bisser *et al*, 2007). Therefore these combinations offer hope.

1.7.5 Angola

The disease due to *T. b. gambiense* is a serious endemic problem for Angola. Angola experienced serious epidemics in the late 1990s with the prevalence of the disease varying between zones (0.9-9.7%) and villages (16%) (Stanghellini & Josenando, 2001). The recurring outbreaks are blamed on wars that hamper surveillance and control activities in the affected regions (Smith *et al*, 1998). With established disease management and control programmes, the prevalence of the disease is reported to have greatly reduced (Ruiz *et al*, 2002;Abel *et al*, 2004). Melarsoprol, which is widely used for the treatment of the late stage disease, is associated with toxicity. A randomised trial was conducted to compare the efficacy and toxicity levels of a 10 day short melarsoprol regimen to the 26 day regimen but the results indicated a similar outcome for both (Burri *et al*, 2000). The short regimen was therefore considered an alternative and is now widely used across Africa. However, the increase in the relapse rate (25%) has led to increased use of the expensive and rarely available alternative eflornithine (Stanghellini & Josenando, 2001). Combination therapy

of melarsoprol and nifurtimox was then thought to be a promising alternative as registered for other countries. Melarsoprol monotherapy has just recently been replaced.

1.8 Diagnosis of Drug Resistance

1.8.1 Treatment and follow-up

Management of the disease, as highlighted earlier, relies on diagnosis and treatment (see section 1.5.5). Patients are treated with the intention to cure taken as the overall outcome. Therefore follow up of patients post-treatment is crucial. This is the current approach used to determine treatment failures. Cut offs are used to determine the effectiveness of the drugs. In *T. b. gambiense* infections a relapse rate of 5-9% is considered normal for melarsoprol as the drug is not always active in cerebral spinal fluid (Wang, 1995;Onyango *et al*, 2000). Relapse rates of 15-30%, as reported in several regions in sub Saharan Africa in the late twentieth century were an indication that the drug was losing efficacy (Legros *et al*, 1999;Burri & Keiser, 2001;Matovu *et al*, 2001a;Brun *et al*, 2001;Moore & Richer, 2001;Balasegaram *et al*, 2006). Such high relapse rates have not been registered for *T. b. rhodesiense*. Patients are requested to return at intervals of 6 months for retesting to check for presence of parasites in any of their body fluids. Treatment is considered successful if no parasites are detected at the end of a 24 months follow up period. However, a draw back with this method, as clear from a number of surveillance studies, relates to the fact that patients are frequently lost at some point during the follow up process making the interpretation of the findings difficult (Burri *et al*, 2000;Robays *et al*, 2004;Lejon & Buscher, 2005). Reports on drug trials carried out in several African countries registered 49.3% of patients at discharge not attending any of the 4 scheduled follow up visits while 50.7% of the patients attended only 1 visit (Schmid *et al*, 2004;Schmid *et al*, 2005). Follow up numbers were reported to drop from 64% at 6 months to 46% at 12 months post treatment (Chappuis *et al*, 2005). This has resulted in restricting the treatment outcome analysis from 24 months to 12 months in particular studies since the numbers drop even lower beyond this period (Balasegaram *et al*, 2006). It is assumed that the loss to follow up patients are possibly cured otherwise they would be passively detected. However, patients could also have died in this period. Patients still showing clinical symptoms, or parasites, at any of the follow up points are assessed and a decision is made. Follow up may continue for some while those regarded as treatment failures are retreated appropriately for example with eflornithine or eflornithine-nifurtimox combination.

1.8.2 PCR genotyping for possible presence of gene mutations

The use of parasitological detection during follow up post-treatment is clearly an unsatisfactorily means of assessing treatment success. Other methods are urgently needed. Point mutations in drug target genes or drug transporter genes have been linked with the development of drug resistance in trypanosomes (Balasegaram *et al*, 2006;Delespaux *et al*, 2006) and other parasites e.g.. *P. falciparum* (Foote *et al*, 1989;Basco *et al*, 1995;Plowe *et al*, 1997;Djimde *et al*, 2001;Babiker *et al*, 2001;Dorsey *et al*, 2001). Genotyping for mutations by PCR is another method used to identify clinical treatment failures that are drug resistant related. The method has been used before for identifying clinical treatment failures with chloroquine (CQ) in malaria. Such studies revealed that clinical treatment failure with CQ was significantly associated with parasites with the mutated forms of both the *P. falciparum* chloroquine resistance transporter gene (*Pfcrt*) and *P. falciparum* multidrug resistance gene (*Pfmdr1*) (Babiker *et al*, 2001;Dorsey *et al*, 2001;Mockenhaupt *et al*, 2001). Similar molecular methods have also been used to detect mutations in the dihydrofolate reductase (*DHFR*) and Dihydropteroate synthetase (DHPS) genes (Peterson *et al*, 1990;Foote *et al*, 1990;Plowe *et al*, 1997;Wang *et al*, 1997), which are associated with sulphadoxine-pyrimethamine resistance using isolates collected in E. Uganda from malaria patients below 5 years infected with *P. falciparum*. The findings revealed that presence of the triple *DHFR* mutant (*DHFR*108Asn/51Ile/59Arg) resulted in earlier treatment failure than the double (*DHFR*108Asn/59Arg) or the single mutant (*DHFR*108Asn) (Nalunkuma-Kazibwe, MSc Thesis, 2003, University of Glasgow).

A PCR/RFLP based method has recently been developed for the detection of specific mutations (Ala^{178} →Thr and Asn^{286} →Ser) in the *T. brucei* purine transporter gene (*TbAT1/P2*) of trypanosomes which are melarsoprol resistant and differentiating them from the melarsoprol sensitive trypanosomes (Mäser *et al*, 1999) as displayed in Figure 1.4 and Figure 1.5. This method has been used to identify similar mutations in *T. b. gambiense* relapse patient isolates from NW Uganda as referred to in section 1.6.1 (Matovu *et al*, 2001b;Nerima *et al*, 2007). The results from PCR/*Sfa NI* RFLP analysis revealed that 30 of 43 relapsed patient isolates possessed the mutated *TbAT1* alleles whilst the remaining 13 possessed wild type isolates which suggested the method may miss detecting some treatment failure cases (Matovu *et al*, 2001b). The method has also been used in S. Sudan (Maina *et al*, 2007). This PCR/*Sfa NI* method was developed to detect only two of melarsoprol resistance associated mutations which suggests it will not detect other

mutations to *TbAT1/P2* such as deletions observed in the *T. brucei* resistant isolate derived by selection *in vitro* to the furamidine DB75 (Lanteri *et al*, 2006).

1.8.2.1 Trypanosome densities in natural infections

Molecular studies such as detection of drug resistance genes, detection of infections and population genetics involving analysis by PCR of field samples, greatly depend on the quantity of available parasite material. Isolates collected from *T. b. gambiense* patients have been reported to have very low parasite densities ranging between 100-1000 trypanosomes/ml (Simarro *et al*, 1999;Garcia *et al*, 2000). It is not known to date why the parasite density is persistently low it could possibly be associated with innate control mechanisms. The fluctuating low parasite levels are related to the presence of the variant surface glycoprotein coat (Cross G, 1975), which plays a huge role in the parasites evasion of the immune system (Cross, 1990;Clayton, 1992;Borst & Rudenko, 1994;Pays *et al*, 1994;Cross, 1996). The low parasite densities in such isolates may result in insufficient material making it impossible to successfully detect the *TbAT1/P2* gene by PCR, which is used as a drug resistance marker in *T. brucei*. The problem can also be enhanced if the primers used for PCR amplification are not so sensitive. This low-density problem may result in false negatives which, although they can be avoided, make the whole mutant detection process more labour intensive. An example of such a false negative would be lack of detection of the DNA of a trypanosome whose *TbAT1* has been deleted (*tbat1*$^{-/-}$), even though such a result would not be associated with low parasite densities.

1.8.2.2 Other methods to detect additional mutations

The PCR/*SfaNI* RFLP method can be used to detect only two of the ten mutations that were found in to melarsoprol resistant strains (Mäser *et al*, 1999). Other alternative methods like the single stranded DNA conformation polymorphism (SSCP)/ Heteroduplex (HD) analysis used in the detection of human diseases (Orita *et al*, 1989;Liechti-Gallati *et al*, 1999) have also been used (Matovu *et al*, 2001b). This sensitive method can be used to detect the difference in the mobility on a polyacrylamide gel of a single stranded DNA whose conformation has been altered by a single mutation. SSCP screening results for the detection of *TbAT1* mutants in *T. b. gambiense* field isolates showed three patterns which corresponded to the wild type, mutant and the mixed patterns observed with the PCR/*SfaNI* RFLP method indicating a correlation between the two methods (Matovu *et al*, 2001b) Screening by the SSCP method was also found to be more sensitive for detection of

mutations in a *T. congolense* adenosine transporter (*TcoAT1*) gene with high similarity to *TbAT1/P2* when comparing diminazene resistant and sensitive *T. congolense* isolates characterised in mice by the single dose mouse test of 20 mg/kg (Delespaux *et al*, 2006).

Direct sequencing (without subcloning into a vector) of DNA (Sanger *et al*, 1977) and sequencing by DNA cloning (Sambrook *et al*, 1989) are the methods most widely used for identifying the position and type of mutation in DNA isolates suspected of being mutated. Changes within the coding region of a sequence as a result of mutations may change the function of the protein or alter its affinity for the inhibiting drug. Direct sequencing of *TbAT1* PCR products from the *T. b. gambiense* isolates of melarsoprol relapsed patients from NW Uganda (with mutant *TbAT1* pattern by PCR/RFLP and SSCP), revealed that eight of the mutations detected were identical to mutations present in the melarsoprol resistant reference stock STIB777R although STIB 777R was found to contain two additional mutations (one synonymous and one non-synonymous) (Matovu *et al*, 2001b). Alternatively, sequencing of the *TbAT1* gene from relapse patient isolates (with wild type *TbAT1* pattern by PCR/RFLP and SSCP) showed that they lacked any of the mutations present in STIB 777R and were confirmed as identical to STIB 777S(Matovu *et al*, 2001b). Sequencing was also carried out for the *TbAT1* gene from *T. b. gambiense* patient isolates from South Sudan (with wild type *TbAT1* pattern by PCR/RFLP), and all of the isolates were almost identical to the melarsoprol sensitive reference stock STIB777S. Although direct sequencing of PCR products revealed one mutation, sequencing of subcloned PCR products revealed 3 more mutations which might have been introduced during PCR by Taq polymerase (Maina *et al*, 2007).

1.8.3 FTA® Card Technology

T. brucei drug resistance field studies especially those involving *T. b. gambiense* rely on very little parasite material as highlighted earlier (section 1.8.2.1). Moreover the convenient storage and transport of such material is crucial for later laboratory analysis. Whole blood samples, often preserved in heparinised capillary tubes, are stored at 4°C before being transported to the laboratory. This may lead to the irrecoverable loss of material in poorly resourced field settings where the power supply may not be constant. The development of a new sampling technology using the FTA® card (Whatman Biosciences) has been a breakthrough. FTA technology is a simple, practical, economical and sensitive method for sampling, storage and retrieval of DNA from parasites or other

biological samples for PCR analysis. The FTA® card method involves application of *T. brucei* infected patient blood samples by spotting on a special card filter or matrix on which the trypanosomes are lysed leading to their DNA being fixed and stabilized on the card matrix for long periods of time. The air dried FTA® card samples can be stored at room temperature in a less humid environment or kept at 4°C before use for DNA extraction as will be described later in chapter 2. Low parasite densities, which are characteristic of *T. b. gambiense,* may result in little amplification material and the use of insensitive PCR primers are some of the drawbacks of the method. However, molecular studies on *T. brucei* amongst many other organisms have shown FTA technology to be an effective method (Becker *et al*, 2004;Picozzi *et al*, 2005;Morrison *et al*, 2007;MacLean *et al*, 2007;Morrison *et al*, 2008).

1.8.4 Fluorescence tests

Fluorescence assays have proved to be important regarding studies on *T. brucei* related to cross resistance to arsenicals and diamidines. A rapid fluorescence assay has been developed as a phenotypic diagnostic tool for the early detection of arsenical and diamidine resistance in *T. brucei* (Stewart *et al*, 2005). The method which was validated on *T. brucei* reference stocks characterised in mice utilises a fluorescent diamidine compound 2,5-bis(4-amidinophenyl)-3,4-dimethylfuran (DB99) which targets the DNA containing compartments and has a TbAT1/P2 mode of uptake. The nucleus and kinetoplast of sensitive parasites with TbAT1/P2 activity were shown to fluoresce within 1 minute under a fluorescence microscope unlike the resistant parasites lacking P2 transport (Stewart *et al*, 2005). A similar study performed by Lanteri and others, (2006) was used to demonstrate the absence of fluorescence in the *TbAT1* null mutant and the *T. brucei* line selected for resistance to the fluorescent diamidine DB75 (prodrug DB289) in comparison to the wild type *T. brucei* 427 parasites. Another fluorescence assay utilising the fluorescent fluorophore 4,6-Diamidino-2-phenylindole dihydrochloride (DAPI) has also been taken advantage of (Frommel & Balber, 1987). Resistance to pentamidine, whose uptake occurs via multiple transporters, has been assessed by utilising DAPI fluorescence and it has been demonstrated that appearance of fluorescence occurred much later (after 30 minutes) in a laboratory derived pentamidine resistant KO-B48 line lacking both TbAT1/P2 and HAPT1 activity while fluorescence occurred much earlier in the wild type *T. brucei* 427 (which has P2 activity) compared to the *TbAT1* null mutant (which lacks P2 activity) (Bridges *et al*,

2007). Fluorescence microscopes suitable for use in African field settings are also under development (Jones *et al*, 2007).

1.9 Background: Project Objectives

The project aims to investigate risk factors, in an African field setting, that might lead to selection of drug resistance in African trypanosomes. This involves four complementary approaches:

1.9.1 Approach 1

To investigate the usefulness of genotyping for mutations in *TbAT1/P2* as a predictive diagnostic and epidemiological tool for the early detection of drug resistance in *T. brucei*. This will offer a lead for making important clinical decisions aimed at reducing the burden of Human African Trypanosomiasis. Moreover, the investigation will be used to extend earlier findings that indicated that mutations in *TbAT1* (related to loss of P2 activity) correlate with melarsoprol treatment outcome in clinical isolates from *T. b. gambiense* sleeping sickness patients in North Western Uganda.

1.9.2 Approach 2

To investigate, by microsatellite analysis, the general population structure of existent *T. b. gambiense* from NW Uganda as a possible way of identifying trypanosome strains that could be associated with drug resistance. Furthermore, to investigate how population structure in NW Uganda compares with other affected areas across Africa.

1.9.3 Approach 3

To investigate, by PCR typing the identity of trypanosome species infective to dogs and humans with the possibility of a veterinary link to human disease. This is a possible route that can lead to selection of drug resistant parasites and cross resistance between human and animal trypanocides which may result in serious implications for disease management.

1.9.4 Approach 4

To investigate, by *in vitro* induction of drug resistance in *T. brucei* the possibility of identifying and characterising other possible markers of resistance to the drugs diminazene aceturate and DB75 both of which enter via the P2 transporter but for which other routes of uptake might exist.

Chapter 2

2 Adaptation of the FTA technology for use in genotypic studies involving *T. brucei*

2.1 Summary

The specific aims of this study were to investigate whether collection of *T. brucei*-infected blood from sleeping sickness patients spotted on to FTA® cards (Whatman) using the FTA method would enable the successful amplification by PCR and analysis of *T. brucei* microsatellite markers, the *T. brucei* drug resistance gene *TbAT1/P2* and the presence of *T. brucei* in blood. Oligonucleotide primer pairs specific for the *T. brucei* multicopy locus (TBR) (Moser *et al*, 1989), the single copy *T. brucei* microsatellite flanking the *Phospholipase C* (*PLC*) gene (Tait *et al*, 2002), the *T. brucei gambiense*-specific glycoprotein gene (*TgsGP*) (Radwanska *et al*, 2002b) and the *T. brucei* adenosine transporter 1 gene (*TbAT1/P2*) (Mäser *et al*, 1999) were used in the sensitivity assay.

The results revealed that the method was very sensitive with the *T. brucei* multicopy locus (TBR) primers and allowed detection of a *T. brucei* infection from a DNA disc punched from an FTA card spotted with the lowest parasite dilution of 1 trypanosome/ml. However, it was observed that the high parasite density required for amplification of the single copy *TbAT1* gene, would be a limitation of the method if *T. b. gambiense* patient samples, which are known to have very low parasitaemia were to be spotted on FTA® cards. Otherwise the method was found to be very useful.

2.2 Introduction

Genotypic studies on *T. brucei,* which involve the determination of parasite population structures, detection of mutations in parasite genes related to drug resistance and diagnosis of parasite infections, require the collection of field isolates. However, the material obtained from these isolates is often of very poor quality. Currently investigations of population genetics and *in vitro* drug susceptibility tests rely on propagation of parasites collected from patients in rodents or the KIVI kit for *in vitro* isolation (Truc *et al*, 1992;Aerts *et al*, 1992). Success in the retrieval of the parasites using these methods has yielded varying results (Jamonneau *et al*, 2002;Jammonneau *et al*, 2003). Detection of

drug resistance, the study of specific mutations in marker genes and the diagnosis of disease all rely on patient isolates which are collected in heparinised vials and stored at 4 °C before transportation to the laboratory for downstream analysis. However, such storage conditions may not be guaranteed under field conditions in the resource-poor setting of sub-Saharan Africa as a result of many factors including an inconsistent power supply. This may lead to the loss of precious material, which may never be recoverable. It is therefore important that any isolates are collected in the most reliable way that will enable the successful retrieval of the material needed later for genotypic analysis in the laboratory. The FTA® card (Whatman) method is a new sampling technology that has been shown to be effective for analysis of human pathogens (Wang *et al*, 2005;Zhong *et al*, 2001), human viruses (Beck *et al*, 2001) and plant viruses (Ndunguru *et al*, 2005) and even specifically for *T. brucei* (Becker *et al*, 2004;Picozzi *et al*, 2005;MacLean *et al*, 2007;Morrison *et al*, 2008). However, I set out to evaluate the sensitivity of this approach independently, in order to investigate whether this technique is suitable for the PCR-based analysis of trypanosome DNA from *T. b. gambiense*-infected patients, many with low parasitaemia.

2.3 Materials and Methods

Study area and population

This section includes a description of the study regions in Central Africa, where the samples for this thesis were collected. This was through collaborations established between Dr. Michael Barrett and Dr. Annette MacLeod (University of Glasgow) and other researchers from Central Africa. Human African Trypanosomiasis (HAT) patient samples from Chad, Cameroon, Ivory Coast and the Democratic Republic Congo were kindly provided to us by Dr. Phillipe Truc (OCEAC, Cameroon)

Field surveys on HAT were carried out in Bodo village (GPS coordinates not available) in Chad in Central Africa during the month of December 2002. Similar activities were carried out in the month of May 2003 in Campo village (GPS coordinates not available) in Southern Cameroon, which is located near the border with Equatorial Guinea. During the month of October 2003, a medical field survey was carried out in the village in Bandundu around Maluku, and Maluku clinical centre (GPS coordinates not available), which is located north of Kinshasa in the Democratic Republic of Congo (Figure 2.1).

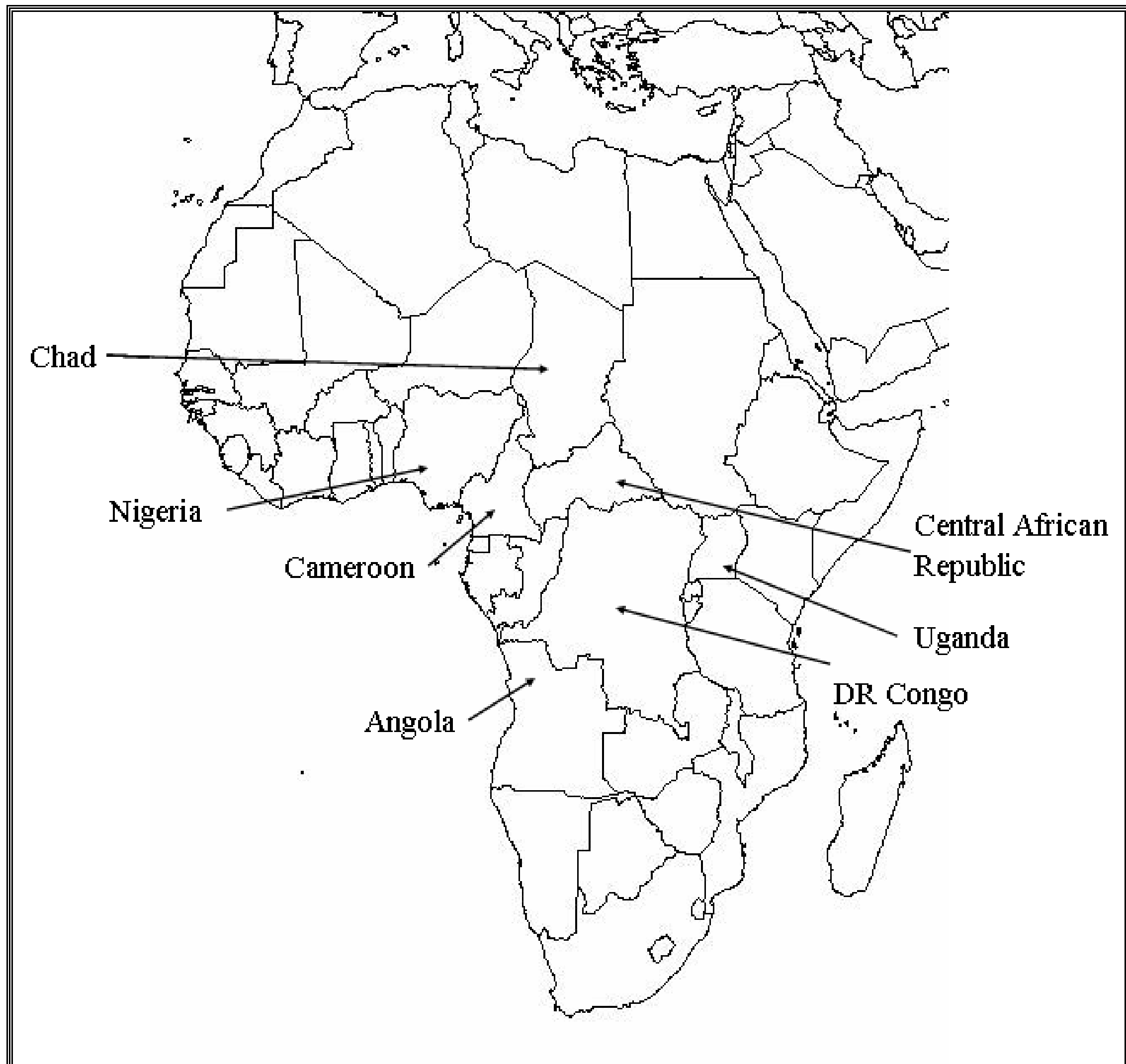


Figure 2.1 Map of Africa showing the locations of this thesis sample study regions **http://geography.about.com/library/blank/blxafrica.htm (with modifications)**

The polymerase chain reaction (PCR) is a basic molecular method that enables the amplification of target genes or DNA fragments along a sequence of interest. FTA sensitivity and detection limit PCR assays using the FTA® cards spotted with known dilutions of infected mouse blood was performed to assess the utility of the FTA method for *T. brucei* sample collection and storage.

T. brucei sample preparation from mice

A female adult ICR mouse was injected intraperitoneally with 2×10^6 trypanosomes/ml of bloodstream form trypanosomes of the *T. brucei brucei* strain 427 (Cross, 1975). At peak parasitaemia (4-5 days post infection) following microscopic examination of smears from tail blood, infected blood was collected through cardiac puncture in a 15 ml falcon tube on ice. Using a start cell density of 1×10^{10} trypanosomes/ml as determined by microscopy with an improved Neubauer counting chamber (WSI, Division of Hawksley Technology, England), the infected blood was diluted in 10-fold serial dilutions in CBSS buffer (Appendix I). 1×10^9 trypanosomes/ml and 1 trypanosome/ml were the highest dilution and lowest dilutions respectively. 500 µl from each blood dilution was spotted directly onto an FTA® card (Whatman), which was allowed to air dry and then stored at room temperature.

Patient sample collection

Blood samples (n=74) from patients from Central Africa were collected as blood spots using the FTA® card (n=54) and whole blood preps (n=20) by trained personnel in the respective regions. The blood preps had been pre-treated with guanidinium chloride for protein denaturation prior to RNA extraction before transportation. The dried cards sealed in plastic bags were transported at room temperature while the whole blood preps were transported on dry ice for molecular analysis at the Institute of Biomedical and Life Sciences, University of Glasgow, UK.

DNA extraction from whole blood spotted on FTA® cards

Extraction and purification of trypanosome DNA from infected blood samples spotted on FTA® cards (Whatman) was performed as described in the FTA® protocols (www.whatman.com/repository/documents/s7/FTAProtocolBook602.pdf). Briefly, a 2.0 mm disc was punched from a dried blood spot using a Harris micro punch (Whatman). The disc was collected in a microfuge tube and washed three times using 200 µl of FTA

purification reagent with incubation and mixing by inversion at intervals for 5 minutes at room temperature between each wash. The FTA purification reagent was removed and the disc was rinsed twice using 200 µl of TE buffer (Appendix I) with incubation mixing for 5 minutes at room temperature. The TE buffer was discarded and the DNA disc air-dried at room temperature for 1 hour. The air dried DNA disk was used immediately in PCR or kept at 4°C.

DNA extraction from whole blood-preps using the Phenol-chloroform method

Genomic DNA of isolates from Chad was extracted from 200 µl of infected whole blood pre-treated with guanidinium chloride, using the phenol-chloroform-isoamyl alcohol method and precipitated using 100% ethanol (Sambrook *et al*, 1989). Extracted DNA was suspended in 50 µl of sterile distilled water. Up to 5 µl of DNA was used immediately in PCR or kept at -20°C.

Polymerase chain reaction amplification

PCR amplification of the *T. brucei* multicopy locus (177 bp repeat or TBR), the *T. brucei* single copy GPI-specific *Phospholipase C* gene (*PLC*) associated microsatellite and the single copy *T. brucei* adenosine transporter 1 gene (*TbAT1/P2*) was performed. However, for the sensitivity assay, PCR amplification of the *T. b. gambiense* specific glycoprotein gene (*TgsGP*) was not included in the assay since the mouse blood used was infected only with *T. b. brucei.* The sequence properties of the TBR multicopy primers TBR-F and TBR-R (Moser *et al*, 1989), the *PLC* gene microsatellite primers PLC-G and PLC-H3 (Tait *et al*, 2002;MacLeod *et al*, 2005a;MacLean *et al*, 2007;Morrison *et al*, 2008) and the specific TbAT1 primers with their various combinations are shown in (Table 2.1). TbAT1 ant-s and TbAT1 ant-as (Mäser *et al*, 1999), TbP2F and TbP2R (Mhairi Stewart., PhD Thesis 2003, University of Glasgow) were designed within the 3' and 5' of the *TbAT1* untranslated regions. Primer pairs P2F(RB1) and P2R(RB2) (Dr. R J S Burchmore, personal communication, University of Glasgow) and TbP2m-3 and TbP2m-4 (Mhairi Stewart, PhD Thesis 2003, University of Glasgow) were designed to amplify within the *TbAT1* ORF as displayed in Figure 2.2.

The analysis of the *T. b. gambiense* patient samples from the Democratic Republic of Congo, Chad and Cameroon stored as blood spots on FTA® cards (Whatman) and whole blood preps followed. Amplification by PCR was performed using the TBR multicopy primers TBR-F and TBR-R, the *PLC* gene microsatellite primers PLC-G and PLC-H3 and

the *T. b. gambiense* specific glycoprotein gene (*TgsGP*) primers TgsGP-F and TgsGP-R (Radwanska *et al*, 2002b).

Each extracted DNA disc on an FTA® card or 1-5 µl of whole blood prep DNA was PCR amplified in a total volume of 20 µl containing a 1 × Custom PCR mastermix (45 mM Tris-HCl (pH 8.8), 4.5 mM $MgCl_2$, 11 mM $(NH_4)_2SO_4$, 0.113 mg/ml BSA, 4.4 µl EDTA and 1 mM each deoxynucleotide triphosphate- dATP, dCTP, dGTP, dTTP) (ABgene®, Advanced Biotechnologies Ltd, UK). Oligonucleotide primer pairs (Table 2.1) synthesised by MWG Biotech-AG each at 0.4 µM and 1.25 units of GoTaq DNA polymerase enzyme (Promega Corporation, Madison, USA) were added to the reaction. TbAT1 primers were added at a concentration of 1 µM. Amplifications were carried out in a PTC-200™ DNA Engine (MJ Research, inc., Waltham, MA, USA) using the following amplification conditions: initial denaturation at 94°C for 4 minutes, followed by denaturation at 94°C for 30seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 1 minute for 30 cycles. This was followed by a last extension at 72°C for 7 minutes. Genomic DNA from the reference strain *T. b. brucei* 427 (Cross, 1975) or the *T. b. gambiense* strain ELIANE (Pays *et al*, 1983) was included in the PCR run were relevant as a positive control. Sterile water with no DNA template was included in the run as a negative control. To increase the sensitivity of each reaction, a second round of PCR, using the same oligonucleotide primer pair, was carried out using 1 µl of the primary PCR product as template under the same conditions.

Primer pair	Primer sequence (5'-3')	T_m	Product size
TBR-F TBR-R	5'-tgc gca gtt aac gct att ata ca-3' 5'-aaa gaa cag cgt tgc aaa ctt-3'	57.1°C 54.0°	177 bp repeat
PLC-G PLC-H3	5'-caa cga cgt tgg aag agt gtg aac-3' 5'-cca ctg acc ttt cat ttg atc gct ttc-3'	62.7°C 63.4°C	177 bp
TgsGP-F TgsGP-R	5'-gct gct gtg ttc gga gag c-3' 5'-gcc atc gtg ctt gcc gct c-3'	61.0°C 63.1°C	308 bp
TbAT1 ant-s* TbAT1 ant-as	5'-gcc cgg atc cgg ctg gtt ttt aga caa aag tga t-3' 5'-gcc cct cga gcc gca tgg agt aag tct ga-3'	70.7°C 72.3°C	1840 bp
TbP2F* TbP2R	5'-cat gcg ctt tgg tgg agg-3' 5'-ttg gcg aat cgg tgt acg-3'	58.2°C 56.0°C	≈2000 bp
TbP2m-3* TbP2m-4	5'-cag agt tcc gat atg cag c-3' 5'- tca tcg cct ccg tgg ggg tc-3'	56.7°C 65.5°C	≈100 bp
P2F(RB1)* P2R(RB2)	5'-gac tgt cga cat gct cgg gtt tga ctc agc c-3' 5'-gac tct gca gta gtg cta ctt ggg aag ccc c-3'	72.1°C 72.1°C	1400 bp
TbP2m-3* TbP2R	5'-cag agt tcc gat atg cag c-3' 5'-ttg gcg aat cgg tgt acg-3'	56.7°C 56.0°C	≈1000 bp
TbP2m-3* P2R(RB2)	5'-cag agt tcc gat atg cag c-3' 5'-gac tct gca gta gtg cta ctt ggg aag ccc c-3'	56.7°C 72.1°C	≈700 bp
P2F(RB1)* TbP2m-4	5'-gac tgt cga cat gct cgg gtt tga ctc agc c-3' 5'- tca tcg cct ccg tgg ggg tc-3'	72.1°C 65.5°C	≈800 bp
TbP2F* TbP2m-4	5'-cat gcg ctt tgg tgg agg-3' 5'- tca tcg cct ccg tgg ggg tc-3'	58.2°C 65.5°C	≈1000 bp

Table 2.1 Oligonucleotide primer sequences, properties and the expected product sizes. The TbAT1 primer pairs* used in the sensitivity assay in different combinations. T_m refers to melting temperature as calculated by the oligonucleotide manufacturer.

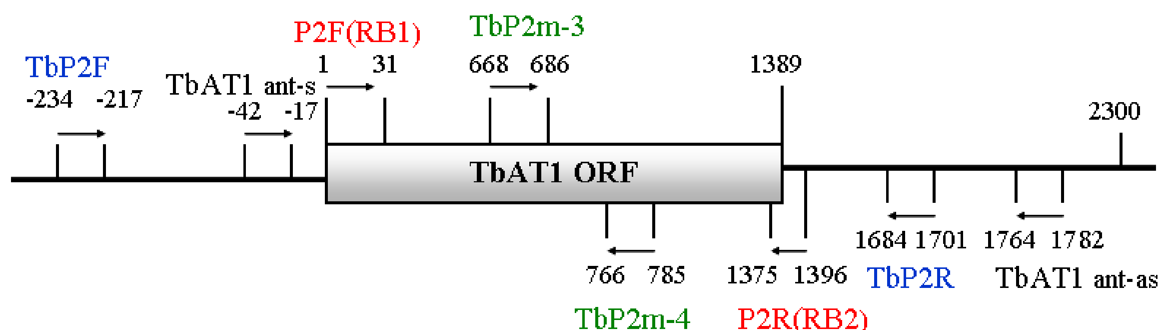


Figure 2.2 A schematic showing the *TbAT1* open reading frame (ORF). (Genbank accession number AF152369). Also shown are the positions of the forward and reverse primers used for PCR along the entire *TbAT1* sequence from the 5'-3' end.

Agarose gel electrophoresis and UV transillumination

10 µl of each primary or second round PCR product was mixed with 1-2 µl of 6 × Blue loading dye (Appendix I) and separated by electrophoresis on either a 2% or 3% Agarose gel stained with 0.7 µg/ml ethidium bromide in 1 × TAE buffer (Appendix I). PCR products were visualised using a high performance ultraviolet transilluminator (Ultraviolet Laboratory Products Ltd, UK) and a digital photograph taken using a digital graphic printer (Sony, UP-D890). The band sizes were determined by comparison with a standard 1-Kb plus DNA size marker (Invitrogen, Life technologies, USA) and then compared to the expected band sizes (Table 2.1).

2.4 Results

2.4.1 Sensitivity and detection limit FTA PCR assays

T. brucei brucei-infected mouse blood prepared in 10-fold serial dilutions and spotted onto FTA® cards (Whatman) as described (section 2.3) was used to determine the sensitivity of the FTA method. Lack of mouse blood infected with *T. b. gambiense* made it impossible to use the TgsGP primers in this sensitivity assay. PCR reactions set up using reference control DNA from the *T. b. brucei* strain 427 (Cross, 1975) and *T. b. gambiense* (ELIANE) (Pays *et al*, 1983) yielded products of the predicted size with the TBR, the *T. brucei PLC*-linked microsatellite, TgsGP and TbAT1 primer sets (Table 2.2). The method was specific with a signal observed only for the *T. b. gambiense* control strain (ELIANE) on amplification with the TgsGP primers set and not for the *T. b. brucei* control strain 427. Amplification was observed for both reference strains with the TBR primer set (Table 2.2).

Primer pairs	***T. brucei brucei* 427**	***T. b. gambiense* ELIANE**
TBR-F and TBR-R	Positive (177 bp repeat)	Positive (177 bp repeat)
PLC-H and PLC-G3	Positive (177 bp)	Positive (177 bp)
TgsGP-R and TgsGP-R	Negative	Positive (308 bp)
TbAT1 ant-s and TbAT1ant-as	Positive (1800 bp)	Positive (1800 bp)

Table 2.2 Control DNA PCR results showing the specificity of the primers. Positive; refers to right size PCR product as observed on agarose gel, with expected size given in brackets. Negative; refers to no PCR product observed on agarose gel.

The TBR primer set was found to be highly sensitive as an amplicon was detected in agarose gel by electrophoresis for the PCR product generated from a DNA disc punched from an FTA card spotted with the lowest dilution of 1 trypanosome/ml. The *TbAT1* and

the *PLC*-linked microsatellite primer sets had detection limits of 10^4 trypanosomes/ml and 10^6 trypanosomes/ml respectively (Table 2.3). It was also observed that the different *TbAT1* based primers had varying sensitivities for this single copy gene and the *PLC*-linked microsatellite as another single copy gene target was also a bit less sensitive (Table 2.3). However, the sensitivity was observed to increase by 10-fold when a second round reaction of PCR was performed as shown (Figure 2.3) for the TbAT1 primer pair P2F(RB1) and P2R(RB2).

The observed results indicate that the TBR primers are highly sensitive with the FTA method at a parasitaemia as low as 1 trypanosome/ml. They can effectively be used for the detection of *T. brucei* in very low parasitaemic samples. The use of the TBR primers has been reported in other studies (Masiga *et al*, 1992;Solano *et al*, 2002;Enyaru *et al*, 2006).

Primer pair	**Dilutions (Trypanosomes/ml)**										**Positive control strain**
	10^9	**10^8**	**10^7**	**10^6**	**10^5**	**10^4**	**10^3**	**10^2**	**10^1**	**1**	
TBR-F TBR-R	+++	+++	+++	+++	++	++	++	++	++	++	++
PLC-G PLC-H3	-	++	++	+	-	-	-	-	-	-	++
TbAT1 ant-s TbAT1 ant-as	-	*	*	*	*	*	-	-	-	-	++
TbP2F TbP2R	-	-	-	-	-	-	-	-	-	-	++
P2F(RB1) P2R(RB2)	-	++++	+++	+++	++	+	-	-	-	-	++
TbP2m-3 TbP2m-4	-	++++	+++	++	+	-	-	-	-	-	++
TbP2m-3 TbP2R	-	++++	+++	+++	++	+	-	-	-	-	++
TbP2m-3 P2R(RB2)	-	+++	++	++	-	-	-	-	-	-	++
P2F(RB1) TbP2m-4	-	*	*	*	*	*	-	-	-	-	++
TbP2F TbP2m-4	-	++++	+++	++	+	-	-	-	-	-	++

Table 2.3 PCR assay primer sensitivities and *T. brucei* DNA detection limits.
Trypanosome dilutions as displayed from the highest (10^9cells/ml) to the lowest (1 cell/ml).
+, positive result showing gel intensity of the PCR product on agarose gel.
-, negative result indicating no signal on agarose gel. *, wrong size product.
TbAT1 primers are listed in bold type.
Positive control reference strain (*T. b. brucei* 427).
Negative control (sterile water with no template) results were not included in the Table.
All results based on second round PCR using the same primer pairs and PCR conditions.

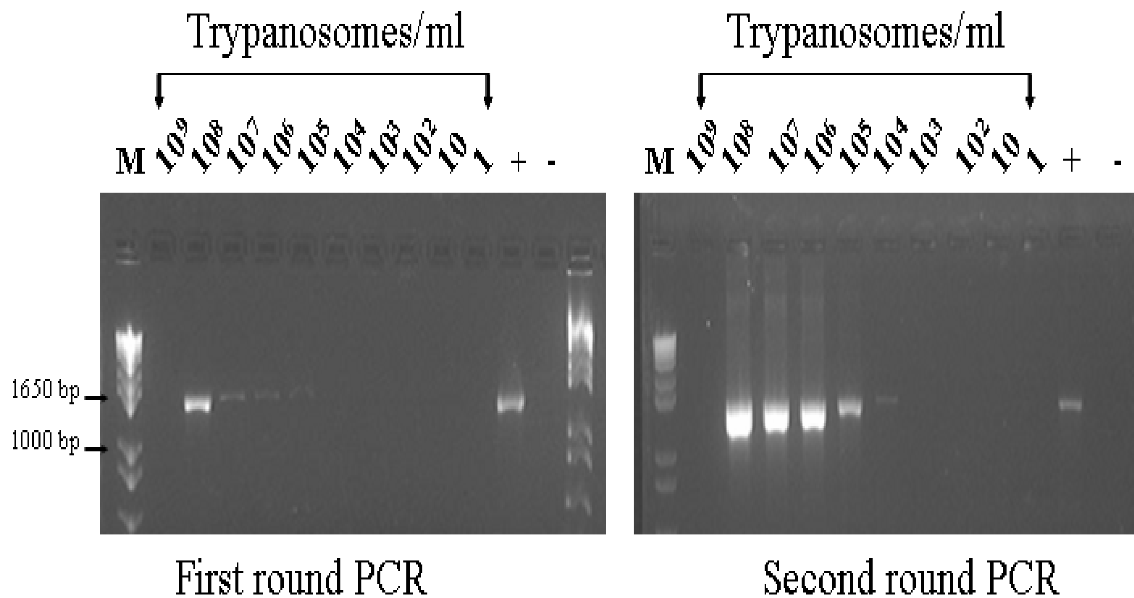


Figure 2.3 The agarose PCR sensitivity results for primers P2F(RB1) and P2R(RB2). Lanes 2-11 are from the highest parasite density (10^9cells/ml) to the lowest dilution (1 cell/ml).
The detection limit increased by 10-fold from 10^5 to 10^4 trypanosomes/ml in a second round PCR using the same primers and PCR conditions.
+, positive control (*T. b. brucei* strain 427). -, negative control (sterile water with no template).
M, 1 Kb plus DNA size marker (Invitrogen, Life technologies, USA).

2.4.2 PCR analysis of T. b. gambiense patient samples

Field samples from Chad (n=20), Cameroon (n=9) and the Democratic Republic of Congo (n=45) provided to us by Dr. Phillipe Truc (OCEAC), were derived from patients confirmed positive by screening using the card agglutination test for trypanosomiasis (CATT) (Magnus *et al*, 1978) as described later in section 3.3 and were analysed by polymerase chain reaction (section 2.3). The patients had also been confirmed parasitologically positive by microscopy using a wet smear of their glandular aspirate and the haematocrit centrifugation technique (HCT) (Woo & Rogers, 1974) on their whole blood as described (section 3.3). The microscopic diagnosis results obtained from Dr. Truc are shown in Table 2.4 and Table 2.5. Out of a total of 74 samples analysed, 54 were blood spots on FTA® cards (Whatman) and 20 were whole blood preps. A total of 53 out of 54 (98%) samples on FTA® cards were successfully amplified for the *T. brucei* multicopy locus (TBR) in comparison to 43 out of 54 (80%) for the single copy *T. brucei PLC* microsatellite (Table 2.4). This result appears to corroborate the earlier finding that TBR primers are more sensitive than the PLC microsatellite primers. A tentative conclusion could also be that 80% of the patients whose samples were observed to be positive with the PLC microsatellite primers likely had a peak parasite density at the time of sampling. In the case of the whole blood prep samples, only 9 out of 20 (45%) and 2 out of 20 (10%)

were successfully amplified for the *T. brucei* multicopy locus and the single copy *T. brucei PLC* microsatellite respectively although the diagnosis results provided indicated that the wet smears were positive (Table 2.5). This finding was an indication that the FTA technology is superior to other sampling methods in terms of preserving parasite DNA material. Amplification of the *TgsGP* gene was unsuccessful for all the FTA® cards (n=54) and whole blood prep (n=20) samples although the assay was positive with the *T. b. gambiense* (ELIANE) reference strain DNA. This result might indicate that the *TgsGP* gene is likely not ubiquitous in *T. b. gambiense* samples. But previous studies have indicated the gene is conserved in *T. b. gambiense* strains (Berberof *et al*, 2001) even those reported earlier to lack the LiTat 1.3 variant surface glycoprotein used in the CATT serological test (Dukes *et al*, 1992). An opportunity to perform an FTA sensitivity assay using the TgsGP primers was not possible as we were unable to successfully grow the *T. b. gambiense* (ELIANE) reference isolate in mice.

Sample code	Origin	Wet smear	HCT (T/ml)	TBR PCR	*PLC* PCR
MAL 1	D.R of Congo	N/A	251	++	++
MAL 2	D.R of Congo	N/A	251	++	++
MAL 3	D.R of Congo	N/A	251	++	+++
MAL 4	D.R of Congo	N/A	1000	++	+++
MAL 5	D.R of Congo	N/A	251	++	+++
MAL 6	D.R of Congo	N/A	1000	++	+++
MAL 7	D.R of Congo	N/A	251	++	-
MAL 8	D.R of Congo	N/A	251	++	+
MAL 9	D.R of Congo	N/A	251	++	++
MAL 10	D.R of Congo	N/A	251	++	-
MAL 11	D.R of Congo	N/A	251	++	+++
MAL 12	D.R of Congo	N/A	501	++	+
MAL 13	D.R of Congo	N/A	501	++	+
MAL 14	D.R of Congo	N/A	251	++	++
MAL 15	D.R of Congo	N/A	501	++	++
MAL 16	D.R of Congo	N/A	1000	-	+++
MAL 17	D.R of Congo	N/A	251	++	+++
MAL 18	D.R of Congo	N/A	501	++	+++
MAL. 19	D.R of Congo	N/A	501	++	+++
MAL 20	D.R of Congo	N/A	501	++	-
MAL 21	D.R of Congo	N/A	1000	++	++
MAL 22	D.R of Congo	N/A	501	++	+
MAL 23	D.R of Congo	N/A	1000	++	++
MAL 24	D.R of Congo	N/A	251	++	-
MAL 25	D.R of Congo	N/A	501	++	++
MAL 26	D.R of Congo	N/A	501	++	+++
MAL 32	D.R of Congo	Positive	N/A	++	+++
MAL 1b	D.R of Congo	Positive	251	++	+
MAL 2b	D.R of Congo	Positive	251	++	+
MAL 3b	D.R of Congo	Positive	501	++	+
MAL 4b	D.R of Congo	Positive	Non	++	+
MAL 5b	D.R of Congo	Positive	501	++	+
MEN 1	D.R of Congo	Positive	251	++	+++
MEN 2	D.R of Congo	Negative	501	++	+
MEN 3	D.R of Congo	Positive	106	++	+
MEN 4	D.R of Congo	Positive	215	++	+
MEN 5	D.R of Congo	Negative	501	++	+++
MEN 6	D.R of Congo	Negative	501	++	+++
MEN 7	D.R of Congo	Negative	251	++	+
MEN 8	D.R of Congo	Negative	251	++	-
MEN 9	D.R of Congo	Negative	251	++	+
MEN 10	D.R of Congo	Negative	251	++	+++
MEN 11	D.R of Congo	Negative	251	++	-
MEN 12	D.R of Congo	Positive	501	++	+
MEN 13	D.R of Congo	Positive	251	++	+
CPO-01	Cameroon	Positive	Not done	++	-
CPO-02	Cameroon	Positive	Not done	++	+
CPO-03	Cameroon	Positive	Not done	++	-
CPO-04	Cameroon	Positive	Not done	++	+
CPO-05	Cameroon	Positive	Not done	++	+
CPO-06	Cameroon	Positive	Not done	++	+
CPO-07	Cameroon	Positive	Not done	++	-
CPO-08	Cameroon	Positive	Not done	++	-
CPO-09	Cameroon	Negative	Positive	++	-

Table 2.4 Microscopic diagnosis and PCR results for the FTA® card field samples. Each PCR reaction was performed on one 2-mm FTA disc. The results were obtained using one round of PCR amplification. D.R, Democratic Republic. HCT, Haematocrit centrifugation test. TBR, *T. brucei* repeat. *PLC, T. brucei PLC* microsatellite. +++, very strongly positive. ++, strongly positive. +, faintly positive. -, negative result. T/ml, number of trypanosomes /ml. N/A, result not available. Wet smear and HCT results as provided by Dr. P.Truc (OCEAC). PCR positive and negative control results were not shown in the Table.

Sample code	Origin	Wet smear	HCT (T/ml)	TBR PCR	*PLC* PCR
TCD 01a	Chad	Positive	Positive	-	-
TCD 01 b	Chad	Positive	Positive	++	-
TCD 07a	Chad	Positive	Positive	+	-
TCD 07b	Chad	Positive	Positive	-	-
TCD 08a	Chad	Positive	Positive	-	-
TCD 08b	Chad	Positive	Positive	-	-
TCD 09a	Chad	Positive	Positive	+	-
TCD 09b	Chad	Positive	Positive	+	-
TCD 10a	Chad	Positive	Positive	++	-
TCD 10b	Chad	Positive	Positive	-	-
TCD 11a	Chad	Positive	Positive	++	-
TCD 11b	Chad	Positive	Positive	-	-
TCD 12a	Chad	Positive	Positive	-	-
TCD 12b	Chad	Positive	Positive	-	-
TCD 14a	Chad	Positive	Positive	-	-
TCD 14b	Chad	Positive	Positive	-	-
TCD 15a	Chad	Positive	Positive	+	-
TCD 15b	Chad	Positive	Positive	-	-
TCD 16a	Chad	Positive	Positive	++	+
TCD 16b	Chad	Positive	Positive	++	++

Table 2.5 Microscopic diagnosis and PCR results for the Whole blood prep field samples. Each PCR reaction was performed twice on each sample. The results were obtained using two rounds of PCR amplification
HCT, Haematocrit centrifugation test. TBR, *T. brucei* repeat. *PLC, T. brucei PLC* microsatellite. ++, strongly positive. +, faintly positive. -, negative result.
Wet smear and HCT results as provided by Dr. P. Truc (OCEAC). PCR positive and negative control results were not shown in the Table.

2.5 Discussion

Collection of trypanosomes on FTA cards is increasingly proving a reliable method to preserve DNA for analysis (Picozzi *et al*, 2005;MacLean *et al*, 2007;Pinchbeck *et al*, 2008;Morrison *et al*, 2008). In our hands, the method was highly sensitive with the *T. brucei* multicopy locus (TBR) primers. With the exception of the TBR primers, no amplification was observed for any of the other primers at the highest concentration of 10^9 trypanosomes/ml. This may have been due to too there being too much DNA in the sample, which resulted in a general failure of the PCR reaction. The TBR primers, which detected an infection from an FTA disc punched from a spot with the lowest dilution of 1 trypanosome/ml, were thought to be highly sensitive because of the presence of several tens of thousands of copies of the repeat sequence in the genome. The FTA method was 100-fold more sensitive in comparison to the whole blood method which could detect 10% of DNA in a single parasite as the lowest limit with TBR primers (Moser *et al*, 1989). However, by Real-time PCR, using DNA extracted from the FTA card with redesigned TBR primers, a detection limit of 10^2 trypanosomes/ml was observed for detection of *T.*

brucei (Becker *et al*, 2004). Our result indicates the method is more sensitive with TBR primers in detecting a *T. brucei* infection in comparison to 1 trypanosome/ 10 ml of cattle blood or 25 trypanosomes/ml of human blood (Fevre *et al*, 2006b). Another new method by PCR followed by oligochromatography using a dipstick is also less sensitive with a detection limit of 1 trypanosome/180 µl of blood (Deborggraeve *et al*, 2006). However, a just recently concluded study has indicated a higher sensitivity with the FTA method using the TBR primers at a detection limit as low as 0.025 of a trypanosome/PCR. This quantity of material using this FTA method is equivalent to 1 µl of DNA that is whole genome amplified from one 2.0-mm disc punched from an FTA® card (Morrison *et al*, 2007).

The negative result obtained for one of the 54 FTA® card field samples (MAL16) using the TBR primers (Table 2.4) was an indication that the sample lacked amplifiable material. The FTA card was designed in such a way that the parasite material spotted on the matrix is immobilised, fixed in place and does not spread elsewhere over the card. The same sample amplified with a positive result using the *T. brucei* single copy *PLC* microsatellite primers indicating presence of amplifiable material. The most plausible explanation for the negative TBR result could be lack of amplifiable *T. brucei* material on the FTA card position where the disc used in the PCR assay was punched. Repeating the PCR assay with another disc(s) may be required on the samples for reproducibility and to rule out sampling errors but this was not done. With the PLC microsatellite primers, it was observed that a high parasite density was required to be able to detect a signal even after performing a second round PCR reaction. This suggested the PLC primers were vastly less sensitive than the TBR primers and therefore required high parasitaemic samples. Results obtained for the FTA® card field samples indicated a similar trend for both TBR and PLC microsatellite primers when compared with FTA sensitivity assay results. Of concern however, was the observation that the PLC-linked microsatellite primers had a detection limit of 10^6 trypanosomes/ml (Table 2.3), which is never achieved in patients and yet these primers were able to clearly detect trypanosomes in the FTA card field samples (80%) provided to us by Dr. Phillipe Truc. It is a possibility that the primer sensitivity was higher than the initial results indicated.

Amplification of whole blood prep samples with both sets of primers was low. Eleven of twenty and 18/20 samples were negative with the TBR and PLC primers respectively. But the wet smear and HCT field diagnosis by microscopy indicated positive samples and yet the PCR technique is more sensitive. It is possible that the DNA extraction may not have been successful or traces of phenol used during the extraction process or guanidinium

chloride, which was used to denature protein in the samples prior to RNA and DNA extraction, may have contributed to inhibiting the Taq polymerase enzyme during PCR (Reviewed by (Radstrom *et al*, 2004)). Alternatively, the sample DNA material could have been degraded or lost due to improper storage conditions prior to transportation to the laboratory; but this was not tested using primers for a human repeat sequence.

The TbAT1 primers used for amplification of the single copy *TbAT1* gene were observed to be slightly more sensitive (10^5-10^4 trypanosomes/ml) than the PLC microsatellite primers used for amplification of the single copy *T. brucei PLC* microsatellite in the FTA sensitivity assay (Table 2.3). The TbAT1 ant primer set amplified different size products than expected, although it gave the right size product (approximately 2000 bp) with the reference strains (Table 2.2) and also for the DNA extracted from a *T. b. gambiense* relapse patient isolate (R015) from NW Uganda (Matovu *et al*, 2001a). It is possible that this set of primers may amplify other *T. brucei* related nucleoside transporters leading to different size products. However, a study performed using this set of TbAT1 ant primers revealed successful amplification of the *TbAT1* gene by nested PCR of DNA extracted from CSF of 65/75 *T. b. gambiense* patient isolates (Matovu *et al*, 2001b). These observations suggest that amplification of the right size fragment of *TbAT1* is less likely to be successful in comparison to smaller size fragments of the gene as shown (see Table 2.1 and Table 2.3). Our results also suggested that PCR detection of *TbAT1* from samples on FTA® cards (Whatman) could be successful at a parasitaemia $\geq 10^4$ trypanosomes/ml. But patient isolates with *T. b. gambiense* infections frequently have very low fluctuating parasitaemia between 10^2 and 10^3 trypanosomes/ml (Garcia *et al*, 2000;Simarro *et al*, 1999). As observed from our results, it was likely that the low parasite densities in the field isolates would make the amplification of *TbAT1* from infected blood spotted on FTA cards more difficult. However, amplification of the *T. brucei* PLC microsatellite with PLC primers was good for the FTA field samples (Table 2.4) even though less sensitive than the TbAT1 primers, which suggested the patients had a high parasitaemia. Although the development work indicated that TbAT1 primers were more sensitive than the PLC microsatellite primers (section 2.4.1), it was decided not to investigate the *TbAT1* status in these samples although the PLC results indicated this was feasible. This was due to the fact Dr. P. Truc was obliged to leave Central Africa prior to having collected any follow up data on treated patients; hence it was not possible to determine whether samples were from treated or relapsed patients and thus no correlation of regarding *TbAT1* status and treatment outcome was possible. The isolates have, however, proven invaluable with regard to development and assessment of the FTA card approach to diagnosis of *T. brucei gambiense*. The

sensitivity of the FTA method using the TbAT1 and PLC primers, which were observed to be less sensitive than the TBR primers, could possibly be increased by nested PCR.

The TgsGP oligonucleotide primer pair was not capable of amplifying the *TgsGP* gene from DNA extracted from any of the FTA® cards (Whatman) and whole blood prep field samples (not even for DNA diluted 1:10, 1:100 or 1:1000 in water). However, the gene was successfully amplified using DNA from the reference *T. b. gambiense* strain (ELIANE) and R015 which is a known *T. b. gambiense* relapse patient isolate from Uganda. The TgsGP primers have successfully been used for amplification of *T. b. gambiense* isolates from Côte d'Ivoire, Democratic Republic of Congo and Cameroon (Radwanska *et al*, 2002b;Agbo *et al*, 2003). The field isolates used in our study were collected from *T. b. gambiense* cases confirmed positive by CATT and parasitologically positive by microscopy. Since the TgsGP primers were not used in the FTA sensitivity assay, as this used *T. brucei brucei* DNA from rodent blood, it was not possible to establish their detection threshold. An alternative could be that these samples were possibly not *T. b. gambiense* type I and therefore did not contain *TgsGP*. The TgsGP primers were confirmed to specifically amplify from *T. b. gambiense* type I strains (Radwanska *et al*, 2002b). It is likely that our samples could have been *T. b. gambiense* type II strains, which are distinct from *T. b. gambiense* type I (Gibson, 1986;Paindavoine *et al*, 1986). *T. b. gambiense* type II strains were shown to be negative by PCR with the TgsGP primers (Radwanska *et al*, 2002b). Yet the *TgsGP* gene was earlier shown to be specific to and conserved in *T. b. gambiense* strains from Cameroon, Côte d'Ivoire, Uganda and the Democratic Republic of Congo (Berberof *et al*, 2001). Possibly even low parasitaemia could have led to lack of amplification but this could not be resolved as explained earlier. However, since amplification of the single copy *T. brucei PLC* microsatellite was successful in many cases, the lack of success with the *TgsGP* gene requires consideration, for example the use of nested PCR.

2.6 Conclusion

Overall, the FTA method was found to be very sensitive with successful amplification of the *T. brucei* multicopy locus (TBR) and this could be useful in detection of infections due to *T. brucei* species. However, the low parasite densities limit the usefulness of the primer sets for the single copy *T. brucei PLC* microsatellite and the single copy *TbAT1* gene. This could potentially restrict the usefulness of *TbAT1* as a putative marker for arsenical and diamidine resistance in the field. The method has so far been utilised for detection of *T.*

brucei infections and distinguishing between infections due to *T. b. gambiense* and *T. b. rhodesiense* in patient isolates collected from Uganda and Sudan (Picozzi *et al*, 2005). However, a more recent technical advance using the whole genome amplification method has been shown to increase the PCR sensitivity of *T. b. gambiense* blood samples spotted on FTA® cards by 20-fold (Morrison *et al*, 2007).

Chapter 3

3 Microsatellite Marker analysis

3.1 Summary

The aim of this part of the thesis was to investigate the population structure of *T. b. gambiense* using patient isolates collected during August 2005-August 2006 from two separate districts, Arua and Moyo, which are located in the same sleeping sickness focus in NW Uganda. A study indicating mutations in the *TbAT1* gene (encoding the P2 transporter) associated with melarsoprol treatment failure in Arua, Omugo (Matovu *et al*, 2001a;Matovu *et al*, 2001b) associated with 30% failure rate (Legros *et al*, 1999) led to a change of treatment to eflornithine as first line treatment for stage II disease. However, melarsoprol is still used in Moyo as first line treatment since no high treatment failure rates have been recorded. The PCR/*Sfa NI* analysis suggested that the *TbAT1* mutations associated with melarsoprol treatment failure were no longer present (Chapter 4). PCR typing of *T. b. gambiense* patient isolates from Arua (N=54) and Moyo (N=17) using 1 minisatellite and 7 microsatellite markers as described (section 3.3) has been attempted to determine the genetic diversity and any existing genotypic differences in these samples. The findings suggest the two *T. b. gambiense* populations are distinct, although the small sample number for Moyo did not enable further statistically significant analysis. Analysis of the genotype frequencies of the *T. b. gambiense* population from Arua, Omugo, showed a deviation from the Hardy-Weinberg model ($P<0.05$) for all markers with evidence for alleles at all loci except one in linkage disequilibrium suggesting a non random mating population. There was evidence for an excess in homozygotes ($F_{is}=0.315$). This barrier to genetic exchange leading to a reduction in heterozygosity might be due to inbreeding or a number of other factors such as population sub-structuring or migration. In conclusion these data suggests that *T. b. gambiense* from Arua is not panmictic, clonal or epidemic but there is evidence of some level of genetic exchange.

3.2 Introduction

Background

Genetics analysis of *T. brucei* is important in determining parasite population structures and thus to understanding their epidemiology. Studies to explain the extent of genetic

exchange are necessary in order to determine how the parasites adapt to the different environments with-in the human and animal host. It is currently hypothesised that *T. brucei* strains with non-desirable selectable traits such as drug resistance (e.g. melarsoprol) or human serum resistance (e.g. *T. b. rhodesiense* and *T. b. gambiense*) could easily spread with in a population as a result of high rates of genetic exchange. In addition, this is of great importance regarding the choice of treatment should there be an overlap of the two diseases given the limited number of drugs used for the treatment of human disease due to *T. b. gambiense* and *T. b rhodesiense*. Moreover, the genetic diversity of *T. brucei* in the natural population is not fully understood. Results from laboratory and field studies give conflicting results (Gibson W & Stevens J, 1999). The population structure has been studied more extensively in *T. b. brucei* and *T. b. rhodesiense* than in *T. b. gambiense*. This is because of the ease with which the former two can be isolated and propagated in mice and culture unlike *T. b. gambiense*, which manifests with low level parasite densities in natural infections. Owing to the current advances in technology, which include the FTA method for sample collection (Chapter 2), population genetic studies involving *T. b. gambiense* in natural populations using microsatellite marker analysis can now be carried out with ease.

Genetic exchange

T. brucei is capable of undergoing genetic exchange. Generation of hybrids resulting from crosses between a reference *T. b brucei* strain (STIB 247) from an animal and a type II *T. b gambiense* strain (STIB 386) from man was first demonstrated to occur in tsetse (Jenni *et al*, 1986). More studies revealed that hybrids could be obtained from successful crosses in tsetse flies between reference strains of *T. b. brucei* with either *T. b. brucei* (Turner *et al*, 1990;Gibson & Garside, 1991;Schweizer *et al*, 1994;Degen *et al*, 1995) or type II *T. b. gambiense* (Paindavoine *et al*, 1986;Sternberg *et al*, 1988) isolated from man, animals or tsetse. Successful crosses were shown to occur between *T. b. rhodesiense* isolated from man with either *T. b. brucei* strains isolated from animals (Gibson, 1989;Gibson & Garside, 1990;Gibson & Whittington, 1993;Gibson & Bailey, 1994;Gibson *et al*, 1992) or type II *T. b. gambiense* from man (Gibson *et al*, 1997). It was also demonstrated that successful crossing can occur as a result of a back cross (Gibson *et al*, 1995) and self-fertilisation (selfing) can occur in the presence of another strain, as demonstrated for the *T. b. brucei* strain STIB 247 (Tait *et al*, 1996) and the human *T. b. gambiense* strain TH2N (Gibson *et al*, 1997). While genetic exchange can occur under laboratory conditions it is not clear to what extent it occurs in natural populations. A prerequisite for genetic exchange to occur in natural populations is the presence of mixed tsetse infections. To

investigate if mixed infections are common, hypervariable *T. brucei* minisatellite markers were used to examine the isolates made from salivary glands of tsetse flies from SE Uganda (Lugala) and Kenya (Kiboko). More than two alleles were observed at each locus in several uncloned field isolates (36% Lugala and 47% Kiboko) corresponding to the presence of several trypanosome genotypes. Analysis of the isolates from one of the two cloned stocks yielded 7 genotypes further supporting the idea that large numbers of alleles were due to mixed infections in the field. In addition the genotypes observed in the isolates from tsetse were more diverse than those observed in man and animals (MacLeod *et al*, 1999).

Different genetic structures

Three different genetic structures have been earlier described for *T. brucei* (Tait, 1980;Tibayrenc *et al*, 1990;Maynard-Smith *et al*, 1993). Unlike the panmictic structure, the clonal and epidemic structures are characterised by linkage disequilibrium between alleles. The panmictic structure was described for *T. b. brucei*, where extensive unrestricted mating occurs (Tait, 1980) and the frequencies of the resulting genotypes of this type of structure follows the Hardy-Weinberg model with the alleles at different unlinked loci always in linkage equilibrium. The clonal structure was demonstrated for *T. b. rhodesiense* (Tibayrenc *et al*, 1990), where mating is limited i.e. little or no recombination occurs. The epidemic structure described for *T. brucei* (Maynard-Smith *et al*, 1993) is an intermediate between the panmictic and clonal type structures where the clonal expansion of a particular genotype conceals most of the underlying unrestricted mating leading to the detection of the deviation from the Hardy-Weinberg model and a lack of association of alleles.

Methods and case studies analysing genetic diversity in African trypanosomes

To investigate the level of diversity in *T. brucei* populations, different molecular methods such as isoenzyme analysis, restriction fragment length polymorphism (RFLP) (Hide *et al*, 1994), random amplification of polymorphic DNA (RAPD) (Stevens and Tibayrenc, 1995), repetitive DNA probes for southern blotting (Hide *et al*, 2000), minisatellite (Barrett *et al*, 1997;MacLeod *et al*, 1999;MacLeod *et al*, 2000;MacLeod *et al*, 2005a) and microsatellite markers (Biteau *et al*, 2000;MacLeod *et al*, 2005a;MacLean *et al*, 2007;Morrison *et al*, 2008) have been applied. Population genetic studies can also be used to reveal variability between parasite strains or species within the same or different foci. This can lead to the identification of new strains associated with different transmission levels or disease patterns. A study by Hide and colleagues (1994), using RFLP, isoenzyme analysis and the human serum resistance test to determine the variation of *T. brucei* in

isolates (n=88) from man, pigs, cattle and tsetse flies collected during a sleeping sickness epidemic in S.E Uganda revealed considerable diversity in the cattle isolates as observed from the different genotypes. The human isolates were found to be genotypically different from the cattle isolates and less diverse. This observation led to the conclusion that extensive genetic exchange was occurring between the cattle strains in comparison to the human infective strains an indication that possibly the transmitting flies had a feeding preference for cattle in comparison to humans (Hide *et al*, 1994). Since genetic exchange occurs in the fly, and mixed infections are necessary for genetic exchange, a feeding preference for cattle could result in mixed cattle genotypes rather than mixed human genotypes. However, it was not clear whether in the same focus the parasite genotypes circulating during an epidemic period are maintained during an endemic period. Other studies were carried out to address this issue. Analysis of animal *T. b. brucei* and *T. b. rhodesiense* (n=47) and human *T. b. rhodesiense* (n=7) isolates collected during an endemic period (1989, 1993-94) from Busia (Uganda-Kenya border) and during an epidemic period (1988, 1990) from Tororo (Uganda) was performed using the RFLP method (Hide *et al*, 1998). The results indicated limited genetic exchange between the animal strains which was in contrast to the previous findings (Hide *et al*, 1998). However, similar genotypes were observed in strains from Tororo (Uganda) suggesting that these strains were common to this region (Hide *et al*, 1998). Another study using RFLP/DNA probes was performed to compare *T. b. rhodesiense* and *T. b. brucei* isolates collected from infected tsetse in a sleeping sickness free region in Kiboko Kenya (n=7) with *T. brucei* isolates from old stocks collected from man and animals in a sleeping sickness foci in Busoga ,Uganda and Zambia (Hide *et al*, 2000). The findings revealed that the genotypes of the trypanosomes transmitted to man in the Busoga focus were similar to those found in Kiboko indicating a persistence of a common genotype. The non-human infective trypanosomes isolated from tsetse flies in this region were more diverse than the human infective trypanosomes. This suggested that circulation of trypanosomes did occur between geographically different foci (Hide *et al*, 2000). But, this did not rule out the existence of similar trypanosome strains occurring in different geographical regions. Observations from these studies seemed to suggest that *T. b. rhodesiense* had a clonal structure while *T. b. brucei* had either a panmictic or an epidemic type of structure. It was not clear whether *T. brucei* can have only one or more than one type of structure within the same or geographically distinct foci at different time points.

Significant work has been carried out assessing *Plasmodium* population structure and efforts were made to see if these could guide inference on the situation regarding *T. brucei*

which is also a human parasite although their genetic systems are different. For example a study using microsatellite markers to determine the population structure of *Plasmodium falciparum* isolates from humans (n=468) collected from South America, Africa, South Asia/ Pacific from 5 regions of high transmission intensity and 4 regions of low level transmission revealed surprising results (Anderson *et al*, 2000). *P. falciparum* was demonstrated to exhibit different population structures with variations in diversity in each of the different geographical regions (Anderson *et al*, 2000). These differences were associated with either geographical factors such as differences in the epidemiological patterns of this species in different foci or thought to be due to migration.

A similar approach was undertaken using minisatellite markers to compare the population structure of *T. b. rhodesiense* and *T. b. brucei* by analysis of isolates collected from three geographically distinct sleeping sickness foci; Uganda (n=64), Zambia (n = 19) and Nyanza (n = 24) (MacLeod *et al*, 2000). The findings suggested *T. b. brucei* had an epidemic structure which was in contrast to earlier findings where *T. b. rhodesiense* was observed to be clonal (MacLeod *et al*, 2000). Such variation in the apparent population structure may be due to differences in the epidemiological patterns of these species. The observed differences which led to that conclusion differentiated between the two sub-species using the human serum resistance test (Jenni & Brun, 1982) prior to the analysis (unlike earlier analyses in which the two sub-species were grouped as one species (MacLeod *et al*, 2000).

<u>New methodologies applied to analysis of population genetics</u>

New methods for analysing population structure have recently become available. These include identification of novel markers for differentiating between *T. b. rhodesiense* (Welburn *et al*, 2001a;Gibson *et al*, 2002;Radwanska *et al*, 2002a) and *T. b. gambiense* (Radwanska *et al*, 2002b), sequencing of the *T. brucei* genome (Berriman *et al*, 2005), novel *T. brucei* microsatellite markers (MacLeod *et al*, 2005b) and the increased sensitivity of the FTA method for sampling *T. b. gambiense* field isolates (Morrison *et al*, 2007). The AFLP clustering method has been used to analyse laboratory derived *T. brucei* clones (n=6) and uncloned stocks (n=22) isolated from pigs (*T. b. brucei*), cattle (*T. b. brucei*) and man (*T. b rhodesiense*) from a sleeping sickness focus in Mukono, S.E Uganda and old reference stocks (*T. b. brucei* (n=5), *T. b. rhodesiense* (n = 2) and type I *T. b. gambiense* (n=6) from East, West and Central Africa. Relatedness existed between the stocks from the same region and small observable differences existed between stocks from different regions (Agbo *et al*, 2003). This gave an insight into the *T. b. gambiense* structure but the

sample numbers used for this analysis were far too small to draw a firm conclusion. It was reported earlier that *T. b. gambiense* had a clonal type of structure (Tibayrenc *et al*, 1990). With the novel FTA sampling method which avoids selection of parasites when compared to other parasite isolation methods (Aerts *et al*, 1992;Truc *et al*, 1992), it was demonstrated, using microsatellite markers, that *T. b. gambiense* from the Democratic Republic of Congo (n=37) and Cameroon (n=9) was sub-structured, genetically distinct and clonal (Morrison *et al*, 2008). This was in agreement with previous studies. Using microsatellite markers to examine the population structure of *T. brucei* may act as a lead to the identification of specific genotypic strains that may be clonal and possibly responsible for drug resistance, disease virulence or human infectivity. Results from a recent study on *T. b. rhodesiense* isolates from two different sleeping sickness regions in the same focus in E Uganda revealed the isolates to be genotypically distinct (MacLean *et al*, 2007). Further analysis, using levels of inflammatory response as a marker for disease virulence indicated separate disease progression patterns for the isolates from the two regions (MacLean *et al*, 2004;MacLean *et al*, 2007). Using a similar approach it was attempted to investigate the structure of *T. b. gambiense* from NW Uganda and to compare with the findings from the Central African study.

3.3 Materials and Methods

Study area and population

This section includes a description of the study regions in NW Uganda from where the samples for this chapter were collected. The NW Uganda patient samples were collected during a field survey that was coordinated by Dr. Enock Matovu (Makerere University, Uganda).

Field activities in areas of Arua and Moyo districts in North Western Uganda were carried out during the period August 2005 to August 2006. Arua and Moyo district neighbour each other and are separated by Yumbe district. Arua district was bordered by the Republic of Sudan in the north, the Democratic Republic of Congo in the west, Yumbe district in the northeast, Nebbi district in the south and Gulu district in the east. Omugo Health Centre (03.27641°N; 31.11522°S), the main sleeping sickness control centre within Arua, is located in Terego County. It is currently divided into three districts; Arua, Nyadri and Koboko (Figure 3.1). Moyo district is bordered by the River Nile in the south and east, Sudan in the north, and Yumbe district in the west (Figure 3.1). Moyo hospital (03.65055°N; 31.68872°S), the main sleeping sickness control centre within this locality, is

located in Obongi County. In 2002, the population in Arua and Moyo districts was estimated at 860,000 and 200,000 respectively (District information Portal, http://www.dip.go.ug). Currently the figures are higher due to the settlement of displaced refugees from Sudan in N Uganda and Chad as result of civil war.(Hovil & Bagenda, 2003;United Nations Report, 2004). Ethnic groups in this area comprise of the Nilotics (Alur), Nilohamites (Kakwa) and the Sudanic (Lugbara, Lendu and Madi). The main economic activity in the two regions is subsistence agriculture.

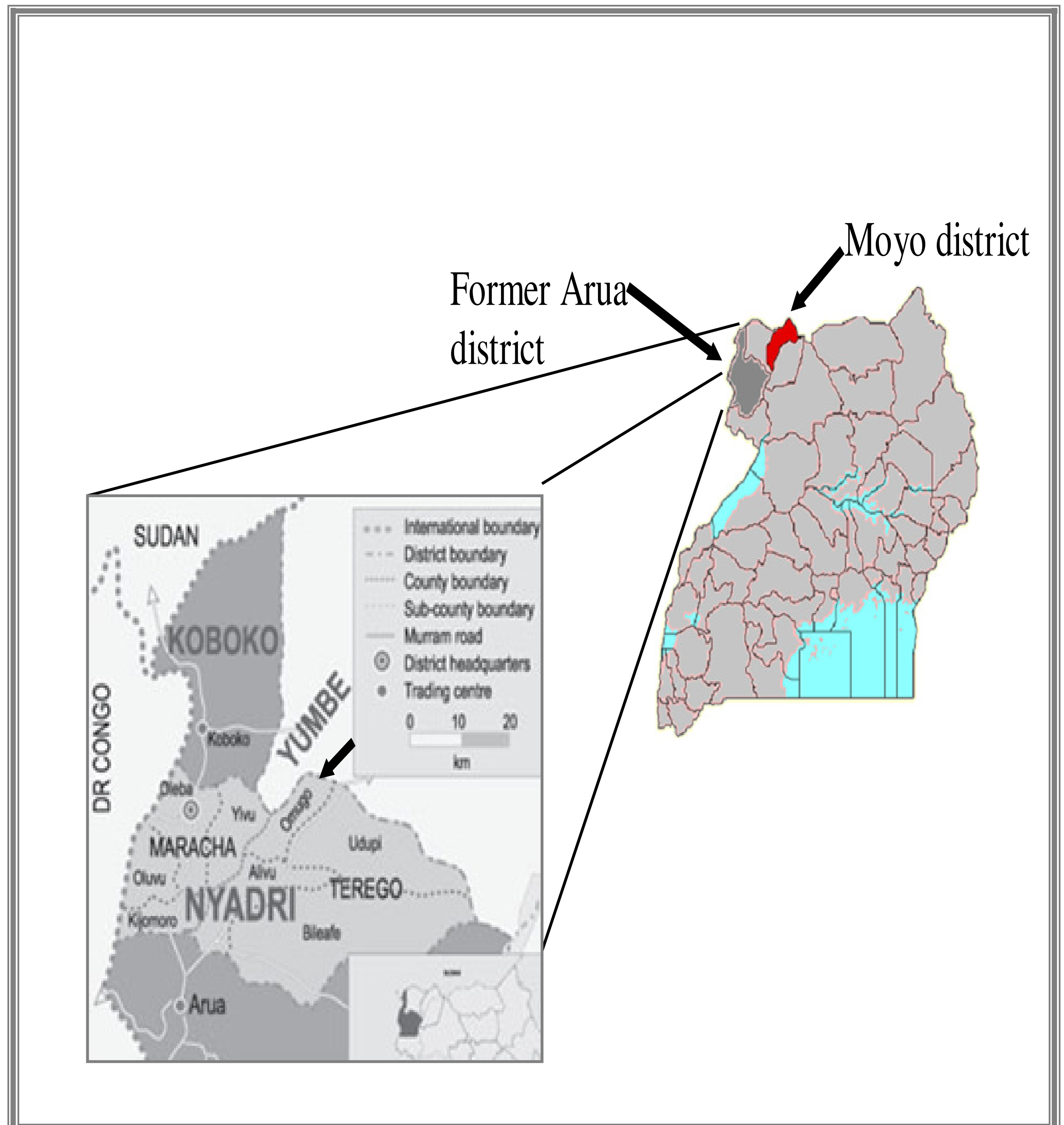


Figure 3.1 Regional Map of Uganda showing the location of the study sites.
Moyo district (shaded red) and former Arua district (shaded dark grey).
The district map of NW Uganda showing the current location of Arua and two other created districts (Nyadri and Koboko).
Omugo Health Centre, the HAT control unit in Omugo (black arrow) is located in Terego County.(www.newvision.co.ug/D/8/13/549160).

Patient screening and diagnosis

In NW Uganda, active surveillance of populations at risk of HAT as determined from hospital records was carried out at designated screening centres in areas of active transmission by Mobile teams made up of personnel from the National Agricultural Livestock Health Research Institute, Vector Control Office, Omugo Health Centre and Moyo Hospital with the help of Sleeping Sickness Assistants (SSAs) and local village leaders. I was not personally involved in the diagnosis of the HAT patients. However, below is a description of the protocol that was used for the diagnosis of the patients that were used for my studies. Screening and diagnosis of patients in northwest Uganda was carried out according to an established diagnostic tree, which was adapted from the Medecens San Frontiers (Figure 3.2). All presenting persons were screened for presence of trypanosome antibodies using the Card Agglutination Test for Trypanosomiasis-CATT (Magnus *et al*, 1978). The cards were obtained from the Institute of Tropical Medicine Antwerp, Belgium (ITM) and provided as a donation by the World Health Organisation (WHO). Whole blood tested by CATT was taken by finger prick and later collected by venipuncture in heparinised vacutainer tubes. The test has been used in a number of studies for the serodiagnosis of *T. b gambiense* sleeping sickness (Blum *et al*, 2005;Robays *et al*, 2004;Balasegaram *et al*, 2006;Garcia *et al*, 2000). Briefly, 50 µl of whole blood was dropped onto a plastic card. A drop of reconstituted CATT reagent (suspension of fixed stained and stabilised blood form trypomastigotes of well-defined serotypes) was added to the blood and mixed well. The card was agitated for 5 minutes at room temperature. Positive and negative controls from the kit were included. Agglutination indicated the presence of trypanosome antibodies suggestive of *T. b. gambiense* while absence of agglutination indicated absence of a trypanosome infection. Antibody levels of the CATT positive individuals were also determined using 1/4 and 1/8 dilutions of their blood plasma diluted in CATT buffer.

Diagnosis of CATT positive persons was carried out using parasitological methods (Microscopy) for the detection of trypanosomes at the respective health unit; Omugo Health Centre (Arua) and Moyo Hospital (Moyo). Wet smears of gland aspirates from individuals with swollen lymph nodes were examined for motile trypanosomes using bright field microscopy under oil immersion at 400 × magnification.

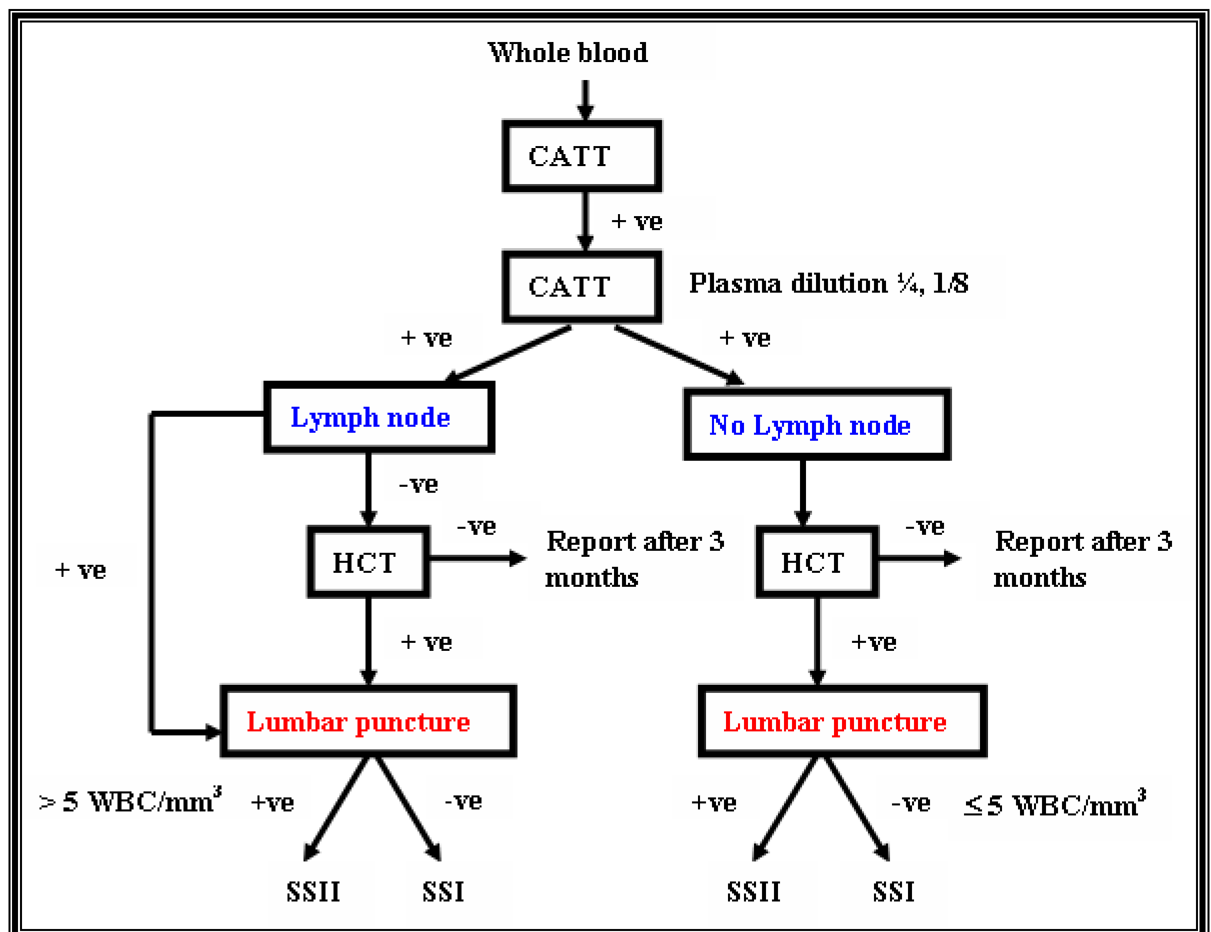


Figure 3.2 A Schematic showing the screening and diagnostic tree used in NW Uganda. CATT, Card Agglutination Test for Trypanosomiasis. HCT, Haematocrit Centrifugation Test. Wet smears were performed on swollen lymph node aspirates and lumbar punctures were for obtaining cerebral spinal fluid.
+ve, positive result for trypanosome antibodies by CATT and/or +ve result for trypanosomes by microscopy. -ve, negative result for trypanosomes by microscopy.
SSI, sleeping sickness stage I. SSII, sleeping sickness stage II.
WBC, white blood cell cerebral spinal fluid count.
(Adapted from Médecins Sans Frontièrs- (Chappuis *et al*, 2005).

Blood from individuals with or without swollen lymph glands was subjected to the Haematocrit Centrifugation Technique (HCT) (Woo & Rogers, 1974). Briefly, blood was drawn into capillary tubes, which were sealed with plasticine and centrifuged in a haematocrit centrifuge at 9000 × g for 5 minutes. The capillaries were examined for the presence of motile parasites by microscopy under oil immersion at 160 × magnification. Presence of trypanosomes in the lymph aspirates or blood was taken as confirmation that the sero-reactive individual was indeed a HAT case. A lumbar puncture for collection of cerebrospinal fluids (CSF) to check for central nervous system (CNS) invasion was performed. Briefly, 3 ml of the CSF was centrifuged at 300 × g for 5 minutes and the pellet was drawn into capillary tubes which were sealed for the second centrifugation followed by microscopic examination. In addition, a 50 µl aliquot of CSF was applied on the

Rosenthal chamber to estimate the white blood cell count (WBC). Patients with demonstrated presence of trypanosomes in CSF or with an elevated WBC of > 5/mm^3 were considered to be late stage cases otherwise they were considered as early stage cases if they had no trypanosomes in CSF and a CSF WBC of ≤ 5/mm^3. Staging of the confirmed HAT patients was used to determine the drug to be administered. Passive surveillance was carried out when individuals feeling unwell reported to the respective health units and found positive after screening and diagnosis. Most of the late stage cases were detected by this mode. Sero-reactive but parasitologically negative individuals were asked to report for re-testing after 3 months. Informed consent was obtained from all confirmed HAT patients before enrollment in this study, who were admitted and hospitalized for the whole duration of treatment.

Patient sample collection

Blood samples for molecular analysis were collected before treatment from parasitologically confirmed HAT patients from NW Uganda from whom informed consent was obtained before enrolment in the study. A total of 133 samples were collected from NW Uganda of which 120 were collected on my behalf by trained personnel. The samples were collected in at least two different forms as either whole blood spotted onto FTA® cards (n=89) or whole blood (n=83) or cerebral spinal fluid (n=90). Briefly, 2 ml of the infected whole blood from each patient was spotted onto 4 spots (500 µl per spot) of an FTA® card (Whatman), air dried and stored in a self-sealing plastic bag at room temperature for later molecular analysis. All samples were then stored at room temperature to await transportation for analysis to the Molecular Biology Laboratory, Makerere University, Kampala, Uganda and the Institute of Biomedical and Life Sciences, University of Glasgow, UK.

DNA extraction

Extraction of DNA from blood spotted on FTA cards was carried out as described (section 2.3). Genomic DNA extraction from whole blood and cerebral spinal fluid was carried out partially for easy transportation to the laboratory using a commercial kit (Puregene®, Gentra Systems, MN, USA). Briefly, to 500 µl of whole blood in a 2 ml microfuge tube was added 1.5 ml of red blood cell (RBC) lysis solution mixed and incubated at room temperature for 10 minutes. This was followed by centrifugation in a desktop centrifuge at 13,000 × g for 5 minutes, the supernatant was discarded and the pellet resuspended in 300 µl of cell lysis solution. The quantity of CSF collected determined how much cell lysis

solution was added. To 50 µl of CSF was added 550 µl of cell lysis solution directly or 300 µl of cell lysis solution was used to resuspend the pellet recovered after centrifugation of the CSF. Extraction was completed using the commercial Puregene® purification kit following the manufacturer's instructions (Gentra Systems, MN, USA). Proteinase K (100 µg/ml) was added to the samples in cell lysis buffer, mixed by pipetting and incubated at 55°C for 1 hour. For removal of protein, 200 µl of protein precipitation solution was added, mixed gently by inversion and the sample was incubated on ice for 5 minutes. After centrifugation of the suspension at 13,000 × g for 5 minutes, the supernatant was collected into a sterile microfuge tube and DNA precipitation and pellet washing was carried out by adding 400 µl each of isopropanol then 70% ethanol and centrifuged at 13,000 × g for 1 min. After air- drying the DNA pellet for 15 minutes, it was dissolved in 20 µl of DNA hydration solution and left to rehydrate overnight at room temperature or at 65°C for 1 hour. Up to 5 µl of the rehydrated DNA was used immediately in PCR or kept at -20°C.

Whole genome amplification

To increase the PCR sensitivity of the DNA samples on FTA® cards (Whatman) and genomic DNA from whole blood or CSF, the whole genome amplification method was carried out as described (Morrison *et al*, 2007) using a commercial kit (GenomiPhi™, Amersham, Biosciences Co., Piscataway, NJ, USA). Reactions were carried out in triplicate for each individual sample following the manufacturer's instructions. Briefly, a DNA sample was denatured prior to amplification. To a DNA sample on an FTA® card disc was added 10 µl of sample buffer in a sterile microfuge tube, which was heated to 95°C for 3 minutes in a heating block and then cooled to 4°C on ice. Alternatively, to 1 µl of DNA sample from whole blood or CSF (at least 1 ng/µl) was added 9 µl of sample buffer. For each amplification reaction, 9 µl of reaction buffer was combined with 1 µl of enzyme mix on ice added to the cooled sample. The sample was then incubated at 30°C in a heating block for 16-18 hours. Following the overnight incubation, the sample was heated to 65°C for 10 minutes in a heating block to inactivate the enzyme and immediately cooled to 4°C. The 3 separate reactions for each individual sample were pooled. 1-2 µl of whole genome amplified DNA was ready for use in PCR or kept at -20°C.

Microsatellite marker typing of NW Uganda field isolates

Microsatellite analysis is a molecular method that has proved useful in determining the genotypic differences or similarities between parasite populations leading to understanding of their population structures (Biteau *et al*, 2000;Hide *et al*, 2000;MacLeod *et al*,

2000;MacLeod *et al*, 2005a). 182 chromosome markers which span all of the 11 megabase chromosomes that make up the *T. brucei* genome have been identified (MacLeod *et al*, 2005b). PCR primers based on some of these individual chromosome markers have been used (MacLeod *et al*, 2005a;MacLean *et al*, 2007;Morrison *et al*, 2008). Nested PCR amplifications of the chromosome (Ch) markers Ch1/18, Ch5/JS2, Ch1/D2/7, Ch11/110 and Ch3/IJ15/1 were carried out using outer and inner microsatellite oligonucleotide primers (MacLean *et al*, 2007;Morrison *et al*, 2008). Amplifications were also carried out for two new microsatellite markers Ch11/51, Ch7/B and one new minisatellite marker Ch10/3778. Outer and nested oligonucleotide primer sequences and the different allele sizes for each chromosome marker are shown in. One of every 5'-primer of each inner primer pair with an exception of the minisatellite marker Ch10/3778, was FAM modified by labelling with a fluorescent dye to enable accurate sizing of products by computer analysis as described below under allele size determination. Out of 133 patient samples collected from NW Uganda, 54 samples from Arua district and 17 from Moyo district were successfully analysed.

In a primary PCR, 2 µl of whole genome amplified DNA was PCR amplified in a total volume of 20 µl containing a custom PCR mastermix (45 mM Tris-HCl (pH 8.8), 4.5 mM $MgCl_2$, 11 mM $(NH_4)_2SO_4$, 0.113 mg/ml BSA, 4.4 µl EDTA and 1 mM each deoxynucleotide triphosphate- dATP, dCTP, dGTP, dTTP) (ABgene®, Advanced Biotechnologies Ltd, UK). For each chromosome marker an outer primer pair (Table 3.1) synthesised by MWG Biotech-AG, each at 1 µM and 1.25 units of GoTaq DNA polymerase enzyme (Promega Corporation, Madison, USA) were added to the reaction. The amplification conditions for the PCR reactions were as follows: initial denaturation at 94°C for 4 minutes, followed by denaturation at 94°C for 30seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 1 minute for 25 cycles. This was followed by a last extension at 72°C for 7 minutes. Amplifications were carried out in a PTC-200™ DNA engine (MJ Research, inc., Waltham, MA, USA). Genomic DNA from reference strains *T. b. gambiense* reference isolate (ELIANE) (Pays *et al*, 1983) or *T. b. brucei* reference isolate (427) (Cross, 1975) was included in each PCR run. An inner PCR was performed for each chromosome marker with a nested oligonucleotide primer pair (Table 3.1) using 1µl of undiluted primary PCR product or 1µl of 1:100 dilution as template under the same primary PCR and amplification conditions.

Primer pair	Sequence (5'-3')	T_m	Allele sizes (approx.)
Ch1/18-outside C	5'-tataatgcgtttgtgagaat-3'	49.1°C	145-224bp
Ch1/18-outside D	5'-gaagggagggaacagaagcaggg-3'	66.0°C	
Ch1/18-nestedA FAM	5'-tgtgagaatggtactcacgcgctg-3'	64.4°C	
Ch1/18-nested B	5'-cctgttggccgcattattcgatgc-3'	64.4°C	
Ch5/JS2-ourtside C	5'-agtaatgggaatgagcgtcaccag-3'	62.7°C	89-253bp
Ch5/JS2-outside D	5'-gatcttcgcttacacaagcggtac-3'	62.7°C	
Ch5/JS2-nested A FAM	5'-gattggcgcaacaactttcacatacg-3'	63.2°C	
Ch5/JS2-nested B	5'-ccctttcttccttggccattgttttactat-3'	64.0°C	
Ch1/D2/7-outside C	5'-acattttggttgtcctgttgg-3'	55.9°C	121-246bp
Ch1/D2/7-outside D	5'-gatcgaagataataaatgcacat-3'	53.5°C	
Ch1/D2/7-nested CC FAM	5'-gttggtcgcattattcgatgc-3'	57.9°C	
Ch1/D2/7-nested DD	5'-gacaaataacacacaggtgcacca-3'	61.0°C	
Ch11/110-outsideC	5'-tttctacgtctcatttcaacg-3'	54.0°C	168-228bp
Ch11/110-outsideD	5'-actgcacaaacaggtaagcag-3'	57.9°C	
Ch11/110-nested A FAM	5'-gatgcgaggattatgactgtagcg-3'	62.7°C	
Ch11/110-nested B	5'-ccaatcttatgcatacatgcaagc-3'	59.3°C	
Ch3/IJ15/1-outside C	5'-aggcttagacgagtgtcagg-3'	59.4°C	102-154bp
Ch3/IJ15/1-outside D	5'-gtaaatagacacagtgaaaccg-3'	56.5°C	
Ch3/IJ15/1-nested A FAM	5'-gttaggttacgcaagtcagt-3'	55.3°C	
Ch3/IJ15/1-nested B	5'-gaaacactcagttccacacc-3'	57.3°C	
Ch11/51-outside A	5'-cttacccacagggccaaa-3'	56°C	92-104 bp
Ch11/51-outside B	5'-tgagatggtacttgaagaaag-3'	54.0°C	
Ch11/51-nested A	5'-aaccgatcattcctgttcc-3'	54.5°C	
Ch/51-nested B FAM	5'-tgagatggtacttgaagaaag-3'	54.0°C	
Ch7/B-outside A	5'-aagtggttgcgatgcggt-3'	56.0°C	133-156 bp
Ch7/B-outside B	5'-cctagcgcataggaatgaaacc-3'	60.3°C	
Ch7/B-nested A	5'-tatgaaatgaagtgaagtg-3'	48.0°C	
Ch7/B-nested B FAM	5'-ctcgcagaagtaaattaaata-3'	52.8°C	
*Ch10/3778-outside A	5'cataagacaccactctccag-3'	57.3°C	Approx. 100 bp size repeats (10)
*Ch10/3778-outside B	5'-cctttgtctgacttaatgcg-3'	55.3°C	
*Ch10/3778-nested A	5'-caacagtgcccggaggaggtacg-3'	67.8°C	
*Ch10/3778-nested B	5'-gcggtgactcgtccgtcttcttgc-3'	67.8°C	

Table 3.1 *T. brucei* oligonucleotide PCR micro/*mini satellite primers and their properties. The Table shows the outside PCR primer pairs (C and D) or (A and B) and the inner or nested primer pairs (A and B or CC and DD) for the different chromosome markers. New chromosome markers (text in green). FAM, fluorescently labelled 5' inner–primer. T_m, primer melting temperature as calculated by the oligonucleotide manufacturer.

Agarose gel electrophoresis and allele size determination

A volume of 8 µl of each inner PCR product, mixed with 1-2 µl of 6 × Blue loading dye (Appendix I), was separated by Agarose gel electrophoresis on a 3% Agarose gel stained with 0.7 µg/ml ethidium bromide in 1 × TAE buffer (Appendix I) and visualised as described (section 2.3). The approximate product band sizes as displayed in Table 3.1 and

were determined by comparison with a standard 100 bp molecular weight DNA marker (Promega Corporation, Madison, USA). For accurate allele size determination to a level of 1 bp, 1µl of each PCR product, which was fluorescent as a result of the FAM-labelled primer was diluted in water (1: 500, 1:250, 1:100 or 1:50 depending on its in gel-intensity) and gene scanned at the Dundee Sequencing services unit. Briefly, 15 µl of diluted PCR product was put in each run and separated in a capillary sequencer (ABI 3100 Genetic Analyser, Applied Biosystems). Fluorescent ROX-labelled standard size markers (GS500 markers, Applied Biosystems) were included in each run for comparison with the product sizes. The Peak Scanner software version 1.0 (Applied Biosystems) was used for reading the peak traces corresponding to each sample allele product size. For each chromosome locus, a single peak represented a homozygous allele while a double peak represented heterozygous alleles for that particular marker.

Microsatellite data analysis

For each isolates' peak trace results at each of the 7 out of 8 chromosome loci, an allele or alleles present were binned in groups according to given specific numbers (Table 3.2). Briefly, a specific allele number was designated to each PCR product or allele (size not always exactly the same for different samples) as detected by the Peak scanner for each locus. For the two populations in NW Uganda, Arua and Moyo, analysis was performed for the 8 chromosome loci using the bin data. To determine the similarities between the genotypes for the two populations, the data analysis was performed using the free Genetic Data Analysis (GDA) program (Lewis & Zaykin, 1999) for the input data to calculate the allele frequencies for each genotype at each loci and to calculate Nei's genetic distance (D) between the Arua (N=54) and Moyo (N=17) populations. The statistical analysis was limited to only the Arua population, which had a reasonable sample size. Using the GDA program, the Fixation index (F_{is}) or inbreeding coefficient for the genotypes at each of the 8 heterozygous chromosome loci (He-Ho)/Ho (where He is the expected allele frequency of heterozygosity and Ho is the observed frequency of heterozygosity) was calculated. Linkage analysis between genotypes at individual loci and the 23 pairwise locus combinations for Hardy-Weinberg equilibrium and linkage disequilibrium to determine the probability of whether mating was random or due to chance was tested using the GDA program. Further comparison between sample genotypes was done using the clustering calculator program http://www2.biology.ualberta.ca/jbrzusto/cluster.php, which generated the bootstrap values and the Phylip drawtree string values for only 29 samples with 5 complete chromosome loci data sets. The TreeView free downloadable program, which converted the drawtree string values, was used for drawing the dendogram

http://taxonomy.zoology.gla.ac.uk/rod/treeview.html. Linkage disequilibrium was also tested using another program Linkage Analysis 3.0 (LIAN) (Haubold and Hudson, 2000) to calculate the Standardised index of Association ($I^{S}A$) as a measure of the level of mating.

Allele No.	1/18	5/JS2	1/D2/7	11/110	3/IJ15/1	10/3778	11/51	7/B
1	160-4 bp	127-34 bp	227-228 bp	168-72 bp	136 bp	**300 bp**	**97-8 bp**	**133 bp**
2	167-71 bp	167-171 bp	298-301 bp	210-2 bp	115 bp	**400 bp**	**104 bp**	**142 bp**
3	224 bp	204-6 bp	231-4 bp	179 bp	144-146 bp	**800 bp**	**92 bp**	**137-9 bp**
4	175-8 bp	95-101 bp	307-308 bp	200 bp	126 bp	**900 bp**		**147-8 bp**
5	145-6 bp	107-110 bp	219-224 bp	174 bp	154 bp			**156 bp**
6	153-4 bp	123-5 bp	147-148 bp	191 bp	**102-104 bp**			
7	219 bp	156 bp	137-139 bp	**187-8 bp**	**132 bp**			
8	**158 bp**	89-92 bp	280 bp	**182 bp**	**120 bp**			
9		162-4 bp	167-73 bp	**228 bp**				
10		182-3 bp	247-51 bp	**197 bp**				
11		104 bp	190 bp	**204 bp**				
12		115 bp	194 bp	**185 bp**				
13		137 bp	**202 bp**					
14		214-18 bp	**242-6 bp**					
15		**141-3 bp**	**180 bp**					
16		**237-9 bp**	**213 bp**					
17		**241-2 bp**	**140-2 bp**					
18		**253 bp**	**176-9 bp**					
19		**137 bp**	**162 bp**					
20		**221 bp**	**207 bp**					
21		**158 bp**	**262 bp**					
22			**107 bp**					
23			**121 bp**					

Table 3.2 Alleles present at each loci and their specific allele bin number (No.)
New microsatellite alleles (black bold) and old existing alleles (not bold**) encountered at 7 chromosome loci from the peak scan traces. Alleles encountered at minisatellite loci 10/3778 based on signal as observed in agarose.**
(Dr A. MacLeod, University of Glasgow, with permission).

3.4 Results

3.4.1 Genetic analysis for variation or diversity

Sleeping sickness patient's trypanosome DNA fixed on FTA® cards (Whatman) was extracted as described (section 2.3) and whole genome amplified to increase the sensitivity (section 3.3). The trypanosome DNA was used in microsatellite/minisatellite analysis to identify those alleles present in parasites in the population. For both populations, Arua, Omugo (n=54) and Moyo (n=17), two or more alleles were observed at each of the eight loci for all of the isolates analysed by PCR typing of microsatellite and minisatellite markers (section 3.3). Determination of accurate allele sizes was performed by the Dundee sequencing services using a capillary based sequencer (ABI 3100 Genetic Analyser, Applied Biosystems) and a Peak Scanner software version 1.0 (Applied Biosystems) was used for reading the peak sizes as described (section 3.3). However, one marker (Ch10/3778) was analysed by agarose gel electrophoresis because it amplified about ten 100 bp repeats, which are difficult to size accurately using the Peak Scanner software. For both populations, the alleles observed for each isolate at each chromosome locus were grouped together by binning and given specific numbers as detailed in Table 3.2. The genotypes present for each individual sample from the Arua and Moyo populations for each of the 8 markers are shown in Table 3.3 and Table 3.4 respectively. A summary of the different genotypes and their frequency for both populations is shown in Table 3.5 and Table 3.6 respectively.

Sample code	Genotype present at each chromosome locus							
	Ch1/18	Ch5/JS2	Ch1/D2/7	Ch11/110	Ch3/IJ15/1	Ch10/3778	Ch11/51	Ch7/B
OM46	1/2	1/1	n.d	7/2	7/7	1/1	1/1	n.d
OM48	1/1	1/1	10/10	1/1	1/1	1/2	2/2	n.d
OM51	1/2	1/14	13/13	n.d	n.d	2/2	1/1	n.d
OM55	1/2	15/16	14/14	1/1	1/7	1/2	1/1	1/1
OM58	1/2	1/1	14/14	n.d	1/1	1/1	n.d	n.d
OM62	1/2	15/16	10/10	1/7	1/7	1/2	1/2	2/2
OM66	1/2	15/15	10/10	8/8	7/7	n.d	n.d	n.d
OM69	1/1	15/15	10/10	1/7	1/1	2/2	1/1	3/3
OM70	1/2	15/15	10/10	7/7	n.d	n.d	n.d	n.d
OM72	2/2	1/14	14/14	1/1	7/7	n.d	2/2	n.d
OM77	1/1	1/1	10/10	1/1	1/7	n.d	n.d	1/1
OM79	1/2	n.d	9/9	7/7	1/1	2/2	3/1	n.d
OM80	1/1	8/4	n.d	9/9	8/8	n.d	3/3	3/4
OM81	1/1	n.d	15/15	1/10	1/1	n.d	2/2	n.d
OM83	1/1	8.4	9/16	9/9	8/8	3/3	n.d	4/5
OM85	1/1	8/4	n.d	n.d	8/1	n.d	n.d	n.d
OM86	1/1	15/14	9/16	1/7	7/1	1/2	1/2	2/2
OM87	1/2	15/17	17/14	1/1	7/1	1/2	1/2	2/2
OM88	1/1	8/4	9/16	9/9	8/8	3/3	3/3	4/5
OM89	n.d	17/17	14/14	1/1	1/1	1/1	2/2	n.d
OM90	1/2	15/17	14/14	1/7	7/7	3/3	n.d	2/2
OM92	1/8	8/4	9/16	1/7	7/7	1/2	1/2	2/2
OM93	1/1	n.d	9/16	1/1	7/7	2/2	2/2	n.d
OM95	1/2	4/4	n.d	7/7	n.d	n.d	n.d	n.d
OM96	1/1	18/18	9/16	1/7	7/7	1/1	1/2	2/2
OM97	1/1	4/4	10/10	1/7	7/1	1/1	n.d	n.d
OM100	1/2	15/15	n.d	1/7	7/7	n.d	1/2	2/2
OM105	1/1	1/1	n.d	7/7	1/1	n.d	1/2	n.d
OM106	2/2	4/4	n.d	7/7	n.d	1/2	n.d	2/2
OM108	1/1	8/4	15/15	n.d	1/1	n.d	n.d	2/2
OM110	1/1	n.d	9/9	1/7	7/7	1/2	n.d	2/2
OM112	1/1	4/4	9/16	9/9	8/8	3/3	3/3	4/5
OM115	1/1	15/16	9/16	1/7	1/7	1/2	1/2	2/2
OM116	1/2	n.d	10/10	1/7	1/7	1/2	1/2	2/2
OM117	1/2	n.d	14/14	1/7	1/7	1/2	1/2	2/2
OM118	1/2	16/16	19/18	7/7	7/7	2/2	2/2	n.d
OM119	1/2	15/14	7/7	1/7	7/7	1/1	1/2	2/2
OM120	n.d	n.d	3/3	n.d	1/1	2/2	1/1	n.d
OM121	1/2	1/1	17/20	1/7	1/1	n.d	n.d	n.d
OM122	1/2	15/14	14/14	1/7	1/7	1/2	n.d	2/2
OM123	1/2	15/16	14/14	1/7	1/7	2/2	n.d	2/2
OM126	1/2	15/15	14/14	1/7	1/7	1/2	1/2	n.d
OM127	1/2	19/20	14/14	1/7	1/7	1/2	1/2	2/2
OM129	1/2	n.d	10/10	1/7	1/7	n.d	1/2	2/2
OM130	1/2	n.d	10/10	1/7	1/7	2/2	1/2	2/2
OM131	1/2	16/16	10/10	1/7	1/7	1/1	n.d	2/2
OM132	1/1	1/20	10/10	7/7	7/7	n.d	n.d	n.d
OM133	1/2	15/15	21/21	7/7	n.d	n.d	n.d	2/2
OM140	1/2	15/16	14/14	7/7	n.d	1/1	n.d	n.d
OM142	1/2	15/15	n.d	1/1	7/7	n.d	n.d	n.d
OM143	1/2	15/15	10/10	1/7	1/1	1/2	1/2	n.d
OM145	1/2	21/21	10/10	7/7	7/7	1/2	n.d	n.d
OM147	1/2	15/15	10/10	1/7	n.d	n.d	1/1	n.d
OM148	1/2	15/21	10/10	1/7	1/7	1/1	1/2	2/2

Table 3.3 Multilocus allele genotype data for the Arua population (N=54) at 8 loci. Allele types for 7 loci (after binning based on signals as determined from Peak scanner trace results) with the exception of locus Ch10/3778 (after binning based on signals as observed in agarose).
Specific allele numbers as allocated to the different size PCR products after binning (Table 3.2). n.d, no product detected in agarose (or missing data).

Sample code	Genotype present at each Chromosome locus							
	Ch1/18	Ch5/JS2	Ch1/D2/7	Ch11/110	Ch3/IJ15/1	Ch10/3778	Ch11/51	Ch7/B
M117	1/2	n.d	n.d	n.d	n.d	2/2	½	2/2
M135	n.d	n.d	n.d	11/11	n.d	2/2	n.d	1/1
M139	1/1	4/4	22/23	10/10	1/7	n.d	2/2	1/1
M140	1/1	n.d	n.d	n.d	1/1	n.d	1/2	2/2
M145	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
M146	2/2	8/6	n.d	n.d	n.d	4/4	n.d	2/2
M148	1/1	n.d	n.d	1/1	1/1	2/2	2/2	2/2
M152	1/2	8/5	n.d	n.d	1/1	n.d	1/1	n.d
M153	1/2	1/1	14/14	10/10	1/1	1/1	n.d	n.d
M155	1/2	n.d	14/14	1/7	n.d	1/2	1/2	n.d
M156	1/1	1/14	n.d	7/7	1/1	n.d	2/2	2/2
M167	1/2	n.d	n.d	n.d	n.d	n.d	n.d	n.d
M169	n.d	4/4	n.d	1/1	n.d	n.d	n.d	n.d
M175	2/2	4/4	10/10	n.d	3/3	n.d	n.d	n.d
M176	1/2	19/19	14/14	n.d	n.d	2/2	n.d	n.d
M177	1/1	1/1	14/14	7/7	1/1	1/1	n.d	n.d
M181	1/2	4/1	n.d	1/1	1/1	1/1	n.d	2/2

Table 3.4 Multilocus allele genotype data for the Moyo population (N=17) at 8 loci
Allele types for 7 loci (after binning based on signals as determined from Peak scanner trace results) with the exception of locus Ch10/3778 (after binning based on signals as observed in agarose).
Specific allele numbers as allocated to the different size PCR products after binning (Table 3.2). n.d, no product detected in agarose (or missing data).

Region	Genotypes at each chromosome locus / (number of samples)							
Arua	**1/18**	**5/JS2**	**1/D2/7**	**11/110**	**IJ15/1**	**3778**	**11/51**	**7/B**
	1/1(18)	1/1(6)	10/10(16)	**7/2**(1)	**7/7**(13)	1/1(9)	1/1(6)	1/1(2)
	1/2(31)	1/14(2)	**13/13**(1)	1/1(8)	1/1(11)	1/2(16)	2/2(6)	2/2(22)
	2/2(2)	**15/16**(5)	14/14(11)	1/7(25)	1/7(18)	2/2(8)	1/2(17)	**3/3**(1)
	1/8(1)	**15/15**(9)	**9/9**(2)	**8/8**(1)	**8/8**(4)	**3/3**(4)	**3/1**(1)	**3/4**(1)
		8/4(6)	**15/15**(2)	7/7(9)	**8/1**(1)		**3/3**(3)	**4/5**(3)
		15/14(3)	**9/16**(8)	**9/9**(4)				
		15/17(2)	**17/14**(1)	**1/10**(1)				
		17/17(1)	**19/18**(1)					
		4/4(4)	**7/7**(1)					
		18/18(1)	**3/3**(1)					
		16/16(2)	**17/20**(1)					
		19/20(1)	**21/21**(1)					
		1/20(1)						
		21/21(1)						
		15/21(1)						
total(N)	**52**	**45**	**46**	**49**	**47**	**37**	**33**	**29**

Table 3.5 Genotypes present in Arua at each individual chromosome locus. Genotypes unique within the Arua *T. b. gambiense* population (Bold red). Genotypes present in both Arua and Moyo *T. b. gambiense* populations (not bold). N, total number of samples successfully analysed for each chromosome marker.

Region	Genotypes at each chromosome locus / (number of samples)							
Moyo	**1/18**	**5/JS2**	**1/D2/7**	**1/110**	**IJ15/1**	**3778**	**11/51**	**7/B**
	1/1(5)	1/1(2)	10/10(1)	1/1(3)	1/1(1)	1/1(3)	1/1(1)	1/1(2)
	1/2(7)	1/14(1)	14/14(4)	1/7(1)	1/7(7)	1/2(1)	2/2(3)	2/2(6)
	2/2(2)	4/4(3)	**22/23**(1)	7/7(2)	**3/3**(1)	2/2(4)	1/2(3)	
		8/6(1)		**10/10**(2)		**4/4**(1)		
		8/5(1)		**11/11**(1)				
		19/19(1)						
		4/1(1)						
total (N)	**14**	**10**	**6**	**9**	**9**	**9**	**7**	**29**

Table 3.6 Genotypes present in Moyo at each individual chromosome locus. Genotypes unique within the *T. b. gambiense* Moyo population (Bold black). Genotypes present in both Arua and Moyo *T. b. gambiense* populations (not bold). N, total number of samples successfully analysed for each chromosome marker.

Microsatellite markers Ch1/18, Ch3/IJ15/1, Ch10/3778, and Ch11/51 were observed to be relatively homogenous for both populations with one or two predominant alleles present in all of the isolates. Markers Ch5/JS2, Ch1/D2/7, Ch11/110 and Ch7/B were more polymorphic, especially within the Arua population, indicating a high level of diversity as observed from the number of alleles and genotypes as summarised (Table 3.7). This difference in the populations could be due to the different sample sizes.

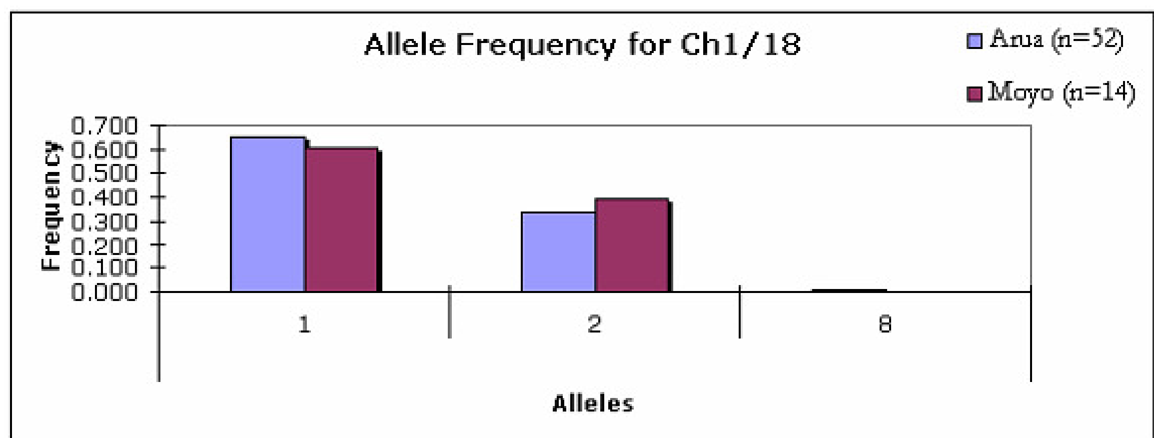
Population	Locus	Sample number (n)	Number of alleles (Na)	Number of genotypes (Ng)
Moyo	Ch1/18	14	2	3
(N=17)	Ch5/JS2	10	7	7
	Ch1/D2/7	6	4	3
	Ch11/110	9	4	5
	Ch3/IJ15/1	9	3	3
	Ch10/3778	9	3	4
	Ch11/51	7	2	3
	Ch7/B	8	2	2
Arua	Ch1/18	52	3	4
(N=54)	Ch5/JS2	45	11	15
	Ch1/D2/7	46	13	12
	Ch11/110	49	6	7
	Ch3/IJ15/1	47	3	4
	Ch10/3778	37	3	4
	Ch11/51	33	3	5
	Ch7/B	29	5	5

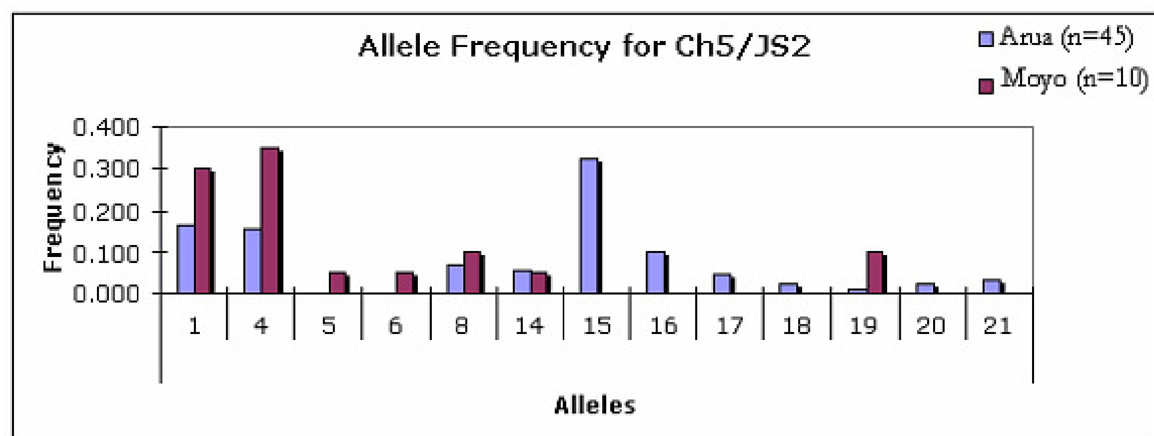
Table 3.7 Diversity of alleles and genotypes observed at each locus in both populations. The data shows the polymorphism of the different markers.
N, Population sample size. n, number of samples with a positive signal.
Na, number of alleles observed based on peak scan traces (7 loci) and in agarose (locus 10/3778). Ng, number of genotypes present at each loci.

The frequencies of the alleles present within each population at each locus which were calculated using the Genetic Data Analysis (GDA) program (Lewis & Zaykin, 1999) http://hydrodictyon.eeb.uconn.edu/people/plewis/software.php and are presented in Table 3.8 and displayed as histograms in Figure 3.3, Figure 3.4 and Figure 3.5.

Locus	Allele	Arua Allele frequency	Moyo Allele frequency
Ch1/18	1	0.654	0.607
	2	0.337	0.393
	8	0.010	0.000
Ch5/JS2	1	0.167	0.300
	4	0.156	0.350
	5	0.000	0.050
	6	0.000	0.050
	8	0.067	0.100
	14	0.056	0.050
	15	0.322	0.000
	16	0.100	0.000
	17	0.044	0.000
	18	0.022	0.000
	19	0.011	0.100
	20	0.022	0.000
	21	0.033	0.000
Ch1/D2/7	3	0.022	0.000
	7	0.022	0.000
	9	0.130	0.000
	10	0.348	0.167
	13	0.022	0.000
	14	0.250	0.667
	15	0.043	0.000
	16	0.087	0.000
	17	0.022	0.000
	18	0.011	0.000
	19	0.011	0.000
	20	0.011	0.000
	21	0.022	0.000
	22	0.000	0.083
	23	0.000	0.083
Ch11/110	1	0.418	0.389
	2	0.010	0.000
	7	0.459	0.278
	8	0.020	0.000
	9	0.082	0.000
	10	0.010	0.222
	11	0.000	0.111
Ch3/IJ15/1	1	0.436	0.833
	3	0.000	0.111
	7	0.468	0.056
	8	0.096	0.000
Ch10/3778	1	0.459	0.389
	2	0.432	0.500
	3	0.108	0.000
	4	0.000	0.111
Ch11/51	1	0.455	0.429
	2	0.439	0.571
	3	0.106	0.000
Ch7/B	1	0.069	0.250
	2	0.759	0.750
	3	0.052	0.000
	4	0.069	0.000
	5	0.052	0.000

Table 3.8 Allele frequencies at each locus for both Arua and Moyo *T. b. gambiense* populations.





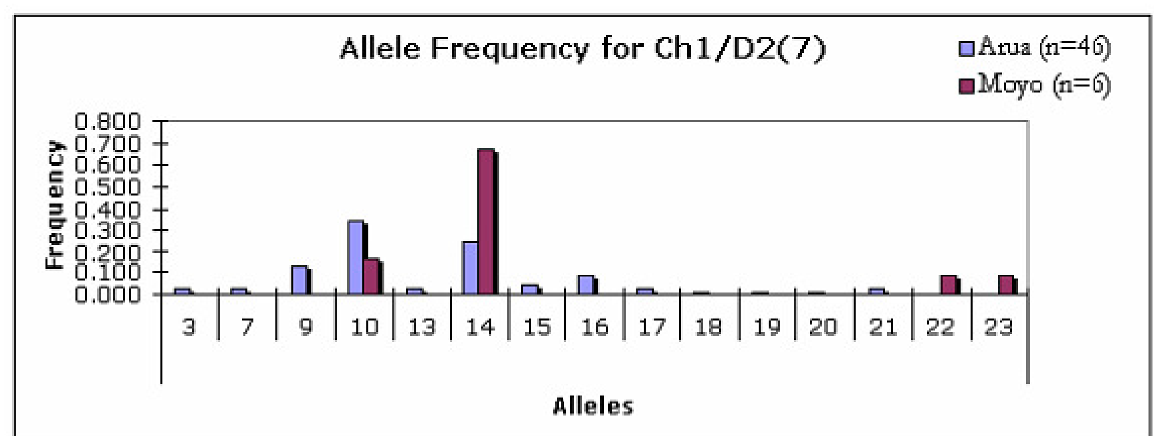
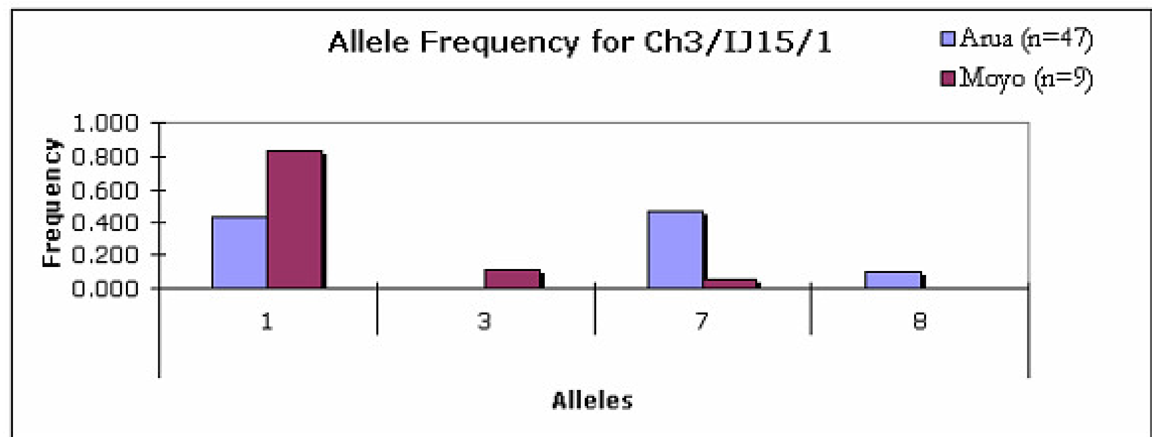
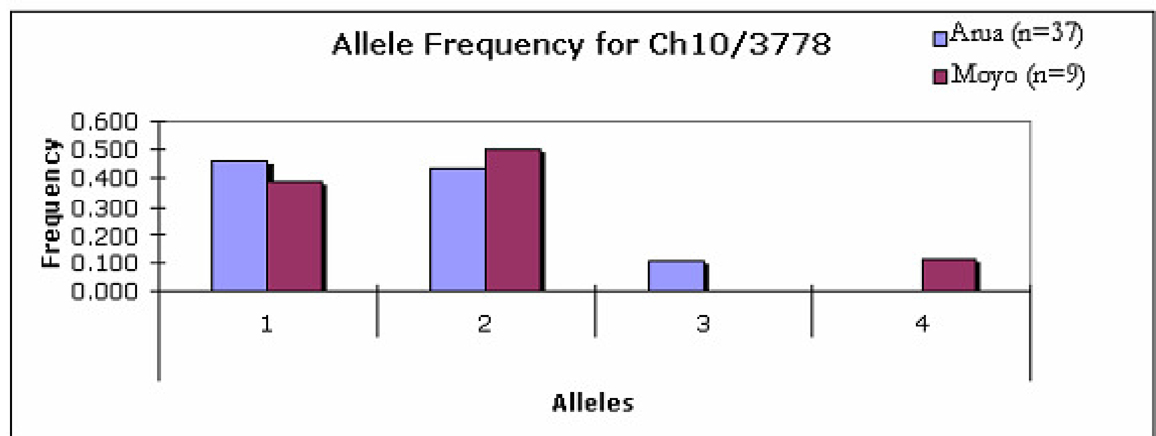


Figure 3.3 Allele frequencies for Ch1/18, Ch5/JS2 and Ch1/D2/7 within Arua and Moyo *T. b. gambiense* populations.
n, number of samples that amplified with a product for each particular marker.
Population size Arua (N=54) and Moyo (N=17).
Allele, specific number allocated to a particular size PCR product after binning based on the peak scanner traces (Table 3.2).





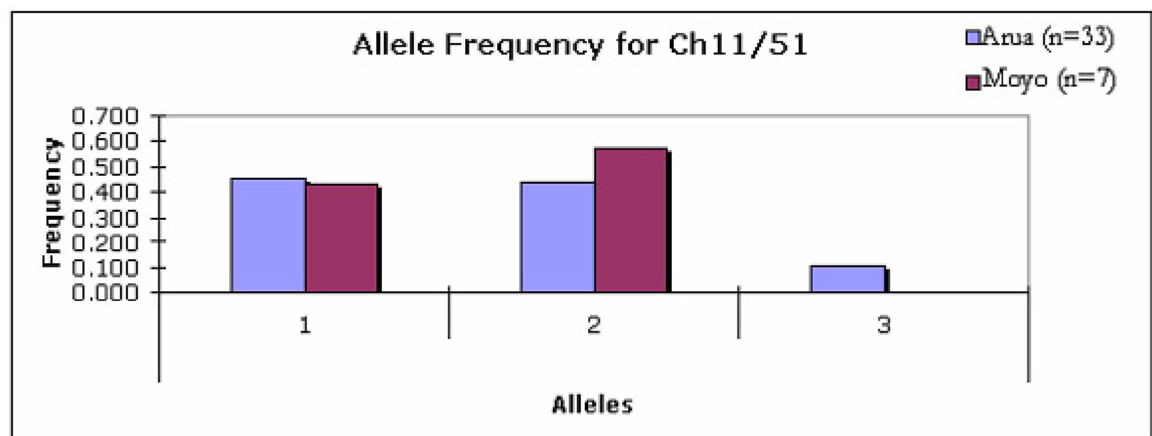
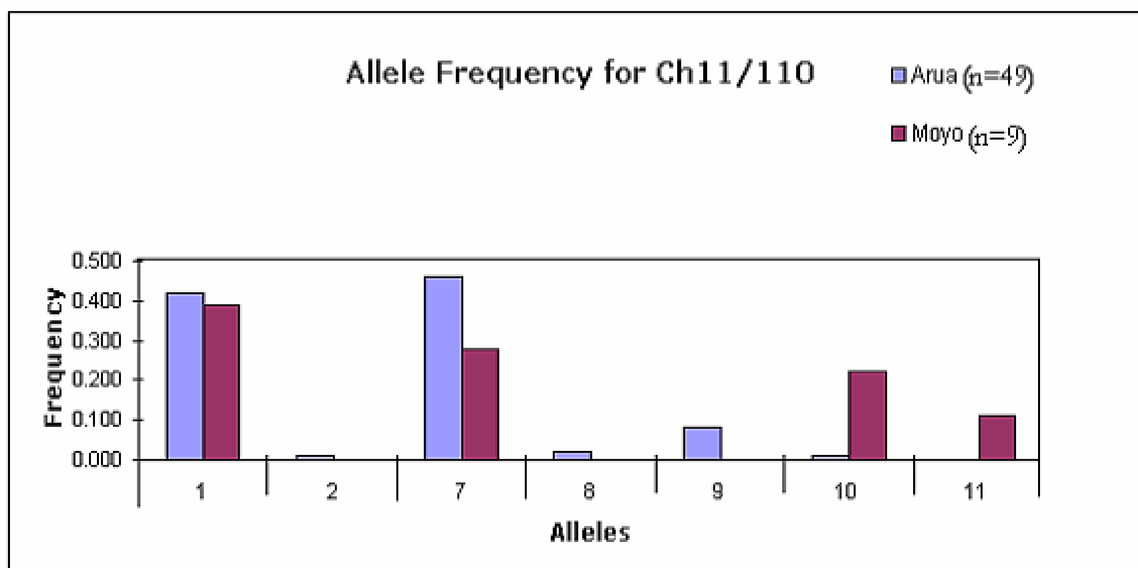


Figure 3.4 Allele frequencies for Ch3/IJ15/1, Ch10/3778 and Ch11/51 within Arua and Moyo *T. b. gambiense* populations.
n, number of samples that amplified with a product for each particular marker.
Population size Arua (N=54) and Moyo (N=17).
Allele, specific allele number allocated to a particular size PCR product after binning based on the peak scanner traces with an exception of minisatellite marker Ch10/3778 (size of PCR products were determined as observed in agarose) [Table 3.2]



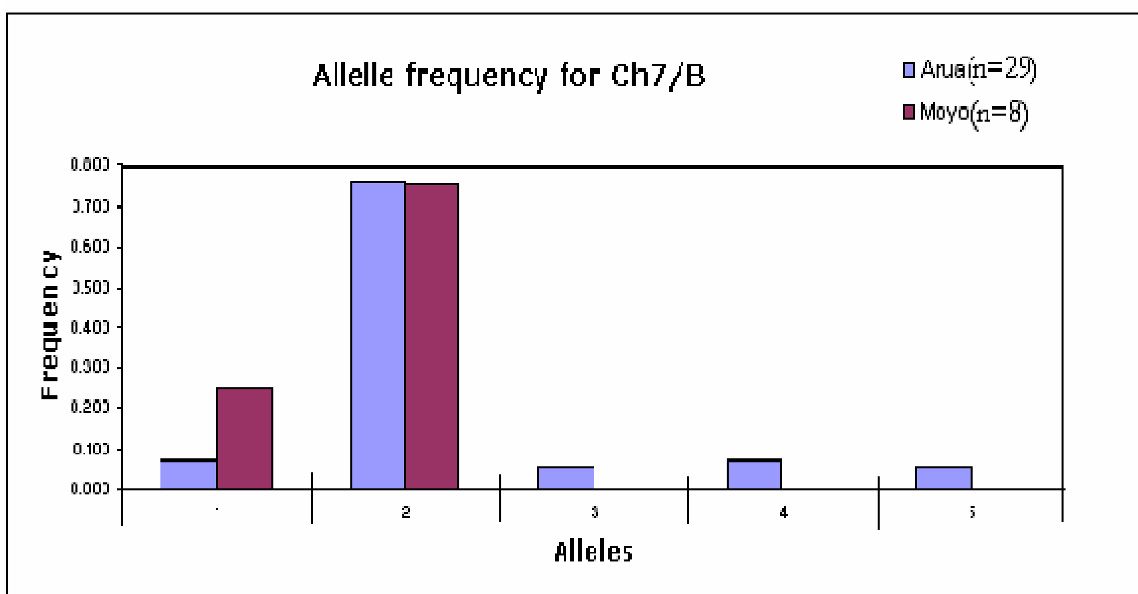


Figure 3.5 Allele frequencies for Ch11/110 and Ch7/B within Arua and Moyo *T. b. gambiense* populations.
n, number of samples that amplified with a product for each particular marker.
Population size Arua (N=54) and Moyo (N=17).
Allele, specific allele number allocated to a particular size PCR product after binning based on peak scanner traces (Table 3.2)

Comparison of the Arua and Moyo *T. b. gambiense* populations

To determine whether a genetic difference existed between the two *T. b. gambiense* populations, the averaged genetic difference was obtained for all the 8 markers. The Genetic Data Analysis (GDA) software program (Lewis & Zaykin, 1999) http://hydrodictyon.eeb.uconn.edu/people/plewis/software.php was used to calculate genetic distance (Nei 1978) using a matrix type input data generated from the multilocus genotype results for Arua and Moyo populations (Table 3.3 and Table 3.4). The result suggested that the two populations were genetically different with a moderate genetic distance (D=0.176). Because of the limitation of the small sample size for the Moyo population (N=17), it has been excluded from further genetic analysis other than for comparison with the Arua population as, after exclusion of samples with missing data at each locus, the sample number was too low.

By comparing the two populations (Table 3.5 and Table 3.6), it is clear that there are a number of alleles unique to each population, referred to as private alleles at each individual locus. These were observed to occur at a very low frequency (<0.1) although five of these (allele 15 and 16 for Ch5/JS2, allele 9 for Ch1/D2/7, allele 3 for Ch10/3778 and allele 3 for Ch11/51) were observed to occur at a frequency ≥0.1 which was suggestive of population sub-structuring (Table 3.9) and is consistent with the moderate genetic distance between the two populations.

Locus	Allele	Frequency
Ch1/18	8	0.009615
Ch1/18	2	0.336538
Ch1/18	1	0.653846
Ch5/JS2	21	0.033333
Ch5/JS2	20	0.022222
Ch5/JS2	19	0.011111
Ch5/JS2	18	0.022222
Ch5/JS2	17	0.044444
Ch5/JS2	***16***	***0.100000***
Ch5/JS2	***15***	***0.322222***
Ch5/JS2	14	0.055556
Ch5/JS2	8	0.066667
Ch5/JS2	4	0.155556
Ch5/JS2	1	0.166667
Ch1/D2/7	21	0.021739
Ch1/D2/7	20	0.010870
Ch1/D2/7	19	0.010870
Ch1/D2/7	18	0.010870
Ch1/D2/7	17	0.021739
Ch1/D2/7	16	0.086957
Ch1/D2/7	15	0.043478
Ch1/D2/7	14	0.250000
Ch1/D2/7	13	0.021739
Ch1/D2/7	10	0.347826
Ch1/D2/7	***9***	***0.130435***
Ch1/D2/7	7	0.021739
Ch1/D2/7	3	0.021739
Ch11/110	10	0.010204
Ch11/110	9	0.081633
Ch11/110	8	0.020408
Ch11/110	7	0.459184
Ch11/110	2	0.010204
Ch11/110	1	0.418367
Ch3/IJ15/1	8	0.095745
Ch3/IJ15/1	7	0.468085
Ch3/IJ15/1	1	0.436170
Ch10/3778	***3***	***0.108108***
Ch10/3778	2	0.432432
Ch10/3778	1	0.459459
Ch11/51	***3***	***0.106061***
Ch11/51	2	0.439394
Ch11/51	1	0.454545
Ch7/B	5	0.051724
Ch7/B	4	0.068966
Ch7/B	3	0.051724
Ch7/B	2	0.758621
Ch7/B	1	0.068966

Table 3.9 Private alleles (shaded grey) at each locus within the Arua *gambiense* population. Private alleles (Bold italics) with an allele frequency ≥0.1. Arua *T. b. gambiense* population size (N=54).

3.4.2 Genetic analysis for Hardy-Weinberg (HW) and linkage disequilibrium

To be able to determine the how frequently genetic exchange was occurring in the *T. b. gambiense* from Arua, statistical analysis was performed on the microsatellite and minisatellite genotype data at each individual locus to test for Hardy-Weinberg equilibrium and whether linkage equilibrium existed between alleles at different loci. The Hardy-Weinberg model predicts random mating should occur in diploid organisms within or between populations under no restrictions (which are either biological or geographical), leading to extensive genetic variation as a result of genetic exchange (Hartl & Clark, 1997). A recently performed study using microsatellite marker analysis on *T. b. gambiense* patient isolates from the Democratic Republic of Congo and Cameroon indicated that very limited genetic exchange goes on between the *T. b. gambiense* populations from within and between the different regions (Morrison *et al*, in press). Apart from being clonal, the *T. b. gambiense* populations from both regions were also clustered or sub-structured in groups based on their hosts geographical locations (Morrison *et al*, in press). It is not yet clear whether *T. b. gambiense* from other geographical regions exhibit the same characteristics in natural populations. A similar approach was used to determine the population structure of *T. b. gambiense* isolates from Arua district, Omugo in NW Uganda.

<u>Determination of the level of heterozygosity in *T. b. gambiense* from Arua</u>

The Genetic Data Analysis (GDA) software program (Lewis & Zaykin, 1999) http://hydrodictyon.eeb.uconn.edu/people/plewis/software.phpwas used for the input data in the form of a matrix generated from the genotype data (Table 3.3) to determine the level of heterozygosity as described under data analysis (section 3.3) and secondly to test for Hardy-Weinberg (HW) equilibrium. The level of heterozygosity is defined by the fixation index (F_{is}), which is the measure of reduction in the observed heterozygosity (Ho) due to inbreeding compared to the expected heterozygosity (He) assuming the HW model. A value Fis >0 indicates an excess of homozygotes (Ho>He) and a negative value Fis<0 indicates an excess of heterozygotes (Ho<He). This analysis was carried out on the Arua data (Table 3.7) as a whole population (i.e. total number of samples and alleles observed at all the 8 chromosome loci) and the result yielded a calculated value greater than 0 (F_{is}=0.315; Ho=0.421<He=0.613) (Table 3.10). This implied an excess of homozygotes and a deviation from the HW model. When the analysis was performed for each locus individually for the Arua population, all loci had an excess of homozygotes except locus

Ch1/18 (F_{is}=-0.332) as shown in Table 3.11. The possible reasons for the deviation from the HW model include inbreeding or other factors will be discussed later.

Population	Sample number (n)	Number of alleles (Na)	Observed heterozygosity (Ho)	Expected heterozygosity (He)	In breeding coefficient (Fis)
Mean	42.25	5.875	0.421	0.613	0.315

Table 3.10 The level of heterozygosity or inbreeding in Arua *T. b. gambiense* population.
The results are based on the mean of all alleles (Na) present at all 8 loci.
n, size obtained as an average of all the sample numbers present for each locus (Table 3.7).
Total number of samples for the Arua *T. b. gambiense* population (N=54).

Locus	Sample number (n)	Number of alleles (Na)	Observed heterozygosity (Ho)	Expected heterozygosity (He)	In breeding coefficient (Fis)
Ch1/18	52	3	0.615	0.464	-0.332
Ch5/JS2	45	11	0.467	0.832	0.441
Ch1/D2/7	46	13	0.239	0.796	0.701
Ch11/110	49	6	0.531	0.613	0.136
Ch3/IJ15/1	47	3	0.404	0.588	0.315
Ch10/3778	37	3	0.432	0.598	0.280
Ch11/51	33	3	0.545	0.598	0.089
Ch7/B	29	5	0.138	0.417	0.673
mean	42.25	5.875	0.421	0.613	0.315

Table 3.11 The level of heterozygosity or inbreeding at each individual locus.
The table shows the level of polymorphism at each locus.
n, number of samples analysed for each locus.
Na, number of alleles present at each locus.
Total number of samples for the Arua *T. b. gambiense* population (N=54).

Hardy-Weinberg disequilibrium in *T. b. gambiense* population from Arua

Deviation from HW equilibrium at each individual locus was tested using the GDA program for the Arua population data (Table 3.3), and the results indicated a deviation from the HW model with $P<0.05$ at all loci although Ch1/18 was not so highly significant (Table 3.12). This was in agreement with the calculated Fis values (Table 3.11) and suggests a non random mating population structure (not panmictic).

Locus	Sample number (n)	Probability (P)
Ch1/18	52	0.031250
Ch5/JS2	45	0.000000*
Ch1/D2/7	46	0.000000*
Ch11/110	49	0.000000*
Ch3/IJ15/1	47	0.000000*
Ch10/3778	37	0.000000*
Ch11/51	33	0.000937*
Ch7/B	29	0.000000*

Table 3.12 Analysis for Hardy-Weinberg (HW) disequilibrium at each locus.
n, number of samples analysed for each locus.
*** very highly significant (P<0.001) deviation from HW (P<0.05).**
Total number of samples for the Arua *T. b. gambiense* population (N=54).

Testing for Linkage disequilibrium in the *T. b. gambiense* from Arua

To determine whether no association existed between alleles at unlinked loci, linkage equilibrium was tested on the matrix input generated from the Arua population multilocus genotype data (Table 3.3) using the GDA program. This was first done by breaking up multilocus genotypes in all different possible allele combinations for two paired loci combinations (23 pairs) and the analysis was performed using 3200 reiterations and Fisher's exact test for significance. As shown in Table 3.13, a lack of random association between alleles or linkage disequilibrium was evident at all the 23 loci combinations with P<0.05. The conclusion was in agreement with the F_{is} values indicating a deviation from HW. However, when the analysis for linkage disequilibrium between alleles was performed while preserving the genotypes (not breaking up the multilocus allele genotypes) of those markers which were not in Hardy-Weinberg there was evidence for linkage equilibrium between alleles at 16 out of the 23 paired loci combinations (P>0.05) as shown in Table 3.14. This indicated some level of genetic exchange.

Locus combination	Sample number (n)	Probability (P)
Ch1/18 and Ch5/JS2	44	0.000000
Ch1/18 and Ch1/D2/7	41	0.000000
Ch1/18 and Ch11/110	48	0.000000
Ch1/18 and Ch3/IJ15/1	45	0.000313
Ch1/18 and Ch10/3778	35	0.001563
Ch1/18 and Ch11/51	31	0.002500
Ch1/18 and Ch7/B	31	0.000000
Ch5/JS2 and Ch1/D2/7	37	0.000000
Ch5/JS2 and Ch11/110	41	0.000000
Ch5/JS2 and Ch3/IJ15/1	38	0.000000
Ch5/JS2 and Ch10/3778	30	0.000000
Ch5/JS2 and Ch11/51	25	0.000000
Ch5/JS2 and Ch7/B	25	0.000000
Ch1/D2/7 and Ch11/110	42	0.000000
Ch1/D2/7 and Ch3/IJ15/1	41	0.000000
Ch1/D2/7 and Ch10/3778	35	0.000000
Ch1/D2/7 and Ch11/51	29	0.000000
Ch1/D2/7 and Ch7/B	35	0.000000
Ch11/110 and Ch3/IJ15/1	35	0.000000
Ch11/110 and Ch10/3778	34	0.000000
Ch11/110 and Ch11/51	31	0.000000
Ch11/110 and Ch7/B	28	0.000000
Ch3/IJ15/1 and Ch10/3778	34	0.000000
Ch3/IJ15/1 and Ch11/51	31	0.000000
Ch3/IJ15/1 and Ch7/B	27	0.000000
Ch10/3778 and Ch11/51	26	0.000000
Ch10/3778 and Ch7/B	23	0.000000
Ch11/51 and Ch7/B	19	0.000000

Table 3.13 Results for the analysis for linkage equilibrium between paired loci. n, number of samples statistically analysed for each locus combination. All multilocus broken genotype combinations in linkage disequilibrium P<0.05. Total number of samples for the Arua *T. b. gambiense* population (N= 54).

Pair wise Locus combination	Sample number (n)	Probability (P)
Ch1/18 and Ch5/JS2	44	0.022187
Ch1/18 and Ch1/D2/7	41	0.000625
Ch1/18 and Ch11/110	48	**0.073125***
Ch1/18 and Ch3/IJ15/1	45	**0.061562***
Ch1/18 and Ch10/3778	35	**0.722187***
Ch1/18 and Ch11/51	31	**0.059062***
Ch1/18 and Ch7/B	31	**0.120313***
Ch5/JS2 and Ch1/D2/7	37	**0.068125***
Ch5/JS2 and Ch11/110	41	**0.409375***
Ch5/JS2 and Ch3/IJ15/1	38	**0.084687***
Ch5/JS2 and Ch10/3778	30	**0.680312***
Ch5/JS2 and Ch11/51	25	**0.466562***
Ch5/JS2 and Ch7/B	25	**0.794375***
Ch1/D2/7 and Ch11/110	42	**0.100937***
Ch1/D2/7 and Ch3/IJ15/1	41	**0.059687***
Ch1/D2/7 and Ch10/3778	35	**0.517813***
Ch1/D2/7 and Ch11/51	29	**0.157188***
Ch1/D2/7 and Ch7/B	35	**0.740625***
Ch11/110 and Ch3/IJ15/1	35	0.000000
Ch11/110 and Ch10/3778	34	0.044687
Ch11/110 and Ch11/51	31	0.000000
Ch11/110 and Ch7/B	28	0.000000
Ch3/IJ15/1 and Ch10/3778	34	0.004375
Ch3/IJ15/1 and Ch11/51	31	0.000000
Ch3/IJ15/1 and Ch7/B	27	0.000000
Ch10/3778 and Ch11/51	26	0.002812
Ch10/3778 and Ch7/B	23	0.014687
Ch11/51 and Ch7/B	19	0.000000

Table 3.14 Analysis of Arua samples for linkage equilibrium between 23 paired loci.
n, number of samples statistically analysed for each locus combination.
***Preserved multilocus genotype combinations (Bold) in agreement with linkage equilibrium P>0.05.**
Total number of samples for the Arua *T. b. gambiense* population (N= 54).

To further determine whether linkage disequilibrium existed between alleles on unlinked loci, another linkage analysis program (LIAN) (Haubold & Hudson, 2000), which is able to determine differences between the predicted and observed frequency of each pair of haplotypes was used for the analysis of the Arua population generated from the multilocus genotype data (Table 3.3). The measure of linkage disequilibrium between genotypes at all paired loci combinations is determined as the Standardisation Index of Association (I^S_A). A value $I^S_A \leq 0$ suggests frequent genetic exchange while $I^S_A > 0$ suggests little or a lack of genetic exchange. The I^S_A at the 5% critical value was calculated from the comparison of the variance of the differences between the paired loci combinations (V_D), to the variance expected (V_e) for linkage equilibrium. The results indicated little linkage disequilibrium (I^S_A=0.0856, V_D=2.2438, Ve=1.4031) with P<0.01. Since the I^S_A value is close to zero, which is the limit for linkage equilibrium, this is suggestive of some genetic exchange.

Further population analysis of the Arua *T. b. gambiense*

Further comparisons of the different multilocus genotypes (Table 3.3) to determine the level of differentiation was undertaken using a free downloadable CLUSTERING CALCULATOR program http://www2.biology.ualberta.ca/jbrzusto/cluster.php for the input data (Appendix II) to produce a matrix of pairwise differences as a draw string of values. To calculate the bootstrap values and generate a dendogram (Figure 3.6) of similarity from the draw string of values (Appendix II), the free downloadable TREE VIEW program http://taxonomy.zoology.gla.ac.uk/rod/treeview.html was used. The calculated bootstrap values were used to determine the statistical robustness of the nodes and the level of similarity or differentiation that existed between the multilocus genotypes generated for each sample analysed.

Only five of the loci (Ch1/18, Ch5/JS2, Ch1/D2/7, Ch11/110 and Ch3/IJ15/1) were considered. Three markers were not included in this analysis due to the high level of missing data for these loci. For the purposes of comparison in this analysis was included three samples from the Moyo population (M139, M153 and M177) (Table 3.4), which had complete multilocus genotype data sets for the five loci. Almost all of the *T. b. gambiense* isolates from the Arua, Omugo (OM) population were closely similar to each other and to the reference strain type I *T. b. gambiense* (ELIANE) with bootstrap values below 60 at the different nodes (not shown). Only nodes with bootstrap values >70 were shown (Figure 3.6). It can be concluded that they probably evolved from the same trypanosome species as the reference strain type I *T. gambiense* which was isolated from a woman in Cote d'Ivoire (Pays *et al*, 1983). The three *T. b. gambiense* samples from Moyo included in this analysis

did not cluster as a separate group and so were not any different from the Arua population but the sample size of three was too low to draw firm conclusions. The earlier result of the genetic difference (D=0.176) between the two populations obtained in the initial analysis is moderate enough to suggest a difference and the presence of some unique alleles found in one population and not in the other further supports this.

While linkage disequilibrium has been detected in the Arua *T. b. gambiense* population, only two isolates OM83 and OM88 were found to have identical genotypes. This is in contrast to the clonal nature of *T. b. gambiense* found in the Democratic Republic of Congo and Cameroon (Morrison *et al*, in press), where a common genotype was repeatedly sampled. The *T. b. gambiense* population from Arua is definitely not clonal. The possible reasons for the linkage disequilibrium detected could be inbreeding, substructuring, null alleles, or migration as will be discussed. The two identical samples (OM83, OM88) and one other sample OM112 were observed to be distinct from the rest of the samples and the reference strain type I *T. b. gambiense* (ELIANE) with bootstrap values of 99-100 (Figure 3.6), which is a significant level of differentiation. It is possibility that the two clustering isolates (OM83 and OM88) and OM112 are a result of recent migration from a different focus of disease for example southern Sudan. These samples are not similar to the *T. b. gambiense* reference control (ELIANE) used in this analysis. This finding is similar to an observation made in a recent study where *T. b. gambiense* isolates from Cameroon were demonstrated to be distinct from type I *T. b. gambiense* (ELIANE) strains and type II *T. b. gambiense* 386 reference strains with bootstrap values of 86 and 91 respectively (Morrison *et al*, in press). As in the Cameroon study, the samples used in this study were all collected from patients who were confirmed seropositive for the presence of the *T. b. gambiense* VSG antigen *LiTat 1.3* which is the basis of the card agglutination test for trypanosomiasis (Magnus et al, 1978). The samples were also parasite-positive using the highly sensitive TBR multicopy primers (Moser *et al*, 1989) for the trypanozoon group as indicated (Chapter 4, Table 4.2). However, a type II *T. b. gambiense* control was not included in the analysis in the current study. Therefore it cannot be ruled out that the three Arua samples could possibly be type II *T. b. gambiense*. Further studies may be required using larger sample numbers to confirm this finding. Alternatively, these three isolates could be responsible for the observed linkage disequilibrium observed in this population. However, exclusion of these samples from the analysis may be required to test whether this is the case.

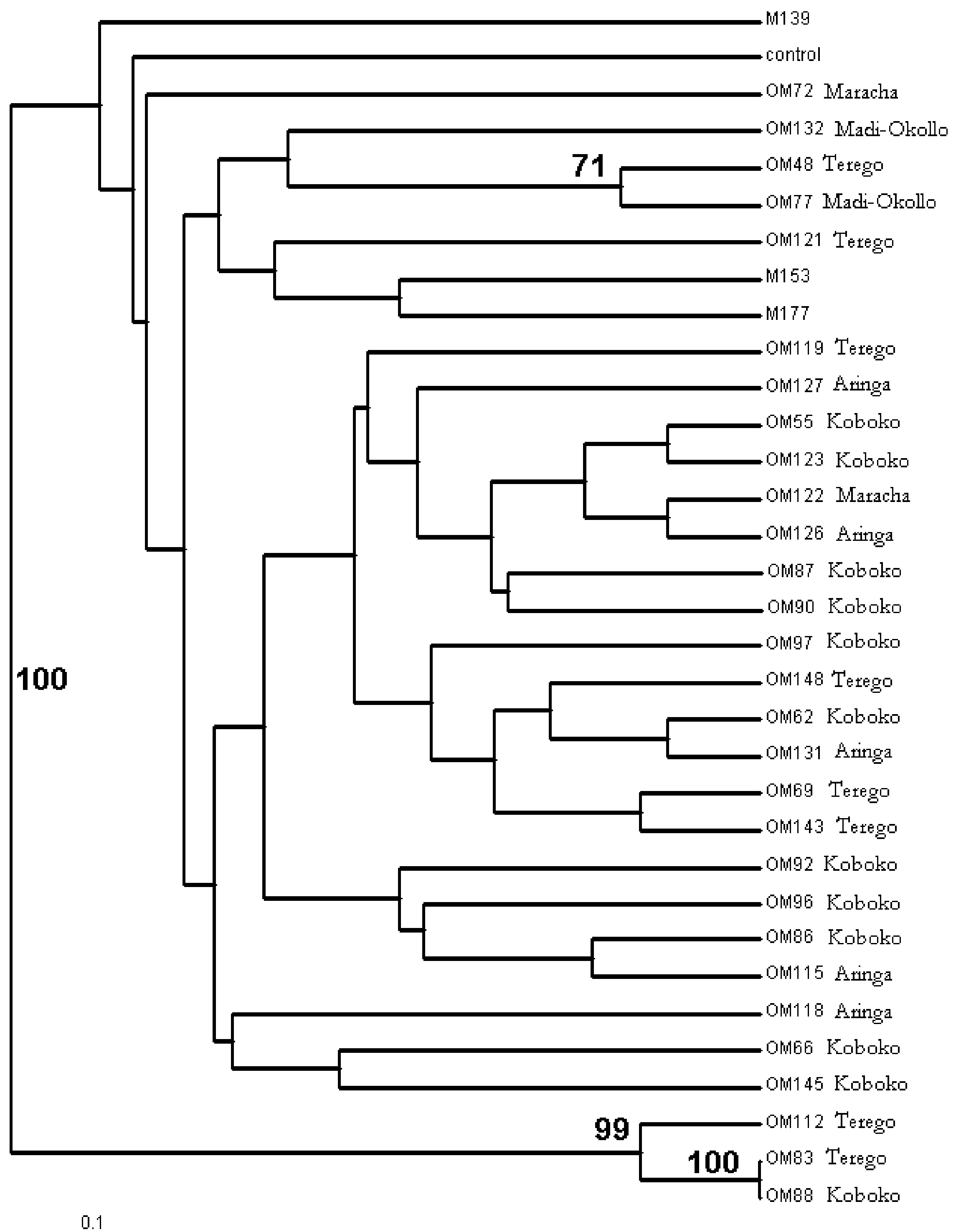


Figure 3.6 Comparison of different Arua sample *T. b. gambiense* genotypes at five loci. The dendogram shows the level of differentiation between the Arua samples. Bootstrap values are indicated at the relevant t node.
OM, *T. b. gambiense* isolates from Omugo, Arua district and their respective hosts' counties.
Control, type I *T. b. gambiense* (ELIANE) (Pays *et al*, 1983).
M139, M153 and M177, *T. b. gambiense* isolates from Moyo with complete data sets.

3.5 Discussion

This aim of the study was to investigate the population structure of *T. b. gambiense* using samples collected from sleeping sickness patients from Arua and Moyo districts both located in NW Uganda. The simple method of sampling using the FTA® cards (Whatman) (rather than propagation in mice and culture) and the availability of microsatellite and minisatellite markers has made this kind of analysis possible. Only one study has previously used this approach and the findings indicated a clonal structure for *T. b. gambiense* isolates from the Democratic Republic of Congo (DRC) and Cameroon taken from patients presenting at a clinic and during a field survey in 2003 (Morrison *et al*, in press). However, it is still not clear whether *T. b. gambiense* from East Africa has a similar or distinct structure. With regard to another protozoan parasite, a study performed by Anderson and colleagues (2000) clearly showed that *P. falciparum* from regions with different disease endemic levels was capable of exhibiting different structures in the same and different geographical foci. It is therefore hypothesised that *T. b. gambiense* from the same or different foci may have distinct structures during an epidemic and a post-epidemic period. This implies that the structure may change over time and it is therefore necessary to determine the structure at various time points in order to make proper comparisons between populations from within the same and distinct geographical foci. The samples used for this study were collected from patients during field surveys (where most patients were in stage I) and from patients reporting to health units (where most patients were in stage II) between 2005 and 2006. The collections were from two regions within a focus of high endemicity during a post epidemic period. Both populations were observed to share at least two alleles between them for all of the markers, with an exception of the Ch5/JS2 marker, which had five alleles in common between the two populations (Figure 3.3). The presence of private alleles at each of the loci especially for Ch5/JS2 and Ch1/D2/7 revealed considerable genetic variation between the two populations.

The Arua and Moyo populations were genetically distinct as determined by the value obtained for the genetic distance. More meaningful analysis could not be done to compare the two populations because of the limitation of the small population sample size for Moyo (N=17), which was reduced further on exclusion of samples with missing data at each locus. Low parasite levels, despite using the whole genome amplification method (Morrison *et al*, 2007), were mostly considered responsible for this lack of microsatellite PCR amplification. Moreover, for both populations the samples that could be amplified for some of the loci could not be amplified alleles for other loci, which led to a further

reduction of sample numbers, especially for the Moyo population. This precluded further analyses on this population. The failure of some markers to amplify was attributed to the low parasite numbers present or the presence of null alleles, possibly due to point mutations in the PCR primer targets such that the primers could not anneal to the template (Jarne & Lagoda, 1996). It is also possible that allele-drop-out occurred during the whole genome amplification process leading to lack of amplification of some alleles. Spotting of the buffy coat on FTA cards from concentrated trypanosome isolates could be used to overcome the low parasitaemia while pooling of whole genome amplified DNA from three individual FTA discs instead of two could reduce on allele drop-out.

Deviation from the Hardy-Weinberg equilibrium (with an excess of homozygotes) and linkage disequilibrium between alleles of paired loci was detected in the Arua population. Hardy-Weinberg and linkage equilibrium was restored at 16 of 23 locus combinations when genotypes that had markers in linkage disequilibrium were preserved without breaking them up. The homozygous excess and linkage disequilibrium observed could be due to a lack of genetic recombination or a number of other reasons as discussed below.

The first reason null alleles associated with genetic differences between samples (Hartl & Clark, 1997) could be among the factors responsible for the excess in homozygosity leading to the deviation from the Hardy-Weinberg equilibrium as observed for the Arua population. Sequence divergence especially at the 3' end of the primer site could also lead to poor primer annealing leading to only one size allele being amplified resulting in the locus being scored a homozygous rather than heterozygous (Reviewed by Dakin & Avise, 2004). Although this may be unlikely since comparison of the sequences of the primers used for the amplifications with the sequences of the relevant loci along the 11 megabase size chromosomes available from the *T. b. brucei* genome project (Berriman *et al*, 2005) or the *T. b. gambiense* genome http://www.genedb.org/ indicated no mismatches. However, sequence differences in the primer sites cannot be ruled out considering the diversity of the genotypes in the natural population coupled with the incomplete sequence of the *T. b. gambiense* genome.

Inbreeding (selfing) or mating between relatives, which is a biological property of a population is another factor which could have played a role in the observed excess of homozygotes. Selfing has been demonstrated to occur in *T. brucei* under laboratory conditions (Tait *et al*, 1996;Gibson *et al*, 1997). However, the extent to which selfing can occur in *T. brucei* under natural field conditions has not been fully explored.

Sub-structuring associated with inbreeding is another possibility for the reduction of heterozygosity. Although all Arua *T. b. gambiense* samples were collected from the same geographical region over the same time period thus reducing the possibility of detecting linkage disequilibrium due to geographical sub-structuring, it is still possible that the population is substructured , possibly due to micro-geographical barriers or host vector dynamics. This substructuring has also been detected in *T. b. gambiense* populations from the Democratic Republic of Congo and Cameroon (Morrison *et al*, in press)

Sampling errors could also lead to a reduction in hetereozygosity especially if there is an animal reservoir within the population. Sampling of the *T. b. gambiense* isolates used in the analysis was by both active and passive surveillance of patients. While the majority of stage II cases were identified at the health units when they turned up on their own (passive), the majority of stage I cases were identified during the field surveys (active) (Patient Raw data, Appendix II). The majority of samples from Arua used in this analysis (N=54) were collected from stage II cases (n=47) already suffering central nervous infection. Only 7 isolates in the analysis were from patients in stage I disease. This may introduce a bias in the data set as possibly only virulent genotypes were sampled. The isolates from stage I cases were also not observed to cluster. The observed reduction in heterozygosity leading to alleles in linkage disequilibrium could also occur as a result of lack of informativeness of the markers e.g. due to the predominance of an allele.

An alternative explanation for the linkage disequilibrium detected and deviation from Hardy-Weinberg equilibrium could possibly be due to migration, (Hartl & Clark, 1997). This is another factor that can contribute to a reduction in heterozygosity due to the introduction of new alleles in the population leading to genetic divergence as observed in the Arua population. Migration is a likely scenario which cannot be ruled out since NW Uganda where Arua and Moyo are located, borders with S Sudan where there is another *T. b. gambiense* focus and a large number of Sudanese refugees have migrated and settled in Northern Uganda as a result of the long term civil war (Hovil & Bagenda, 2003;United Nations Report, 2004). The human populations in Arua and Moyo regions are of different ethnic backgrounds. It is possible that host differences exist between the two populations, which may enable some trypanosome genotypes to survive in one host population and not the other leading to the difference in diversity. This may require further investigation with larger sample numbers and also looking at isolates from tsetse flies in these regions.

3.6 Conclusion

I tested the overall genotypes using microsatellite and minisatellite markers in samples collected in Arua and Moyo. Using 8 different markers it can tentatively be concluded that the *T. b. gambiense* population from Arua seems to be different from the *T. b. gambiense* population from Moyo although this may necessitate further investigations with larger sample numbers to confirm this finding. Deviation from the Hardy-Weinberg equilibrium with an excess of homozygotes and the evidence for alleles at pairs of loci in linkage disequilibrium points to the fact that the *T. b. gambiense* population from Arua is not panmictic. The lack of a high frequency common genotype indicates that the population is not epidemic or clonal. The most parsimonious explanation for the observed linkage equilibrium is that some degree of genetic exchange is occurring within the Arua *T. b. gambiense* population. The observed linkage disequilibrium could be due to factors such as bias sampling (due to predominance of late stage), cryptic population sub-structuring, migration or selfing.

Chapter 4

4 Genotypic status of the *TbAT1/P2* of *T. b. gambiense* isolates from NW Uganda

4.1 Summary

The aim of this study was to investigate the current status of *TbAT1* alleles in *T. b. gambiense* isolates collected from sleeping sickness patients from areas within Arua and Moyo districts of NW Uganda. This was carried out using the PCR/RFLP based method to determine if it can be useful as a predictive tool for the early detection of treatment failure or developing drug resistance and hence guide the decision making process regarding the treatment of Human African Trypanosomiasis. Moreover, not only can the method be used for predicting melarsoprol treatment failures but it may also be utilised in the case of diamidines including pentamidine whose uptake is also mediated partly through the same P2 transporter (de Koning & Jarvis, 2001;de Koning, 2001b). Out of a total of 133 isolates (collected as either blood spots on FTA cards, whole blood or CSF) analysed, 91 (68 %) were successfully amplified by PCR using TbAT1 primers and all possessed the *TbAT1* wild type alleles after RFLP analysis with *Sfa NI* endonuclease, implying that trypanosomes in circulation have *TbAT1* alleles which showed normal melarsoprol transport as far as can be assessed with the *Sfa NI* analysis. This finding indicates a shift from previous results (58.5%) of the presence of *tbat1* mutant alleles or melarsoprol resistant alleles in Omugo, NW Uganda (Matovu *et al*, 2001b). It could be speculated that this reduction of *TbAT1* mutant alleles in the field might be linked with the withdrawal of the drug from Omugo health centre. The melarsoprol *TbAT1* resistant alleles may have a fitness cost disadvantage and therefore cannot compete against wild type for long in the absence of drug pressure. In a recent study in South Sudan, where melarsoprol was withdrawn, the findings revealed the presence of only *tbat1* wild type alleles (Maina *et al*, 2007), which is in agreement with our findings. This also led these authors' to conclude that the *tbat1* mutant alleles associated with melarsoprol resistance did not occur in S. Sudan. However, they did not collect samples for analysis at the peak of relapses before drug withdrawal as in the case of NW Uganda situation.

4.2 Introduction

The *Trypanosome brucei* adenosine transporter 1 gene encoding P2 activity (*TbAT1/P2*) is known to mediate the cellular uptake of melaminophenyl arsenicals and diamidines. Moreover, loss of drug uptake by this P2 purine transporter as a result of alterations in the transporter has been associated with the development of arsenical and diamidine resistance in *Trypanosoma brucei* (Carter & Fairlamb, 1993;Carter *et al*, 1995;Carter *et al*, 1999;Barrett *et al*, 1995;Scott *et al*, 1997). Resistance has also been suggested to occur through other mechanisms (see section 1.4.2). The *TbAT1/P2* gene was identified by complementation in the yeast *Saccharomyces cerevisiae* as a single copy gene encoding 463 amino acids that should fold into 11 transmembrane domains (Mäser *et al*, 1999). Identification of this drug transporter gene has led to elucidation of many mechanisms responsible for development of drug resistance in *T. brucei*. Mäser and colleagues (1999) were able to demonstrate, by sequencing, that ten point mutations which were homozygous were present in the open reading frame of the cloned *TbAT1* gene of the laboratory-derived melarsen oxide cysteamine (Melcy) resistant stock (STIB 777R) when compared to the *TbAT1* gene of the *T. brucei* drug sensitive parent stock (STIB 777S). This further implicated *TbAT1* involvement in drug resistance. One of the mutations caused an amino acid change [alanine to threonine (A178T)] associated with a base change (G to A) at position 532 and another [asparagine to serine (A286S)] associated with a base change (A to G) at position 857. The former mutation destroyed a *Sfa NI* site in the *TbAT1* of the wild type *T. brucei* and the latter created a new *Sfa NI* site. Some of the remaining mutations caused amino acid substitutions while others were silent (Mäser *et al*, 1999).

These two mutations were used to differentiate between the drug sensitive and resistant *T. brucei* stocks by a PCR/RFLP-based method using the restriction endonuclease *Sfa NI* (Mäser *et al*, 1999). This development offers a potential tool for the diagnosis of drug resistance in *T. brucei*. The method required validation by determining whether the same mutations occurred in field isolates. Analysis of *T. b. gambiense* isolates (n=65) from Omugo, NW Uganda, collected from sleeping sickness patients who relapsed after melarsoprol treatment as well as isolates from newly infected patients, using the PCR/*Sfa NI* based method indicated the presence of *TbAT1* mutant alleles in 50% of the melarsoprol treatment failure patient isolates (Matovu *et al*, 2001b). However, the presence of only *TbAT1* wild type alleles in 30% of the isolates from relapsed patients which was also confirmed by sequencing, was an indication that other factors such as biochemically mediated resistance could also play a role in the development of melarsoprol treatment

failure (Matovu *et al*, 2001a;Matovu *et al*, 2001b). The isolates analysed were collected from patients treated in Omugo, Arua district, during an outbreak when melarsoprol relapses had reached alarming levels (30%) (Legros *et al*, 1999). Such treatment failure levels were not observed in Moyo, a neighbouring district also located in NW Uganda. These findings led to the replacement of melarsoprol with DFMO (Eflornithine) as first line treatment for stage II disease at Omugo health centre located in Arua district and this took effect in 2001.

In South Sudan, where melarsoprol treatment failures were reported at 20% (Moore & Richer, 2001), melarsoprol was also withdrawn. Two years later, isolates were collected from *T. b. gambiense* sleeping sickness patients (n=18) from the same region and a study carried out using the same PCR/RFLP approach to determine the existence of such *TbAT1* mutant alleles which were reported in NW Uganda (Maina *et al*, 2007). The results indicated only the presence of *TbAT1* wild type alleles and sequencing the *TbAT1* from these isolates confirmed their finding which led the authors to suggest that the *TbAT1* mutant alleles had never occurred (Maina *et al*, 2007). In this study, I revisited NW Uganda where melarsoprol was withdrawn to determine the *TbAT1* status by PCR/RFLP and sequencing and to confirm earlier findings of its role in melarsoprol treatment failure.

4.3 Materials and Methods

Study area and population

As described in chapter 3 (see section 3.3).

Patient sample collection

Blood samples from confirmed stage I and stage II HAT patients (Table 4.1) were collected as described (section 3.3).

Disease Stage	Arua district (n) (Omugo health Centre)	Moyo district (n) (Moyo hospital)	Total (N)
Early stage (SSI)	11	0	11
Late stage (SSII)	88	34	122
Total (N)	99	34	133

Table 4.1 *T. b. gambiense* sleeping sickness isolates collected from NW Uganda.
Omugo health centre and Moyo hospital are the sleeping sickness control centres located in Arua and Moyo district respectively.
SSI, sleeping sickness stage I disease. SSII, sleeping sickness stage II.
N, total number of isolates collected in both districts.
n, number of isolates collected in each district under each respective group.
Patients screening by the Card agglutination test for trypanosomiasis (Magnus *et al*, 1978) Diagnosis confirmed by microscopy using wet smear, haematocrit centrifugation technique (Woo & Rogers, 1974) and lumbar puncture (section 3.3).

Patient treatment and follow-up

Qualified medical personnel at Omugo Health Centre and Moyo Hospital in NW Uganda carried out the monitored drug administration. The drugs used were a donation by the World Health Organisation (WHO). Stage I patients at both health units were treated with pentamidine isethionate (4 mg/kg body weight/day) administered intramuscularly for 10 days. Stage II patients at Moyo Hospital were treated with a melarsoprol schedule of 3 series each as four daily injections administered intravenously with an increasing dosage from 1.2 to 3.6 mg/kg body weight with a 7-day rest interval between each series. Unlike in Moyo, stage II patients at Omugo Health Centre, were treated with 400 mg/kg body weight/day of eflornithine (DFMO), which was administered at 100 mg/kg body weight intravenously with 4 daily infusions each lasting 6 hours for 14 days. On completion of treatment, patients were discharged and requested to return every 6 months for follow up for a period of 2 years.

Follow up post treatment was supposed to take place at 6, 12, and 24 months to monitor for any treatment failures. During this time patients were retested by microscopy for presence of trypanosomes in any of their body fluids. Patients showing signs of infection were re-admitted and retreated with an appropriate drug, which depended on the stage of the disease and the region of readmitting health unit. An individual can be declared fully recovered with no signs of infection after two years of follow up.

DNA extraction

Extraction of DNA from blood spotted on FTA® cards (Whatman) was performed as described (section 2.3).

DNA extraction from whole blood and CSF

Partial extraction of trypanosome genomic DNA for easy transportation to the laboratory was performed using a commercial kit (Puregene®, Gentra Systems, MN, USA) on CSF and whole blood samples. Briefly, 1.5 ml of red blood cell (RBC) lysis solution was added to 500 µl of whole blood in a 2 ml microfuge tube, mixed and incubated at room temperature for 10 minutes. This was followed by centrifugation in a desktop centrifuge at 13,000 × g for 5 minutes, the supernatant was discarded and the pellet resuspended in 300 µl of cell lysis solution. The quantity of CSF collected determined how much cell lysis solution wad added. To 50 µl of CSF was added 550 µl of cell lysis solution directly or 300 µl of cell lysis solution was used to resuspend the pellet recovered after centrifugation of the CSF. Extraction was completed using the commercial purification kit following the manufacturer's instructions (Puregene®, Gentra Systems, MN, USA). Proteinase K (100 µg/ml) was added to the samples in cell lysis buffer, mixed by pipetting and incubated at 55°C for 1 hour. For removal of protein, 200 µl of protein precipitation solution was added, mixed gently by inversion and the sample was incubated on ice for 5 minutes. After centrifugation of the suspension at 13,000 × g for 5 minutes, the supernatant was collected into a sterile microfuge tube and DNA precipitation and pellet washing was carried out by adding 400 µl each of isopropanol then 70% ethanol and centrifuged at 13,000 × g for 1 min. After air-drying the DNA pellet for 15 minutes, it was dissolved in 20 µl of DNA hydration solution and left to rehydrate overnight at room temperature or at 65°C for 1 hour. Up to 5 µl of the rehydrated DNA was used immediately in PCR or kept at -20°C.

TbAT1 genotyping by PCR amplification

Detection of *TbAT1* mutants associated with melarsoprol treatment failures in NW Uganda was performed in Uganda during the period July 2006 to September 2006 using the PCR /RFLP method as described (Mäser *et al*, 1999;Matovu *et al*, 2001b). The *TbAT1* RFLP fragment was successfully amplified from extracted DNA by nested PCR (Mäser *et al*, 1999;Matovu *et al*, 2001b). The method has also been used on samples from Moyo, NW Uganda (Nerima *et al*, 2007) and in South Sudan to clarify the existence of *TbAT1* mutant alleles linked to melarsoprol treatment failure (Maina *et al*, 2007).

In a primary PCR to amplify the *TbAT1* gene each DNA disc on an FTA® card or 5 µl of whole blood or CSF genomic DNA was amplified in a total volume of 25 µl, containing a 1 × Custom PCR Mastermix (45 mM Tris-HCl (pH 8.8), 4.5 mM $MgCl_2$, 11 mM $(NH_4)_2SO_4$, 0.113 mg/ml BSA, 4.4 µM EDTA and 1 mM of each deoxynucleotide triphosphate- dATP, dCTP, dGTP, dTTP) (ABgene®, Advanced Biotechnologies Ltd, UK). The oligonucleotide primer pair TbAT1 ant-s (5'-gcc cgg atc cgg ctg gtt ttt aga caa aag tga t-3') and TbAT1 ant-as (5'-gcc cct cga gcc gca tgg agt aag tct ga-3') (Mäser *et al*, 1999) (MWG Biotech-AG) each at 1 µM, and 1.25 units of Taq DNA polymerase (Promega Corporation, Madison, USA) were added to the reaction. The reaction was performed in a PTC-100™ DNA Engine (MJ Research, inc., Waltham, MA, USA) under the following conditions: initial denaturation at 94°C for 4 minutes; denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 1 minute for 30 cycles with a final extension at 72°C for 7 minutes. Genomic DNA of a *T. brucei* reference clone STIB 950 (isolated from a cow in Somalia, resistant to diminazene, isometamidium and quinapyramine) (Kaminsky *et al*, 1989) and sterile water (no template) were included in each run as a positive and negative control respectively. To amplify the *TbAT1* RFLP 677 bp fragment, 2 µl of primary PCR product was used as template in an inner PCR with the oligonucleotide primer pair Sfa-s or ATF-2 (5'-cgc cgc act cat cgc ccc gtt-3') and Sfa-as or ATR-2 (5'-cca ccg cgg tga gac gtg at-3') (Mäser *et al*, 1999;Matovu *et al*, 2001b) under the same conditions with the annealing temperature increased to 65°C (Matovu *et al*, 2001b).

<u>RFLP analysis using *SfaNI* endonuclease</u>

The RFLP analysis of *TbAT1* with the restriction endonuclease enzyme *Sfa NI* was shown to distinguish between melarsoprol sensitive and resistant *T. brucei* (Mäser *et al*, 1999;Matovu *et al*, 2001b). For each individual sample, 5 µl of a successfully amplified *TbAT1* 677 bp PCR product analysed by agarose gel electrophoresis was digested with the enzyme *SfaNI*. The reaction which was incubated at 37°C overnight was carried out in a total volume of 20 µl containing 1 × NEBuffer 3 (100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT) (pH 7.9) and 1 unit of *Sfa NI* enzyme (New England Biolabs).

<u>Agarose gel electrophoresis and UV transillumination</u>

A volume of 10 µl of each *TbAT1* nested PCR product was mixed with 1-2 µl of 6 × Blue loading dye (Appendix I) and separated by electrophoresis on a 3% Agarose gel stained with 0.7 µg/ml ethidium bromide in 1 × TAE buffer (Appendix I). The products were

visualised under an ultraviolet transilluminiscence box (Synegene GelVue model no. GVM20, Synoptics Ltd. UK) and pictures taken on polaroid film (Polaroid 667, Polaroid corporation, Mexico) with a polaroid camera (DS34 Polaroid). The product band size was determined by comparison with a standard 1-Kb (Gene Ruler™, Fermentas, Life Sciences) molecular weight DNA marker. A 677 bp size fragment should be observed in agarose.

20 µl of each *SfaNI* digested reaction was analysed by electrophoresis on a 2% Agarose gel and the different size fragments visualised under UV light as described above. Any of the three patterns signifying wild type *TbAT1* alleles (566 bp and 111 bp) or mutant *TbAT1* alleles (435 bp and 242 bp) or a mixture of both patterns (wild type *TbAT1* together with mutant *TbAT1*) should be observed in agarose.

Cloning and sequencing

The *TbAT1* RFLP fragments (677 bp) of 5 randomly selected samples from NW Uganda were sub-cloned (Sambrook *et al*, 1989) and the sequencing was performed by MWG Biotech AG (Germany). Preparation of the samples for sequencing was carried out as described below.

Agarose purification of PCR products

Briefly, two independent 25 µl PCR reactions for each individual sample were pooled and separated on an Agarose gel as described above. The PCR fragment was purified from an Agarose gel using a Qiaquick gel purification kit (Qiagen) following the manufacturer's instructions.

Ligation into a vector

In a sterile microfuge tube, 7 µl of each purified PCR product was ligated with 25 ng of pGEM®-T Easy vector (Promega corporation, Madison, USA) in a total reaction volume of 20 µl containing 1 × ligation buffer (30 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 1mM ATP 5% PEG), and 1 unit of ligase enzyme (10 mM Tris-HCl pH 7.4, 50 mM KCl, 1 mM DTT, 0.1 mM EDTA. 50% glycerol (Promega). The reaction was incubated at room temperature overnight.

Transformation into bacteria

Transformation of the ligated purified PCR products was carried out using competent *Eschericia coli* bacteria. Briefly, 10 µl of the ligation reaction was added to 50 µl of

competent *E. coli* strain DH5-α cells (donation from G. Campbell, University of Glasgow) in a sterile microfuge tube on ice and the reaction incubated for 30 minutes. Following incubation the reaction was heat-shocked at 42°C for 45 seconds followed by immediate cooling on ice for 2 minutes. 1 ml of SOC medium (Appendix I) was added and the reaction incubated at 37°C with shaking for 45-60 minutes.

Plating on Luria Broth (LB) Agar under blue/white selection

Following incubation, the reaction was centrifuged briefly at 13,000 × g. Most of the medium was removed leaving behind about 100 µl in which the pellet was resuspended. Under sterile conditions the suspension was plated onto LB agar/Ampicillin (50 µg/ml) prepared on a petri dish (Appendix I) and spread with 100 µl X-Gal (100 mM) and 50 µl IPTG (50 mg/ml) blue/white marker compounds. The plate was incubated at 37°C for 15-16 hours (preferably overnight) for colony growth.

Plasmid DNA preparation

Under sterile conditions, two colonies of the transformed bacteria containing insert were picked from the plate and each inoculated separately into 5 ml of sterile LB medium (Appendix I) containing Ampicillin (50 µg/ml). The cultures were incubated for 15-16 hours at 37°C. Following incubation, plasmid DNA was prepared from the two cultures using a plasmid purification kit following the manufacturer's instructions (Qiagen). To check for the presence of the plasmid DNA, 2 µl was analysed on an agarose gel. To check for successful cloning of the *TbAT1* fragment, 10 µl of plasmid DNA was digested with the endonuclease Not I enzyme (Promega Corporation, Madison, USA) in a total volume of 20 µl containing 1 × Buffer D (6 mM Tris-HCl (pH 7.9), 0.15 M NaCl, 6.0 mM $MgCl_2$ and 1 mM DTT) 10 µg/µl BSA, and 5 units of enzyme. The reactions were incubated at 37°C for 3 hours. 20 µl of the digested reaction was analysed by electrophoresis on a 2.0% Agarose gel and visualised as described (section 2.3). The two independent plasmid DNA preparations were pooled. 20µl of plasmid DNA in duplicate were vacuum-dried in a speed vac concentrator (Thermo Electronic Corporation, model DNA Speed Vac System 120-230, USA) and sent off for T7 and SP6 promoter sequence determination at MWG Biotech AG (Germany).

4.4 Results

4.4.1 PCR amplification of the TbAT1 gene

PCR amplification of DNA extracted from the samples was performed to confirm whether the patients were infected with *T. brucei* using the highly sensitive *T. brucei* multicopy repeat (TBR) primers specific for the trypanozoon group (Moser *et al*, 1989) as described (section 2.3). PCR amplification of the ubiquitous single copy *T. brucei Phospholipase C* linked microsatellite was also performed as a control using *PLC* linked microsatellite based primers (Tait *et al*, 2002;MacLeod *et al*, 2005b) as described (section 2.3).

The RFLP bearing fragment (677 bp) of the *TbAT1* gene (Figure 1.5) was amplified by nested PCR using primers specific for the *TbAT1* gene as described (section 4.3). The *TbAT1* 677 bp fragment was successfully PCR amplified from 65 of the 133 samples analysed and, when the whole genome amplification method was employed (Morrison *et al*, 2007) as described in section 4.3, the number of samples from which the *TbAT1* gene was amplified increased giving a total of 91 samples analysed.

The PCR results with the TBR primers revealed that all of the isolates (99.3%) except one isolate from Moyo were positive for the *T. brucei* group (Table 4.2). It is unclear why the single isolate was not positive for the T. brucei repeat and it is proposed that the analysis should be repeated and if necessary checked for other markers, rather than suggest, on the basis of this one piece of evidence, that this patient was infected with a non-*brucei* trypanosome species. Using the single copy *PLC*–linked microsatellite primers, only 45.8% of the isolates were detectable by PCR post whole genome amplification while 68.4% were successfully amplified for the single copy *TbAT1* gene (Table 4.2).

PCR target	Arua district		Moyo district	Total
	SSI (n=11)	SSII (n=88)	SSII (n=34)	(N=133)
T. brucei repeat	11	88	33	132 (99.3%)
T. brucei PLC	8	42	11	61 (45.8%)
TbAT1 (677 bp)	8	74	9	91 (68.4%)

Table 4.2 PCR analysis positive results of the NW Uganda *T. b. gambiense* patient isolates. The Table shows a summary of the results following amplification of the *T. brucei* 177 bp repeat, the single copy *T. brucei Phospholipase C*-linked microsatellite and the adenosine transporter gene (*TbAT1*).
SSI, sleeping sickness stage I. SSII, sleeping sickness stage II. N, total number of samples analysed. n, number of samples analysed under each group.
TBR-F and TBR-R primers (Moser *et al*, 1989) used to amplify the *T. brucei* repeat (section 2.3).
PLC-G and PLC-H3 primers (Tait *et al*, 2002) used to amplify the *T. brucei Phospholipase C* microsatellite (section 2.3).
TbAT1 ant-s and TbAT1 ant-as outer primers and ATF-2 and ATR-2 nested primers (Mäser *et al*, 1999;Matovu *et al*, 2001b) used to amplify the *TbAT1* 677 bp fragment (section 4.3)

These results are consistent with our earlier findings from the sensitivity assay (Chapter 2) which indicated that the high sensitivity of the *T. brucei* repeat (TBR) primers makes them better at reporting on infection. Even in this study, the TbAT1 primers are more sensitive than the PLC-linked microsatellite primers. Only one isolate from Moyo failed amplification with the highly sensitive TBR primers following two attempts of DNA extraction from two separate discs punched from the blood spot on an FTA card. This was possibly due to lack of trypanosome DNA on the cut FTA card discs used for DNA extraction or presence of extremely little amplifiable material. The finding also suggested that the isolates that failed to amplify for the single copy *T. brucei PLC* -linked microsatellite and the *TbAT1* gene most likely had very low parasite densities below the threshold for detection by PCR. However, on comparison of the PCR results for each of the isolates with both sets of primers, 42 isolates (Arua n=37and Moyo n=5) had been successfully amplified for the *TbAT1* gene but not for the *T. brucei PLC* like microsatellite (Table 4.3). And yet 12 isolates (Arua n=5 and Moyo n=7) were successfully amplified for the *T. brucei PLC*-linked microsatellite but not for the *TbAT1* gene (Table 4.3). While low parasite densities may be responsible for the former situation, it could not be ruled out that the complete loss or deletions within the *TbAT1* gene could be responsible for the later situation which is likely. An example is the complete loss of the *TbAT1* gene which was demonstrated in 1 Angolan *T. brucei* stock (K001) isolated from a melarsoprol relapsed

patient (Matovu *et al*, 2001b). However, some of the PCRs were done only once and some twice. Alternatively, other point mutations that influence the ability of the primers to bind template DNA could have affected the results.

PCR target	Arua district (n=99)		Moyo district (n=33)		Total
	PLC (+ ve)	*PLC* (-ve)	*PLC* (+ve)	*PLC* (-ve)	(N=132)
TbAT1 (+ ve)	45	37	4	5	91
TbAT1 (-ve)	5	12	7	17	41
Total	50	49	11	22	132

Table 4.3 The *TbAT1* and the single copy *T. brucei PLC* microsatellite PCR results.
The Table shows how lack of amplification of the *T. brucei Phospholipase C* (*PLC)* like gene possibly due to low parasite densities influenced the amplification of *T. brucei adenosine transporter gene* (*TbAT1*).
+ve, PCR product detected in agarose gel. –ve, no PCR product detected in agarose gel.
N, total number of samples analysed. n, number of samples from each respective district.
Cells (shaded grey), no *TbAT1* PCR product detected (possibly lost or deleted *TbAT1*).
Cells (diagonal pattern), no *PLC* PCR product detected (most probably due to low parasitaemia)
***TbAT1* PCR results based on one experiment and *PLC* PCR results based on two experiments.**

4.4.2 Sfa NI analysis of the TbAT1 gene

The 677 bp PCR fragment (Figure 4.1) of the *TbAT1* gene from each of the 91 successfully amplified isolates (Table 4.2) was digested with the *Sfa NI* restriction endonuclease enzyme as described (section 4.3) to determine the *TbAT1* genotype based on the fragment sizes generated. All of the 91 isolates (Arua n=82 and Moyo n=9) possessed *TbAT1* wild type alleles corresponding to the presence of the 566 bp and 111 bp band size pattern similar to that shown earlier for the *T. brucei* melarsoprol sensitive stock STIB 777 (Figure 4.2). The *TbAT1* mutant positive control a *T. brucei* reference clone STIB 950 (multidrug resistant to diminazene aceturate, isometamidium chloride and quinapyramine) (Kaminsky *et al*, 1989), displayed the *TbAT1* mutant pattern (425 bp and 242 bp) (Figure 4.2) similar to that reported for the *T. brucei* melarsoprol resistant reference clone STIB 777R (Mäser *et al*, 1999). These results suggested the patients were infected with trypanosomes possessing *TbAT1* melarsoprol sensitive alleles.

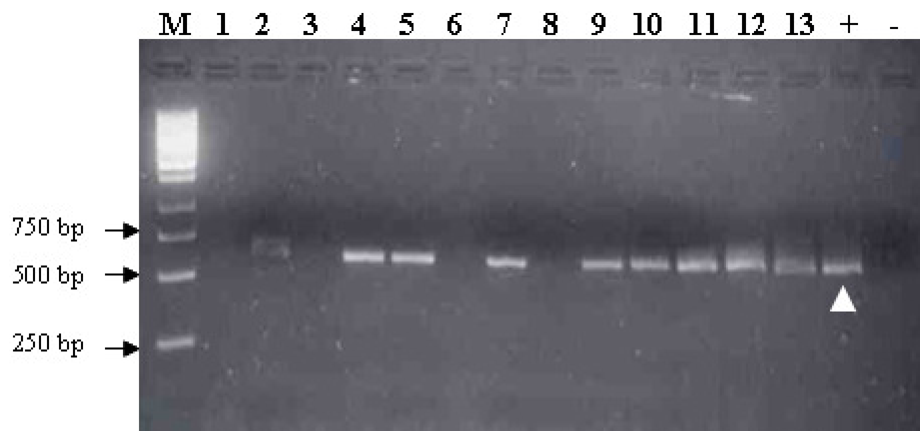


Figure 4.1 A representative Agarose gel showing the *TbAT1* nested PCR results.
The Figure shows the 677 bp *TbAT1* gene RFLP fragment (White arrow head).
Lanes 2, 4, 5, 7, 9 and 10-13 representative patient samples positive for the *TbAT1* gene.
Lanes 1, 3, 6 and 8 patient samples negative for the *TbAT1* gene.
M, 1 Kb molecular weight DNA marker (Gene Ruler™, Fermentas, Life Sciences).
+, *TbAT1* mutant positive control (multidrug resistant reference clone STIB 950 identical to STIB 777R).
-, negative control (sterile water no template).
A *TbAT1* wild type positive control e.g. the melarsoprol sensitive reference clone (STIB 777S) was not included in the PCR run.

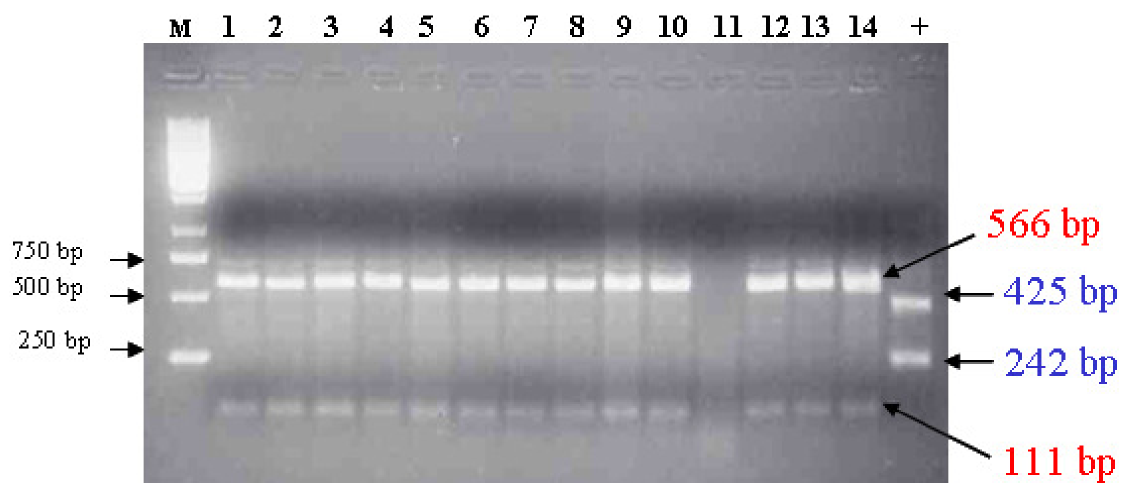


Figure 4.2 A representative Agarose gel showing the *TbAT1* (677 bp) *Sfa NI* digest results.
The Figure shows the *TbAT1* wild type pattern (566 bp and 111 bp) and the *TbAT1* resistant pattern (425 bp and 242 bp) size bands.
Lanes 1-9 and 12-14 representative patient samples with *tbat1* wild type alleles.
M, 1 Kb molecular weight DNA marker (Gene Ruler™, Fermentas, Life Sciences).
+, *TbAT1* mutant positive control, a multidrug resistant reference clone STIB 950 identical to STIB 777R
A *TbAT1* wild type positive control was not included in the *SfaNI* RFLP digest reaction

4.4.3 Sequencing of the TbAT1 gene

In a previous study, Matovu and others (2001b) demonstrated by sequencing 13 isolates from melarsoprol relapsed patients from Omugo, the presence of *TbAT1* wild type alleles confirming the PCR/RFLP results. In the current study, only *TbAT1* wild type alleles were observed by PCR/RFLP in all the 91 patient isolates successfully analysed (section 4.4.2). Sequencing was therefore carried out on a few samples to confirm this observation.

The *TbAT1* PCR products (677 bp fragment) from 5 randomly selected patient isolates were cloned as described (section 4.3) and the plasmid DNA sequenced at MWG Biotech from the forward and reverse directions using the T7 and SP6 primers. The 677 bp fragment of *TbAT1* of the positive control reference strain STIB 950 (Kaminsky *et al*, 1989) used in the RFLP analysis was cloned as well and sequenced for comparison with the sequences of the patient isolates. The cloning and sequencing was carried out once for each patient isolate. Using the blast analysis tool from the National Centre for Biotechnology Institute (NCBI) database http://www.ncbi.nih.gov/, the patient isolate *TbAT1* sequences were 99% identical to the *TbAT1* gene sequence of the wild type *T. brucei* reference strain STIB 427 (Genbank Accession number AF152369). The *TbAT1* sequence of the positive control STIB 950 was 99% identical to the *TbAT1* gene of the melarsen oxide cysteamine resistant *T. brucei* reference strain STIB 777R (Genbank Accession number AF152370). A multiple alignment of the sequences was performed using the alignment tool at http://www.expasy.ch/ (Figure 4.4). Four of the five isolates lacked the 10 mutations reported earlier to be present in the *TbAT1* gene sequence of the drug resistant stock STIB777R. This included the two mutations Ala178Thr and Asn276Ser at nucleotide positions G532A and A858G respectively, which were used in the PCR/RFLP analysis as the basis for differentiating between the *TbAT1* genes of the melarsoprol sensitive (STIB777S) and resistant (STIB777R) trypanosomes (Mäser *et al*, 1999;Matovu *et al*, 2001b). The fifth isolate OM62 was found to possess one of the silent mutations C502T reported to be present in STIB777R but not any of the other reported mutations including those that could be detected by PCR/RFLP. This however, indicated it had one characteristic of a mutant. The RFLP analysis, which detects only the two mutations Ala178Thr and Asn276Ser, indicated it was wild type. Moreover all the isolates had other changes compared to the original wild type occurring in different positions in their *TbAT1* sequences (Figure 4.3 and Table 4.4). Isolate OM72 had a mutation C1062T

causing amino acid change Ser354Leu. Another mutation led to a dinucleotide deletion in isolate OM62 at positions 1087 and 1088, while isolate M148 had one nucleotide deletion at position 1088. However these deletions may or may not alter the conformation of amino acid Leu 363 since a base change T1089A observed in the rest of the isolates that occurred within the same codon did not code for another amino acid (Table 4.4). Another mutation A1090T occurred in all the isolates causing amino acid change Thr364Ser. The *tbat1* mutant control used in this analysis (STIB950) was also found to have a mutation in its sequence at nucleotide positions 950-952 leading to the deletion of an entire codon Phe317 which was similar to the deletion observed in field isolates from melarsoprol relapsed patients but not in the mutated *TbAT1* gene sequence of STIB777R (Matovu *et al*, 2001b). This result is in agreement with earlier findings (Mäser *et al*, 1999;Matovu *et al*, 2001b). Some of the observed changes in these isolates like the deletions would change the open reading frame by frame shift therefore altering the protein and its function or they may not necessarily affect the function of the protein. The new mutations may also be due to sequencing errors or changes introduced during the amplification process by Taq polymerase. However, the sequencing, which was performed at MWG Biotech was carried out only once so reamplification and reanalysis would be required to confirm these results.

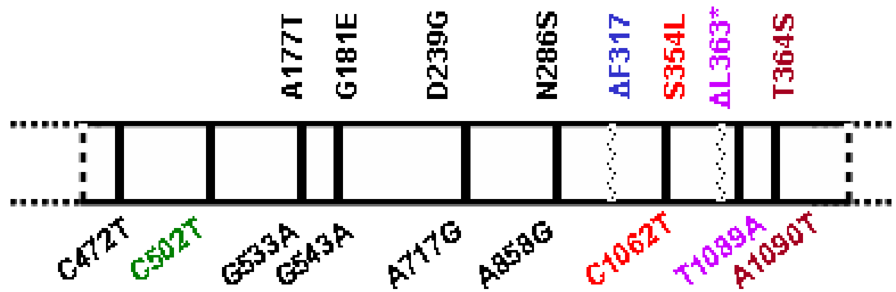


Figure 4.3 The positions of mutations present in the RFLP fragment of field isolates and STIB950.
The schematic shows (in bold black) mutations in sequenced STIB950 also present in STIB777R including G543A and A858G used for *tbat1* mutant allele detection in RFLP.
(in bold blue), deletion observed only in STIB950 but reported absent in STIB777R
(in bold green), silent mutation observed in OM62 but reported to exist in STIB777R.
(in bold red), mutation present in isolate OM 72.
(in bold purple), mutations/deletions present in OM62 & M148 or silent mutation in OM72, OM141 and M176.
(in bold brown) mutation present in all isolates (OM62, OM72, OM141, M148 and M176).
[Adapted from (Matovu *et al*, 2001b)]

∗Reference clone/ •Field sample code	Base positions in the *TbAT1* gene					
	502	1062	1087	1088	1089	1090
∗STIB427 wild type	C	C	C	T	T	A
∗STIB950 mutant	C	C	C	T	**A***	**T****
•OM62	**T***	C	**Δ**	**Δ**	**A***	**T****
•OM72	C	**T****	C	T	**A***	**T****
•OM141	C	C	C	T	**A***	**T****
•M148	C	C	C	**Δ**	**A***	**T****
•M176	C	C	C	T	**A***	**T****

Table 4.4 Comparison *TbAT1* gene sequences at positions 503, 1063 and 1087-1090.
The Table shows base changes at the different positions in the *TbAT1* of the field isolates in comparison to the *TbAT1* of the reference clones, STIB 427 wild type (Genbank accession number AF152639) and STIB 950 mutant (identical to STIB 777R Genbank accession number AF152370).
OM, Omugo (patient sample code for Arua, Omugo health centre).
M, Moyo (patient sample code for Moyo hospital).
***, silent mutation. **, coding mutation. Δ, base deletion.**

```
STIB427_wildtype TGCTGGAAACGCCGCACTCATCGCCCCGTTTCCAACGAAATTTTATAGCTCCGTCGTGTG 480
OM141            --------TCGCCGCACTCATCGCCCCGTTTCCAACGAAATTTTATAGCTCCGTCGTGTG 52
M148             --------TCGCCGCACTCATCGCCCCGTTTCCAACGAAATTTTATAGCTCCGTCGTGTG 52
OM62             --------TCGCCGCACTCATCGCCCCGTTTCCAACGAAATTTTATAGCTCCGTCGTGTG 52
M176             --------TCGCCGCACTCATCGCCCCGTTTCCAACGAAATTTTATAGCTCCGTCGTGTG 52
OM72             AATTCGATTCGCCGCACTCATCGCCCCGTTTCCAACGAAATTTTATAGCTCCGTCGTGTG 62
STIB950_mutant   --------TCGCCGCACTCATCGCCCCGTTTCCAACGAAATTTTATAGCTCTGTCGTGTG 52
                          ****************************************** ********

STIB427_wildtype GGGTATCGCTGTGTGCGGCGTCGTCACATCTTTCTTCTCGATCGTCATAAAAGCATCCAT 540
OM141            GGGTATCGCTGTGTGCGGCGTCGTCACATCTTTCTTCTCGATCGTCATAAAAGCATCCAT 112
M148             GGGTATCGCTGTGTGCGGCGTCGTCACATCTTTCTTCTCGATCGTCATAAAAGCATCCAT 112
OM62             GGGTATCGCTGTGTGCGGCGTTGTCACATCTTTCTTCTCGATCGTCATAAAAGCATCCAT 112
M176             GGGTATCGCTGTGTGCGGCGTCGTCACATCTTTCTTCTCGATCGTCATAAAAGCATCCAT 112
OM72             GGGTATCGCTGTGTGCGGCGTCGTCACATCTTTCTTCTCGATCGTCATAAAAGCATCCAT 122
STIB950_mutant   GGGTATCGCTGTGTGCGGCGTCGTCACATCTTTCTTCTCGATCGTCATAAAAACATCCAT 112
                 ********************* ****************************** *******

STIB427_wildtype GGGAGGCGGTTATCACAACATGCTCATACAGTCGCGCATATACTTTGGATTGGTCATGTT 600
OM141            GGGAGGCGGTTATCACAACATGCTCATACAGTCGCGCATATACTTTGGATTGGTCATGTT 172
M148             GGGAGGCGGTTATCACAACATGCTCATACAGTCGCGCATATACTTTGGATTGGTCATGTT 172
OM62             GGGAGGCGGTTATCACAACATGCTCATACAGTCGCGCATATACTTTGGATTGGTCATGTT 172
M176             GGGAGGCGGTTATCACAACATGCTCATACAGTCGCGCATATACTTTGGATTGGTCATGTT 172
OM72             GGGAGGCGGTTATCACAACATGCTCATACAGTCGCGCATATACTTTGGATTGGTCATGTT 182
STIB950_mutant   GGAAGGCGGTTATCACAACATGCTCATACAGTCGCGCATATACTTTGGATTGGTCATGTT 172
                 ** *********************************************************

STIB427_wildtype TATGCAGGTGATATCTTGCGCCCTTTTAGTGTTGCTAAGGAAGAACCCTTACGCCCAAAA 660
OM141            TATGCAGGTGATATCTTGCGCCCTTTTAGTGTTGCTAAGGAAGAACCCTTACGCCCAAAA 232
M148             TATGCAGGTGATATCTTGCGCCCTTTTAGTGTTGCTAAGGAAGAACCCTTACGCCCAAAA 232
OM62             TATGCAGGTGATATCTTGCGCCCTTTTAGTGTTGCTAAGGAAGAACCCTTACGCCCAAAA 232
M176             TATGCAGGTGATATCTTGCGCCCTTTTAGTGTTGCTAAGGAAGAACCCTTACGCCCAAAA 232
OM72             TATGCAGGTGATATCTTGCGCCCTTTTAGTGTTGCTAAGGAAGAACCCTTACGCCCAAAA 242
STIB950_mutant   TATGCAGGTGATATCTTGCGCCCTTTTAGTGTTGCTAAGGAAGAACCCTTACGCCCAAAA 232
                 ************************************************************

STIB427_wildtype GTACGCGGCAGAGTTCCGATATGCAGCGAGGAAAGGGATTGATGATAAGGGCGCAGATGG 720
OM141            GTACGCGGCAGAGTTCCGATATGCAGCGAGGAAAGGGATTGATGATAAGGGCGCAGATGG 292
M148             GTACGCGGCAGAGTTCCGATATGCAGCGAGGAAAGGGATTGATGATAAGGGCGCAGATGG 292
OM62             GTACGCGGCAGAGTTCCGATATGCAGCGAGGAAAGGGATTGATGATAAGGGCGCAGATGG 292
M176             GTACGCGGCAGAGTTCCGATATGCAGCGAGGAAAGGGATTGATGATAAGGGCGCAGATGG 292
OM72             GTACGCGGCAGAGTTCCGATATGCAGCGAGGAAAGGGATTGATGATAAGGGCGCAGATGG 302
STIB950_mutant   GTACGCGGCAGAGTTCCGATATGCAGCGAGGAAAGGGATTGATGATAAGGGCGCAGGTGG 292
                 ******************************************************** ***

STIB427_wildtype TGACGAAGGAAACGGCGCAGCAAAAGGGCCGGCCGATCAGGATGATGACCCCCACGGAGG 780
OM141            TGACGAAGGAAACGGCGCAGCAAAAGGGCCGGCCGATCAGGATGATGACCCCCACGGAGG 352
M148             TGACGAAGGAAACGGCGCAGCAAAAGGGCCGGCCGATCAGGATGATGACCCCCACGGAGG 352
OM62             TGACGAAGGAAACGGCGCAGCAAAAGGGCCGGCCGATCAGGATGATGACCCCCACGGAGG 352
M176             TGACGAAGGAAACGGCGCAGCAAAAGGGCCGGCCGATCAGGATGATGACCCCCACGGAGG 352
OM72             TGACGAAGGAAACGGCGCAGCAAAAGGGCCGGCCGATCAGGATGATGACCCCCACGGAGG 362
STIB950_mutant   TGACGAAGGAAACGGCGCAGCAAAAGGGCCGGCCGATCAGGATGATGACCCCCACGGAGG 352
                 ************************************************************

STIB427_wildtype CGATGATACTGACAAAGGAAATGTAATGACCGCCACTGTAGATCCTGACACAATGAAGGA 840
OM141            CGATGATACTGACAAAGGAAATGTAATGACCGCCACTGTAGATCCTGACACAATGAAGGA 412
M148             CGATGATACTGACAAAGGAAATGTAATGACCGCCACTGTAGATCCTGACACAATGAAGGA 412
OM62             CGATGATACTGACAAAGGAAATGTAATGACCGCCACTGTAGATCCTGACACAATGAAGGA 412
M176             CGATGATACTGACAAAGGAAATGTAATGACCGCCACTGTAGATCCTGACACAATGAAGGA 412
OM72             CGATGATACTGACAAAGGAAATGTAATGACCGCCACTGTAGATCCTGACACAATGAAGGA 422
STIB950_mutant   CGATGATACTGACAAAGGAAATGTAATGACCGCCACTGTAGATCCTGACACAATGAAGGA 412
                 ************************************************************

STIB427_wildtype CATGGACCAGGTGGAAAACATCACGACTTCGCAGCAGATGTTAATGGCAAGGGTATGGAA 900
OM141            CATGGACCAGGTGGAAAACATCACGACTTCGCAGCAGATGTTAATGGCAAGGGTATGGAA 472
M148             CATGGACCAGGTGGAAAACATCACGACTTCGCAGCAGATGTTAATGGCAAGGGTATGGAA 472
OM62             CATGGACCAGGTGGAAAACATCACGACTTCGCAGCAGATGTTAATGGCAAGGGTATGGAA 472
M176             CATGGACCAGGTGGAAAACATCACGACTTCGCAGCAGATGTTAATGGCAAGGGTATGGAA 472
OM72             CATGGACCAGGTGGAAAACATCACGACTTCGCAGCAGATGTTAATGGCAAGGGTATGGAA 482
```

```
STIB950_mutant    CATGGACCAGGTGGAAAGCATCACGACTTCGCAGCAGATGTTAATGGCAAGGGTATGGAA 472
                  **************** *******************************************

STIB427_wildtype  TGTGTTCTGGCGCGTTTGGCCCATGCTGTTCGCATGCTTCATGGTTTTCTTCACCACATT 960
OM141             TGTGTTCTGGCGCGTTTGGCCCATGCTGTTCGCATGCTTCATGGTTTTCTTCACCACATT 532
M148              TGTGTTCTGGCGCGTTTGGCCCATGCTGTTCGCATGCTTCATGGTTTTCTTCACCACATT 532
OM62              TGTGTTCTGGCGCGTTTGGCCCATGCTGTTCGCATGCTTCATGGTTTTCTTCACCACATT 532
M176              TGTGTTCTGGCGCGTTTGGCCCATGCTGTTCGCATGCTTCATGGTTTTCTTCACCACATT 532
OM72              TGTGTTCTGGCGCGTTTGGCCCATGCTGTTCGCATGCTTCATGGTTTTCTTCACCACATT 542
STIB950_mutant    TGTGTTCTGGCGCGTTTGGCCCATGCTGTTCGCATGCTTCATGGTTTTC---ACCACATT 529
                  *************************************************   ********

STIB427_wildtypeTCTCGTCTACCCTGCCGTGTACTTCGCCATCAAGGCAGATACGGGTGACGGCTGGTACTT 1020
OM141           TCTCGTCTACCCTGCCGTGTACTTCGCCATCAAGGCAGATACGGGTGACGGCTGGTACTT 592
M148            TCTCGTCTACCCTGCCGTGTACTTCGCCATCAAGGCAGATACGGGTGACGGCTGGTACTT 592
OM62            TCTCGTCTACCCTGCCGTGTACTTCGCCATCAAGGCAGATACGGGTGACGGCTGGTACTT 592
M176            TCTCGTCTACCCTGCCGTGTACTTCGCCATCAAGGCAGATACGGGTGACGGCTGGTACTT 592
OM72            TCTCGTCTACCCTGCCGTGTACTTCGCCATCAAGGCAGATACGGGTGACGGCTGGTACTT 602
STIB950_mutant  TCTCGTCTACCCTGCCGTGTACTTCGCCATCAAGGCAGATACGGGTGATGGCTGGTACTT 589
                ************************************************ ***********

STIB427_wildtypeGACGATCGCTGCCGCATTGTTCAATTTGGGTGATTTCTTGTCGCGTCTTTGCCTTCAGTT 1080
OM141           GACGATCGCTGCCGCATTGTTCAATTTGGGTGATTTCTTGTCGCGTCTTTGCCTTCAGTT 652
M148            GACGATCGCTGCCGCATTGTTCAATTTGGGTGATTTCTTGTCGCGTCTTTGCCTTCAGTT 652
OM62            GACGATCGCTGCCGCATTGTTCAATTTGGGTGATTTCTTGTCGCGTCTTTGCCTTCAGTT 652
M176            GACGATCGCTGCCGCATTGTTCAATTTGGGTGATTTCTTGTCGCGTCTTTGCCTTCAGTT 652
OM72            GACGATCGCTGCCGCATTGTTCAATTTGGGTGATTTCTTGTTGCGTCTTTGCCTTCAGTT 662
STIB950_mutant  GACGATCGCTGCCGCATTGTTCAATTTGGGTGATTTCTTGTCGCGTCTTTGCCTTCAGTT 649
                ***************************************** ******************

STIB427_wildtypeCAAAGCCTTACACGTCTCACCGCGGTGGGTTCTGATTGGGACATTTGCGCGTATGCTGCT 1140
OM141           CAAAGCCTATCACGTCT------------------------------------------- 669
M148            CAAAGCC-ATCACGTCTCACCGCGGTGGA------------------------------- 680
OM62            CAAAGC--ATCACGTCTCACCGCGGTGGA------------------------------- 679
M176            CAAAGCCTATCACGTCTCACCGCGGTGGA------------------------------- 681
OM72            CAAAGCCTATCACGTCTCACCGCGGTGGA------------------------------- 691
STIB950_mutant  CAAAGCCTATCACGTCTCACCGCGGTGGAA------------------------------ 679
                ******    *******
```

Figure 4.4 Comparison of *TbAT1* gene sequences (RFLP fragment) by multiple alignments. OM62, OM72 and OM141 are isolates from sleeping sickness patients treated at Omugo health centre.
M148 and M176 are isolates from sleeping sickness patients treated at Moyo hospital.
wild type *T. brucei TbAT1* (STIB427 Genbank accession number AF152369)
mutant *T. brucei TbAT1* STIB950 identical to (STIB777R Genbank accession number AF152370).

4.4.4 TbAT1 genotype and patient treatment outcome

Patients had to be followed according to protocol at 6, 12 and 24 months after treatment to detect any treatment failures or relapses (section 4.3). However, correlation of the *TbAT1* genotype with treatment outcome would only apply to melarsoprol and pentamidine-treated cases and not eflornithine where, unlike the other two drugs, resistance is not mediated through the TbAT1/P2 transporter (Delespaux & De Koning, 2007). The correlation greatly depends on the patient follow up data and successful amplification of the *TbAT1* gene. By the time of writing this thesis follow up data from Omugo, Arua was available for 25 of 88 stage II patients (Table 4.5) and 5 of 11 stage I patients (Table 4.6). Follow up data was available for only 2 patients M135 and M177 (Table 4.7) out of 34

patients from Moyo who had at most attended only one follow up visit (Patient Raw data, Appendix II). However the patient follow up diagnosis results for one of the patients (OM95) treated with eflornithine indicated an increase in the cerebral spinal fluid (CSF) white blood cell count (WBC) from 10 to 81/mm^3 in the CSF but with no detectable trypanosomes in the CSF (Table 4.5). A cut-off of 5WBC/mm^3 in the CSF and/or presence of trypanosomes in any of the body fluid is considered a late stage case at Omugo, Arua NW Uganda. No trypanosomes were observed in the CSF possibly because of very low parasitaemia and the low sensitivity of microscopy. The result would suggest that the patient failed treatment. For eflornithine to be effective, it requires an intact immune system that can recognise and fight infection after it has exerted its trypanostatic effect (Burri & Brun, 2003). It is possible that this patient was Immune-suppressed or had another secondary infection e.g. with Human Immune deficiency Virus (HIV) rendering the drug ineffective (Pepin *et al*, 1992). HIV is known to be prevalent in Uganda. It is also a possibility that the patient was infected with drug resistant trypanosomes. However, HIV does attack the T-lymphocytes (CD4 cells) in the immune system while trypanosome resistance may be due to B cells and innate factors. The drug is also difficult to administer since it requires 4 daily infusions of 100 mg/kg body weight for 14 days, which in some instance may be unbearable particularly if the patient is a child.

Patient code	Date Admitted	CSF WBC & tryps yes or no	Date followed	CSF WBC & tryps yes or no	*TbAT1* PCR/*SfaNI*	*PLC* PCR
OM46	Aug `05	205/ yes	Feb `06	5/ no	Wild type	n. d
OM47	Aug `05	247/ yes	Mar `06	43/ no	n. d	n. d
OM50	Aug `05	363/ yes	Mar `06	16/ no	n. d	n. d
OM54	Sep `05	283/ yes	Mar `06	20/ no	Wild type	n. d
OM55	Sep `05	45/ yes	Apr `06	22/ no	Wild type	Positive
OM56	Sep `05	383/ yes	Jun `06	12/ no	Wild type	n. d
OM57	Sep `05	99/ yes	Apr `06	1/ no	n. d	n. d
OM62	Oct `05	295/ yes	May `06	7/ no	Wild type	Positive
OM64	Nov `05	93/ yes	Jun `06	3/ no	Wild type	n. d
OM68	Nov `05	170/ yes	Jun `06	5/ no	Wild type	n. d
OM69	Nov `05	529/ yes	Aug `06	9/ no	Wild type	Positive
OM73	Nov `05	571/ yes	Jun `06	7/ no	Wild type	n. d
OM75	Nov `05	318/ yes	Aug `06	9/ no	Wild type	n. d
OM79	Jan `06	107/ yes	Aug `06	2/ no	Wild type	Positive
OM82	Jan `06	20/ yes	Aug `06	5/ no	n. d	n. d
OM83	Jan `06	617/ yes	Aug `06	3/ no	Wild type	n. d
OM84	Jan `06	963/ no	Jun `06	7/ no	Wild type	n. d
OM85	Jan `06	1025/ no	Aug `06	7/ no	Wild type	n. d
OM86	Jan `06	7/ no	Aug `06	0/ no	Wild type	Positive
OM89	Jan `06	21/ no	Aug `06	3/ no	n. d	n. d
OM90	Jan `06	103/ no	Aug `06	74/ no	Wild type	Positive
OM94	Jan `06	61/ no	Aug `06	3/ no	Wild type	n. d
OM95*	Jan `06	10/ no	Aug `06	81/ no	n. d	n. d
OM96	Jan `06	259/ no	Aug `06	64/ no	Wild type	Positive
OM101	Jan `06	137/ no	Jul `06	39/ no	Wild type	n. d

Table 4.5 Follow up and PCR results for stage II patients (n=25) treated with DFMO at Omugo, Arua.
"CSF WBC", cerebral spinal fluid-white blood cell count (number of cells/mm^3 CSF).
tryps, trypanosomes.
yes, presence of trypanosomes detected by microscopy
no, no trypanosomes detected by microscopy.
Haematocrit centrifugation results by microscopy were negative at follow up
Parasitological tests as determined by trained personnel at Omugo health centre
n. d, no PCR product detected in agarose.
***Patient (OM95) had an increased CSF WBC count (10 to 81/mm^3) post treatment and was readmitted and treated with melarsoprol.**

Of the 11 pentamidine treated stage I patients from Omugo health centre in Arua, follow-up data 6 months post treatment, was available for only 5 patients (Table 4.6). Of these patients, 4 had no trypanosomes in their body fluids with <5WBC/mm^3 in the CSF or none after parasitological diagnosis. Isolates from these patients possessed *TbAT1* wild type alleles according to the *Sfa NI* enzyme analysis (Table 4.6). This suggested the TbAT1/P2 transporter was not defective which may be in agreement with the hypothesis that the *TbAT1* genotype should correlate with the treatment outcome if the transporter is responsible for treatment failure or resistance.

Patient code	Date Admitted	CSF WBC & tryps yes or no	Date followed	CSF WBC & tryps yes or no	*TbAT1* PCR/*SfaNI*	*PLC* PCR
OM65*	Nov `05	3/ no	Jul. `06	39/ yes	n. d	n. d
OM88	Jan. `06	5/ no	Aug. `06	3/ no	Wild type	Positive
OM93	Jan. `06	1/ no	Aug. `06	0/ no	Wild type	Positive
OM97	Jan. `06	4/ no	Aug. `06	1/ no	Wild type	Positive
OM98	Jan. `06	1/ no	Aug. `06	3/ no	Wild type	Positive

Table 4.6 *TbAT1* status and diagnosis results post treatment of Arua stage I patients.
The Table shows results of the comparison between pentamidine treatment outcome at 6 months follow up and the PCR/RFLP results.
The *T. brucei* Phospholipase C linked microsatellite (*PLC*) PCR was used as a single copy gene control.
Haematocrit centrifugation test follow up results all negative by microscopy (not shown).
"CSF WBC", cerebral spinal fluid-white blood cell count (number of cells/mm^3 CSF).
tryps, trypanosomes.
yes, presence of trypanosomes detected by microscopy. no, no trypanosomes detected by microscopy.
Parasitological tests as determined by trained personnel at Omugo health centre.
n. d, no PCR product detected in agarose.
***Patient (OM65) failed treatment during follow up and was readmitted and retreated with eflornithine.**

The problem of low parasite densities encountered with most isolates (Table 4.3) made it impossible to determine the *TbAT1* status of the isolate from patient OM65 who had progressed to stage II disease. This patient who had an elevated CSF cell count (from 3 to 39 WBC/mm^3) also had trypanosomes in CSF after diagnosis by microscopy (Table 4.6). This patient was readmitted and treated with eflornithine. This patient's follow-up results after retreatment are not yet available. It was not possible to draw any conclusions from

these results regarding the role of *TbAT1* in relation to pentamidine. Pentamidine resistance in the field is not considered a problem because it is imported via multiple transporters, which is considered a rationale for the observed high efficacy in the field (de Koning & Jarvis, 2001;de Koning, 2001b;Matovu *et al*, 2003). It is therefore more likely that this pentamidine relapse patient (OM65) was a misdiagnosed late stage case which is a possibility especially if the cell counts are at the borderline. The weakness of this argument though, is from a study that indicated that patients in the early late stage of phase II are likely to benefit from pentamidine therapy (Doua *et al*, 1996), invoking the consideration that this observed failure as is the case with Omugo in NW Uganda could as well be a result of genuine pentamidine resistance. Indeed, results from a more recent study demonstrated increased resistance to pentamidine *in vitro* following the loss of both TbAT1/P2 and HAPT1 transport in a laboratory pentamidine resistance induced strain B48 (Bridges *et al*, 2007). However, there is no evidence to date of the loss of HAPT1 in field trypanosome isolates because the molecular characterisation of HAPT1 is not yet undertaken. Moreover, at least in mice, the B48 strain was of greatly reduced virulence and could only infect if given at a greatly elevated inoculum (Bridges *et al*, 2007).

Follow up data (6 months) was available for 2 out of the 34 melarsoprol treated stage II patients enrolled in the study from Moyo hospital. The problem of low parasite densities, made it impossible to determine the *TbAT1* status by PCR/RFLP of most of the isolates and yet for the isolates (n=9) whose *TbAT1* genotype was determined their follow up data was unavailable except for one (M135) which was wild type (Table 4.7). Patient M135 had no trypanosomes in any of the body fluids and the CSF cell count had decreased from 240 to 2/mm^3 following diagnosis by microscopy (Table 4.7). It was not possible to draw conclusions from this data. Otherwise, it would have been of interest to compare the melarsoprol treatment outcome results and *TbAT1* PCR/RFLP results of isolates from two patients M173 (relapsed earlier after melarsoprol treatment) and M174 (reinfected after 4 years of first admission) who were enrolled in this study from a previous survey (Table 4.7). For melarsoprol, a 9% treatment failure rate is regarded as normal (Legros *et al*, 1999). One would suggest that patient M173 might not be a treatment failure in this study based on the *TbAT1* genotype by PCR/RFLP further suggesting that the initial situation falls among the expected treatment failure cases regarded as normal. On the other hand, knowing the results for two other patients (M165 and M166) who had earlier failed treatment with pentamidine would have ruled out whether they were misdiagnosed late stage cases as explained earlier on for patient OM65 (Table 4.6) or genuine pentamidine resistant.

Patient code	Date Admitted	CSF WBC & tryps Yes or no	Date followed	CSF WBC & tryps Yes or no	*TbAT1* PCR/*SfaNI*	*PLC* PCR
M117	Feb `05	186/ yes	N/A	N/A	Wild type	Positive
M135	Apr `05	240/ yes	Nov `05	2/ no	Wild type	n. d
M146	Jul `05	88/ yes	N/A	N/A	Wild type	n. d
M148	Aug. `05	66/ yes	N/A	N/A	Wild type	Positive
M150	Aug `05	122/ yes	N/A	N/A	Wild type	n. d
M160	Sept `05	82/ yes	N/A	N/A	Wild type	Positive
M162	Oct `05	16/ yes	N/A	N/A	Wild type	n. d
M168	Dec `05	116/ yes	N/A	N/A	Wild type	Positive
M161*	Oct `05	15/ yes	N/A	N/A	n. d	n. d
M165*	Oct `05	10/ yes	N/A	N/A	n. d	n. d
M173§	Feb `06	13/ yes	N/A	N/A	Wild type	n. d
M174✝	Feb `06	16/ yes	N/A	N/A	n. d	n. d
M177	Feb `06	136/ yes	N/A	1/ no	n. d	n. d

Table 4.7 *TbAT1* status and diagnosis results post-treatment of Moyo stage II patients.
The Table shows PCR/RFLP results but comparison with melarsoprol treatment outcome not conclusive due to the unavailability of adequate data.
The *T. brucei Phospholipase C* (*PLC*) microsatellite PCR was used as a single copy gene control.
CSF, cerebral spinal fluid. WBC, white blood cell count. tryps, trypanosomes.
no, no trypanosomes detected by microscopy. yes, trypanosomes detected by microscopy.
Parasitological results as determined by trained personnel at Moyo hospital.
N/A, follow up results not available. n.d, PCR product not detected in agarose possibly due to low parasitaemia.
***Patient relapsed earlier following pentamidine treatment and was readmitted.**
✝Patient relapsed earlier following melarsoprol treatment and was readmitted.
§Patient reinfected (2nd admission 4 years after first admission following melarsoprol treatment) (Dr.E Matovu, personal communication).

4.5 Discussion

The alarming increase in incidence of treatment failure following the use of melarsoprol, in several HAT foci, has led to concerns that drug resistance has emerged and may be spreading (Legros *et al*, 1999;Moore & Richer, 2001;Stanghellini & Josenando, 2001). Resistance to melarsoprol has been linked to mutations in the *TbAT1* gene that encodes the P2 amino purine transporter that can also carry melaminophenylarsenical and diamidine drugs (Carter & Fairlamb, 1993;Carter *et al*, 1995;Carter *et al*, 1999;Mäser *et al*, 1999;Matovu *et al*, 2001b). Melarsoprol was replaced, in 2001, by eflornithine (DFMO) as first-line treatment for late stage disease at Omugo health centre, Arua due to high levels of treatment failure in this area (Legros *et al*, 1999). However, this was not the case in Moyo and therefore melarsoprol is still used at this centre as first-line treatment for late stage disease. Moreover, detection of treatment failures or developing drug resistance relies on the diagnosis of the presence of trypanosomes in body fluids using microscopy, a notoriously insensitive technique and dependent on long-term follow-up of patients, which is logistically fraught. A PCR/*Sfa NI* RFLP based method was devised by Mäser and colleagues (1999) to demonstrate the presence of two of the mutations in the *TbAT1* gene of the arsenical resistant *T. b. brucei* stock STIB 777R. Similar mutations were identified in isolates from *T. b. gambiense* melarsoprol relapsed (n=30) and patients infected for the first time (n=8) who were hospitalised at Omugo health centre. These manifested mainly as mixed banding patterns (26/38) reflecting the presence of both mutant and wild genotypes in the same patient (Matovu *et al*, 2001b). However, the presence of the wild type pattern alone observed in a limited number of isolates from melarsoprol relapsing patients (n=13) was suggestive that the method failed to detect all relapse cases (Matovu *et al*, 2001b). It remains possible that trypanosome numbers in the relapse cases were below detection levels, thus preventing detection of the so-called resistance alleles. One would also suggest that these wild type drug failures are due to inadequate administration or poor drug batches implying failure may not be due to true drug resistance but possibly other biochemically mediated factors. But the patients were treated under careful supervision by trained medical personnel for the whole duration of hospitalisation and only drugs donated by WHO were used. Further analyses using *in vitro* drug susceptibility assays on the isolates from NW Uganda revealed that treatment failures in this region were most likely due to decreased melarsoprol susceptibility (Matovu *et al*, 2001a) although results from a pharmacokinetic study suggested melarsoprol treatment failures in this region could be due to other factors (Brun *et al*, 2001). The treatment policy was changed through mediation by the Médecins Sans Frontières (MSF), who then worked with Ministry of Health, which

granted permission for the replacement of melarsoprol with DFMO for Omugo Health centre, and Yumbe hospital which took effect in 2001. However, monitoring of the *TbAT1/P2* status in the *T. b. gambiense* isolates continued in order to investigate possible changes in genotypic composition over time. Furthermore, although eflornithine does not utilize the P2 transporter, monitoring of *TbAT1/P2* status was deemed important for pentamidine (therapy for *T. b. gambiense* stage I disease) whose uptake occurs via the same P2 transporter (Carter *et al*, 1995;Barrett & Fairlamb, 1999) in addition to other transporters (de Koning & Jarvis, 2001;de Koning, 2001b;Matovu *et al*, 2003) whose loss has been shown to lead to high level resistance *in vitro* (Bridges *et al*, 2007). Besides, the same P2 transporter is the targeted route for delivery of furamidine [DB75 (2,5-bis(4-amidinophenyl)furan] a diamidine whose prodrug DB289 is the O-methyl amidoxime derivative (pafuramidine) oral drug that was until recently undergoing phase III clinical trials for the treatment of early stage disease (Lanteri *et al*, 2006;Jannin & Cattand, 2004).

While the previous study(Matovu *et al*, 2001b) utilized isolates from one HAT focus (Omugo), this study has been performed using isolates from the same region as well as from the neighbouring foci in Moyo district. The PCR/*Sfa NI* RFLP based method was used, to determine the current status of *TbAT1/P2* in isolates from Omugo and whether the *TbAT1* mutant type alleles observed previously are still in existence after a prolonged absence of treatment with melarsoprol. We further aimed to generate more evidence of a correlation between the *TbAT1* genotype and treatment outcome, using isolates collected from Moyo where melarsoprol is still used as first-line treatment for stage II disease. Equal importance was also attached to observation of pentamidine treatment outcome.

In the present study, analysis of all the successfully amplified 677bp *TbAT1* fragments revealed one banding pattern with 566 and 111bp fragments matching with the wild type *TbAT1* of the melarsoprol sensitive strain (STIB 777S) which was present in all samples from Arua, Omugo (n=82) and Moyo (n=9). It is clear that the *TbAT1* mutant alleles at Omugo have dwindled (0%) compared to about 30% overall prevalence observed earlier when the treatment failure rate (also 30%) was at its highest (Matovu *et al*, 2001b). This absence of the *TbAT1* mutant type alleles may be due to a shift in Arua (currently Nyadri district) foci from Terego where most cases previously originated, to Koboko that is the current source of patients treated at Omugo (Figure 3.1). A more recent report did indicate *TbAT1* mutant alleles were present in isolates collected from Moyo in the period 2003-2004 albeit at low prevalence that coincides with the relatively low melarsoprol relapse rates (9%) at the focus (Nerima *et al*, 2007). Their approach was based on using an allele-

specific PCR but the results were in agreement with the PCR/RFLP based method. This method is worth considering, since it eliminates the problem of differentiating between a single infection due to a trypanosome that is *TbAT1* heterozygous for wild type and mutant alleles and a mixed infection due to a trypanosome that is either *TbAT1* homozygous/hemizygous for wild type or mutant alleles. The current analysis using the PCR/RFLP method had been completed by the time *tbat1* allele specific PCR report was published and therefore I did not take a similar approach. Comparison of the current findings with the results from previous studies does suggest a decline in *TbAT1* PCR/*SfaNI* mutant alleles over the years with a possibility of few or no mutant alleles in circulation now (Table 4.8).

Study period/ sample number (N)	Melarsoprol relapse rate (%)	*TbAT1* mutant alleles prevalence (%)	References
1999-2001 (65)	30	59	Matovu *et al*, 2001b
2003-2004 (80)	10	14	Nerima *et al*, 2007
2005-2006 (91)	***	0	Kazibwe A, this Thesis

Table 4.8 The observed prevalence of *TbAT1* mutant alleles in NW Uganda *T. b. gambiense.* The Table shows a declining trend in the number of PCR/RFLP mutant alleles when results from previous studies and this study are compared for patient samples (N) collected in the presence and absence of melarsoprol at the different time periods.
***; **Relapse rate not registered since follow up data incomplete at the time of writing this thesis.**

To determine whether a difference existed between the results obtained during each study period, and the sample numbers, the different *TbAT1* alleles were either grouped separately (wild type alleles pure, mutant alleles pure and mixed alleles) or combined (wild type total or mutant total) and analysed using the Chi square test. There was a significant difference (i.e. $P<0.05$) with $X^2=87.5$, P=0.000 and $X^2=57.1$, P=0.000 respectively (Table 4.9 and Table 4.10). The result suggests a difference exists between the sample numbers analysed for the different alleles results obtained during each study period.

Study period	**TbAT1* wild type alleles pure**	**TbAT1* mutant alleles pure**	**Mixed alleles (WT & Mut)**	**Total (N)**
1999-2001	27	12	26	**65**
2003-2004	69	0	11	**80**
2005-2006	91	0	0	**91**
Total (N)	**187**	**12**	**37**	**236**

Table 4.9 *TbAT1* alleles and corresponding sample numbers analysed over three study periods.
X^2=87.5 (P=0.000) when alleles are grouped separately.
WT, *TbAT1* wild type alleles. Mut, *TbAT1* mutant alleles.
***Analysis based on PCR/*Sfa NI* analysis.**

Study period	**TbAT1* wild type alleles total**	**TbAT1* mutant alleles total**	**Total (N)**
1999-2001	53	38	91
2003-2004	80	11	91
2005-2006	91	0	91
Total (N)	**224**	**49**	**273**

Table 4.10 *TbAT1* alleles present (by *PCR/Sfa NI*) grouped together over three study periods X^2=57.1, (P=0.000)

The low relapse rates reported for Moyo (9%) in comparison to Omugo (30%) suggest that the Moyo trypanosome population may be entirely different from the Omugo, Arua population since such high relapse rates were never observed at Moyo. Findings from the microsatellite analysis study in Chapter 3 gave an indication that the two populations are different (Nei's genetic distance 0.176). However, this result is based on the analysis of low sample numbers for Moyo (N=17). It is possible, given the known focal nature of HAT, that even the populations within the different Arua regions Terego, Koboko are different. Although the Arua trypanosome genotypes were observed to be diverse by microsatellite marker analysis, the trypanosomes isolated from the regions within Arua are not any different or clustered based on location (Figure 3.6) but a difference does exist between the Arua and Moyo populations. It was observed that there were villages within

the endemic areas where CATT reactions were negative, while other villages only a few kilometres away had positive patients in abundance (Dr E. Matovu, personal communication). It is therefore possible that just a few tsetse flies concentrated around a watering hole may propagate an epidemic within a limited geographical range. Genetic exchange between parasites occurs through mating within the tsetse populations. This might be of concern based on the finding of *tbat1* mutants in some of the isolates as revealed by the sequencing results, which would suggest that such trypanosomes might be able to repopulate the region quickly. This might have implications for treatment with 'P2 drugs' especially if the identified mutations alter the function of TbAT1/P2 and/or have a fitness cost to the trypanosomes, either in the mammalian host or in the tsetse fly. The results from the microsatellite analysis (Chapter 3) however, indicated some degree of genetic exchange within the Arua population based on analysis of samples collected between 2005 and 2006 period. The situation may be different now.

The results from this PCR/RFLP analysis also suggest that the patients treated at Omugo and Moyo were exclusively infected with potentially melarsoprol sensitive trypanosomes. The absence of the PCR/RFLP *TbAT1* mutant type alleles from the area seems to correlate with withdrawal of melarsoprol from Omugo health centre, although this could be a result of the coincidental shift of the focus to Koboko. The majority of the patients used to come from the immediate surroundings of Terego sub-county during the late 1990s, when melarsoprol treatment failures where common but from 2003, the majority of the patients who received treatment at Omugo originated from Koboko which is 40 km away.

Drug resistance-associated alleles can carry a fitness cost and can thus be lost from a population when selective drug pressure is removed. For example *Plasmodium falciparum* having mutations in the genes (*Pfmdr1 and Pfcrt*) associated with chloroquine resistance was observed *in vivo* and *in vitro* in the absence of drug (Walliker *et al*, 2005;Hayward *et al*, 2005). This observation had been noted earlier in the field following the withdrawal of chloroquine which corresponded with disappearance of the resistance genotype (*Pfcrt 76T*) and reversal of resistance within 10 years of this strategy in Malawi (Kublin *et al*, 2003). It should be noted that in the case of *P. falciparum* this is possible since resistant parasites can be easily spread by mosquito bites to treated patients thereby encouraging development of resistance by transmission of drug exposed pathogens. In the case of HAT, this is less likely since patients are treated under carefully monitored hospital settings and do not resume normal activities that would expose them to tsetse flies, at least not to such a degree as to mosquitoes, which is in contrast to tsetse infest indoors, in the case of malaria.

Further *Plasmodium* undergoes obligate sexual reproduction in the mosquito whereas genetic exchange between trypanosomes in the tsetse vector is optional and thought to be relatively rare. It is therefore unlikely that the vector plays a significant role in the spread of trypanocidal resistance mutants arising from drug exposure. The most probable explanation may be consistent with the hypothesis that melarsoprol resistant trypanosomes that possess these *TbAT1* mutant alleles are unstable in the field or declining in the population in the absence of drug pressure. In South Sudan where the drug was withdrawn in 2001 because of high failure rates (Moore & Richer, 2001), a similar situation was observed. No *TbAT1* mutant alleles were detected by PCR/RFLP and sequencing in any of the *T. b. gambiense* collected in 2003 (Maina *et al*, 2007) which led to the authors to speculate that *TbAT1* mutants never occurred. However, in the previous study in Omugo, NW Uganda, (Matovu *et al*, 2001b), "mutant" alleles, were present but they appear to have disappeared since treatment with melarsoprol was terminated. It is therefore possible that "resistant" alleles could also have been circulating in South Sudan when melarsoprol acted as a selective force although this cannot be proven either way as no samples were collected at that time. A recent report indicates the presence of *TbAT1* mutant alleles at a prevalence of 14% in isolates collected between 2003 and 2004 from Moyo, NW Uganda where melarsoprol is still in use to the present day further supporting the existence of such alleles (Nerima *et al*, 2007). The small sample number successfully analysed by PCR/RFLP from Moyo (9 of 34) may possibly be responsible for the lack of observation or current absence of resistant alleles from this region.

The PCR/*Sfa NI* RFLP based method is useful as a field tool for detection of *TbAT1/P2* defective trypanosomes especially to monitor for emerging resistance to drugs that use the same P2 transporter. However, the method does not reveal anything about other mutations and deletions. The PCR/RFLP analysis reported here, based on the *Sfa NI* profiles indicated that all the parasites identified here were wild type at that marker. However, sequence analysis (verified on both strands) indicated other changes. For example in the *TbAT1* sequence of isolate OM72 at position 1062 is a base change from C to T but more dramatically are two nucleotides missing at positions 1087 and 1088 in isolate OM62, which should yield a frame shift. This would dramatically alter the protein most likely leading to a loss of activity. A single base deletion in M148 would create a similar frame shift. Sequencing was performed on both strands but just once. It would require re-amplification and reanalysis to confirm these important results that further expand the ways to which diverse alterations to *TbAT1* may contribute to loss of P2 activity in clinical settings. Since the PCR/RFLP method does not detect other mutations which could also

lead to loss of P2 function, phenotypic tests such as a-fluorescence based test would be useful. One such test for detection of loss of P2 function has been developed (Stewart *et al*, 2005).

A limitation of this study was the low patient return for follow up visits especially in Moyo, making the analysis difficult. In a previous field survey taking into account all patients that were followed up at least once, patients who felt better after treatment had to be convinced to return for another lumbar puncture (Dr. E. Matovu personal communication). Similar problems have been encountered in related field studies where patients are impossible to follow through the 24 months leading to a lack of complete post treatment data (Lejon & Buscher, 2005;Burri *et al*, 2000). This has been associated with waiting periods, which stretch beyond 3 months. Yet 49.3% of patients at discharge not attending any of the 4 scheduled follow up visits and 50.7% of the patients attending only 1 visit have been reported (Schmid *et al*, 2005). Follow up numbers have been reported to drop from 64% at 6 months to 46% at 12 months post treatment (Chappuis *et al*, 2005), which has resulted in restricting the treatment outcome analysis to 12 months in particular studies since the numbers drop even lower beyond this period (Balasegaram *et al*, 2006).

4.6 Conclusion

The PCR/RFLP method detected only *TbAT1* wild type alleles and not the mutant allele that was in circulation in 1999. The limited number of followed up patients means that the correlation between the *TbAT1* genotype and treatment outcome do not permit tests for statistical significance. However, the data suggests that melarsoprol sensitive trypanosomes are in circulation and therefore melarsoprol could still be a useful drug in the treatment of stage II disease, possibly even at Omugo where it was withdrawn 6 years ago. But to ascertain its usefulness at the centre, a rapid and specific test easily adaptable to a field setting is required to identify potential melarsoprol treatment outcome. Results from the use of such a test would guide the clinical decision process regarding treatment using this drug. The detection of homozygous resistance alleles would be grounds for treatment failures with either eflornithine or with nifurtimox as a compassionate treatment, or with a combination of both (Matovu *et al*, 2001a). However, the absence of the particular mutation detected by the PCR-RFLP method (apparent wild type alleles) would not necessarily guarantee the presence of a functional P2 transport activity, due to the possibility of other mutations or the low abundance of resistant parasites in a mixed population. Nifurtimox/Eflornithine Combination Trials (NECT) are on-going in Uganda

and Congo. On the other hand, (Bisser *et al*, 2007) have demonstrated successful combination of melarsoprol with Nifurtimox, although this may not be a lasting solution at foci where melarsoprol is already failing. A fluorescence based method for detection of P2 transport activity (Stewart *et al*, 2005) appears to be a promising option for assisting in the determination of the specific drug treatment.

Chapter 5

5 Detection of animal and human infective trypanosome species in dogs as possible reservoirs

5.1 Summary

As discussed in the introduction (section 1.4.1) diminazene enters trypanosomes predominantly via the P2 transporter and loss of P2 correlates to drug resistance. There is a risk that treatment of animals with diminazene aceturate (to clear babesiosis or trypanosomiasis) could therefore contribute to selection of P2 defective trypanosomes, resistant to other drugs should human infectious trypanosomes infect these animals. Species typing by PCR is the most sensitive tool for the detection of trypanosome infections including those caused by *T. b gambiense,* which is known to manifest at very low parasite densities making microscopic detection insensitive. Apart from confirming presence of trypanosomes by microscopy, distinguishing between human and animal trypanosomes is dependent on qualified personnel whose work is made particularly difficult if faced with low parasitaemic infections. With the availability of species specific markers, PCR typing using species specific primers can now be used to identify the presence of the human infective *T. b. gambiense* in isolates from animals capable of acting as a reservoir (Radwanska *et al*, 2002b). The aim of this study was to use species specific PCR typing to detect the different trypanosome species known to be infective to dogs (*T. brucei*, *T. vivax*, *T. congolense*) and humans (*T. b. gambiense*) in samples (N=190) collected from dogs (Mongrels) from the tsetse fly endemic areas of the Jos High Plateau area, North Central Nigeria, West Africa. These were provided to us by our collaborator the late Dr. E. Yanan (NITOR). The success rate for the detection of animal trypanosomes (*T. brucei*, *T. vivax*, *T. congolense*) in this region has been recorded at 27.9% by microscopy using the wet smear and the haematocrit centrifugation technique (Dede et al, unpublished). Samples were collected in a field survey as dried blood spots on FTA® cards (Whatman) between the months of August and November 2006 from the local government areas of the plateau state during an animal trypanosomiasis outbreak (Dede *et al*, unpublished). Field diagnosis results by microscopy carried out by qualified personnel were later provided and these indicated an absence of trypanosomes (Appendix III). The dog samples were also analysed by PCR for infection due to babesiosis since field diagnosis of the dogs indicated they had clinical symptoms. This was confirmed using

microscopy which indicated infections due to *Babesia gibsoni* by thin smear (23/190). Some of the dogs were domestic and used for hunting, guarding or kept as pets while some were free roaming and originated from typical rural, urban or near urban areas. Unlike dogs from around urban areas, which are near veterinary clinics, dogs from the typical rural areas do not have access to treatment (Dr. E Yanan, personal communication). All the sampled dogs were treated for babesiosis or trypanosomiasis using a single dose of diminazene aceturate (Trypadim®, Merial, France) at 3.5 mg/Kg body weight. The PCR results indicated that 180 of 190 (95%) of the analysed dog samples were infected with trypanosomes from the *T. brucei* group and 169 of 180 (94%) were co-infected with *T. vivax*. 59.5% and 51% of the 190 dog samples were, also infected with *T. congolense* subtypes (savannah (59.5%) or forest (51%)). None of the dogs sampled (N=190) was positive for *T. b. gambiense*. 73 of the 190 (38%) samples were found to be PCR positive for *Babesia* using non species-specific babesiosis primers. Sequencing for *Babesia* species confirmation of four samples, positive by PCR, indicated that two of the samples were 99% similar to the reference *Babesia canis rossi* 18s ribosomal RNA gene sequence (GenBank accession number DQ111760.1) by blast search. The remaining two samples were 99% similar to the reference *Hepatozoon canis* 18s ribosomal RNA gene sequence (GenBank accession number DQ439543). The analysis revealed that the dogs were co-infected with several parasite species, which may complicate the successful treatment of the disease. Moreover, using the same treatment for both trypanosomiasis and babesiosis as was the case for these dogs might result in selection for resistance to diminazene especially if the dogs are treated for only babesiosis. This is likely to be a problem due to the lack of sensitivity of detection of trypanosomes by microscopy. I was interested to learn to what extent misdiagnosis could underlie introduction of inappropriate treatment options.

5.2 Introduction

The control and management of Human African Trypanosomiasis involves screening of people at risk, diagnosis of the disease, treatment of infected people and reducing tsetse fly numbers. However, animals acting as a reservoir represents one of the ways in which these trypanosomes are maintained in circulation (Gibson *et al*, 1978;Mehlitz *et al*, 1982;Zillman *et al*, 1984). Most important are domestic animals which include cattle, pigs, goats,(Njiru *et al*, 2004;Waiswa *et al*, 2003;Welburn *et al*, 2001a) and even dogs (Gibson & Gashumba, 1983). Of the three *T. brucei* sub-species only *T. b. brucei* does not infect humans but does infect animals. *T. b. rhodesiense* infects both humans and domestic animals especially cattle (Welburn *et al*, 2001a). *T. b. rhodesiense* is typically known to cause acute disease in

humans but there is evidence that it can cause different disease patterns as either acute (SE Uganda) or mild (Malawi) in humans (MacLean *et al*, 2004). This is an indication that animals and humans can both act as major reservoirs. Humans are the major reservoir of *T. b. gambiense* which typically causes the chronic form of the disease (Welburn *et al*, 2001b). It is of concern that the same vector *Glossina palpalis* transmits *T. b. gambiense* to both humans and animals (Waiswa *et al*, 2003). Studies looking at *T. brucei* isolates from man and animals have revealed more mammalian hosts acting as reservoirs for *T. b. gambiense* (Gibson *et al*, 1978;Mehlitz, 1986;Penchenier *et al*, 1999). It has also been speculated that animals could have a veterinary link to human *T. b. gambiense* disease (Barrett, 2001). Diagnosis of these parasites has always depended on microscopy, which apart from being less sensitive is unable to differentiate *T. b. brucei*, *T. b. rhodesiense* and *T. b. gambiense* morphologically. The diagnosis of both animal and human trypanosomes has improved over time with new methods which include use of species specific PCR primers based on markers to specifically identify each trypanosome group or species (Moser *et al*, 1989;Masiga *et al*, 1992;MacLeod *et al*, 1999;Welburn *et al*, 2001a;Gibson *et al*, 2002;Radwanska *et al*, 2002a;Radwanska *et al*, 2002b;Simo *et al*, 2008a). The *T. b. gambiense* specific glycoprotein (TgsGP) PCR primers (Radwanska *et al*, 2002b) have been used to demonstrate the presence of *T. b. gambiense* in wild (rodents, ungulates, primates, carnivores, reptiles) and domestic animals especially pigs from Cameroon and Central Africa (Nkinin *et al*, 2002;Herder *et al*, 2002;Simo *et al*, 2006;Simo *et al*, 2005). Moreover these animals were also found co-infected with other animal trypanosome species, as demonstrated by the use of species specific primers for *T. b. brucei*, *T. vivax*, and *T. congolense* (Herder *et al*, 2002;Simo *et al*, 2006). Genetic analysis using a recently developed method, Amplified Fragment Length Polymorphism (AFLP) has been used to demonstrate that *T. b. gambiense* isolates collected in Central Africa from pigs were of the same genotype as *T. b. gambiense* isolated from man in the same and different geographical regions (Simo *et al*, 2008a).

The animal trypanocide diminazene aceturate like the human trypanocides melarsoprol and pentamidine is known to have a dependency on the same *T. brucei* adenosine transporter (TbAT1/P2) for uptake (Matovu *et al*, 2003;de Koning *et al*, 2004) (section 1.4.2). Moreover, it has been demonstrated that the same transporter is the major route of uptake of furamidine (DB75) whose prodrug DB289 was until recently under development for the treatment of human disease (Jannin & Cattand, 2004;Lanteri *et al*, 2006). Different sub-Saharan African countries are experiencing parasite resistance to animal trypanocides (Delespaux & De Koning, 2007). Trypanosome infected animals (e.g. cattle, pigs, dogs)

from tsetse endemic regions in Nigeria are mostly treated using diminazene aceturate, but the problem of resistance has rendered treatment less effective leading to the use of isometamidium chloride in some regions (Kalu, 1995;Anene *et al*, 2001;Anene *et al*, 2006). This continuous treatment of infected animals that are capable of acting as reservoirs of human infection with such trypanocides has been considered as a possible route that may lead to selection of human infective P2 drug resistant trypanosomes. This may eventually result in cross resistance between animal and human trypanocides which utilize the same P2 transporter (Barrett & Fairlamb, 1999). Not only is diminazene aceturate used for treatment of animal canine trypanosomiasis but it is also used for treatment of babesiosis which is another parasitic disease that affects animals (Brüning, 1996), including dogs http://www.addl.purdue.edu/newsletters/1999/fall/cb.shtml. It is likely that animals treated for *Babesia canis* may also harbour human infective trypanosomes, which could result in the selection of drug resistant human infective trypanosomes. In a recent study a *T. b. brucei* stock resistant to diminazene aceturate was isolated from an infected dog in Nigeria and was found to be cross-resistant to pentamidine when tested *in vivo* in albino rats (Anene *et al*, 2006). A more recent study has indicated that melarsoprol resistant *T. b. rhodesiense* isolates from sleeping sickness patients from Kenya and Uganda were found to be cross resistant to diminazene aceturate when tested *in vivo* in mice (Kagira & Maina, 2007). Melarsoprol resistant parasites which resulted from selection pressure were also found cross-resistant to diminazene possibly due to loss of the same transporter system. The concept of licensing diminazene aceturate for treatment of human disease should be considered with caution and preferably not implemented.

5.3 Materials and Methods

Study area and population

This section includes a description of the study regions in Nigeria from where the samples for this chapter were collected. This was through collaborations established between Dr. Michael Barrett and other researchers from West Africa. Dr. Everestus Yanan-Deceased (NITOR, Nigeria) provided us with samples collected from trypanosomiasis and babesiosis infected dogs in Jos High Plateau, North Central Nigeria.

A pilot survey of Canine Trypanosomiasis in dogs was carried out in Jos East, Bassa, Riyom, Barkin Ladi and Mangu Local Government areas which are located between latitudes 8.65° and 9.13°N and longitude 9.55° and 9.68°E and altitudes between 998 to

1600 metres above sea level on the of Jos High Plateau of Northern Nigeria. Some of these regions are urban and others are typically rural. The region is comprised of the semi nomadic (Fulani) ethnic group whose main occupation is hunting. Most of the dogs in the survey were hunting dogs, guard dogs or kept as pet dogs. These dogs had owners or some were in transit for sale to hunters and people who wished to keep pets or guard dogs. However, although these dogs had owners, some could move freely in search of prey.

Sample collection

Blood samples were taken before treatment from all the dogs examined during the survey in Nigeria, Jos Plateau. The blood samples (N=190) were collected using the FTA® card method by field personnel. The air-dried FTA card samples were transported for analysis at the Institute of Biomedical and Life Sciences, University of Glasgow, UK. The dogs diagnosed as positive for trypanosomaisis or babesiosis were treated using the same dosage of diminazene aceturate (Berenil) at 3.5 mg/kg body weight.

DNA extraction

Extraction of DNA from dog blood spotted on FTA cards was performed as described (section 2.3).

Species typing by PCR of dog samples from Nigeria

It was of interest to investigate whether *T. b. gambiense* might be present in domestic animals from the collection of dog derived blood in Nigeria. Microscopy is the main method used for diagnosis of *T. brucei* infections in the field. However, its lack of sensitivity means it may fail to detect some infections. Moreover, microscopy cannot distinguish *T. brucei brucei* and *T. b. gambiense* and other trypanosome species (*T. congolense* and *T. vivax*) can only be distinguished by trained personnel. Analysis using a more sensitive method by PCR on infected dog samples from Nigeria was performed to identify the presence of any infection due to human infective *T. b. gambiense* parasites using specific primers (Radwanska *et al*, 2002b). These dog samples were also analysed for detection of infective trypanosome species *T. brucei*, *T. vivax*, and *T. congolense* (Masiga *et al*, 1992) and infection due to *Babesia* (Foldvari *et al*, 2005) since many of the dogs were presented at the clinic with suspected babesiosis.

Detection of the *T. vivax* West Africa, *T. congolense* forest and *T. congolense* savannah by PCR amplification of satellite DNA repeats

In a first round PCR, a DNA disc on an FTA® card was PCR amplified in a total volume of 20 µl containing a 1 × PCR buffer (10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl) and 200 µM each deoxynucleotide triphosphate- dATP, dCTP, dGTP, dTTP) (Promega Corporation, Madison, USA). Species-specific oligonucleotide primer pairs (Figure 5.1) synthesised by MWG Biotech-AG each at 1 µM and 1.25 units of GoTaq DNA polymerase enzyme (Promega Corporation, Madison, USA) were added to the reaction. The reaction was performed in a PTC-200™ DNA Engine (MJ Research, inc., Waltham, MA, USA) under the following conditions: initial denaturation at 94°C for 3 minutes, followed by denaturation at 94°C for 1 minute, annealing at 60°C for 2 minutes, and extension at 74°C for 30 seconds for 30 cycles. 1 µl of genomic DNA from reference strain *T. vivax* (Y486) (Masiga *et al*, 1992), *T. congolense* forest (TSW 103) (Masiga *et al*, 1992) and *T. congolense* savannah (WG 81) (Masiga *et al*, 1992) was included in each respective PCR run as a positive control otherwise it was included as a negative control. Sterile water without DNA template was also included in each run as a negative control. To increase the sensitivity of the reaction, a second round PCR was performed using 1 µl of the first round PCR product as template with the same oligonucleotide primer pair under the same PCR and amplification conditions.

Detection of *T. brucei* group species by PCR amplification of the *T. brucei* repeat

PCR amplification of the *T. brucei* multicopy locus (177 bp repeat) was performed as described (section 2.3) using the *T. brucei* group oligonucleotide primer pair TBR-F and TBR-R (Moser *et al*, 1989) (Table 2.1).

Attempts to detect *T. b. gambiense* by PCR amplification of *TgsGP*

Each DNA disc on an FTA® card was amplified in a total volume of 20 µl containing 1 × PCR buffer (20 mM Tris-HCl (pH 8.7), 100 mM KCl, 50 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$ and Q solution) (Qiagen), and 200 µM each dinucleotide triphosphate- dATP, dCTP, dGTP, dTTP) (Promega Corporation, Madison, USA). *T. b. gambiense* specific oligonucleotide primer pair TgsGP-F and TgsGP-R (Radwanska *et al*, 2002b) (Table 2.1) synthesised by MWG Biotech-AG each at 0.5 µM and 1.25 units of HotStarTaq DNA polymerase (Qiagen) were added to the reaction. The reaction was performed in a PTC-200™ DNA Engine (MJ Research, inc., Waltham, MA, USA) under the following conditions: initial denaturation at 95°C for 15 minutes, followed by denaturation at 94°C

for 1 minute, annealing at 63°C for 1 minute, and extension at 72°C for 1 minute for 45 cycles. This was followed by a last extension at 72°C for 10 minutes. 1 µl of genomic DNA (5 ng/µl) or a DNA disc from an FTA® card (Whatman) from *T. b. gambiense* reference strain (ELIANE) (Pays *et al*, 1983) was included in the PCR run as a positive control. 1µl (1:100 dilution) of genomic DNA from a *T. b. brucei* reference strain (427), *T. b. rhodesiense* reference strain (Nyanza-1052) (Baldry, 1972), *P. falciparum* reference strain (7G8) (Su *et al*, 1997) and sterile water (no DNA template) were included in the run as negative controls. To increase the sensitivity of the reaction 1 µl of the primary PCR product was used as template in a second round reaction under the same PCR and amplification conditions.

Detection of *Babesia* species by PCR amplification of the 18S ribosomal RNA gene

Each DNA disc on an FTA® card (Whatman) was amplified in a total volume of 20 µl containing 1 × PCR buffer (10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl) and 200 µM each dinucleotide triphosphate-dATP, dCTP, dGTP, dTTP) (Promega Corporation, Madison USA). A non species-specific oligonucleotide primer pair (Table 5.1) synthesised by MWG Biotech-AG each at 0.5 µM and 1.25 units of GoTaq DNA polymerase (Promega Corporation, Madison, USA) were added to the reaction. The reaction was performed in a PTC-200™ DNA Engine (MJ Research, inc., Waltham, MA, USA) under the following conditions: initial denaturation at 94°C for 10 minutes, followed by denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 30 seconds for 30 cycles. This was followed by a last extension at 72°C for 5 minutes. *T. b. gambiense* reference strain (ELIANE) DNA from an FTA® card (Whatman) disc and sterile water (no template) was included in the PCR run as a negative control. Because of the difficulty encountered in acquiring a known *Babesia* reference strain, it was not possible to include a positive control in the assay. Four PCR products from the *Babesia* amplified samples were sequenced following the protocol as described (section 4.3) to confirm the infecting species.

Species/sub species	Primer pair	Sequence (5'-3')	T_m	Product size
T. vivax West Africa	TVW1 TVW2	5'-ctg agt gct cca tgt gcc ac-3' 5'-cca cca gaa cac caa cct ga-3'	61.4°C 59.4°C	≈150 bp
T. congolense forest	TCF1 TCF2	5'-gga cac gcc aga agg tac tt-3' 5'-gtt ctc gca cca aat cca ac-3'	59.4°C 57.3°C	≈350 bp
T. congolense savannah	TCS1 TCS2	5'-cga gaa cgg cac ttt gcg a-3' 5'-gga caa aca aat ccc gca ca-3'	61.4°C 57.3°C	316 bp
Babesia	PIRO A1 PIRO B	5'-agggagcctgagagacggctacc-3' 5'-ttaaatacgaatgcccccaac-3'	65.2°C 42.9°C	≈ 450 bp

Table 5.1 Animal trypanosome oligonucleotide PCR primer sequences and their properties. The *Babesia* primer pair PIRO A1 and PIRO B is non-species specific.
T_m, primer melting temperature as calculated by the oligonucleotide manufacturer.

Agarose gel electrophoresis and UV transillumination

A volume of 18 µl of each TgsGP PCR product and 10-15 µl of each of the PCR products from the rest of the amplification reactions was analysed on a 2.0% Agarose gel and visualised as described (section 2.3).

5.4 Results

5.4.1 PCR species typing for detection of T. vivax, and T. congolense satellite DNA in dog isolates

The dogs originated from areas with a high prevalence (approximately 27.9%) of animal trypanosomiasis as detected by microscopy (Dede *et al*, unpublished). The PCR technique, which is more sensitive than microscopy, was therefore utilised to detect the presence of trypanosome infections and to differentiate between the infecting trypanosome species. DNA extracted from FTA® cards (Whatman) from dog samples (N=190) collected by Dr. Yanan was performed as described (section 2.3). Detection of *T. vivax* and *T. congolense* infections was performed by PCR amplification of satellite DNA from dog samples (N=190) using species specific primer pairs TVW1 and TVW2 for *T. vivax* (Masiga *et al*, 1992), TCS1 and TCS2 or TCF1 and TCF2 for the *T. congolense* subtypes savannah and forest (Masiga *et al*, 1992) respectively as described (section 5.3). Results indicated that infection due to *T. vivax* showed the highest prevalence (91%), followed by infection due to *T. congolense* forest (59.5%) while the prevalence of *T. congolense* savannah was 51%

(Table 5.2). The findings also revealed that the dogs infected with *T. vivax* were also co-infected with either one or both of the *T. congolense* subtypes (Table 5.2).

5.4.2 Detection of T. brucei by PCR in dog isolates

Trypanozoon detection was carried out prior to PCR typing for the *T. b. gambiense* infections to determine if the dogs were infected with any trypanosomes from the *T. brucei* group other than the tested animal trypanosomes (section 5.3). DNA extraction from FTA® cards (Whatman) containing dog blood samples (N=190) was performed as described (section 2.3). PCR amplification of the *T. brucei* 177 bp repeat using the specific trypanozoon group multicopy *T. brucei* primers TBR-F and TBR-R (Moser *et al*, 1989) was performed as described (section 2.3). These primers were demonstrated to be highly sensitive and therefore useful for reporting on *T. brucei* infections in samples with a low parasite density (1 trypanosome /ml) using the FTA method (Chapter 2). Separation of the PCR products in agarose by electrophoresis followed by observation under U.V light showed signals corresponding to the *T. brucei* 177 bp repeat for 180 of 190 (94.7%) dog (Table 5.2 and Table 5.4). This result confirmed the dogs were infected with *T. brucei* species and at the same time co-infected with the other animal trypanosome species. *T. brucei* co-infections with *T. vivax* had the highest prevalence of 89% (Table 5.2 and Table 5.4). Lack of amplification material was ruled out for the ten TBR PCR negative samples, which were found positive for infections from the non *T. brucei* group or babesia (Table 5.4). The presence of multiple animal trypanosome species as observed in this study is in agreement with findings from other studies (Herder *et al*, 2002;Simo *et al*, 2006;Pinchbeck *et al*, 2008).

5.4.3 PCR typing for detection of the TgsGP gene in dog isolates

This was carried out to determine if there could be a possibility of this human infective parasite being present in these dog samples. DNA extracted from FTA® cards (Whatman) containing blood from dog samples (N=190) was performed as described (section 2.3). PCR analysis was performed to amplify a 306 bp fragment of the *T. brucei gambiense* specific glycoprotein gene (Berberof *et al*, 2001) using the PCR *T. b. gambiense* specific glycoprotein primers TgsGP-F and TgsGP-R (Radwanska *et al*, 2002b) as described (section 2.3). Preliminary results indicated that three of the first 50 samples analysed were possibly positive for the *TgsGP* gene on amplification. However, these results were not reproducible (DNA extraction and PCR repeated 5 times) even after increasing sensitivity

using the whole genome amplification method (Morrison *et al*, 2007). The same *T. b. gambiense* (ELIANE) reference strain used as a control, along with FTA® cards (Whatman) containing human blood (Chapter 2) was used. The preliminary results were therefore disregarded. Overall, all of the dog samples (N=190) were found to be negative by PCR of the *TgsGP* gene, which suggested that these dogs were not infected with *T. b. gambiense* parasites. Amplification of the single copy *T. brucei PLC* microsatellite linked gene as a positive control was not performed in this study. Determination of the presence of human disease in this region is currently under investigation. The samples were not analysed using the serum resistance antigen (SRA) primers specific for *T. b. rhodesiense* (Welburn *et al*, 2001a;Radwanska *et al*, 2002a;Gibson *et al*, 2002) since their source of origin is Nigeria, West Africa where only *T. b. gambiense* is found (Figure 1.1). Lack of *T. b. gambiense* in the dog samples therefore indicated that the observed TBR PCR positive *T. brucei* infections were due to *T. b. brucei*. Dogs have been shown to carry *T. b. gambiense* in other regions (Gibson *et al*, 1978;Mehlitz *et al*, 1982;Zillman *et al*, 1984;Schares & Mehlitz, 1996) but not, to date, in the Jos Plateau of Nigeria.

Trypanosome species or subspecies	Sample number (Prevalence)
T. brucei	180 (94.7%)
T. vivax	171 (90%)
T. congolense forest	113 (59.5%)
T. congolense savannah	97 (51%)
T. b. gambiense	0
T. brucei & *T. vivax*	169 (89%)
T. brucei & *T. congolense* forest	102 (53.7%)
T. brucei & *T. congolense* savannah	96 (50.5%)
T. brucei & T congolense forest & *T. congolense* savannah	46 (24.2%)
T. brucei & *T. vivax* & *T. congolense* forest	94 (49.5%)
T. brucei & *T. vivax* & *T. congolense* savannah	89 (46.8%)
T. brucei & *T. vivax* & *T. congolense* savannah & forest	41 (21.6%)

Table 5.2 Prevalence of animal and human trypanosomes in Nigerian dogs by PCR typing. Results show the number of dogs infected with one or more than one trypanosome species. Total number of dog samples analysed by PCR (N=190).

5.4.4 PCR typing for detection of the *Babesia* 18s rRNA gene in dog isolates

It is speculated that treatment of animals with diminazene aceturate for babesiosis without knowing whether they could be infected with trypanosomiasis (which is likely to be missed on detection by microscopy) could provide a stronger selection for P2 drug resistant trypanosomes. The dogs in this study had signs of babesiosis infection which was later identified by laboratory microscopic diagnosis of the dog samples by qualified personnel at the veterinary clinic in Nigeria to be due to *Babesia gibsoni* in 23 of 190 (12%) dog samples (Table 5.5). Diagnosis of infection by the more sensitive PCR amplification technique was therefore applied to detect and confirm the presence of infection due to babesiosis (By Clare McCann, Project student, University of Glasgow). It was not possible in this study to use *Babesia* (*B. canis canis*, *B canis rossi*, *B. canis vogeli* or *B. gibsoni*) species specific primers but instead universal *Babesia* parasite 18s rRNA specific primers were used. DNA extraction from FTA® cards (Whatman) containing dog blood samples (N=190) was performed as described (section 2.3). The DNA from the dog samples was PCR amplified for an approximately 450 bp size fragment of the 18s ribosomal RNA gene using *Babesia* parasite 18s rRNA specific primers PIRO-A1 and PIRO B (Foldvari *et al*, 2005) as described (section 5.3). However, different size products between 450-700 bp were obtained for different samples (Table 5.3) and (*Babesia* PCR data, Appendix III). 38.4% (73/190) of the dog samples were positive by PCR compared to 12% by microscopy indicating a 3-fold increase in sensitivity by the PCR method. The results also revealed that the dogs infected with babesiosis were also infected with animal trypanosomes (Table 5.4).

Dog samples (n)	**PIRO-A1 and PIRO B PCR product size (bp)**
32	450
33	500
5	600
3	700

Table 5.3 18s rRNA PCR amplification product sizes obtained for the dog samples. Total number of dog samples positive (N=73) (Data from Clare McCann).

Species or subspecies	**Sample number (Prevalence)**
T. brucei	180 (94.7%)
T. vivax	171 (90%)
T. congolense forest	113 (59.5%)
T. congolense savannah	97 (51%)
Babesia	73 (38.4%)
T. brucei & *T. vivax*	169 (89%)
T. brucei & *Babesia*	70 (36.8%)
T. brucei & *T. vivax* & *Babesia*	64 (33.7%)
T. brucei & *T. vivax* & *T. congolense* & *Babesia*	53 (27.9%)

Table 5.4 Prevalence of animal trypanosomes and babesia parasites in dogs by PCR. Results show number of infections due to one or more parasite species
Total number of isolates analysed by PCR (N=190)

Sample no.	HCT	Thin smear	TBR	TVW	TCS	TCF	PIRO
138	-ve	-ve	+ve	+ve	-ve	+ve	+ve
139	-ve	-ve	+ve	+ve	-ve	+ve	-ve
140	-ve	-ve	+ve	+ve	-ve	+ve	+ve
141	-ve	-ve	+ve	+ve	+ve	+ve	-ve
142	-ve	-ve	+ve	+ve	-ve	-ve	+ve
143	-ve	-ve	+ve	+ve	+ve	+ve	-ve
144	-ve	-ve	+ve	+ve	-ve	-ve	-ve
145	-ve	-ve	+ve	+ve	-ve	+ve	-ve
146	-ve	-ve	+ve	+ve	-ve	+ve	-ve
147	-ve	-ve	+ve	+ve	-ve	+ve	+ve
148	-ve	-ve	+ve	+ve	-ve	+ve	-ve
149	-ve	-ve	+ve	+ve	-ve	+ve	+ve
150	+/mf	-ve	+ve	+ve	+ve	-ve	-ve
151	-ve	-ve	+ve	+ve	-ve	+ve	-ve
152	-ve	-ve	+ve	+ve	+ve	-ve	+ve
153	-ve	*B. gibsoni*+	+ve	+ve	+ve	-ve	-ve
154	-ve	-ve	+ve	+ve	-ve	-ve	+ve
155	-ve	-ve	+ve	+ve	+ve	+ve	-ve
156	-ve	-ve	+ve	+ve	+ve	-ve	-ve
157	-ve	-ve	+ve	+ve	+ve	-ve	-ve
158	-ve	-ve	+ve	+ve	-ve	+ve	-ve
159	-ve	*B. gibsoni*+	+ve	+ve	-ve	+ve	+ve
160	-ve	-ve	+ve	+ve	+ve	-ve	+ve
161	-ve	-ve	+ve	-ve	-ve	+ve	+ve
162	-ve	-ve	+ve	+ve	+ve	-ve	-ve
163	-ve	-ve	+ve	+ve	+ve	+ve	-ve
164	-ve	-ve	+ve	+ve	+ve	-ve	-ve
165	-ve	-ve	+ve	+ve	-ve	-ve	+ve
166	-ve	-ve	+ve	+ve	+ve	-ve	+ve
167	-ve	-ve	+ve	+ve	+ve	+ve	-ve
168	-ve	-ve	+ve	-ve	+ve	-ve	-ve
169	-ve	-ve	+ve	-ve	+ve	+ve	-ve
170	-ve	*B. gibsoni*+	+ve	+ve	-ve	-ve	-ve
171	-ve	-ve	+ve	+ve	-ve	+ve	-ve
172	-ve	-ve	+ve	+ve	-ve	+ve	+ve
173	-ve	-ve	+ve	+ve	+ve	+ve	+ve
174	-ve	-ve	+ve	-ve	+ve	+ve	+ve
175	-ve	-ve	+ve	-ve	+ve	+ve	-ve
176	-ve	-ve	+ve	+ve	-ve	+ve	-ve
177	-ve	-ve	+ve	+ve	-ve	+ve	+ve
178	-ve	-ve	+ve	+ve	+ve	+ve	-ve
179	-ve	-ve	+ve	+ve	-ve	-ve	+ve
180	-ve	-ve	+ve	+ve	+ve	-ve	-ve
181	-ve	-ve	+ve	+ve	-ve	-ve	+ve
182	-ve	-ve	+ve	+ve	+ve	-ve	-ve
183	-ve	-ve	+ve	+ve	-ve	-ve	-ve
184	-ve	-ve	+ve	+ve	-ve	-ve	-ve
185	-ve	-ve	+ve	+ve	-ve	-ve	+ve
186	-ve	-ve	+ve	+ve	+ve	-ve	+ve
187	-ve	-ve	+ve	+ve	+ve	+ve	-ve
188	-ve	-ve	+ve	+ve	+ve	+ve	-ve

Sample no.	HCT	Thin smear	TBR	TVW	TCS	TCF	PIRO
189	-ve	-ve	+ve	+ve	-ve	+ve	+ve
190	-ve	*B. gibsoni*+	+ve	-ve	-ve	+ve	+ve
191	-ve	-ve	+ve	+ve	-ve	+ve	+ve
192	-ve	-ve	+ve	+ve	-ve	+ve	+ve
193	-ve	-ve	+ve	+ve	-ve	+ve	+ve
194	-ve	-ve	+ve	+ve	+ve	+ve	+ve
195	-ve	*B. gibsoni*+	+ve	+ve	-ve	+ve	+ve
196	-ve	-ve	+ve	+ve	-ve	+ve	-ve
197	-ve	*B. gibsoni*+	+ve	+ve	-ve	+ve	-ve
198	-ve	-ve	+ve	+ve	-ve	+ve	-ve
199	-ve	-ve	+ve	+ve	-ve	+ve	-ve
200	-ve	-ve	+ve	+ve	-ve	+ve	-ve
201	-ve	*B. gibsoni*+	+ve	+ve	-ve	+ve	-ve
202	-ve	-ve	+ve	+ve	-ve	+ve	-ve
203	-ve	*B. gibsoni*+	+ve	+ve	-ve	+ve	-ve
204	-ve	-ve	+ve	+ve	+ve	+ve	-ve
205	-ve	*B. gibsoni*+	+ve	+ve	-ve	+ve	-ve
206	-ve	-ve	+ve	+ve	-ve	+ve	-ve
207	-ve	-ve	+ve	+ve	-ve	+ve	-ve
208	-ve	-ve	+ve	+ve	+ve	+ve	+ve
209	-ve	-ve	+ve	+ve	-ve	-ve	+ve
210	-ve	*B. gibsoni*+	+ve	+ve	-ve	+ve	-ve
211	-ve	-ve	+ve	+ve	-ve	+ve	-ve
212	-ve	-ve	+ve	+ve	-ve	+ve	+ve
213	-ve	*B. gibsoni*+	+ve	+ve	-ve	+ve	+ve
214	-ve	-ve	+ve	+ve	-ve	-ve	-ve
215	-ve	-ve	+ve	+ve	-ve	-ve	-ve
216	-ve	-ve	+ve	+ve	-ve	+ve	+ve
217	-ve	-ve	+ve	-ve	-ve	-ve	-ve
218	-ve	-ve	+ve	+ve	-ve	-ve	-ve
219	-ve	-ve	+ve	+ve	-ve	-ve	+ve
220	-ve	-ve	+ve	+ve	-ve	+ve	-ve
221	-ve	*B. gibsoni*+	+ve	+ve	-ve	+ve	-ve
222	-ve	-ve	+ve	+ve	-ve	+ve	-ve
223	-ve	-ve	+ve	+ve	-ve	+ve	-ve
224	-ve	-ve	+ve	+ve	-ve	+ve	-ve
225	-ve	-ve	+ve	+ve	-ve	+ve	+ve
226	-ve	-ve	+ve	+ve	-ve	-ve	+ve
227	-ve	-ve	+ve	+ve	+ve	-ve	-ve
228	-ve	-ve	+ve	+ve	-ve	+ve	+ve
229	-ve	*B. gibsoni*+	-ve	-ve	-ve	+ve	-ve
230	-ve	-ve	+ve	+ve	-ve	+ve	-ve
231	-ve	-ve	+ve	+ve	-ve	+ve	+ve
232	-ve	-ve	+ve	-ve	-ve	+ve	-ve
233	-ve	-ve	+ve	+ve	-ve	-ve	-ve
234	-ve	-ve	+ve	+ve	-ve	+ve	-ve
235	-ve	*B. gibsoni*+	+ve	+ve	-ve	+ve	-ve
236	-ve	-ve	+ve	-ve	-ve	+ve	-ve
237	-ve	-ve	+ve	+ve	-ve	+ve	-ve
238	-ve	-ve	+ve	+ve	-ve	-ve	+ve
239	-ve	-ve	+ve	+ve	-ve	+ve	+ve
240	-ve	-ve	+ve	+ve	-ve	-ve	-ve

Sample no.	HCT	Thin smear	TBR	TVW	TCS	TCF	PIRO
241	-ve	*B. gibsoni*+	+ve	+ve	-ve	+ve	-ve
242	-ve	-ve	+ve	+ve	+ve	+ve	+ve
243	-ve	-ve	+ve	+ve	+ve	+ve	+ve
244	-ve	-ve	-ve	-ve	-ve	+ve	-ve
245	-ve	-ve	-ve	-ve	-ve	+ve	-ve
246	-ve	-ve	+ve	+ve	+ve	-ve	+ve
247	-ve/mf	*B. gibsoni*+	+ve	+ve	+ve	+ve	-ve
248	-ve	-ve	+ve	+ve	+ve	+ve	+ve
249	-ve	-ve	+ve	+ve	+ve	+ve	+ve
250	-ve	-ve	+ve	+ve	+ve	+ve	+ve
251	-ve	-ve	-ve	+ve	+ve	+ve	-ve
252	-ve	-ve	+ve	+ve	+ve	+ve	+ve
253	-ve	-ve	+ve	+ve	+ve	-ve	+ve
254	ve/mf++	*B. gibsoni*+	+ve	-ve	+ve	+ve	-ve
255	-ve	-ve	+ve	+ve	+ve	+ve	-ve
256	-ve	-ve	+ve	+ve	+ve	+ve	-ve
257	-ve	-ve	+ve	+ve	+ve	-ve	-ve
258	-ve	-ve	+ve	+ve	+ve	-ve	-ve
259	-ve	-ve	-ve	+ve	-ve	+ve	-ve
260	-ve	*B. gibsoni*+	+ve	+ve	-ve	+ve	-ve
261	-ve	-ve	+ve	+ve	+ve	+ve	+ve
262	-ve	-ve	-ve	+ve	-ve	+ve	-ve
263	-ve	-ve	+ve	+ve	+ve	-ve	+ve
264	-ve	-ve	+ve	+ve	+ve	-ve	+ve
265	-ve	-ve	-ve	-ve	-ve	+ve	-ve
266	-ve	-ve	-ve	+ve	+ve	-ve	-ve
267	-ve	-ve	+ve	+ve	+ve	-ve	-ve
268	-ve	-ve	+ve	+ve	+ve	-ve	-ve
269	-ve	-ve	+ve	+ve	+ve	-ve	-ve
270	-ve	-ve	+ve	+ve	+ve	-ve	-ve
271	-ve	-ve	+ve	+ve	+ve	+ve	-ve
272	-ve	-ve	+ve	+ve	+ve	+ve	-ve
274	-ve	-ve	+ve	+ve	+ve	+ve	-ve
275	+ve	-ve	+ve	+ve	+ve	+ve	-ve
276	-ve	N/A	+ve	+ve	+ve	-ve	-ve
277	-ve	*B. gibsoni*+	+ve	+ve	+ve	+ve	-ve
278	-ve	*B. gibsoni*+	+ve	+ve	+ve	+ve	+ve
279	-ve	-ve	+ve	+ve	+ve	-ve	-ve
280	-ve	-ve	+ve	+ve	+ve	-ve	+ve
281	-ve	-ve	+ve	+ve	+ve	+ve	-ve
282	-ve	-ve	+ve	+ve	+ve	-ve	+ve
283	-ve	-ve	+ve	+ve	+ve	+ve	-ve
284	-ve	-ve	+ve	+ve	+ve	+ve	+ve
285	-ve	-ve	+ve	+ve	+ve	+ve	+ve
286	-ve	-ve	-ve	-ve	-ve	+ve	-ve
287	-ve	-ve	+ve	+ve	+ve	-ve	+ve
288	-ve	-ve	+ve	+ve	+ve	-ve	+ve
289	-ve	-ve	+ve	+ve	+ve	+ve	+ve
290	-ve	-ve	+ve	+ve	+ve	+ve	+ve
291	-ve	*B. gibsoni*+	+ve	+ve	+ve	-ve	-ve
292	-ve	-ve	+ve	+ve	+ve	-ve	-ve
293	-ve	-ve	+ve	+ve	+ve	-ve	+ve

Sample no.	HCT	Thin smear	TBR	TVW	TCS	TCF	PIRO
294	-ve	-ve	+ve	+ve	-ve	-ve	-ve
295	-ve	-ve	+ve	+ve	-ve	+ve	-ve
296	-ve	-ve	+ve	+ve	+ve	-ve	-ve
297	-ve	-ve	+ve	+ve	+ve	-ve	-ve
298	-ve	-ve	+ve	+ve	+ve	-ve	-ve
299	-ve	-ve	+ve	+ve	-ve	-ve	-ve
300	-ve	*B. gibsoni*+	+ve	+ve	+ve	-ve	+ve
301	-ve	-ve	+ve	+ve	+ve	-ve	-ve
302	N/A	N/A	+ve	+ve	+ve	-ve	-ve
303	-ve	-ve	+ve	+ve	+ve	-ve	+ve
304	-ve	-ve	+ve	+ve	+ve	+ve	+ve
305	-ve	-ve	+ve	+ve	-ve	-ve	-ve
306	-ve	-ve	+ve	+ve	+ve	+ve	+ve
307	-ve	-ve	-ve	+ve	-ve	+ve	-ve
308	-ve	-ve	+ve	+ve	+ve	+ve	-ve
309	-ve	-ve	+ve	+ve	+ve	-ve	+ve
310	-ve	-ve	+ve	+ve	-ve	+ve	+ve
311	-ve	-ve	+ve	+ve	-ve	-ve	+ve
312	-ve	-ve	+ve	+ve	-ve	-ve	+ve
313	-ve	-ve	+ve	+ve	-ve	-ve	+ve
314	-ve	-ve	+ve	+ve	+ve	+ve	+ve
315	-ve	-ve	+ve	+ve	+ve	-ve	-ve
316	-ve	-ve	+ve	-ve	+ve	-ve	+ve
317	-ve	-ve	+ve	+ve	+ve	-ve	-ve
318	-ve	-ve	+ve	+ve	+ve	-ve	+ve
319	-ve	-ve	+ve	+ve	+ve	+ve	-ve
320	-ve	-ve	+ve	+ve	+ve	-ve	-ve
321	-ve	-ve	+ve	+ve	+ve	+ve	-ve
322	-ve	*B. gibsoni*+	+ve	+ve	+ve	+ve	-ve
323	-ve	-ve	+ve	+ve	+ve	-ve	-ve
324	-ve	-ve	+ve	+ve	-ve	-ve	-ve
325	-ve	-ve	+ve	+ve	+ve	-ve	-ve
326	-ve	-ve	+ve	+ve	+ve	-ve	-ve
327	-ve	-ve	+ve	+ve	+ve	+ve	-ve
328	-ve	-ve	+ve	+ve	+ve	-ve	-ve

Table 5.5 PCR analysis results of the dog isolates in comparison to microscopy results. HCT; haematocrit centrifugation test (parasites/ml), +ve; positive result, -ve; negative result, no.; number, N/A; microscopy result not available; mf; microfilaria
TBR; *T. brucei* repeat PCR (section 2.3)
TVW; *T. vivax* satellite DNA PCR (section 5.3),
TCS; *T. congolense* savannah satellite DNA PCR (section 5.3)
TCF; *T. congolense* forest satellite DNA PCR (section 5.3)
PIRO; *Babesia* 18s ribosomal RNA PCR (section 5.3)
Microscopy diagnosis results provided by Dr. Yanan.

Amplification of different size bands ranging between 450-700 bp was observed in different samples with one sample amplifying both the 450 and 600 bp amplicons. One sample amplified both 450 and 700 bp amplicons, 6 samples amplified both 500 and 700 bp amplicons and 4 samples amplified both 600 and 700 bp amplicons. The difficulty in obtaining *Babesia* parasite reference strains made it impossible to have them included in the PCR as positive controls in order to confirm the PCR results. Amplicons from samples JSK177 and JNK219 (approximately 450 bp), JNK166 (approximately 500 bp) and JSK189 (approximately 600 and 700 bp) were sequenced. Sub-cloning of the PCR products of the 18s rRNA gene prior to sequencing was carried out as described (section 4.3). Blast search analysis of the National Centre for Biotechnology Information data base http://www.ncbi.nih.gov/ showed that nucleotide sequences of the cloned amplicons from DNA samples JSK177 and JNK219 (500 bp after purification) were 99% similar with the reference sequence of the 18s ribosomal RNA gene of *Babesia canis rossi* (GenBank accession number DQ111760.1) by (Figure 5.1). Surprisingly nucleotide sequences for cloned amplicons from DNA samples JNK166 and JSK189 (550 bp after purification) were 99% similar to the reference sequence of the 18s ribosomal RNA gene of *Hepatozoon canis* (GenBank accession number DQ439543) (Figure 5.2). However, very few samples were cloned and sequenced but the sequencing results possibly indicated that the dogs were not only infected with *Babesia canis* but also *Hepatozoon canis*. The presence of *Hepatozoon canis* in these dog isolates is in agreement with a study by Foldavari and others (2005) in which they demonstrated infection due to both *Babesia canis canis* and *Hepatozoon canis* in dogs from Hungary following amplification using the same set of primers and sequencing. Ticks as the major vector transmit these two parasite species. According to the symptoms and results from the field diagnosis (Nigerian dog sample Raw data, Appendix III), *Babesia* parasite or trypanosome infections were suspected. The identification of both *Babesia* and *Hepatozoon* parasite species in addition to multiple animal trypanosome species has revealed that dogs can harbour several parasites species, which might compromise the unsuccessful treatment for animal trypanosomiasis.

```
DQ439543.1  GGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGG 420
JSK_189     ----------------AGGGAGCCTGAGAGACGGCTACCACATCTAAGGAAGGCAGCAGG 44
                            ************* ******************************

DQ439543.1  CGCGCAAATTACCCAATTCTAACAGTTTGAGAGAGGTAGTAACAAGAAATAACAATACAA 480
JSK_189     CGCGCAAATTACCCAATTCTAACAGTTTGAGAGAGGTAGTAACAAGAAATAACAATACAA 104
            ************************************************************

DQ439543.1  GGCAATTAAAATGCTTTGTAATTGGAATGATAGAAATTTAAACCCTTTTTAAAGTATCAA 540
JSK_189     GGCAATTAAAATGCTTTGTAATTGGAATGATAGAAATTTAAACCCTTTTTAAAGTATCAA 164
            ************************************************************

DQ439543.1  TTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAA 600
JSK_189     TTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAA 224
            ************************************************************

DQ439543.1  AATTGTTGCAGTTAAAAAGCTCGTAGTTGAAGTTCTGCTAAAAGTAACCGGTCTGCTTTT 660
JSK_189     AATTGTTGCAGTTAAAAAGCTCGTAGTTGAAGTTCTGCTAAAAGTAACCGGTCTGCTTTT 284
            ************************************************************

DQ439543.1  AATAAAAGTGGTATCTTGGTGTGTATTTAGCAATGATGTCCTTTGAAGTGTTTTTTACTT 720
JSK_189     AATAAAAGTGGTATCTTGGTGTGTATTTAGCAATGATGTCCTTTGAAGTGTTTTTTACTT 344
            ************************************************************

DQ439543.1  TATTGTAATAAAGCATATTCAGGACTTTTACTTTGAGAAAATTAGAGTGTTTCTAGCAGG 780
JSK_189     TATTGTAATAAAGCATATTCAGGACTTTTACTTTGAGAAAATTAGAGTGTTTCTAGCAGG 404
            ************************************************************

DQ439543.1  CCGACGCTTTGAATACTGCAGCATGGAATAATAAGATAGGATTTTAGTTCTACATTATTG 840
JSK_189     CCGACGCTTTGAATACTGCAGCATGGAATAATAAGATAGGATTTTAGTTCTACATTATTG 464
            ************************************************************

DQ439543.1  GTTTTAAGAGCTAAATTAATGATTGATAGGGACAGTTGGGGGCATTTGTATTTAACTGTC 900
JSK_189     GTTTTAAGAGCTAAATTAATGATTGATAGGGACAGTTGGGGGCATTCGTATTTAAA-ATC 523
            ********************************************** ********   **

DQ439543.   AGAGGTGAAATTCTTAGATTTGTTAAAGACAAACTACTGCGAAAGCATTTGCCAAAGATG 960
JSK_189     ACTAGTG----------------------------------------------------- 530
            *   ***
```

Figure 5.1 Sequence alignments between a dog isolate and the reference *Hepatozoon canis* isolate (Genbank accession number DQ439543)

```
DQ111760.1  AGCAACGGGTAACGGGGAATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAGACGGCTAC 360
JSK_177     -------------------------------------TAGGGAGCCTGAGAGACGGCTAC 23
                                                  **********************

DQ111760.   CACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCCGACACGGGGAGGTAGTGA 420
JSK_177     CTCATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCCGACACGGGGAGGTAGTGA 83
            * **********************************************************

DQ111760.   CAAGAAATAACAATACAGGGCTAATGTCTTGTAATTGGAATGATGGTGACTTAAACCCTC 480
JSK_177     CAAGAAATAACAATACAGGGCTAATGTCTTGTAATTGGAATGATGGTGACTTAAACCCTC 143
            ************************************************************

DQ111760.1  ACCAGAGTAGCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAA 540
JSK_177     ACCAGAGTAGCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAA 203
            ************************************************************

DQ111760.   TAGCGTATATTAAACTTGTTGCAGTTAAAAAGCTCGTAGTTGTATTTTTGCTTGGCGGTT 600
JSK_177     TAGCGTATATTAAACTTGTTGCAGTTAAAAAGCTCGTAGTTGTATTTTTGCTTGGCGGTT 263
            ************************************************************

DQ111760.   TGTTGCCTTTGTGGCTTGATTCCGCTTGGCTTTTGGCTTTTTGCCTTATTACTTTGAGAA 660
JSK_177     TGTTGCCTTTGTGGCTGTATCCCGCTTGGCTTTTGGCTTTTTGCCTTATTACTTTGAGAA 323
            ****************  ** ***************************************

DQ111760.1  AATTAGAGTGTTTCAAGCAGACTTTTGTCTTGAATACTGTAGCATGGAATAATAGAGTAG 720
JSK_177     AATTAGGGTGTTTCAAGCAGACTTTTGTCTTGAATACTGTAGCATGGAATAATAGAGTAG 383
            ****** *****************************************************

DQ111760.   GACTTTGGTTCTATTTTGTTGGTTTGGGAACCTTGGTAATGGTTAATAGGAACGGTTGGG 780
JSK_177     GACTTTGGTTCTATTTTGTTGGTTTGGGAACCTTGGTAATGGTTAATAGGAACGGTTGGG 443
            ************************************************************

DQ111760.1  GGCATTCGTATTTAACTGTCAGAGGTGAAATTCTTAGATTTGTTAAAGACGAACTACTGC 840
JSK_177     GGCATTCGTATTTAAA-ATCACTAGTG--------------------------------- 469
            ***************   ***   ***
```

Figure 5.2 Sequence alignments between a dog isolate and the reference *Babesia canis* isolate (Genbank accession number DQ111760.1)

5.5 Discussion

Domestic animals acting as a reservoir of human infective *T. b. gambiense* parasites, pose a risk to humans in disease endemic regions. This might also have serious implications regarding the control and management of the human disease if it involves transmission of P2 transporter lacking trypanosomes selected as a result of drug pressure to animals. However, in this study, none of the dog samples had *T. b. gambiense* parasites, which indicated these human infecting parasites may not be involved in the animal-tsetse transmission cycle in this Jos Plateau region of Nigeria. Alternatively, the *T. b. gambiense* parasites may currently not be in circulation in this part of Nigeria. The status of human disease in these areas is not known due to lack of adequate surveillance systems, although the surrounding areas Quan-Pan, Bachit and Wamba in the Kaduna region were previously known to be endemic HAT foci (Ukah, 1989, NITR Unpublished). Determination of human disease status in the study areas is currently under investigation as a result of efforts initiated by Dr. M. Barrett (University of Glasgow) and our recently deceased collaborator Dr. E. Yanan (NITOR). According to the analysis all of the dogs infected with babesiosis were also infected with animal trypanosomes. Moreover, both diseases are treated using the same dosage (3.5 mg/Kg body weight) of diminazene aceturate which has been reported as less effective in Nigeria for treatment of domestic animals (Anene *et al*, 2006). Imidocarb dipropionate is an alternative drug used for treatment of babesiosis in this part of Nigeria but this depends on its availability. Diminazene aceturate is the drug mostly commonly used. Because of the low sensitivity of parasite detection by microscopy as observed from the negative wet smear and HCT results (Nigerian dog sample Raw data, Appendix III), trypanosome parasites are most likely to be missed and instead the animals could be treated only for babesiosis. There is a likelihood that this could also lead to selection of drug resistant, *TbAT1/P2* defective, trypanosomes as a result of the treatment. Although the chance that this may happen is low, since generally only animals near or within urban centres have access to treatment in comparison to animals from the typical rural areas which have no access at all. Nevertheless, the possibility of a veterinary link associated with selection of drug resistant P2 defective trypanosomes cannot be ruled out since in previous and recent studies it was demonstrated that *T. b. gambiense* species identified in dogs, pigs, wild animals and bovids in Liberia, Cote d'Ivoire and Burkina Faso. Moreover, pigs in Cameroon were found to carry *T. b. gambiense* identical to the human gambiense isolates from the same focus which strengthens the notion that animals

can act as a reservoir for *T. b. gambiense* (Mehlitz *et al*, 1982;Mehlitz, 1986;Simo *et al*, 2008a).

PCR technology, using specific primers, is a more sensitive method that has successfully been used for detection of various infections and species (Moser *et al*, 1989;Masiga *et al*, 1992;Morlais *et al*, 1998;Penchenier *et al*, 2000;Herder *et al*, 2002;Nkinin *et al*, 2002;Njiru *et al*, 2004;Radwanska *et al*, 2002a;Radwanska *et al*, 2002b;Picozzi *et al*, 2005;Simo *et al*, 2006). From the PCR analysis, every trypanosome positive sample had previously been observed to be negative by wet smear and HCT which might possibly suggest problems with the PCR method (i.e. false positives as a result of contamination). Reference control strains and a no template (water) control were included in the analysis (section 5.3) but a parasitologically diagnosed control was unavailable for inclusion in the assays. However, microscopy has a low sensitivity in comparison to the PCR method and is therefore likely to miss the detection of trypanosome parasites as observed in this case. On the other hand, some dogs diagnosed as positive for *Babesia* parasites by microscopy were found negative by PCR which could also be due to methodology problems, degradation or improper storage of sample material during transportation to the laboratory. A reference control strain to resolve this issue was lacking. However, sequencing of a few *Babesia* PCR positive samples (negative by microscopy) was carried out for confirmation as indicated earlier.

Overall, the study revealed that dogs were infected with multiple species of animal trypanosomes which is consistent with findings from previous and recent studies involving other wild animals and other domestic animals such as pigs, horses and donkeys (Njiokou *et al*, 2004;Simo *et al*, 2006;Pinchbeck *et al*, 2008). The findings indicated that most of the dogs were co-infected with both *T. brucei* and *T. vivax*. The dogs infected with these two species were also observed to be co infected with either subtypes of *T. congolense* savannah or *T. congolense* forest or both.

In addition to animal trypanosomes, the dogs were also infected with *Babesia canis* and *Hepatozoon canis* parasite species. Due to the nature of the survey it was not possible to determine the exact proportions of the dogs that recovered, relapsed or died after treatment with diminazene aceturate although earlier communication indicated four of the dogs did not survive while more than 90% of the dogs improved clinically apart from those infected with both trypanosome and *Babesia* parasites (follow-up data not available). Because of this information gap it has not been possible to have a definitive answer on whether both

the relapsed and dead dogs were infected with drug resistant trypanosomes to necessitate molecular typing the isolates for drug resistance. Therefore the PCR/*SfaNI* analysis for detection of *TbAT1/P2* mutant alleles associated with drug resistance could not be done. Comparison of the treatment outcome data for each dog sampled with the type and number of infective parasite species obtained from the PCR typing results could possibly have revealed whether successful treatment is compromised by the type or number of infective parasite species.

5.6 Conclusion

The PCR method was able to show that the dog isolates were lacking *T. b. gambiense* parasites. The method was able detect the presence of *T. b brucei*, *T. vivax* and *T. congolense* animal trypanosomes and *Babesia* parasite infections which were not detected by microscopy. This confirms the supremacy of PCR based diagnostics over microscopy. The findings also revealed the presence of mixed parasite infections. Simultaneous infection with several parasite species, as observed in the dog samples in this study, may complicate the determination of correct clinical diagnosis, which might affect the success of treatment. Moreover, although *T. b. gambiense* were not identified in this small study it is certainly of importance to consider that treatment of babesiosis with diminazene would risk selection of P2 deficient (and drug resistant) trypanosomes should it emerge that *T. b. gambiense* infection is more widespread than suspected hitherto.

Chapter 6

6 *In vitro* selection for resistance to high levels of diminazene aceturate and DB75 resistance in *T. brucei*

6.1 Summary

The problem of drug resistance is a threat to the successful treatment of animal and human African trypanosomiasis. The development of new trypanocides is dependent on understanding the mechanisms of drug resistance. Trypanosomes with a deleted *TbAT1* gene that encodes the P2 transporter, although significantly resistant to diminazene and DB75 (furamidine), are relatively sensitive to some diamidines suggesting other routes of entry. The aim of this study was to select for increased levels of diminazene and DB75 resistance in trypanosomes lacking the *TbAT1* gene, for the purpose of identifying additional markers for drug resistance. A *T. brucei* line (ABR) that was more resistant to diminazene than the parent *tbat1*$^{-/-}$ strain, (Matovu *et al*, 2003) was isolated. This strain also proved to be highly resistant to DB75. Uptake of radiolabeled pentamidine in the ABR strain was significantly reduced and this was shown to be the result of loss of the High Affinity Pentamidine Transporter (HAPT1) while the Low Affinity Pentamidine Transporter (LAPT1) was not affected. However, transport of radiolabeled diminazene was still possible at 1 µM in the ABR line, implicating LAPT1 as another possible uptake route. Selection for DB75 resistance proved difficult. The findings from this study revealed that selection for high level resistance to diminazene in the *T. brucei* line lacking TbAT1/P2 transport resulted into the loss of HAPT1 which suggested HAPT1 was a secondary route for diminazene transport. The *in vitro* drug selection for resistance in *T. brucei* resulted into a phenotype similar to the B48 strain with increased resistance and reduced uptake of pentamidine (Bridges *et al*, 2007) but with a different diminazene and DB75 resistance profile.

6.2 Introduction

The uptake of trypanocidal melaminophenyl arsenicals and diamidines in *T. brucei* involves the P2 adenosine transporter, encoded by TbAT1. The development of resistance to the arsenicals and the diamidine group of compounds is associated with the loss of this

membrane transporter as previously reviewed (Maser *et al*, 2003;Delespaux & De Koning, 2007). However, using the 72 hour Alamar blue assay only a 2-3–fold resistance to melarsoprol and the diamidine pentamidine was observed in *T. brucei* with a deleted *TbAT1* gene that encodes P2 transport (Raz *et al*, 1997;Matovu *et al*, 2003). The slightly resistant *T. brucei* with the deleted *TbAT1* gene were still sensitive (at sub micromolar concentrations) to these drugs, which suggested other routes of uptake (Matovu *et al*, 2003). However, *T. brucei* lacking the *TbAT1* gene were previously shown to be significantly resistant to the diamidine diminazene (approximately 18-fold compared to wild type *T. brucei* with P2 transport) with hardly any detectable transport of radiolabeled diminazene, which confirmed its dependency on TbAT1/P2 transport (Matovu *et al*, 2003;de Koning *et al*, 2004).

Pentamidine has been shown to utilise TbAT1/P2 and two other transporters (HAPT1 and LAPT1) which accounts for the slight 2-3 fold resistance observed in the *tbat1*$^{-/-}$ strain (de Koning & Jarvis, 2001;de Koning, 2001b;Matovu *et al*, 2003). On the other hand, DB75 (furamidine) has been demonstrated to have a major dependency on the TbAT1/P2 transport (10-fold resistance in the *tbat1*$^{-/-}$ strain) although another uptake route is suspected since resistance was increased to 20-fold in a *T. brucei* line selected against DB75 (Lanteri *et al*, 2006). However, the increased resistance could be based on other modifications than reduced uptake across the plasma membrane. *In vitro* selection for high level resistance using pentamidine in the laboratory has been shown to lead to 130-fold increase in resistance in a *T. brucei* B48 line that was derived from *tbat1*$^{-/-}$ (Bridges *et al*, 2007). This pentamidine resistant line was found to have lost HAPT1 activity but not LAPT1 (Bridges *et al*, 2007). Unlike HAPT1 and LAPT1, which have not yet been characterised at the molecular level, TbAT1/P2 has been characterised and targeted as a route for the uptake of the novel diamidine DB75 (Mäser *et al*, 1999;Barrett & Gilbert, 2006). Identifying resistance markers for diminazene or DB75 by selecting for high level resistance *in vitro* in *T. brucei* will aid to determine whether a secondary route exists for the transport of these drugs and whether this can possibly be exploited for the delivery of new drugs into trypanosomes. It will also provide essential insights into the likelihood of resistance developing against new diamidine drugs. Moreover, given the fact that diminazene is used in treating animals, further insight into possible cross-resistance risks with human parasites requires attention.

6.3 Materials and Methods

Cultivation of bloodstream *T. brucei in vitro* has contributed greatly to the understanding of trypanosomes cell and their molecular biology (Baltz *et al*, 1985). Previously it has been shown that trypanosomes deleted in the *TbAT1* gene that encodes the P2 transporter develop significant resistance to diminazene and to furamidine in a 72 hour Alamar blue assay (Lanteri *et al*, 2006;Matovu *et al*, 2003). However, even these "resistant" cells are relatively sensitive (at sub micromolar minimum inhibitory concentrations) to some diamidines. Thus we aimed to select higher level resistance with an aim to detect markers of high level resistance.

Briefly, to a sterile 25 cm^3 vented tissue culture flask (Corning Incorporation) containing 5 ml of pre-warmed (room temperature) HMI-9 medium (Appendix I) with 10% fetal calf serum (FCS) (Biosera) was inoculated with bloodstream form *T. brucei* from an existing cell line *tbat1*$^{-/-}$ (Matovu *et al*, 2003) culture at a start cell density of 0.5×10^6 cells/ml as determined using an improved Neubauer counting chamber (WSI, Division of Hawksley Technology, England). Bloodstream form *T. brucei* from the wild type reference strain 427 were set up in a similar manner and the flasks incubated in a low pressure CO_2 incubator at 37°C and 5% CO_2. The cells were left to grow to a density of 2-3 $\times 10^6$ cell/ml before they were seeded into fresh HMI-9 medium with 10% FCS.

To confirm the identity of the strain, a series of short experiments as described below were performed prior to starting the *in vitro* drug selection process.

Analysis of the *tbat1*$^{-/-}$ cell line

Under sterile conditions, 500 µl from an established culture of *T. brucei* trypanosomes from the *tbat1*$^{-/-}$ cell line and wild type s427 was each spotted on a separate FTA® card (Whatman). The cards were allowed to air dry at room temperature and extraction of DNA following the FTA protocol was performed as described (section 2.3).

Detection of the neomycin resistance marker gene by PCR

Generation of the *tbat1*$^{-/-}$ cell line by gene knock out were constructed in a plasmid containing antibiotic resistance markers neomycin and puromycin (Matovu *et al*, 2003) Based on this, PCR amplification using primers designed to amplify the *NEO* gene was

carried out on the *tbat1*$^{-/-}$ cell line. Briefly each extracted DNA disc on an FTA® card was amplified in a total volume of 25 µl containing 1 × custom PCR mastermix (45 mM Tris-HCl (pH 8.8), 4.5 mM $MgCl_2$, 11 mM $(NH_4)_2SO_4$, 0.113 mg/ml BSA, 4.4 µl EDTA and 1 mM each deoxynucleotide triphosphate-dATP, dCTP, dGTP, dTTP) (ABgene®, Advanced Biotechnologies Ltd, UK). Neomycin oligonucleotide primer pair (Neo-s 5'-atg att gaa caa gat gga ttg c- 3' and Neo-as 5'-tca gaa gaa ctc gtc aag aag-3' (Dr. P Alibu, personal communication, University of Glasgow), synthesised by MWG Biotech-AG each at 1 µM and 1.25 units of GoTaq DNA polymerase (Promega Corporation, Madison, USA) were added to the reaction. PCR conditions were initial denaturation at 95°C for 4 minutes, followed by denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 2 minute for 30 cycles. This was followed by a last extension at 72°C for 7 minutes. DNA on an FTA® card (Whatman) disc from *T. brucei* wild type 427 and sterile water (no DNA template) were included in the run as negative controls.

Testing for resistance of *tbat1*$^{-/-}$ trypanosomes to neomycin in culture

Culturing of the *T. brucei tbat1*$^{-/-}$ cell line in presence of neomycin antibiotic was performed in parallel with the neomycin PCR assay. A start cell density of 0.5×10^6 cells/ml from an on-going culture of the *tbat1*$^{-/-}$ cell line was inoculated into 10 ml of fresh HMI-9 medium containing 10% FCS and 1.5 µg/ml of neomycin (G418 sulphate) in a 25 ml culture flask. A *T. brucei* wild type 427 culture was set up under the same conditions as a negative control. Both cultures in flasks were incubated at 37°C and 5% CO_2 for 3-5 days. After 3 to 5 days incubation, the flasks containing the cultures were viewed under a light microscope (Leitz Wietzler, Germany) at 20 × magnification. Observation of motile trypanosomes in the *tbat1*$^{-/-}$ cell culture flask should confirm their resistance to antibiotic. Absence of motile trypanosomes in the *T. brucei* wild type 427 culture flask should confirm their susceptibility to antibiotic.

Detection of the *TbAT1/P2* gene by PCR

To confirm absence of the *TbAT1* gene in the *tbat1*$^{-/-}$ cell line, PCR amplification of the entire *TbAT1* using an outer oligonucleotide primer pair TbAT1 ant -s and TbAT1 ant-as (Mäser *et al*, 1999) was performed as described (section 4.3). An FTA® card (Whatman) DNA disc from the *T. brucei* wild type strain 427 was included in the run as a positive control. Sterile water (no template) was used as a negative control. A nested PCR using 5 µl of the outer PCR product as template with a TbAT1 nested oligonucleotide primer pair

ATF-1 5'-gaa agc tta atc aga agg atg ctc ggg ttt gac tca-3'and ATR-1 5'-gag gat cct gaa cag tat tcg tat gac gat tag tgc tac-3' to amplify the *TbAT1* open reading frame (ORF) was performed (Matovu *et al*, 2001b). The PCR and amplification conditions were similar but the annealing temperature was increased to 60°C for the inner reaction.

Agarose gel electrophoresis and UV transillumination

10 µl of each of the PCR products was analysed by electrophoresis on a 2.0% Agarose gel as described (section 2.3). Amplification of the *TbAT1* and *NEO* genes should yield 1400 bp and 800 bp size products on agarose respectively.

Initiation of *T. brucei* drug resistance selection *in vitro*

In vitro drug selection in *T. brucei* has made possible the understanding of possible resistance mechanisms, leading to the identification of putative resistance markers such as *TbAT1*, HAPT1, LAPT1, TbMRPA (Carter & Fairlamb, 1993;Barrett *et al*, 1995;Carter *et al*, 1999;Mäser *et al*, 1999;de Koning *et al*, 2000;de Koning & Jarvis, 2001;de Koning, 2001b;Shahi *et al*, 2002;Matovu *et al*, 2003;de Koning *et al*, 2004;Luscher *et al*, 2006;Lanteri *et al*, 2006). A similar approach of subjecting *T. brucei brucei* already deficient in the TbAT1 (P2) transporter was attempted for diminazene aceturate (berenil) and furamidine [2,5-bis (4-amidinophenyl) furan (DB75)], which both enter via P2 (De Koning *et a*l, 2004; Lanteri *et al*, 2006). This aimed to lead to an increased level of resistance for these diamidines and possibly to the identification of the unknown additional route postulated for DB75 uptake (Lanteri *et al*, 2006).

In vitro drug selection in the presence of P2-mediated trypanocides diminazene and DB75 in stepwise small increments was carried out in duplicate on *T. brucei* bloodstream trypanosomes from the *tbat1*$^{-/-}$ cell line (Matovu *et al*, 2003). The selection process was initiated using the doubling dilution method on a 96 well plate. The drugs were prepared by dissolving in sterile water and kept at stock concentrations of 50 mM and 0.5 mM for diminazene and DB75 respectively. Diminazene aceturate was used at a start concentration of 1.6 µM and DB75 at 0.85 µM - both concentrations equivalent to their respective IC_{50} values. Briefly, 1.6 µM of diminazene aceturate and 0.85 µM of DB75, each made up in 200 µl of HMI-9 medium with 10% FCS (Appendix I), was each added to one of the first wells in the first column of a 96-well culture plate (Corning Incorporation) in duplicate. To the following 11 wells along each row was added 100 µl of HMI-9 medium with 10% FCS. 100 µl of drugged medium from the first well was transferred with mixing to the next

well and the doubling dilution process repeated to the 11th well leaving out the last well (no drug control). The *tbat1*$^{-/-}$ cells at a start cell density of 0.5×10^6cells/ml were aliquoted into each of these wells. The plates were incubated at 37°C and 5% CO_2. After 3-5 days, incubation, cell growth was monitored by observation of the plates under an inverted light microscope (Motic, AET31) at 20 × magnification. Motile cells with a normal morphology were seeded again in fresh HMI-9 medium with 10% FCS at the same drug concentration of that particular well into a fresh well and incubated under the same conditions. The same cells were also seeded at twice, a-half, and a tenth of that drug concentration in fresh HMI-9 medium with 10% FCS. Cells established as growing stably in drug were transferred and seeded into 1 ml volume cultures on 24 well plates and the selection continued with gradual increase in drug concentration. The drug selected cells were later transferred into 5 ml volumes of HMI-9 medium with 10% FCS containing drug in sterile 25 cm^3 vented tissue culture flasks and incubated as before. The whole selection process took a period of 5 months for each of the two independent lines. The two *tbat1*$^{-/-}$ diminazene selected lines each at 0.1µM and the *tbat1*$^{-/-}$ DB75 selected lines each at 0.08 µM were then tested for their susceptibility to drug using the Alamar Blue assay as described below.

<u>*In vitro* drug testing using the Alamar Blue assay</u>

In vitro drug testing using a derivative the 72-hour Alamar Blue method (Raz *et al*, 1997) was carried out after 5 months of selecting the *tbat1*$^{-/-}$ lines in diminazene and the furamidine DB75 to measure their susceptibility to the drugs. This was done to determine the inhibitory concentration of the drugs that affect viability by 50% (IC_{50}). The assay utilizes a blue coloured dye (Resazurin or Alamar Blue), which is used to measure the level of survival of the trypanosomes. This dye (Appendix I) is non fluorescent and when added to the medium containing live trypanosomes a reduction process due to an unspecified cellular dehydrogenase takes place leading to a colour change from blue to pink (Resofurin). Resofurin is fluorescent and therefore the amount of fluorescence due to this process can be measured in a fluorimeter at a wavelength of 530 nm excitations and emission of 590 nm. Since conversion of the dye is a function of cell viability, the degree to which the non-fluorescent compound is converted is taken as a measure of viability (but confirmation by microscopy is required).

The reference strain, wild type 427, and the parent *tbat1*$^{-/-}$ line were used as controls in each assay, which was carried out three separate times. Each assay was set up in duplicate using the serial doubling dilution method. Briefly, 200 µl of a 200 µM working drug stock

concentration was aliquoted into the first well of a white 96-well flat-bottomed plate (Greiner-bio-one, Germany). 100 µl of sterile HMI-9 medium with 10% FCS was aliqouted into the following wells in a row from well 2 to 23. 100 µl of drug from the first well was added with mixing to the next well bringing the concentration of the drug in the second well to 100 µM. This serial dilution was repeated across the plate leaving the last well free with no drug as a negative control. To each of the wells was added 100 µl of bloodstream form trypanosomes in HMI-9 medium with 10% FCS at a density of 2×10^5 cells/ml bringing the final cell density to 1×10^5 cells/ml. The plate was incubated at 37°C and 5% CO_2 for 48 hours at which time, 20 µl of Alamar Blue dye (10% resazurin (Sigma) was added to each well and the plate incubated for another 24 hours at 37°C and 5% CO_2. The fluorescence was measured using a fluorescence spectrophotometer at an excitation wavelength of 530 nm and emission wavelength of 590 nm. The same assay was also performed using a lower cell density of 4×10^4 cells/ml bringing the final density to 2×10^4 cells/ml after addition of 100 µl of cells. Using the high and low cell densities was done to compare the IC_{50} values obtained for each drug in order to determine whether an off set in P2 expression is a likely factor that could lead to the low increment in the IC_{50} value observed in wild type cells in comparison to the P2-deficient cells which had pronounced elevated IC_{50} values at high cell densities especially for diminazene (Dr. C Ward, University of Glasgow, personal communication).

The assays were repeated on the lines selected against high concentration of diminazene after several passages in drug free HMI-9 medium with 10% FCS for a period of 3 months to determine whether the acquired resistance was stable.

Alamar Blue assay data analysis

The fluorescence data was analysed using the Prism software package version 5.0 for determination of the IC_{50} values. The IC_{50} value for each test drug, which is referred to as the inhibitory concentration that reduces the fluorescence by 50% was calculated by nonlinear regression. Graphs were generated which showed the amount of absorbed fluorescence as a function of the trypanosomes' response to the test drug plotted against the log_{10} values of each of the different test drug concentrations.

Cross-resistance using the Alamar Blue assay

Testing for cross-resistance to other P2 drugs pentamidine, cymelarsan (melarsoprol), and the furamidine DB75, was carried out for one of the *tbat1*$^{-/-}$ lines selected against increased diminazene concentration (ABR) using the Alamar Blue assay as described earlier in this section. Diminazene was included among the P2 drugs and a non-P2 trypanocide phenylarsine oxide (PAO), which diffuses across the trypanosome membrane (Scott *et al*, 1997) was included in each assay as a drug control. *T. brucei* 427 (P2 wild type), *tbat1*$^{-/-}$ (P2-deficient) whose susceptibility profiles to the mentioned P2 drugs are known (Lanteri *et al*, 2006) and the pentamidine resistant stock KO-B48 which is another P2-deficient line but also lacking HAPT1 activity (Bridges *et al*, 2007) were included in each of the three independent assays as controls. This was carried out to compare their drug susceptibility profiles with that of the *tbat1*$^{-/-}$ cell line selected against diminazene (ABR).

Pentamidine transport assays

Pentamidine transport assays using high and low concentrations of radiolabeled pentamidine and propamidine have been used to assess the status of the high affinity and low affinity pentamidine transporters (HAPT1 and LAPT1) in *T. brucei* (de Koning & Jarvis, 2001;de Koning, 2001b). In the laboratory *T. b. brucei* line KO-B48 (derived from the *tbat1*$^{-/-}$ line through selection for pentamidine resistance), uptake of [^{3}H]-pentamidine label was reduced and HAPT1 was demonstrated to be missing (de Koning, 2001b;Bridges *et al*, 2007). The KO-B48 cell line was also shown to be highly cross resistant (15-fold less susceptible than wild type *T. brucei* 427) to the arsenical melarsoprol in comparison to *tbat1*$^{-/-}$ (2-3 fold less susceptible than wild type *T. brucei* 427) (Bridges *et al*, 2007).

Due to time constraints, I could not personally perform the transport assays, which were instead carried out by Dr. Nasser El Sabbagh, University of Glasgow. However, the assays were performed as described below.

Assays involving the uptake of [^{3}H]-pentamidine label (Appendix I) by the *T. b. brucei tbat1*$^{-/-}$ diminazene selected line were performed using the rapid oil–stop method (Figure 6.1) as described (de Koning, 2001b;Bridges *et al*, 2007). Assays were carried out in triplicate and on the same day for the diminazene-selected line and bloodstream form wild type *T. brucei brucei* 427 which was used as a control. Briefly, *tbat1*$^{-/-}$ cells adapted to increased diminazene concentrations (ABR) cultured to a high density in HMI-9 with 10% FCS were aliquoted in 50 ml falcon tubes and centrifuged at 2,500g for 10 minutes at room

temperature. The medium was aspirated off and the cells were washed twice in assay buffer pH 7.3 (Appendix I) by spinning under the same conditions. The cells were resuspended in assay buffer at a cell density of 1×10^8 cells/ml as determined by microscopy using an improved Neubauer counting chamber (WSI, Division of Hawksley Technology, England). 100 µl of the cell suspension at room temperature was added to each microfuge tube already containing a mixture of 200 µl of dibutyl-phthalate oil/mineral oil (v/v 7:1) (Sigma) and 100 µl of assay buffer with [^{3}H]-pentamidine label at 60 nM for HAPT1 determinations and 2 µM for LAPT1 assays, as well as any competitive inhibitors being tested, such as unlabelled pentamidine and propamidine. The uptake reactions were initiated by the addition of 100 µl of assay buffer containing 10^7 trypanosomes and terminated after 60 seconds using 1 ml of ice cold unlabelled pentamidine stop solution (Appendix I) at a saturation concentration of 4 mM. The cells were centrifuged through oil at 13,000 rpm for 30 seconds in a microfuge. The tubes were snap frozen immediately in liquid nitrogen and the bottom end, containing the cell pellet, cut off and the pellet solubilised in 2% SDS (Appendix I). A volume of 3 ml of scintillation fluid (Optiphase HiSafe III) was added to the reactions, mixed and incubated overnight at room temperature. Control reactions to measure the radiolabel associated with the cell pellet at zero-uptake conditions (T_0) were determined by adding cells kept on ice to a mix of [^{3}H]-pentamidine label and unlabelled pentamidine also kept on ice simultaneous with stop solution and spinning the cells immediately through oil. Radioactivity in the pellet, as a measure of uptake, was determined using a scintillation counter (1450 MicroBeta Trilux). Transport values expressed as pmol(10^7 cells)$^{-1}$)s^{-1} were determined after non-mediated transport values at time zero (T_0) were subtracted.

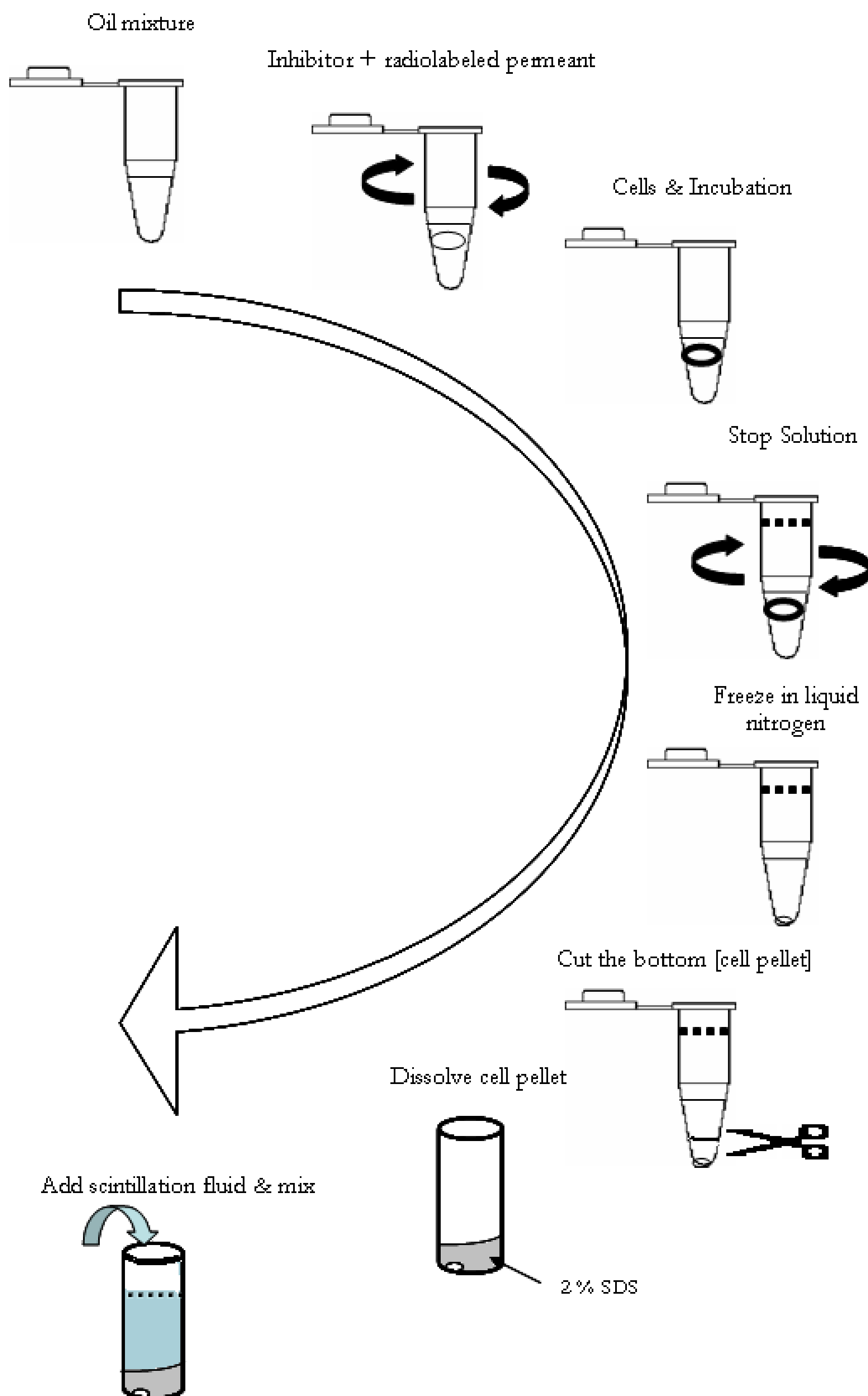


Figure 6.1 A schematic of the rapid oil-stop method used in the pentamidine transport assays.
(Adapted from Al Salabi Mohammed, PhD Thesis, 2006, University of Glasgow)

6.4 Results

6.4.1 In vitro selection process

T. brucei bloodstream form trypanosomes lacking the *TbAT1* gene were chosen for the *in vitro* selection process. To confirm their identity, DNA extracted from an FTA® card (Whatman) spotted with a bloodstream form culture was PCR amplified for the neomycin gene, which is one of the markers that was used in the plasmid construction of the *TbAT1* knock-out cell line (Matovu *et al*, 2003). Amplification of the *TbAT1* gene was also carried out as described (section 6.3). The *TbAT1* knock-out cell line was also tested against neomycin in culture as described (section 6.3). Wild type *T. brucei* (s427) with TbAT1/P2 activity was included in each test as a control. Amplification of the 800 bp size fragment of the neomycin gene (Figure 6.2 panel A) and lack of amplification of the 1400 bp fragment which corresponds to the *TbAT1* gene open reading frame (Figure 6.2 panel B) confirmed the identity of the *T. brucei TbAT1* knock-out cell line. The *T. brucei TbAT1* knock-out cell line was resistant to 1.5 µg/ml of neomycin in culture, unlike wild type *T. brucei* which was susceptible, thus confirming its identity.

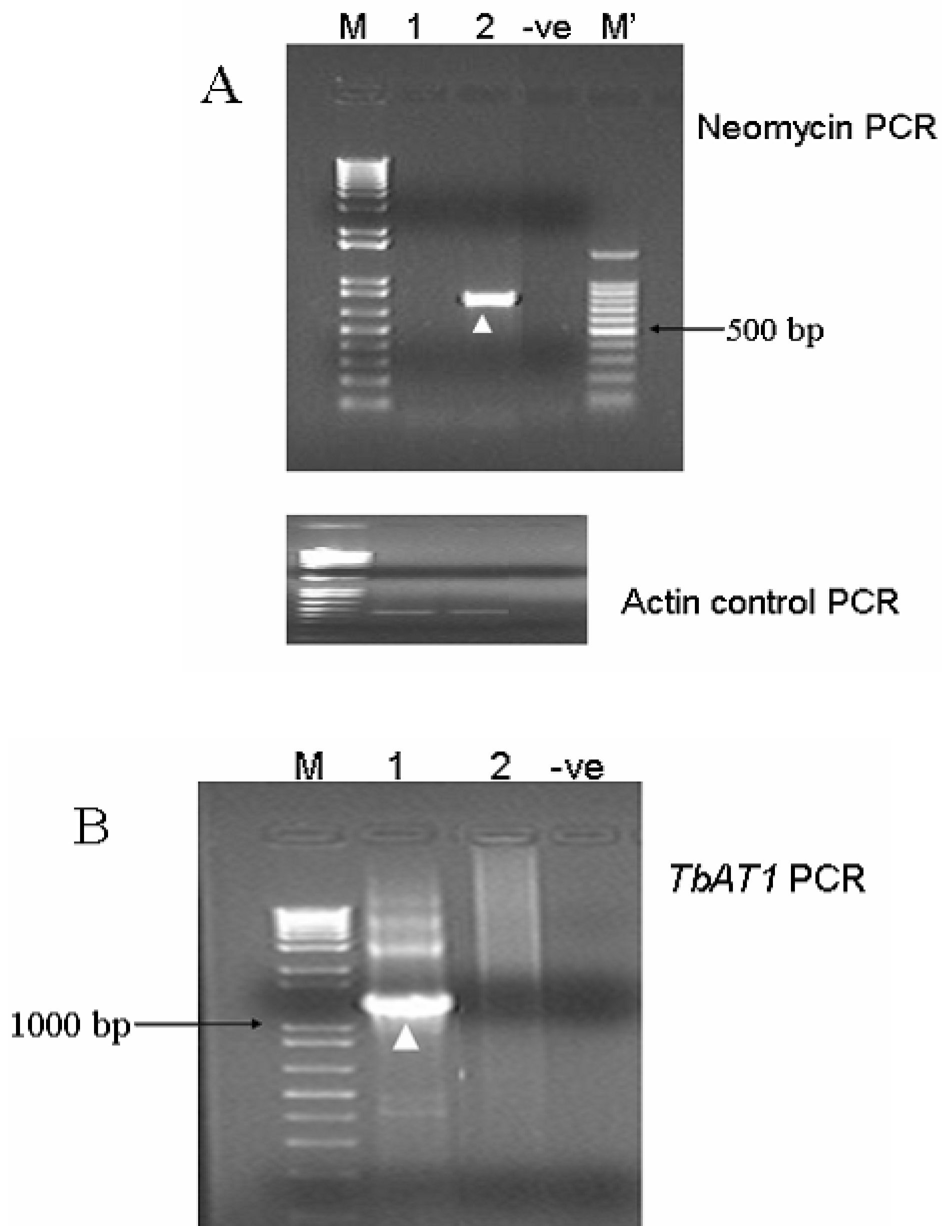


Figure 6.2 PCR amplification results to confirm identity of *T. brucei tbat1*$^{-/-}$
Panel A, a neomycin 800 bp PCR product (white arrow head).
Panel B, a *TbAT1* 1400 bp PCR product (white arrow head).
Lane 1, *T. brucei* s427 (wild type with P2 activity).
Lane 2, *tbat1*$^{-/-}$ (mutant lacking P2 activity).
-ve, no template (water) control.
M, 1 Kb plus DNA size marker (Invitrogen, Life technologies, USA).
M', 100 bp DNA size marker (Promega).

The *in vitro* selection for high level resistance against the diamidine drugs diminazene and DB75 was carried out for 5-6 months on two independent *T. brucei tbat1*$^{-/-}$ cell cultures in a stepwise manner with gradual increment of drug as described (section 6.3). The *tbat1*$^{-/-}$ cell cultures selected against high level resistance to diminazene (ABR) and DB75 (ADB75) were then tested for their susceptibility to the two drugs using the Alamar Blue assay after a total of 55-60 passages in diminazene and 49-58 passages in DB75. The *tbat1*$^{-/-}$ cultures in diminazene were observed to be more resistant compared to wild type strain 427 (Figure 6.3) and the *tbat1*$^{-/-}$ parent strain possibly indicating an additional resistance mechanism. The cultures grown in DB75 were passaged about the same number of times as the cultures grown in diminazene. However, the *tbat1*$^{-/-}$ cultures in DB75 did not acquire any significant increase in the level of resistance in comparison to the parent strain *tbat1*$^{-/-}$. The same level of resistance as for the parent strain was observed for these *tbat1*$^{-/-}$ cultures in DB75 in comparison to wild type strain 427 (Figure 6.3). Alamar Blue assays were performed using both a high and low cell density (Table 6.1 and Table 6.2) to determine if the IC_{50} values vary as a result of different seeding densities. The experiment might further address the possibility that P2 expression is down regulated in captured trypanosomes, (observation by Dr. C. Ward and Dr. Pui Wong) leading to a low estimate of resistance compared to *in vivo* studies, especially for diminazene which is known to be highly dependent on P2 (Matovu *et al*, 2003;de Koning *et al*, 2004). As observed from the results (Table 6.1 and Table 6.2), the IC_{50} values were slightly increased for both drugs at the high cell seeding density for the wild type strain 427 and *tbat1*$^{-/-}$ strain but more pronounced for diminazene than DB75 which is suspected to have a secondary import route independent of P2 (Lanteri *et al*, 2006). These results were obtained directly after the selection process was complete without allowing the cells to proliferate without drug.

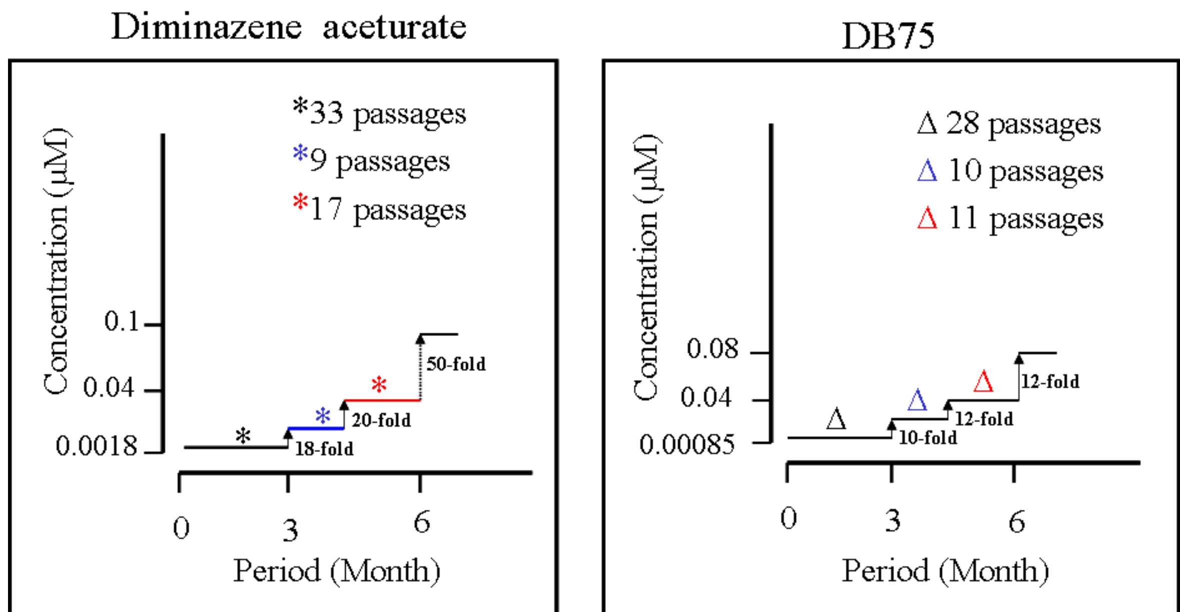


Figure 6.3 Selection for resistance in the *tbat1*$^{-/-}$ cell line cultured under drug pressure. Observed increments in resistance in the *tbat1*$^{-/-}$ cell line in comparison to wild type s427 when cultured in increasing drug concentrations over a 6 month period. The obtained IC_{50} values are not shown.

Drug	Cell line	IC_{50} (µM)* (1x10^{4}cells/ml)	IC_{50} (µM)∗ (1x10^{5}cells/ml)	RF*	RF∗	RF (published)
Diminazene	WT s427	0.00535 (n=2)	0.0343 (n=1)			
	tbat1$^{-/-}$	0.0121 (n=2)	0.0894 (n=1)	2.2	2.6	17.8
	ABR	0.511 (n=2)	1.73 (n=1)	95.5	43	

Table 6.1 Summary of diminazene Alamar Blue results at different cell seeding densities. ABR, *T. brucei tbat1*$^{-/-}$ cell line grown in high concentration of diminazene (up to 0.1 µM). RF, resistance factor in comparison to wild type (WT) s427.
n, number of experiments performed. (*) refers to seeding cells at 10^4 cells per ml and (∗) refers to seeding cells and 10^5 per ml.

Drug	Cell line	IC_{50} (µM)* (1x10^{4}cells/ml)	IC_{50} (µM)∗ (1x10^{5}cells/ml)	RF*	RF∗	RF (published)
DB75	WT s427	0.00581 (n=2)	0.00903 (n=1)			
	tbat1$^{-/-}$	0.0279 (n=2)	0.0746 (n=1)	4.79	8.26	10.6
	ADB75	0.0395 (n=2)	0.111 (n=1)	6.79	12.3	

Table 6.2 Summary of DB75 Alamar Blue results at different cell seeding densities. ADB75, *T. brucei tbat1*$^{-/-}$ cell line grown in high concentration of DB75 (up to 0.08 µM). RF, resistance factor in comparison to wild type (WT) s427.
n, number of experiments performed. (*) refers to seeding cells at 10^4 cells per ml and (∗) refers to seeding cells and 10^5 per ml.

Resistance phenotype

The cultures selected against high level resistance to diminazene were withdrawn from drug and passaged 27 times in drug-free medium for a period of 3 months. To determine the cross-resistance profile and to compare with the *tbat1*$^{-/-}$ strain, which is only slightly resistant to melarsoprol and pentamidine (2-3 fold) but significantly resistant (17.8 fold) to diminazene (Matovu *et al*, 2003), and the B48 strain, which lacks P2 and HAPT1 with no increased resistance to diminazene but significant resistance to pentamidine and melarsoprol (Bridges *et al*, 2007), the Alamar Blue assay was carried out. The results are summarised in Table 6.3. The raw data can be found in Appendix IV. The results indicated the resistance phenotype of the ABR cell line was somewhat different from the B48 cell line. The ABR cell line was more resistant to diminazene and DB75 than either the parent strain *tbat1*$^{-/-}$ or the B48 cell line, although some variations (possibly due to technical errors) were observed for the *tbat1*$^{-/-}$ and B48 cell lines with DB75 at the high cell seeding density (1×10^5 cells/ml). The ABR cell line was also found to be more resistant to pentamidine than to cymelarsan but not as highly resistant to pentamidine as B48 cell line.

Cell line	Expt (n)	IC_{50}(μM) 1×10^5 cells/ml	RF	Expt (n)	IC_{50}(μM) 2×10^4 cells/ml	RF
Diminazene						
WT s427	6	0.63	**1.0**	6	0.224	**1.0**
tbat1$^{-/-}$	5	6.40	**10**	4	2.58	**11**
B48	5	8.98	**14**	5	1.58	**7.1**
ABR	6	12.5	**20**	6	5.98	**27**
Pentamidine						
WT s427	6	0.00685	**1.0**	6	0.00206	**1.0**
tbat1$^{-/-}$	5	0.0149	**2.2**	3	0.00491	**2.4**
B48	5	0.368	**54**	5	0.278	**135**
ABR	5	0.342	**50**	6	0.0529	**26**
DB75						
WT s427	5	0.279	**1.0**	6	0.0202	**1.0**
tbat1$^{-/-}$	5	1.67	**6**	4	0.434	**21**
B48	5	1.16	**4.2**	5	0.225	**11**
ABR	5	4.56	**16**	6	0.753	**37**
Cymelarsan						
WT s427	6	0.00408	**1.0**	6	0.00222	**1.0**
tbat1$^{-/-}$	6	0.0130	**3.2**	3	0.00938	**4.2**
B48	6	0.0623	**15**	3	0.0294	**13**
ABR	6	0.0268	**6.6**	3	0.0167	**7.5**

Table 6.3 *In vitro* susceptibility Alamar Blue results comparing resistance phenotypes. WT s427, *T. brucei* (with *TbAT1/P2* activity). B48, *T. brucei* (lacking *TbAT1/P2* and HAPT1 activity). ABR, *T. brucei* (lacking *TbAT1/P2* activity) selected for high level resistance to diminazene. Expt (n), number of experiments performed. RF, resistance factor compared to WT s427. IC_{50} values are an average of each of the recorded number of experiments (n).

The *in vitro* test results suggested an additional uptake route for diminazene in the ABR line already lacking P2 transport leading to the findings as below.

6.4.2 Pentamidine and diminazene transport in the T. brucei *ABR strain selected against high level diminazene*

Additional work was carried out using radiolabeled transport assays to determine whether HAPT1 and LAPT1 activity could be detected in the ABR line. The ABR cell line was similarly tested similarly for diminazene transport. Due to time constraints this work was carried out by Nasser El Sabbagh (Pentamidine transport) and Ibrahim Teka (Diminazene transport) but the results are included in this thesis (Appendix IV) as they offer an explanation to the phenotypes described in section 6.4.1.

6.5 Discussion

Alterations in drug targets or loss of drug uptake by membrane transporters is associated with development of trypanocidal resistance in *T. brucei* (Maser *et al*, 2003). Loss of TbAT1/P2 led to 2-3 fold resistance to melarsoprol and pentamidine but a significantly higher level of resistance to diminazene (18-fold) (Matovu *et al*, 2003) and DB75 (11-fold) (Lanteri *et al*, 2006). However, the *tbat1*$^{-/-}$ mutant was still sensitive at sub-micromolar concentrations to diminazene and DB75 suggesting the drugs either get into the trypanosomes through other routes or by diffusion or endocytosis (or act at the cell surface). HAPT1 and LAPT1 are other routes for the uptake of pentamidine and possibly other diamidines (de Koning, 2001b;de Koning & Jarvis, 2001). Adaptation of ABR to a high concentration of diminazene resulted in more resistance to diminazene and cross-resistance to pentamidine and DB75 in comparison to the parent *tbat1*$^{-/-}$ strain. Pentamidine transport associated with loss of HAPT1 was abolished but pentamidine transport through LAPT1 was not affected. Diminazene transport was not totally abolished even in the B48 strain which also lacks HAPT1 implicating another mode of entry which could be transporter related or by endocytosis or diffusion although the latter possibilities are less likely as uptake of 1 µM [^{3}H]-diminazene was saturable, i.e. completely inhibited by 1 mM unlabelled diminazene. Evidence has emerged that, at least in procyclic trypanosomes, which are used as a model, diminazene is primarily taken up by HAPT1 (De Koning, unpublished). While procyclics do not express TbAT1/P2 (de Koning *et al*, 1998) they do express HAPT1 (K_m for diminazene 27 ± 5 µM (n=4) and LAPT1. Evidence for loss of

HAPT1 in the ABR diminazene resistant derived line implicates HAPT1 as a second route, which is in agreement with the procyclic model.

The *in vitro* selection of ABR thus appears to have led to loss of HAPT1 activity, which is a phenotype similar to B48. However, there is no increased diminazene resistance in the B48 strain as is observed in the ABR strain. This could be due to point mutations in the B48 strain that could have affected high affinity binding of pentamidine by HAPT1 but not the low affinity binding by diminazene. On the other hand, a different set of mutations could have affected binding by both pentamidine and diminazene in ABR although even in the ABR line the increase in pentamidine resistance was very much higher than the loss in diminazene sensitivity (Table 6.3). It was observed that the level of cross-resistance to pentamidine attained in the ABR strain was of a high level than the level of resistance to diminazene and DB75. Moreover, no increased resistance to diminazene or DB75 was reported for the highly pentamidine resistant strain B48 (Bridges *et al*, 2007) and (Table 6.3). This difference may be due to differences in the 3-dimensional structures of the three compounds (Figure 6.4). Pentamidine, which displays has a significant level of resistance in the adapted *tbat1*$^{-/-}$ lines is more flexible (frame C) and may owe its high affinity (Km = 0.0035 ± 0.005 μM) to HAPT1 to its ability to engage in multiple hydrogen-bond interactions with the transporter. In contrast, the rigid (and very similar) structures of diminazene (K_m=62.48 ± 3 μM) and DB75 (K_m=38.25 ± 10 μM) allow the formation of fewer bonds (frame A and B), reflected in the much lower binding energy. Thus it is conceivable that some mutations in HAPT1 might affect pentamidine binding while not affecting diminazene binding, or to a far lesser extent.

An alternative model that would also explain the non-reciprocal cross-resistance profiles seen in the B48 line compared with ABR line would relate to HAPT1 activity being dependent upon accessory factors. Loss of either of two accessory factors could yield pentamidine resistance while only loss of one particular factor would give diminazene resistance. Further work would be required to distinguish these models.

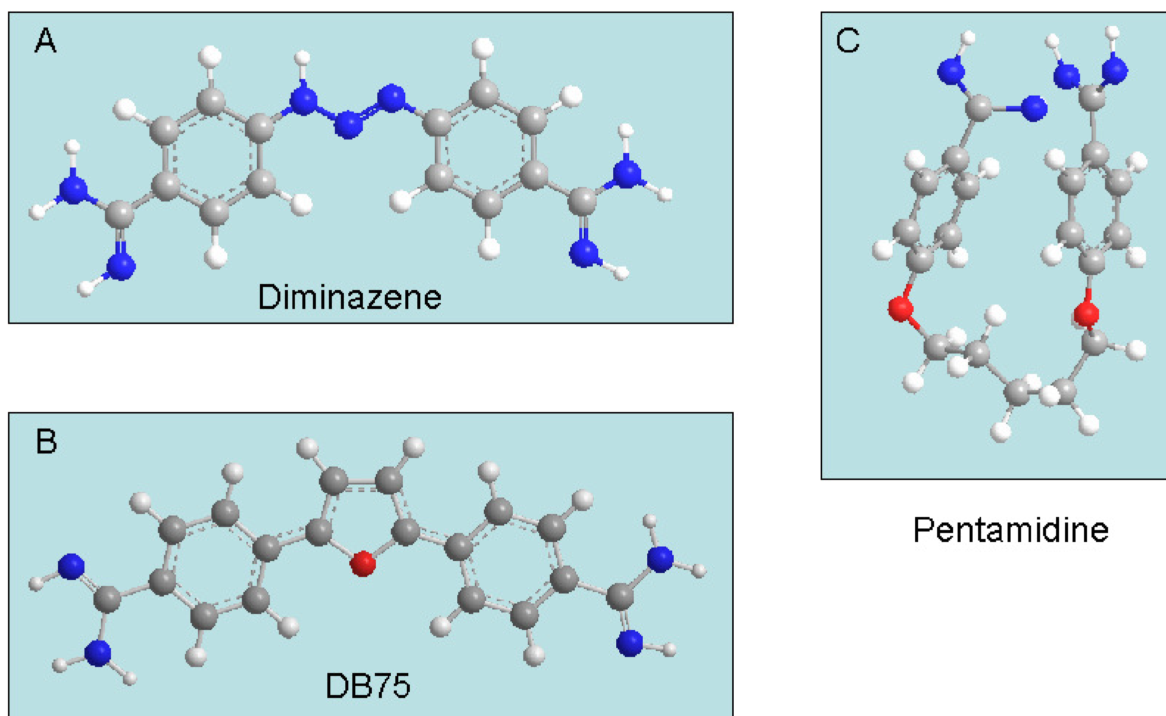


Figure 6.4 Rigid structures of diminazene and DB75 and flexible structure of pentamidine. Images produced with Chem3D Ultra software version.10 (Cambridge soft) after minimalisation of energy.
(Dr.Harry De Koning, University of Glasgow, with permission).

6.6 Conclusion

Adaptation of the *tbat1*$^{-/-}$ line to a high concentration of diminazene leads to loss of HAPT1. This changes the previous model of P2 as the sole route for diminazene uptake and instead the new model shows HAPT1 as a secondary route (Figure 6.5). Uptake of diminazene in the ABR line is still possible in the absence of P2 and HAPT1, which indicates at least one additional means of drug entry. TbAT1/P2 has been cloned and is being exploited for the delivery of drugs such as DB75. Molecular characterisation of HAPT1 has not yet been accomplished and neither is the function and physiological substrate of both HAPT1 and LAPT1 known to date. Nevertheless, HAPT1 offers hope as a lead target for the delivery of new trypanocidal compounds into trypanosomes.

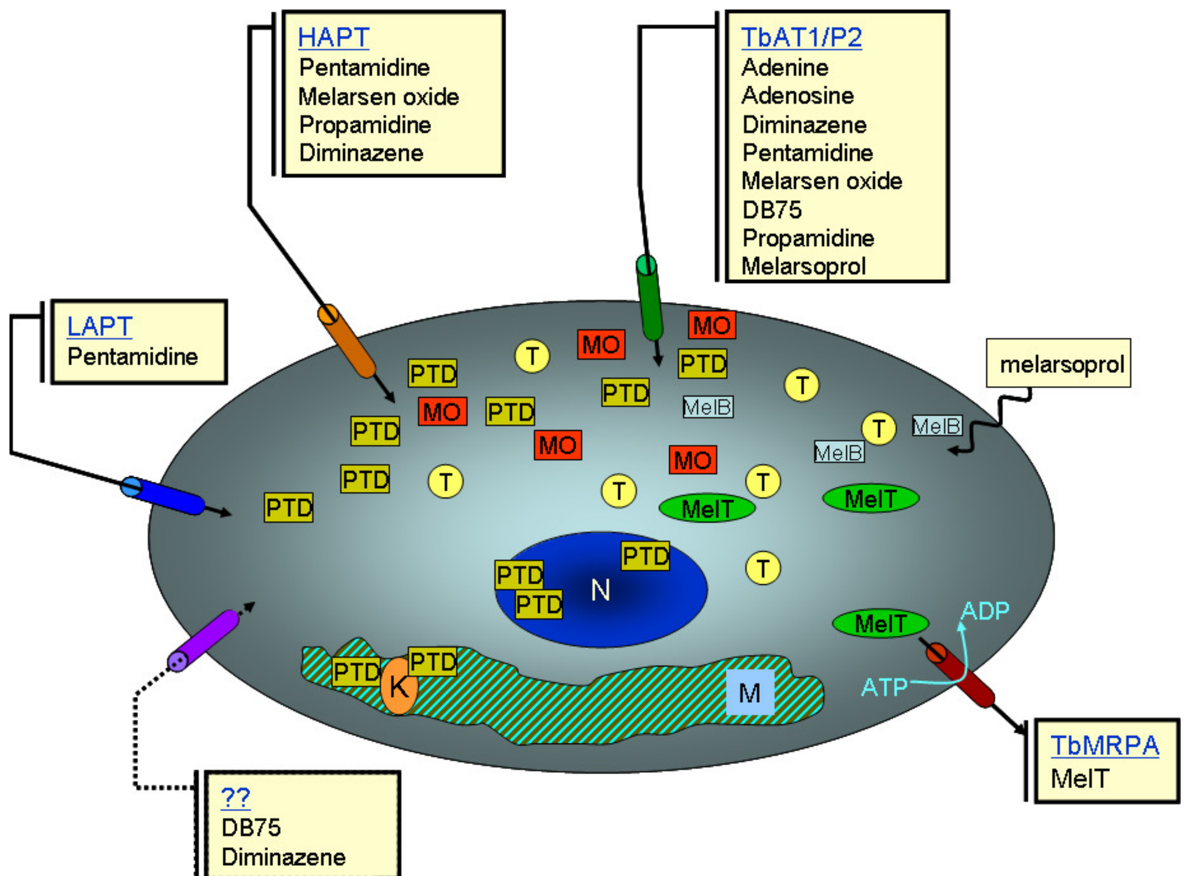


Figure 6.5 Current model showing mechanisms for drug uptake in the trypanosome.
***TbAT1/P2*, T. brucei adenosine transporter 1 encoding P2 activity.**
HAPT1, High Affinity Pentamidine Transporter.
LAPT1, Low Affinity Pentamidine Transporter.
TbMRPA, *T. brucei* multidrug resistance protein A.
PTD, pentamidine. MelB, melarsoprol. MO, Melarsen oxide. T, trypanothione.
MelT, melarsoprol-trypanothione complex. N, nucleus. K, kinetoplast. M, mitochondria.
(De Koning, 2008, in press).

Chapter 7

7 General discussion

Human African Trypanosomiasis; a problem exacerbated by increasing drug resistance.

HAT is a public health problem and occurs in 36 countries in sub-Saharan Africa with the most severely affected including Uganda, Angola, the Democratic Republic of Congo (DRC), Sudan, the Central African Republic (CAR), and the Republic of Congo. The disease, which mostly affects the rural poor, is fatal if left untreated and it has a major impact on social and economic development.

The problem of drug resistance and the existence of an animal reservoir have made efforts to manage the disease difficult. The existence of parasite strains resistant to the cheapest and non-species specific drug melarsoprol recently led to the withdrawal of the drug as first-line therapy for stage II disease and its replacement with eflornithine in some regions of Uganda, Sudan and Angola. More alarming yet are anecdotal reports of eflornithine treatment failures in the field, which have started to emerge.

This project thesis reports work aimed at studying several factors influencing the spread and selection of drug resistance in Human African Trypanosomiasis.

FTA card technology and its application to the analysis of *T. brucei* in the field

I have developed and expanded the FTA® card (Whatman) technique, which was found effective for sampling and retrieval of DNA material for analysis from *T. brucei* blood isolates. Amplification by PCR of the multicopy *T. brucei* 177 bp repeat, the single copy *T. brucei* adenosine transporter 1 (*TbAT1/P2*) gene and the single copy *T. brucei PLC*-linked microsatellite was possible using serially diluted *T. brucei* blood from a rodent.

T. b. gambiense patient samples from the Democratic Republic of Congo, Cameroon and Chad, provided to us by Dr. Phillipe Truc, were used in a pilot study to assess the effectiveness of the method for field studies. The results indicated that the method was useful in detecting *T. brucei* infections and low level parasitaemic infections with the highly sensitive multicopy *T. brucei* TBR primers.

This work was important in establishing an experimental platform to enable me to study field isolates transferred to a laboratory setting. I adapted the method to study the population genetics and the *TbAT1* gene status of sleeping sickness patient samples collected from Arua and Moyo districts in North-western Uganda, a region endemic for *T. brucei gambiense* disease.

Population genetics analysis of *T. b. gambiense* from North-western Uganda

Analysis of patient blood isolates revealed the population structure of *T. b. gambiense* to be stable with the level of genetic diversity more intra than inter regional. But some genetic exchange was observed to be taking place in the Arua *T. b. gambiense* population indicating some risk of transfer and spread of resistance genes. Conversely, the same mechanism could contribute to the loss of resistance alleles in the absence of drug pressure, especially if there is a fitness cost to the resistance.

Melarsoprol was withdrawn from Arua, Omugo health centre in 2001 because of a high treatment failure rate (30%) associated with the problem of resistance, but the drug is still used in the neighbouring district in Moyo, where such a high treatment failure rate has not been registered. The population genetics analysis did indicate that the Arua and Moyo *T. b. gambiense* populations are genotypically distinct possibly accounting for the different patient responses to the drug melarsoprol although further work would be needed to verify this.

***T. brucei adenosine transporter 1* (*TbAT1*) status determination**

Alterations in TbAT1/P2 leading to the loss of drug uptake have been implicated in the development of resistance to melaminophenyl arsenicals and diamidines. Mutations in the *TbAT1* gene associated with melarsoprol resistance were found to be present in isolates from melarsoprol relapsed patients from Omugo when treatment failures were at their peak.

Determination of the *TbAT1* gene status of *T. b. gambiense* patient isolates collected from Omugo, NW Uganda in 2005-2006 when melarsoprol was no longer in use revealed the absence of *TbAT1* mutant alleles. This is an important finding which indicates that melarsoprol can possibly be reintroduced as first-line for treatment of stage II disease in Omugo. Since eflornithine resistance has been reported, this fact would be of great significance. With drug trials involving the use of combination therapies as a means of preserving the efficacy of the few existing drugs still underway, the rational use of

melarsoprol with eflornithine in this region could be taken advantage of at this point because of the lack of new drugs. Meanwhile monitoring for *TbAT1* status needs to continue, in order to avoid a return to the high relapse rates of the late 1990's.

Dogs as a potential reservoir of human *T. b. gambiense*

Animals are known to act as host reservoirs of the zoonotic parasite *T. b. rhodesiense*, which implicates them in the transmission of the human disease. *T. b. gambiense* occurs in humans as the major reservoir, but it does also infect domestic animals (mostly pigs and dogs) and wild animals, since it is transmitted by the same tsetse fly vector, *Glossina palpalis*, that transmits to humans.

Dogs acting as a host reservoir of *T. b. gambiense* (or other parasites e.g. *Babesia*) coupled with their treatment using "P2" trypanocides, raises a risk of a possible veterinary link to selecting resistance in human disease. The diamidine, diminazene aceturate, which is dependent on TbAT1/P2 transport, is becoming less effective in the tsetse-fly endemic Jos Plateau, Nigeria. The drug is also used to treat canine babesiosis. Continuous treatment of dogs with diminazene could eventually lead to selection and spread of P2-defective trypanosomes, which could end up being transmitted to humans, resulting in cross-resistance to human trypanocides that utilise the same P2 transporter e.g. pentamidine and melarsoprol.

Trypanosome-infected dog isolates from Nigeria collected on FTA cards, which were provided to us by the late Dr. Everestus Yanan were used in this study and the findings revealed the absence of *T. b. gambiense* in this particular region. However, the parasites could exist in animals in other places, which could complicate treatment of human disease. Multiple infections with other animal trypanosomes and other parasite species, which included *Babesia canis* and *Hepatozoon canis*, as revealed in the study, could complicate proper diagnosis and the successful treatment of animal trypanosomiasis.

In vitro* selection for high-level resistance in *T. brucei

TbAT1/P2 is a known and characterised marker associated with the development of diamidine resistance in *T. brucei*. However, its loss does not lead to the complete loss of sensitivity for some diamidines nor melamine-based arsenicals. Identification of additional markers for diamidine resistance is therefore required particularly since DB289 (pafuramidine) and other diamidines are under consideration as next generation

trypanocides. These markers can be exploited as possible routes for the delivery of new diamidine compounds into the trypanosomes and critically provide better diagnostics for field drug-resistance.

The diamidine, diminazene aceturate, relies largely on P2 for its uptake but adaptation to high concentration of diminazene in the known *T. brucei tbat1*$^{-/-}$ cell line, which is already significantly resistant to diminazene, resulted in increased diminazene resistance and cross-resistance to pentamidine. The High Affinity Pentamidine Transporter (HAPT1) appeared to be lost in these cells. This finding suggested HAPT1 as a possible secondary route for diminazene uptake. The preliminary model emerging from this work is that diamidines may be taken up by P2 as well as HAPT1 and possibly LAPT1, but that different diamidines displaying different affinity for each transporter utilise the various uptake routes to a different extent.

The next priority is the characterisation of HAPT1 at molecular level and the identification of more resistance markers as the basis for future work.

7.1 Conclusion

This work investigated a series of factors that can impact on the selection and propagation of drug resistance in trypanosomes and made some key findings. The absence of *TbAT1* mutant alleles associated with melarsoprol resistance following the withdrawal of melarsoprol from Omugo, NW Uganda shows the effect drug pressure possibly has on the selection of melarsoprol resistant *TbAT1* alleles. Moreover, the finding that some low-level mating occurs in the Omugo *T. b. gambiense* population highlights the risk of spreading undesirable traits such as those associated with drug resistance should drug resistant trypanosomes exist in this population.

The use of the PCR technique using the FTA method proved superior to microscopy for the identification of low parasitaemic trypanosome infections in dogs suspected of having a possible veterinary link to human *T. b. gambiense* disease. *In vitro* drug selection for drug resistance led to the identification of HAPT1 as a marker for diamidine resistance and offers hope for the development of new trypanocides targeting HAPT1 for uptake. It is anticipated that such drugs would display at most a modest cross-resistance with the current treatments that rely mostly on P2 for their activity.

Most of the above insights have been made possible because of the simple and convenient FTA method of sampling.

Appendix I

1 × TE buffer (pH 8.0) (Tris-EDTA buffer)

10 mM Tris-HCl pH 8.0,

0.1 mM EDTA pH 8.0

6 × Blue loading dye

0.25% bromophenol blue

0.25% xylene cyanol FF

30% glycerol in water

50 × TAE buffer (pH 8.5) (Tris acetate-EDTA buffer) (1 Litre)

Tris- base	242 g
Glacial acetic acid	57.1 ml
0.5 M EDTA (pH 8.0)	100 ml

1 × TAE buffer (1 litre)

50 × TAE buffer	20 ml
Double distilled water	980 ml

LB medium (Luria broth) pH 7.0 (1 Litre)

LB powder (Sigma, USA)	25 g
Double distilled water	1 L

LB Agar (1 Litre)

Luria Agar (Sigma, USA)	35 g
LB medium	1 L

SOC Medium pH 7.0 (500 ml)

LB medium	482.5 ml
250 mM KCl	5 ml

2 M $MgCl_2$	2.5 ml
1 M Glucose	10 ml

HMI-9 medium pH 7.4 (5 Litres) (Based on Hirumi and Hirumi, 1989)

HMI-9 powder 1 sachet (Biosera)	500 g
Double distilled water	4.5 L
10% Heat inactivated fetal calf serum (FCS) (Biosera)	500 ml
β-mercapto-ethanol (200 mM)	71.5 µl

CBSS buffer pH 7.4 (1 Litre)

25 mM Hepes	5.96 g
120 mM Nacl	7.01 g
5.4 mM KCl	0.4 g
0.55 mM $CaCl_2$	81 mg
0.4 mM $MgSO_4$	98.6 mg
5.6 mM NaH_2PO_4	0.79 g
11.1 mM D-Glucose	1.44 g

TELT (Lysis buffer)

50 mM Tris-Cl pH 8.0

62.5 mM EDTA pH 9.0

2.5 mM LiCl

4% Triton × 100

Alamar Blue dye (0.49 nM) (pH 7.4)

Resazurin sodium salt (FW 251.18 g/ml)	12.5 mg
1 × Phosphate buffered saline (PBS)	100 ml

Transport assay buffer pH 7.3 (grams/Litre)

Glucose	2.53 g
HEPES	8.0 g
MOPS	5.0 g

$NaHCO_3$	2.0 g
KCl	347.5 mg
$MgCl.6H_20$	62.5 mg
NaCl	5.7 g
$NaH_2PO4_2H_20$	913.5 mg
$CaCl_2.2H_2O$	40.7 mg
$MgSO_4.7H_2O$	19.9 mg

Transport assay stop solution

4 mM unlabelled Pentamidine

ImM diminazene aceturate

2% SDS (Sodium Dodecyl Sulfate) (g/100ml)

SDS 2 g

Radiolabel

[^{3}H] Pentamidine isethionate- specific activity 3.40 TBq/mmol; 92 Ci/mmol (Amersham)

Radioactive concentration 7.4 MBq/ml; 2 mCi/ml

[^{3}H] diminazene aceturate Radioactive concentration 83 Ci/mmol

Appendix II

Input for clustering data

36												
	1	2	8	1	4	8	9	10	14	15	16	
	17	18	19	20	21	7	9	10	14	16	17	
	18	19	20	22	23	1	7	8	9	10	12	1
	7	8										
OM48	1	0	0	1	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	1	0	0	0	0	0
	0	0	0	1	0	0	0	0	0	1	0	0
OM55	1	1	0	0	0	0	0	0	0	1	1	0
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	0	0	0	1	0	0	0	0	0	1	1	0
OM62	1	1	0	0	0	0	0	0	0	1	1	0
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	0	0	0	1	1	0	0	0	0	1	1	0
OM66	1	1	0	0	0	0	0	0	0	1	0	0
	0	0	0	0	0	0	1	0	0	0	0	0
	0	0	0	0	0	1	0	0	0	0	1	0
OM69	1	0	0	0	0	0	0	0	0	1	0	0
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OM77	1	0	0	1	0	0	0	0	0	0	0	0
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	0	0	0	1	0	0	0	0	0	1	1	0
OM83	1	0	0	0	1	1	0	0	0	0	0	0
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	0	0	0	0	0	0	1	0	0	0	0	1
OM86	1	0	0	0	0	0	0	0	1	1	0	0
	0	0	0	0	0	1	0	0	1	0	0	0
	0	0	0	1	1	0	0	0	0	1	1	0
OM87	1	1	0	0	0	0	0	0	0	1	0	1
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	0	0	0	1	0	0	0	0	0	1	1	0
OM88	1	0	0	0	1	1	0	0	0	0	0	0
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	0	0	0	0	0	0	1	0	0	0	0	1
OM90	1	1	0	0	0	0	0	0	0	1	0	1
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	0	0	0	1	1	0	0	0	0	0	1	0
OM92	1	0	1	0	1	1	0	0	0	0	0	0
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	0	0	0	1	1	0	0	0	0	1	1	0
OM96	1	0	0	0	0	0	0	0	0	0	0	0
	1	0	0	0	0	1	0	0	1	0	0	0
	0	0	0	1	1	0	0	0	0	0	1	0

OM97 1 0 0 0 1 0 0 0 0 0 0 0
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0 0 0 1 1 0 0 0 0 1 1 0
OM112 1 0 0 0 1 0 0 0 0 0 0
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OM123 1 1 0 0 0 0 0 0 0 1 1
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OM126 1 1 0 0 0 0 0 0 0 1 0
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OM127 1 1 0 0 0 0 0 0 0 0 0
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0
OM131 1 1 0 0 0 0 0 0 0 0 1
0 0 0 0 0 0 0 1 0 0 0 0
0 0 0 0 1 1 0 0 0 0 1 1
0
OM132 1 0 0 1 0 0 0 0 0 0 0
0 0 0 1 0 0 0 1 0 0 0 0
0 0 0 0 0 1 0 0 0 0 0 1
0
OM143 1 1 0 0 0 0 0 0 0 1 0
0 0 0 0 0 0 0 1 0 0 0 0
0 0 0 0 1 1 0 0 0 0 1 0
0
OM145 1 1 0 0 0 0 0 0 0 0 0
0 0 0 0 1 0 0 1 0 0 0 0

	0	0	0	0	0	1	0	0	0	0	0	1
	0											
OM148		1	1	0	0	0	0	0	0	0	1	0
	0	0	0	0	1	0	0	1	0	0	0	0
	0	0	0	0	1	1	0	0	0	0	1	1
	0											
M139	1	0	0	0	1	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0
	0	1	1	0	0	0	0	1	0	1	1	0
M153	1	1	0	1	0	0	0	0	0	0	0	0
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M177	1	0	0	1	0	0	0	0	0	0	0	0
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	0	0	0	0	1	0	0	0	0	1	0	0
control	1	1	0	0	0	0	0	0	1	0	0	0
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	0	0	0	0	0	0	0	0	1	1	0	0

Tree Draw string

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NW Uganda Patient Raw data

Code	Sex	Age (yrs)	Village	Parish	County	DOA	GP	HCT	WBC	CSF Tryps	Stage/F-up	DOD	Treatment
OM46	F	25	Sinyaki	Alirubu	Koboko	09.08.05	+ ve	N	205	+ ve	SSII	02.09.05	DFMO
						13.02.06	N	N	5	- ve	TLA 6 mths		
						08.08.06	N	N	2	- ve	TLA 1 yr		
OM47	F	12	Kumoyo	Erea	Terego	8.08.05						05.09.05	DFMO
						9.08.05	+ ve	N	247	+ ve	SSII		
						23.03.06	N	N	23	- ve	TLA 6 mths		
OM48	M	13	Luo	Ayuri	Terego	17.08.05						08.09.05	DFMO
						09.08.05	+ ve	N	452	+ ve	SSII		
OM50	F	30	Jilla	Yiddu	Terego	11.08.05						01.09.05	DFMO
						10.08.05	N	- ve	363	+ ve	SSII		
						03.03.05	N	N	16	- ve	TLA 6 mths		
OM51	M	25	Ndeku	Otumbari	Terego	11.08.05	N	+ ve	293	+ ve	SSII	29.08.05	DFMO
OM52	F	38	Yosini	Lobule	Koboko	30.08.05	+ ve	N	2075	+ ve	SSII	15.09.05	DFMO
OM53	M	8	Otumbari	Otumbari	Terego	13.09.05						07.10.05	DFMO
						02.09.05	+ ve	N	455	+ ve	SSII		
OM54	F	30	Kimuru	Pabura	Maracha	09.09.05	N	+ ve	283	+ ve	SSII	27.09.05	DFMO
						07.03.06	N	N	20	- ve	TLA 6 mths		
OM55	M	19	Bamure	Gurepi	Koboko	12.09.05	+ ve	N	41	+ ve	SSII	01.10.05	DFMO
						10.04.06	N	N	22	- ve	TLA 6 mths		
OM56	F	21	Komindaku	Lobule	Koboko	23.09.05	N	+ ve	383	+ ve	SSII	29.09.05	DFMO
						21.06.06	N	N	12	- ve	TLA 6 mths		
OM57	F	27	Oduzia	Alirubu	Koboko	23.09.05	N	- ve	99	+ ve	SSII	19.10.05	DFMO
						25.04.06	N	N	1	- ve	TLA 6 mths		
OM58	M	12	Ocea R/S	Ocea	Madi-Okolo	28.09.05	+ ve	N	507	+ ve	SSII	24.10.05	DFMO
OM59	F	12	Yosini	Lurujo	Koboko	03.10.05	N	- ve	367	+ ve	SSII	23.10.05 (Died)	DFMO
OM60	F	45	Kigemero	Padrombu	Koboko	03.10.05	N	- ve	203	+ ve	SSII	26.10.05	DFMO
OM61	F	24	Kigemero	Padrombu	Koboko	03.10.06	N	- ve	165	- ve	SSII	26.10.06	DFMO

Code	Sex	Age (yrs)	Village	Parish	County	DOA	GP	HCT	WBC	CSF Tryps	Stage/F-up	DOD	Treatment
OM62	M	13	Adologo	Alirubu	Koboko	14.10.05						07.11.05	DFMO
						13.10.05	+ ve	N	295	- ve	SSII		
						29.05.06	N	N	7	- ve	TLA 6 mths		
OM63	F	43	Gbogby	Lobule	Koboko	14.10.05	N	+ ve	7	- ve	SSII	02.11.05	DFMO
OM64	F	50	Alutura	Lurujo	Koboko	08.11.05	N	+ ve	93	+ ve	SSII	05.12.05	DFMO
						05.06.06	N	- ve	3	- ve	TLA 6 mths		
OM65	F	3	Alutura	Lurujo	Koboko	08.11.05	N	+ ve	3	- ve	SSI	22.11.05	Pentamidine
						07.07.06	N	N	39	+ ve	SSII TLA 6 mths	Re-admitted	DFMO
OM66	F	32	Alutura	Lurujo	Koboko	08.11.05	+ ve	N	2	- ve	SSI	23.11.05	Pentamidine
OM67	F	9	Kabure	Lurujo	Koboko	19.09.03						06.10.03	DFMO
						10.11.05	N	N	229	+ ve	SSII	30.11.05	
OM68	F	19	Kolo	Lurujo	Koboko	10.11.05	N	- ve	170	+ ve	SSII	05.12.05	DFMO
						05.06.06	N	- ve	5	- ve	TLA 6 mths		
OM69	F	23	Akua	Ayuri	Terego	11.11.05	+ ve	N	529	+ ve	SSII	05.12.05	DFMO
						22.08.06	N	- ve	9	- ve	TLA 6 mths		
OM70	M	14	Kabule	Lurujo	Koboko	11.11.05	+ ve	N	243	+ ve	SSII	30.11.05	DFMO
OM71	F	9	Alutura	Lurujo	Koboko	15.11.05	+ ve	N	5	- ve	SSI	27.11.05	Pentamidine
OM72	M	13	Okele	Okuvu	Maracha	15.11.05	+ ve	N	211	+ ve	SSII	04.12.05	DFMO
OM73	F	20	Bamure	Gurepi	Koboko	21.11.05						09.12.05	DFMO
						16.11.05	+ ve	N	571	+ ve	SSII		
						09.06.06	N	- ve	7	- ve	TLA 6 mths		
OM74	F	26	Erea	Ayuri	Terego	28.11.05	+ ve	N	777	+ ve	SSII	3.02.06	DFMO
OM75	F	37	Ibia	Otumbari	Terego	29.11.05	N	+ ve	318	+ ve	SSII	17.12.05	DFMO
						28.11.06	N	- ve	9	- ve	TLA 6 mths		
OM76	F	6	Okele	Okuvu	Maracha	01.12.05	+ ve	N	159	+ ve	SSII	18.01.06	DFMO
OM77	F	30	Radulu	Aliba	Madi-Okolo	02.12.05	+ ve	N	359	+ ve	SSII	13.02.06	DFMO
OM78	M	22	Rugbuja	Ginyako	Koboko	05.12.05	+ ve	N	483	+ ve	SSII	07.02.06	DFMO
OM79	M	7	Alio.N	Egamara	Maracha	25.01.06						09.02.06	DFMO
						20.12.05	N	+ ve	107	+ ve	SSII		
						11.08.06	N	N	2	- ve	TLA 6 mths		

Code	Sex	Age (yrs)	Village	Parish	County	DOA	GP	HCT	WBC	CSF Tryps	Stage/F-up	DOD	Treatment
OM80	F	55	Abaa	Anufira	Terego	20.12.05	N	- ve	429	+ ve	SSII	Died before Txt	
OM81	F	47	Bamure	Gurepi	Koboko	06.01.06	N	+ ve	554	+ ve	SSII	Referred to Yumbe	DFMO
OM82	M	22	Erea	Ogunu	Terego	17.01.06						05.02.06	DFMO
						10.01.06	+ ve	N	20	+ ve	SSII		
						22.08.06	N	- ve	5	- ve	TLA 6 mths		
OM83	F	23	Ayiziveko	Otumbari	Terego	12.01.06	- ve	+ ve	617	+ ve	SSII	11.02.06	DFMO
						22.08.06	N	- ve	3	- ve	TLA 6 mths		
OM84	M	15	Milia	Gojuru	Aringa	.06.02 (Yumbe)						Re-admitted	Pentamidine
						13.01.06	N	- ve	963	+ ve	SSII	31.01.06	DFMO
						30.06.06	N	N	7	- ve	TLA 6 mths		
OM85	F	35	Kimu	Lobule	Koboko	17.01.06	+ ve	N	1025	+ ve	SSII	05.02.06	DFMO
						10.08.06	N	- ve	7	- ve	TLA 6 mths		
OM86	F	35	Ija	Ludara	Koboko	20.01.06	+ ve	+ ve	7	- ve	SSII	05.02.06	DFMO
						16.08.06	N	N	0	- ve	TLA 6 mths		
OM87	F	12	Ija	Ludara	Koboko	20.01.06	N	+ ve	4	- ve	SSI	31.01.06	Pentamidine
OM88	M	9	Ija	Ludara	Koboko	20.01.06	N	+ ve	5	- ve	SSI	31.01.06	Pentamidine
						16.08.06	N	N	3	- ve	TLA 6 mths		
OM89	F	34	Ija	Ludara	Koboko	20.01.06	+ ve	+ ve	21	- ve	SSII	13.02.06	DFMO
						16.08.06	N	- ve	3	- ve	TLA 6 mths		
OM90	F	9	Ija	Ludara	Koboko	20.01.06	+ ve	+ ve	103	- ve	SSII	06.02.06	DFMO
						16.08.06	N	N	74	- ve	TLA 3 mths		
OM91	F	49	Ija	Ludara	Koboko	20.01.06	+ ve	+ ve	3	- ve	SSI	31.01.06	Pentamidine
OM92	F	10	Ija	Ludara	Koboko	23.01.06						02.02.06	Pentamidine
						21.01.06	N	+ ve	3	- ve	SSI		
OM93	F	40	Morodo	Gurepi	Koboko	23.01.06						02.02.06	Pentamidine
						21.01.06	N	+ ve	1	- ve	SSI		
						16..08.06	N	N	0	- ve	TLA 6 mths		
OM94	M	20	Rukaku	Gichara	Aringa	23.01.06						13.02.06	DFMO
						21.01.06	+ ve	+ ve	61	- ve	SSII		
						18.08.06	N	- ve	3	- ve	TLA 6 mths		

Code	Sex	Age (yrs)	Village	Parish	County	DOA	GP	HCT	WBC	CSF Tryps	Stage/F-up	DOD	Treatment
OM95	M	9	Gimere	Gurepi	Koboko	23.01.06						08.02.06	DFMO
						21.01.06	N	+ ve	10	- ve	SSII		
						18.08.06	N	- ve	81	- ve	TLA 6 mths	Re-admitted	
OM96	F	22	Ija	Ludara	Koboko	23.01.06	N	+ ve	259	+ ve	SSII	14.02.06	DFMO
						16.08.06	N	- ve	64	- ve	TLA 6 mths		
OM97	M	20	Ija	Ludara	Koboko	23.01.06	N	+ ve	4	- ve	SSI	02.02.06	Pentamidine
						16.08.06	N	N	1	- ve	TLA 6 mths		
OM98	F	8/12 mths	Ija	Ludara	Koboko	25.01.06	N	+ ve	1	- ve	SSI	02.02.06	Pentamidine
						16.08.06	N	N	3	- ve	TLA 6 mths		
OM99	M	3 2/12 yrs	Ayizeveku	Otumbari	Terego	01.02.06						19.02.06	DFMO
						25.01.06	N	+ ve	59	+ ve	SSII		
OM100	F						- ve	+ ve	1	- ve	SSI		Pentamidine
OM101	M	15	Lobule	Lobule	Koboko	.05.04 (Yumbe)							DFMO
						27.01.06	N	- ve	137	- ve	SSII	12.02.06 Re-admitted	DFMO
						18.07.06	N	N	39	- ve	TLA 3 mths		
OM102	M	14	Nabogo	Aupi	Aringa	.01.05 (Yumbe)							DFMO
						01.02.06						15.02.06	DFMO
						30.01.06	N	N	119	+ ve	SSII	Re-admitted	
OM104	M	29	Loronga	Gojuru	Yumbe	13.02.06	+ ve	N	506	+ ve	SSII	08.03.06	DFMO
OM105	F	11	Pasulu	Luba	Madi-Okolo	16.02.06	- ve	+ ve	77	+ ve	SSII	07.03.06	DFMO
OM106	M	39	Arina R/S		Madi-Okolo	02.03.06	N	+ ve	672	+ ve	SSII	18.03.06	DFMO
OM107	M	25	Alutura	Lurujo	Koboko	03.03.06						24.03.06	DFMO
						02.03.06	+ ve	+ ve	761	+ ve	SSII		
OM108	M	25	Onzoro	Aroi	Maracha	08.03.06						28.03.06	DFMO
						03.03.06	+ ve	+ ve	219	+ ve	SSII		

Code	Sex	Age (yrs)	Village	Parish	County	DOA	GP	HCT	WBC	CSF Tryps	Stage/F-up	DOD	Treatment
OM109	M	14	Drandua	Ayuri	Terego	10.03.06 06.03.06	+ ve	N	1121	+ ve	SSII	26.03.06	DFMO
OM110	M	9	Dondo	Lurujo	Koboko	07.03.06 06.03.06	+ ve	N	317	+ ve	SSII	26.03.06	DFMO
OM111	M	18		Nyori	Yumbe	26.03.06 09.03.06	+ ve	N	251	+ ve	SSII	16.04.06	DFMO
OM112	F	29	Drandua	Ayuri	Terego	18.03.06 17.03.06	N	+ ve	893	+ ve	SSII	08.04.06	DFMO
OM113	F	26	Robu	Orinzi	Aringa	26.03.06 21.03.06	N	+ ve	90	- ve	SSII	16.04.06	DFMO
OM114	M	28	Ija	Ludara	Koboko	2004 (Yumbe)							
						21.03.06	N	- ve	58	- ve	SSII	08.04.06 Re-admitted	DFMO
OM115	M	13	Elekele	Nyori	Aringa	26.03.06 24.03.06	+ ve	+ ve	195	+ ve	SSII	08.04.06	DFMO
OM116	F	21	Madikini	Ludara	Koboko	27.03.06	+ ve	+ ve	497	+ ve	SSII	19.04.06	DFMO
OM117	F	1 5/12 yrs	Madikini	Ludara	Koboko	27.03.07	+ ve	N	6	+ ve	SSII	13.04.06	DFMO
OM118	M	18	Ilekile	Nyori	Aringa	08.04.06	- ve	+ ve	183	+ ve	SSII	03.05.06	DFMO
OM119	M	16	Obayia	Okavu	Terego	10.04.06	- ve	+ ve	434	+ ve	SSII	28.04.06	DFMO
OM120	F	11	Erekpea	Orivu	Terego	25.04.06 10.04.06	N	+ ve	99	+ ve	SSII	10.05.06	DFMO
OM121	M	25	Okavu	Paranga	Terego	13.04.06 11.04.06	- ve	+ ve	948	+ ve	SSII	30.04.06	DFMO
OM122	M	18	Oreku	Okuvu	Maracha	26.04.06						17.05.06	DFMO
						21.04.06	+ ve	+ ve	1206	+ ve	SSII		
OM123	F	40	Kimu	Kijiriba	Koboko	13.05.06		+ ve	767	+ ve	SSII	05.06.06	DFMO
OM124	M	17	Kumiyo	Erea	Terego	30.06.05 24.05.06	N	- ve	159	- ve	SSII	20.01.05 11.06.06 Re-admitted	DFMO DFMO
OM125	M	24	Aliowaku	Ayuri	Terego	24.05.06	+ ve	+ ve	212	+ ve	SSII	18.06.06	DFMO

Code	Sex	Age (yrs)	Village	Parish	County	DOA	GP	HCT	WBC	CSF Tryps	Stage/F-up	DOD	Treatment
OM126	F	22	Igamara	Olube	Aringa	29.05.06	+ ve	+ ve	372	+ ve	SSII	18.06.06	DFMO
OM127	M	19	R/S	Arina	Aringa	.01.05 (Yumbe)							DFMO
						31.05.06	N	N	221	+ ve	SSII	11.06.06 Re-admitted	DFMO
OM128	M	34	Nakamure	Angazi	Terego	6.06.06						23.06.06	DFMO
						5.06.06	N	- ve	238	+ ve	SSII		
OM129	F	19	Nyatu	Lobule	Koboko	08.06.06	+ ve	+ ve	573	+ ve	SSII	05.07.06	DFMO
OM130	F	2 1/2 yrs	Egamara	Oluba	Aringa	09.06.06	+ ve	+ ve	163	+ ve	SSII	23.06.06	DFMO
OM131	F	34	Rigboa	Rogale	Aringa	09.06.06	- ve	- ve	578	+ ve	SSII	09.07.06	DFMO
OM132	M	4	Kwili	Aliba	Madi-Okolo	.12.04							DFMO
						12.06.06	N	N	259	+ ve	SSII	13.06.06 Re-admitted	DFMO
OM133	F	6	Agodonga	Gurepi	Koboko	18.06.06	+ ve	N	199	+ ve	SSII	20.06.06	DFMO
OM134	F	25	Guruki	Lurujo	Koboko	15.10.04						05.11.04	DFMO
						19.06.06	N	N	193	+ ve	SSII	Re-admitted	DFMO
OM135	F	28	Nagulu	Ponyura	Koboko	05.07.06	N	+ ve	137	+ ve	SSII	01.08.06	DFMO
OM136	M	15	K.T.C	K.T.C	Koboko	.12.05 (Sudan)							Arsobal
						07.07.06	N	- ve	257	+ ve	SSII	24.07.06 Re-admitted	DFMO
OM137	F	32	Alutura	Lurujo	Koboko	07.07.06	- ve	+ ve	169	- ve	SSII	28.07.06	DFMO
OM138	F	3 1/2 yrs	Alutura	Lurujo	Koboko	08.11.05						22.11.05	Pentamidine
						07.07.06	N	N	39	+ ve	SSII	26.07.06 Re-admitted	DFMO
OM139	F	12	Itia	Erea	Terego	14.07.06						30.07.06	DFMO
						13.07.06	N	+ ve	218	- ve	SSII		
OM140	F	30	Goromonye	Rugbuja	Koboko	17.07.06	N	+ ve	99	- ve	SSII	08.08.06	DFMO
OM141	F	6	Jamure	Alirubu	Koboko	21.12.04						08.01.05	DFMO
						26.07.06	N	N	75	+ ve	SSII	Re-admitted	DFMO

Code	Sex	Age (yrs)	Village	Parish	County	DOA	GP	HCT	WBC	CSF Tryps	Stage/F-up	DOD	Treatment
OM142	F	36	Jamure	Alirubu	Koboko	26.07.06	N	+ ve	267	+ ve	SSII	18.08.06	DFMO
OM143	M	60	Kimiro	Olua	Terego	12.08.06	N	+ ve	1995	+ ve	SSII		DFMO
OM144	M	9	Gimere	Gurepi	Koboko	23.01.06 18.08.06	 N	 - ve	 81	 - ve	 SSII	08.02.06 Re-admitted	DFMO DFMO
OM145	F	24	Ija	Ludara	Koboko	18.08.06	N	+ ve	6	- ve	SSII		DFMO
OM146	M	31	Licobe	Gurepi	Koboko	22.08.06	N	+ ve	793	+ ve	SSII		DFMO
OM147	M	19	Drandua	Ayuri	Terego	22.08.06	+ ve	+ ve	11	- ve	SSII		DFMO
OM148	F	21	Drandua	Ayuri	Terego	22.08.06	N	+ ve	1	- ve	SSI		Pentamidine
M117	F	38	Patabo	Pamujo	N/A	25.02.05			186	+ ve	SSII	13.03.05	Melarsoprol
M135	M	49	Ramogi. N	Arra	N/A N/A	15.04.05 9.11.05		+ ve	240 2	+ ve - ve	SSII	8.05.05	Melarsoprol
M138	M	13	Kendi	Vura	N/A	17.06.05	+ ve		134	+ ve	SSII	10.07.05	Melarsoprol
M139	F	14	Erepi. E	Pameri	N/A	17.03.04		+ ve	41	- ve	SSII	Re-admitted	Melarsoprol
									248	+ ve	SSII	10.07.05	
M140	F	Adult	Central II	Central	N/A	22.06.05		+ ve	48	- ve	SSII	16.07.05	Melarsoprol
M145	F	13	Erepi-Loliwe	Pameri	N/A	15.07.05	+ ve		78	- ve	SSII	9.08.05	Melarsoprol
M146	M	22	Patabo	Pamujo	N/A	27.07.05	+ ve		88	+ ve	SSII	13.08.05	Melarsoprol
M148	M	46	Ginyi East	Pamuyi	N/A	4.08.05		+ ve	66	+ ve	SSII	31.08.05	Melarsoprol
M149	F	4	Elegu	Pamujo	N/A	12.08.05	+ ve		2	- ve	SSI	3.09.05	Melarsoprol
M150	F	28	Palorinya E	Palorinya	N/A	12.08.05		+ ve	122	- ve	SSII	3.09.05	Melarsoprol
M152	M	47	Aya	Pajakiri	N/A	24.08.05	+ ve		164	+ ve	SSII	16.09.05	Melarsoprol
M153	M	6	Arra	Arra	N/A	24.08.05						16.09.05	Melarsoprol
M154	M	17	Parego	Ebihwa	N/A	24.08.05	+ ve		88	- ve	SSII		Melarsoprol
M155	F	36	Indridri	Dufile	N/A	2.09.05		+ ve	42	+ ve	SSII	25.09.05	Melarsoprol
M156	M	28	Padiga	Pamujo	N/A	2.09.05						25.09.05	Melarsoprol
M160	F	24	Arapi	Pamujo	N/A	30.09.05		+ ve	82	- ve	SSII	1.11.05	Melarsoprol
M161	F	9	Erepi E	Pamujo	N/A	21.10.05	- ve	- ve	15	+ ve	SSII Formerly SSI relapse	13.11.05	Melarsoprol
M162	M	14	Gbari	Pamujo	N/A	21.10.05	+ ve		16	- ve	SSII	13.11.05	Melarsoprol
M163	M	28	Agugwe	Pajakiri	N/A	26.10.05		+ ve	176	+ ve	SSII	17.11.05	Melarsoprol

Code	Sex	Age (yrs)	Village	Parish	County	DOA	GP	HCT	WBC	CSF Tryps	Stage/F-up	DOD	Treatment
M165	M	8	Oruba	Pamujo	N/A	28.10.05	- ve	- ve	10	+ ve	SSII Relapse	23.11.05	Melarsoprol
M166	M	21	Parego	Ebihwa	N/A	23.11.05	+ ve		130	- ve	SSII	16.12.05	Melarsoprol
M167	F	16	Goopi W	Pamujo	N/A	29.11.05	+ ve		348	+ ve	SSII	2.01.06	Melarsoprol
M168	F	10	Indridri	Dufile	N/A	29.12.05	+ ve		116	+ ve	SSII	22.01.06	Melarsoprol
M169	F	16	Ibihwa	Ibihwa	N/A	4.01.06	+ ve		86	- ve	SSII	29.01.06	Melarsoprol
M170	M	21	Padiga	Pamujo	N/A	18.01.06	+ ve		110	+ ve	SSII	9.02.06	Melarsoprol
M171	M	26	Ramogi N	Arra	N/A	18.01.06	+ ve		38	- ve	SSII	9.02.06	Melarsoprol
M172	M	39	Ramogi N	Arra	N/A	18.01.06	+ ve		74	+ ve	SSII	9.02.06	Melarsoprol
M173	M	13	Oruba	Pamujo	N/A	8.02.06 No control done after discharge, either a relapse or re-infection			13	+ ve	SSII	5.03.06	Melarsoprol
M174	F	17	Oruba	Pamujo	N/A N/A	1.02.06			16	+ ve	SSII Relapse	25.02.06	Melarsoprol
M175	M	9	Indriba	Pameri	N/A	25.01.06	+ ve		66	- ve	SSII	18.02.06	Melarsoprol
M176	F	40	Oruba	Pamujo	N/A	17.02.06		+ ve	54	- ve	SSII	17.03.06	Melarsoprol
M177	M	20	Parego	Ebihwa	N/A	22.02.06 14.10.06	+ ve		136 1	+ ve - ve	SSII	17.03.06	Melarsoprol
M178	M	13	Iyua	Pamujo	N/A	19.05.06		+ ve	26	- ve	SSII	13.06.06	Melarsoprol
M179	M	46	Kegburu	Kohci	N/A	2.06.06	+ ve		480	+ ve	SSII	29.06.06	Melarsoprol
M180	M	6	Erepi E	Pamujo	N/A	23.06.06	+ ve		49	+ ve	SSII	24.07.06	Melarsoprol
M181	F	30	Malingu	Ebwea	N/A	23.06.06	+ ve		426	+ ve	SSII	15.07.06	Melarsoprol

NW Uganda, Omugo and Moyo *T. b. gambiense* Patients Raw data
DOA, Date of admission. GP, glandular puncture. HCT, haematocrit centrifugation test. WBC, white blood cell count/mm^3. CSF, cerebral spinal fluid. F-up, follow up period. DOD, date of discharge. DFMO, eflornithine. +ve, positive result for trypanosomes by microscopy . –ve, negative result for trypanosomes by microscopy. N, test not performed.
SSI, stage I disease. SSII, stage II disease. N/A, detail not available.
Field surveys conducted by members of the mobile team: Margaret Akol, Charles Sebikali (National and Agricultural Livestock Health Research Institute-NALIRI), Nicholas Ogweng the vector control officer (Moyo). Data collection and Microscopy diagnosis by Albino Louga (Omugo health centre) and Phillip Dravu (Moyo Hospital), Clinicians: Dr. Charles Wamboga (Omugo health centre) and Dr. Richard Mangwi, (Moyo hospital)

Appendix III

Nigerian Dog sample Raw data

Sample No.	Origin	PCV	HCT	Thin smear	RBC(X10^4)	WBC
138	JNK-9	39	-ve	-ve	471	1010
139	JNK-9	41	-ve	-ve	486	1150
140	JNK-9	40	-ve	-ve	407	1150
141	JNK-9	41	-ve	-ve	431	815
142	JNK-9	38	-ve	-ve	476	1300
143	JNK-9	37	-ve	-ve	473	1950
144	JNK-9	38	-ve	-ve	366	975
145	JNK-9	40	-ve	-ve	443	930
146	JNK-9	55	-ve	-ve	201	975
147	JNK-9	43	-ve	-ve	315	1005
148	JNK-9	39	-ve	-ve	317	1350
149	JNK-9	30	-ve	-ve	415	1440
150	JNK-9	43	+/mf	-ve	335	851
151	JNK-9	35	-ve	-ve	325	1030
152	JNK-9	33	-ve	-ve	195	940
153	JNK-9	23	-ve	*B. gibsoni*+	305	1115
154	JNK-9	30	-ve	-ve	335	850
155	JNK-9	38	-ve	-ve	351	653
156	JNK-9	37	-ve	-ve	470	758
157	JNK-9	50	-ve	-ve	455	878
158	JNK-9	47	-ve	-ve	295	735
159	JNK-9	35	-ve	*B. gibsoni*+	359	187
160	JNK-9	34	-ve	-ve	359	187
161	JNK-9	34	-ve	-ve	431	755
162	JNK-9	20	-ve	-ve	211	1105
163	JNK-9	54	-ve	-ve	485	160
164	JNK-9	57	-ve	-ve	490	402
165	JNK-9	43	-ve	-ve	443	954
166	JNK-9	49	-ve	-ve	435	957
167	JNK-9	33	-ve	-ve	335	1035
168	JNK-9	35	-ve	-ve	401	720
169	JNK-9	30	-ve	-ve	395	865
170	JNK-9	40	-ve	*B. gibsoni*+	388	735
171	JNK-9	37	-ve	-ve	401	1115
172	JNK-9	41	-ve	-ve	379	719
173	JNK-9	37	-ve	-ve	201	198
174	JNK-9	27	-ve	-ve	289	178
175	JNK-9	51	-ve	-ve	301	335
176	JSK-9	40	-ve	-ve	325	215
177	JSK-9	53	-ve	-ve	488	701
178	JSK-9	66	-ve	-ve	415	1001
179	JSK-9	45	-ve	-ve	321	635
180	JSK-9	43	-ve	-ve	401	875
181	JSK-9	25	-ve	-ve	398	788
182	JSK-9	34	-ve	-ve	198	753
183	JSK-9	43	-ve	-ve	501	85
184	JSK-9	44	-ve	-ve	515	987

Sample No.	Origin	PCV	HCT	Thin smear	RBC(X10^4)	WBC
185	JSK-9	50	-ve	-ve	415	857
186	JSK-9	42	-ve	-ve	498	874
187	JSK-9	49	-ve	-ve	419	1116
188	JSK-9	51	-ve	-ve	469	904
189	JSK-9	37	-ve	-ve	289	830
190	JSK-9	62	-ve	*B. gibsoni*+	541	780
191	JSK-9	48	-ve	-ve	538	790
192	JNK-9	47	-ve	-ve	411	895
193	JNK-9	44	-ve	-ve	315	1011
194	JNK-9	36	-ve	-ve	398	950
195	JNK-9	40	-ve	*B. gibsoni*+	412	880
196	JNK-9	48	-ve	-ve	398	795
197	JNK-9	52	-ve	*B. gibsoni*+	491	585
198	JNK-9	47	-ve	-ve	411	1025
199	JNK-9	45	-ve	-ve	389	1015
200	JNK-9	47	-ve	-ve	389	985
201	JNK-9	11	-ve	*B. gibsoni*+	98	1105
202	JNK-9	37	-ve	-ve	273	805
203	JNK-9	25	-ve	*B. gibsoni*+	119	855
204	JNK-9	45	-ve	-ve	385	890
205	JNK-9	28	-ve	*B. gibsoni*+	291	750
206	JNK-9	49	-ve	-ve	491	1225
207	JNK-9	33	-ve	-ve	349	985
208	JSK-9	35	-ve	-ve	325	730
209	JNK-9	30	-ve	-ve	319	905
210	JSK-9	40	-ve	*B. gibsoni*+	415	795
211	JSK-9	37	-ve	-ve	373	975
212	JSK-9	41	-ve	-ve	471	1150
213	JSK-9	37	-ve	*B. gibsoni*+	354	1090
214	JNK-9	27	-ve	-ve	231	775
215	JNK-9	51	-ve	-ve	512	870
216	JNK-9	40	-ve	-ve	419	740
217	JNK-9	53	-ve	-ve	521	355
218	JNK-9	66	-ve	-ve	611	1020
219	JNK-9	45	-ve	-ve	412	885
220	JNK-9	43	-ve	-ve	431	1065
221	JNK-9	25	-ve	*B. gibsoni*+	213	850
222	JNK-9	34	-ve	-ve	315	1015
223	JNK-9	43	-ve	-ve	455	497
224	JNK-9	44	-ve	-ve	395	350
225	JNK-9	50	-ve	-ve	388	579
226	JEK-9	42	-ve	-ve	488	185
227	JEK-9	49	-ve	-ve	496	173
228	JEK-9	51	-ve	-ve	401	505
229	JEK-9	37	-ve	*B. gibsoni*+	599	1028
230	JSK-9	62	-ve	-ve	418	350
231	JSK-9	48	-ve	-ve	401	295
232	JSK-9	47	-ve	-ve	398	915
233	JSK-9	44	-ve	-ve	316	800
234	JSK-9	36	-ve	-ve	415	420
235	JSK-9	40	-ve	*B. gibsoni*+	417	858
236	JNK-9	48	-ve	-ve	413	459
237	JNK-9	52	-ve	-ve	413	720

Sample No.	Origin	PCV	HCT	Thin smear	RBC(X10^4)	WBC
238	JNK-9	47	-ve	-ve	515	450
239	JNK-9	45	-ve	-ve	401	965
240	JNK-9	47	-ve	-ve	398	840
241	JNK-9	11	-ve	*B. gibsoni*+	416	165
242	JNK-9	37	-ve	-ve	101	972
243	JNK-9	25	-ve	-ve	348	935
244	BSAK-9	45	-ve	-ve	201	720
245	BSAK-9	28	-ve	-ve	138	350
246	BSAK-9	47	-ve	-ve	415	735
247	BSAK-9	19	-ve/mf	*B. gibsoni*+	105	1120
248	BSAK-9	55	-ve	-ve	498	845
249	BSAK-9	46	-ve	-ve	387	749
250	BSAK-9	50	-ve	-ve	599	738
251	BSAK-9	44	-ve	-ve	405	813
252	BSAK-9	65	-ve	-ve	897	915
253	BSAK-9	57	-ve	-ve	491	815
254	BSAK-9	47	ve/mf++	*B.gibsoni*+	435	1035
255	BSAK-9	49	-ve	-ve	415	735
256	BSAK-9	38	-ve	-ve	375	920
257	BSAK-9	48	-ve	-ve	398	935
258	BSAK-9	27	-ve	-ve	205	850
259	BSAK-9	18	-ve	-ve	98	98
260	BSAK-9	61	-ve	*B. gibsoni*+	587	870
261	BSAK-9	63	-ve	-ve	594	789
262	BSAK-9	60	-ve	-ve	598	1105
263	BSAK-9	37	-ve	-ve	373	1031
264	BSAK-9	33	-ve	-ve	401	950
265	BSAK-9	28	-ve	-ve	139	101
266	BSAK-9	59	-ve	-ve	485	1135
267	BSAK-9	68	-ve	-ve	598	788
268	JEK-9	36	-ve	-ve	356	1020
269	JEK-9	58	-ve	-ve	489	1215
270	BSAK-9	38	-ve	-ve	289	1025
271	BSAK-9	52	-ve	-ve	445	1150
272	BSAK-9	52	-ve	-ve	448	870
274	JEK-9	50	-ve	-ve	511	1015
275	JEK-9	38	+ve	-ve	298	1120
276	JEK-9	68	-ve	N/A	589	870
277	JEK-9	46	-ve	*B. gibsoni*+	416	1115
278	JEK-9	31	-ve	*B. gibsoni*+	291	1115
279	JEK-9	60	-ve	-ve	551	1025
280	JEK-9	62	-ve	-ve	536	1030
281	JEK-9	43	-ve	-ve	451	11400
282	JEK-9	52	-ve	-ve	438	6800
283	JEK-9	30	-ve	-ve	288	10400
284	JEK-9	63	-ve	-ve	538	10250
285	JEK-9	48	-ve	-ve	415	9200
286	JEK-9	52	-ve	-ve	478	4600
287	JEK-9	58	-ve	-ve	495	13250
288	JEK-9	55	-ve	-ve	483	7420
289	JEK-9	45	-ve	-ve	439	9400
290	JEK-9	52	-ve	-ve	448	8505
291	JEK-9	51	-ve	*B. gibsoni*+	479	8600

Sample No.	Origin	PCV	HCT	Thin smear	RBC(X10^4)	WBC
292	JEK-9	61	-ve	-ve	589	7400
293	JEK-9	45	-ve	-ve	491	1500
294	JEK-9	55	-ve	-ve	498	2512
295	JEK-9	64	-ve	-ve	551	3100
296	JEK-9	53	-ve	-ve	485	6500
297	JEK-9	47	-ve	-ve	457	7400
298	JEK-9	65	-ve	-ve	589	5450
299	JEK-9	55	-ve	-ve	480	1400
300	JEK-9	17	-ve	*B. gibsoni*+	118	14500
301	JEK-9	62	-ve	-ve	598	9510
302	JEK-9	N/A	N/A	N/A	N/A	N/A
303	JEK-9	18	-ve	-ve	145	784
304	JEK-9	37	-ve	-ve	367	635
305	JEK-9	42	-ve	-ve	389	867
306	JEK-9	43	-ve	-ve	415	194
307	JEK-9	38	-ve	-ve	401	1108
308	JEK-9	43	-ve	-ve	389	1109
309	JEK-9	48	-ve	-ve	391	718
310	JEK-9	45	-ve	-ve	554	345
311	JEK-9	31	-ve	-ve	309	665
312	JEK-9	50	-ve	-ve	595	870
313	JEK-9	50	-ve	-ve	68	780
314	JEK-9	12	-ve	-ve	N/A	N/A
315	JEK-9	33	-ve	-ve	434	984
316	JEK-9	22	-ve	-ve	245	1030
317	JEK-9	29	-ve	-ve	384	870
318	JEK-9	37	-ve	-ve	398	720
319	JEK-9	42	-ve	-ve	403	178
320	JEK-9	45	-ve	-ve	389	350
321	JEK-9	23	-ve	-ve	434	350
322	JEK-9	18	-ve	*B. gibsoni*+	245	218
323	JEK-9	57	-ve	-ve	384	1005
324	JEK-9	62	-ve	-ve	398	870
325	JEK-9	32	-ve	-ve	403	857
326	JEK-9	45	-ve	-ve	389	874
327	JEK-9	53	-ve	-ve	2423	795
328	JEK-9	43	-ve	-ve	124	1025

Microscopy data for the dog isolates from Nigeria (by Dr. Yanan-NITOR).
HCT, haematocrit centrifugation test (parasites/ml). PCV, packed cell volume (%).
RBC, red blood cell count (M/µL). WBC, white blood cell count (/µL).
Mf, microfilariae. N/A, result not available.
JNK, Jos North Kaduna. JSK, Jos south Kaduna. JEK, Jos East Kaduna. BSA, Bakin South Kaduna.

Nigerian dog *Babesia* PCR data

Sample number	PCR product size (bp) with *Babesia* spp. primers			
	450	**500**	**600**	**700**
138	-	-	-	-
138	-	-	-	+
139	-	-	-	-
140	-	+	-	-
141	-	-	-	-
142	-	-	-	-
142	-	+	-	-
142	-	+	-	-
143	-	-	-	-
144	-	-	-	-
145	-	-	-	-
146	-	-	-	-
147	-	-	+	+
148	-	-	-	-
149	+	-	-	-
150	-	-	-	-
151	-	-	-	-
152	+	-	-	-
153	-	-	-	-
154	+	-	+	-
155	-	-	-	-
156	-	-	-	-
157	-	-	-	-
158	-	-	-	-
159	+	-	-	-
160	+	-	-	-
161	+	-	-	-
162	-	-	-	-
163	-	-	-	-
164	-	-	-	-
165	-	+	-	-
166	-	+	-	-
167	-	-	-	-
168	-	-	-	-
169	-	-	-	-
170	-	-	-	+
171	-	-	-	-
172	+	-	-	-
173	+	-	-	-
174	-	+	-	-
175	-	-	-	-
176	-	-	-	-
177	+	-	-	-
178	-	-	-	-
179	-	+	-	-
179	-	+	-	-

Sample number	PCR product size (bp) with *Babesia* spp. primers			
	450	500	600	700
180	-	-	-	-
181	-	+	-	-
182	-	-	-	-
183	-	-	-	-
184	-	-	-	-
185	-	+	-	+
186	-	-	+	+
187	-	-	-	-
188	-	-	-	-
189	-	-	+	+
190	-	-	+	+
191	-	-	-	+
192	-	-	+	-
193	-	+	-	+
194	+	-	-	+
195	-	+	-	-
196	-	-	-	-
197	-	-	-	-
198	-	-	-	-
199	-	-	-	-
200	-	-	-	-
201	-	-	-	-
202	-	-	-	-
203	-	-	-	-
204	-	-	-	-
205	-	-	-	-
206	-	-	-	-
207	-	-	-	-
208	+	-	-	-
209	+	-	-	-
210	-	-	-	-
211	-	-	-	-
212	-	+	-	-
213	-	+	-	-
214	-	-	-	-
215	-	-	-	-
216	+	-	-	-
217	-	-	-	-
218	-	-	-	-
219	+	-	-	-
220	-	-	-	-
221	-	-	-	-
222	-	-	-	-
223	-	-	-	-
224	-	-	-	-
225	+	-	-	-
226	-	+	-	-
227	-	-	-	-
228	-	+	-	-

Sample number	PCR product size (bp) with *Babesia* spp. primers			
	450	500	600	700
229	-	-	-	-
230	-	-	-	-
231	+	-	-	-
232	-	-	-	-
233	-	-	-	-
234	-	-	-	-
235	-	-	-	-
236	-	-	-	-
237	-	-	-	-
238	-	+	-	-
239	+	-	-	-
240	-	-	-	-
241	-	-	-	-
242	-	+	-	+
243	+	-	-	-
244	-	-	-	-
245	-	-	-	-
246	+	-	-	-
247	-	-	-	-
248	-	+	-	+
249	-	+	-	-
250	+	-	-	-
251	-	-	-	-
252	+	-	-	+
253	-	+	-	+
254	-	-	-	-
255	-	-	-	-
256	-	-	-	-
257	-	-	-	-
258	-	-	-	-
259	-	-	-	-
260	-	-	-	-
261	+	-	-	-
262	-	-	-	-
263	-	+	-	+
264	-	+	-	-
265	-	-	-	-
266	-	-	-	-
267	-	-	-	-
268	-	-	-	-
269	-	-	-	-
270	-	-	-	-
271	-	-	-	-
272	-	-	-	-
274	-	-	-	-
275	-	-	-	-
276	-	-	-	-
277	-	-	-	-
278	-	+	-	-

279	-	-	-	-
280	-	+	-	-
281	-	-	-	-
282	-	+	-	-
283	-	-	-	-
284	-	+	-	-
285	-	+	-	-
286	-	-	-	-
287	+	-	-	-
288	+	-	-	-
289	+	-	-	-
290	-	+	-	-
291	-	-	-	-
292	-	-	-	-
293	+	-	-	-
294	-	-	-	-
295	-	-	-	-
296	-	-	-	-
297	-	-	-	-
298	-	-	-	-
299	-	-	-	-
300	+	-	-	-
301	-	-	-	-
302	-	-	-	-
303	-	+	-	-
304	-	+	-	-
305	-	-	-	-
306	-	+	-	-
307	-	-	-	-
308	-	-	-	-
309	-	+	-	-
310	+	-	-	-
311	+	-	-	-
312	+	-	-	-
313	+	-	-	-
314	-	+	-	-
315	-	-	-	-
316	+	-	-	-
317	-	-	-	-
318	-	+	-	-
319	-	-	-	-
320	-	-	-	-
321	-	-	-	-
322	-	-	-	-
323	-	-	-	-
324	-	-	-	-
325	-	-	-	-
326	-	-	-	-
327	-	-	-	-
328	-	-	-	-

PCR amplification results for the dog samples with *Babesia* non-specific primers. +, positive. -, negative. (Data from Clare McCann). Total number of dog samples (N=190).

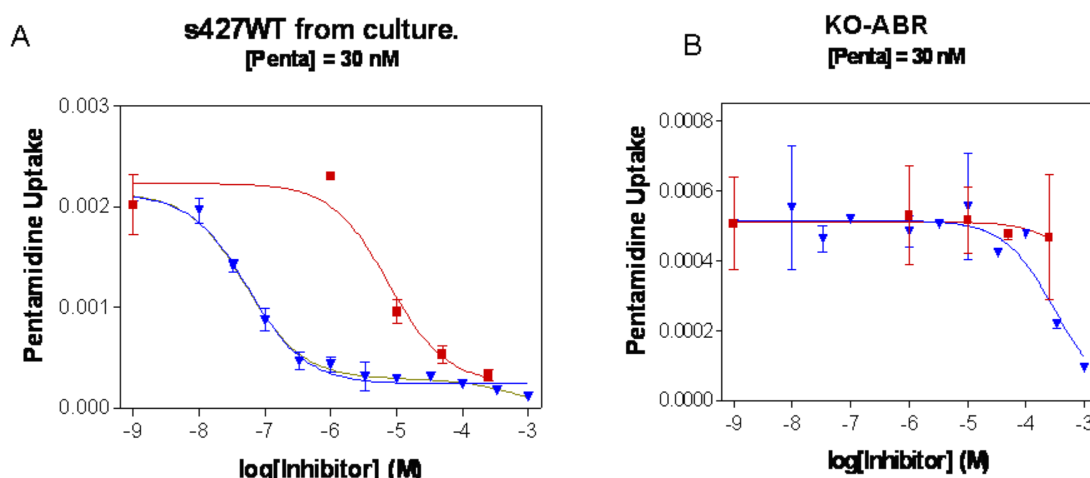
Appendix IV

This section of the thesis refers to the pentamidine and diminazene transport assay results obtained from the ABR strain selected against high level diminazene (Chapter 6, section 6.4.1).

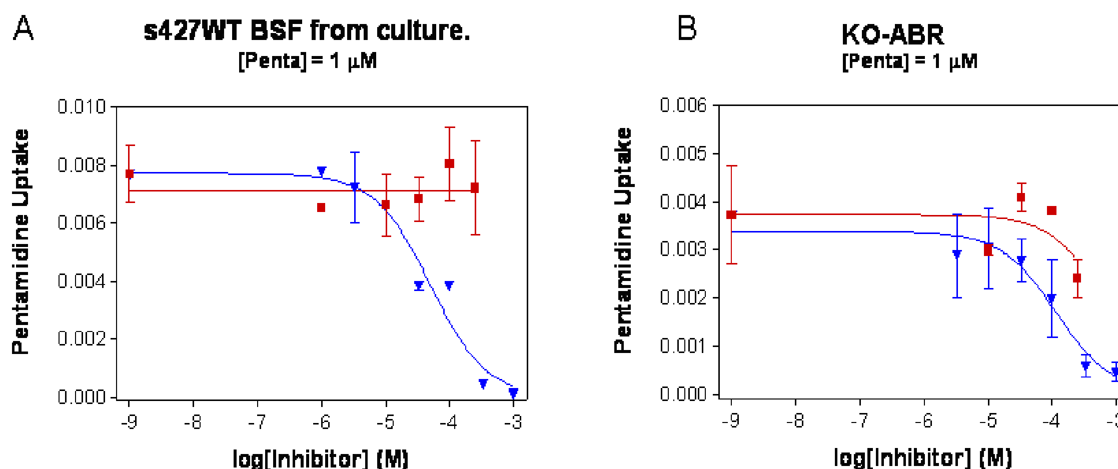
Pentamidine transport in the ABR strain selected against high level diminazene

The KO-B48 strain selected against pentamidine (130-fold) did not display a detectable HAPT1 activity (Bridges *et al*, 2007). HAPT1 and LAPT1 are responsible for pentamidine transport in the *tbat1*$^{-/-}$ line. The ABR line selected for growth in high levels of diminazene was found to be cross-resistant to pentamidine (Table 6.3). To determine whether the observed resistance correlated with reduced HAPT1 or LAPT1 activity, pentamidine transport assays were performed using [^{3}H]-pentamidine at 60 nM for HAPT1 and 2 µM for LAPT1 respectively as described (section 6.3). HAPT1 activity was found to be absent in the ABR strain in which [^{3}H]-pentamidine was not measurably inhibited by less than 10 µM unlabelled pentamidine (Graph 1, panel B). In contrast, [^{3}H]-pentamidine uptake in WT s427 was potently inhibited by unlabelled pentamidine, with typical IC_{50} values between 35 and 55 nM (Graph 1, panel A), which is in close agreement with the reported K_m for HAPT1 of 36 nM (de Koning, 2001b). Similarly, [^{3}H]-pentamidine uptake in wild type cells was inhibited by propamidine, a selective inhibitor of HAPT1 but not of LAPT1 (de Koning, 2001b), whereas propamidine had no effect on pentamidine uptake in the ABR strain,

LAPT1 mediated pentamidine uptake was observed to be present in both the ABR strain as in WT s427 as shown on page 216 (Graph 2, panel A and B). The K_m value for [^{3}H]-pentamidine uptake in the ABR strain was 59±11 µM (n=3), identical to the published value for LAPT1 of 56±8 µM (de Koning, 2001b). The V_{max} value of 1.2 ± 0.4 pmol/10^7 cells)$^{-1}$s^{-1} was also identical to the published value for LAPT1, showing that LAPT1 in contrast to P2 is not down regulated in cultured cells.
The above results strongly suggested that the pentamidine cross-resistance in ABR strain adapted to high diminazene concentration was due to loss of HAPT1 transport.



Graph 1 HAPT1 activity in WT s427 (panel A) but not in the ABR strain (panel B). Uptake of 30 nM [^{3}H]-pentamidine over 60 seconds in the presence of propamidine (■) or unlabelled pentamidine (▼). Results shown are representative of three independent experiments at various concentrations as indicated.
Transport measured as [(pmol/10^7 cells)$^{-1}$s^{-1}].

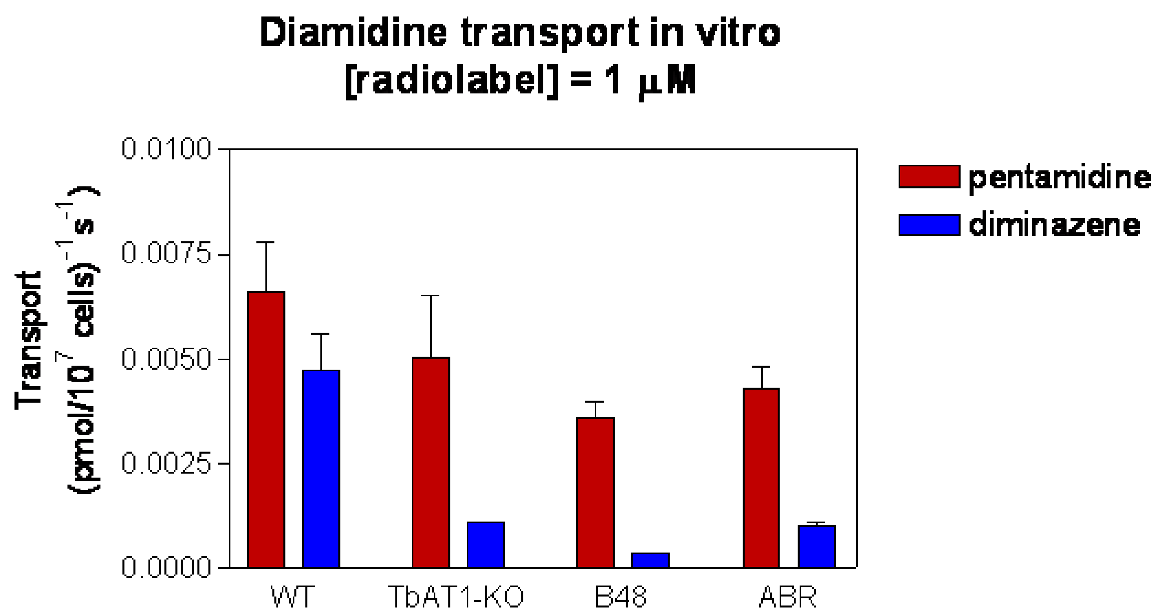


Graph 2 LAPT1 activity in WT s427 (panel A) and the ABR strain (panel B) Uptake of 1 µM [^{3}H]-pentamidine over 60 seconds in the presence of propamidine (■) or unlabelled pentamidine (▼). Results shown are representative of three independent experiments at various concentrations as indicated.
Transport measured as [(pmol/10^7 cells)$^{-1}$s^{-1}].

Diminazene transport in the ABR strain selected against high level diminazene

Previous studies had only shown that diminazene uptake in *T. brucei* is dependent on P2 transport (Matovu *et al*, 2003;de Koning *et al*, 2004). The loss of HAPT1 in the ABR strain adapted to high diminazene concentration suggests that this could be a second route for diminazene uptake. Therefore no diminazene uptake should take place in the ABR strain if diminazene resistance correlates to loss of both TbAT1/P2 and HAPT1. To determine whether this is the case, the ABR cell line was subjected to a diminazene transport assay

using 1 µM [^{3}H]-diminazene as described for pentamidine transport (section 6.3). The B48 strain (adapted to high pentamidine concentration and lacking both P2 and HAPT1 activity is not more resistant to diminazene than *tbat1*$^{-/-}$ (Bridges *et al*, 2007), was included in the assay for comparison. The results obtained revealed that diminazene transport was not completely abolished in the ABR and B48 cell lines at 1 µM [^{3}H]-diminazene (Graph 3). Uptake of 1 µM [^{3}H]-pentamidine was only 24% reduced in *tbat1*$^{-/-}$ compared to WT s427 and by 40% and 35% in the B48 and ABR strains respectively (n=3-4). This is explained by the fact that at this concentration, pentamidine is mostly transported by LAPT1 (Bray *et al*, 2003), which is present in all four strains. However, uptake of 1 µM [^{3}H]-diminazene was more dramatically affected by the loss of P2 activity (68%) and reduced to just 12% or 20% of WT s427 uptake rates in the B48 and ABR strains, respectively. This clearly is consistent with the expected higher proportion of diminazene uptake through P2, even at this high concentration of radiolabel. It further shows that the reported loss of HAPT1 activity in B48 (Bridges *et al*, 2007) and ABR (this result section) correlates with reduced diminazene uptake. However, a measurable rate of [^{3}H]-diminazene uptake even in these strains shows that, like pentamidine, diminazene must have at least three routes by which it can enter trypanosomes, and the rate by which it is able to utilise each transporter is a function of its concentration. Preliminary results from the De Koning group indicate that the third (and least important) diminazene transporter is probably LAPT1 (results not shown).



Graph 3 Pentamidine and diminazene transport in the different *T. brucei* strains
Rates were determined from the slopes of time courses with 6-7 points spread over 10 minutes. Slopes were determined by linear regression with r^2 values >0.9. Data shown are the average and standard errors of 3-4 independent experiments, each performed in triplicate.

Alamar Blue assay Raw data

Glasgow start cell density (2 x 10^5 cells/ml)

	ak expt 1							
	Pentamidine	RF	Diminazene	RF	DB75	RF	Cymelarsan	RF
WT s427	3.67E-09	**1.00**	8.80E-08	**1.00**	1.03E-08	**1.00**	3.21E-09	**1.00**
KO	3.94E-09	**1.07**	2.57E-07	**2.92**	7.95E-08	**7.72**	7.94E-09	**2.47**
KO-B48	1.84E-07	**50.14**	5.78E-08	**0.66**	3.11E-08	**3.03**	1.33E-07	**41.36**
WT s427	4.26E-09	**1.00**	4.69E-08	**1.00**	4.72E-09	**1.00**	2.55E-09	**1.00**
KO	1.03E-08	**2.42**	2.57E-07	**5.48**	5.84E-08	**12.39**	9.40E-09	**3.69**
KO-ABR	7.23E-08	**16.97**	1.79E-06	**38.08**	2.28E-07	**48.38**	2.32E-08	**9.08**

	ak expt 2									
	Pentamidine	RF	Diminazene	RF	DB75	RF	Cymelarsan	RF	PAO	RF
WT s427	6.48E-09	**1.00**	5.44E-07	**1.00**	2.20E-07	**1.00**	3.66E-09	**1.00**	1.06E-09	**1.00**
KO	5.98E-09	**0.92**	4.67E-06	**8.58**	1.10E-06	**4.98**	6.80E-09	**1.86**	8.49E-10	**0.80**
KO-B48	3.03E-07	**46.75**	1.28E-06	**2.36**	2.66E-07	**1.21**	4.23E-08	**11.57**	4.88E-10	**0.46**
WT s427	5.05E-09	**1.00**	3.93E-07	**1.00**	1.34E-07	**1.00**	3.68E-09	**1.00**	1.29E-09	**1.00**
KO	1.28E-08	**2.54**	3.59E-06	**9.15**	5.78E-07	**4.32**	1.37E-08	**3.72**	7.30E-10	**0.57**
KO-ABR	1.25E-07	**24.80**	8.44E-06	**21.51**	1.34E-06	**10.00**	2.01E-08	**5.45**	8.68E-10	**0.67**

	ak expt 3									
	Pentamidine	RF	Diminazene	RF	DB75	RF	Cymelarsan	RF	PAO	RF
WT s427	1.36E-08	**1.00**	8.66E-07	**1.00**	4.15E-07	**1.00**	6.33E-09	**1.00**	6.74E-10	**1.00**
KO	7.03E-09	**0.52**	2.93E-06	**3.39**	6.69E-07	**1.61**	7.61E-09	**1.20**	8.23E-10	**1.22**
KO-B48	5.72E-07	**42.14**	2.51E-06	**2.90**	6.92E-07	**1.67**	6.32E-08	**9.99**	8.66E-10	**1.28**
WT s427	1.23E-08	**1.00**	1.71E-06	**1.00**	7.72E-07	**1.00**	4.53E-09	**1.00**	9.33E-10	**1.00**
KO	7.05E-08	**5.71**	7.51E-06	**4.40**	1.84E-06	**2.38**	2.44E-08	**5.39**	1.32E-09	**1.42**
KO-ABR	4.57E-07	**37.04**	1.29E-05	**7.56**	5.23E-06	**6.77**	2.86E-08	**6.31**	1.08E-09	**1.16**

Glasgow start cell density (2 x 10^5 cells/ml)

	cpw expt 1							
	Pentamidine	**RF**	**Diminazene**	**RF**	**DB75**	**RF**	**Cymelarsan**	**RF**
WT s427	7.44E-09	**1.00**	5.86E-07	**1.00**	1.35E-07	**1.00**	4.39E-09	**1.00**
KO	8.13E-09	**1.09**	3.73E-06	**6.37**	4.13E-06	**30.70**	1.37E-08	**3.11**
KO-B48	1.55E-06	**208.85**	3.41E-06	**5.83**	4.03E-06	**29.93**	6.09E-08	**13.87**
WT s427	1.26E-08	**1.00**	3.81E-07	**1.00**	2.03E-07	**1.00**	6.69E-09	**1.00**
KO	3.77E-08	**3.00**	3.51E-06	**9.21**	4.47E-06	**22.00**	3.47E-08	**5.18**
KO-ABR	6.06E-07	**48.08**	8.00E-06	**21.02**	9.62E-06	**47.37**	4.49E-08	**6.72**

	cpw expt 2									
	Pentamidine	**RF**	Diminazene	**RF**	DB75	**RF**	Cymelarsan	**RF**	PAO	**RF**
WT s427	3.84E-09	**1.00**	1.10E-06	**1.00**	1.61E-07	**1.00**	3.28E-09	**1.00**	2.57E-10	**1.00**
KO	2.87E-09	**0.75**	1.63E-05	**14.84**	7.97E-07	**4.94**	4.92E-09	**1.50**	4.95E-10	**1.92**
KO-B48	3.82E-07	**99.32**	3.42E-05	**31.08**	3.31E-07	**2.05**	2.94E-08	**8.96**	1.93E-10	**0.75**
WT s427	4.34E-09	**1.00**	7.86E-07	**1.00**	1.70E-07	**1.00**	3.44E-09	**1.00**	4.67E-10	**1.00**
KO	5.75E-09	**1.32**	1.64E-05	**20.80**	6.14E-07	**3.62**	1.06E-08	**3.07**	8.38E-10	**1.80**
KO-ABR	1.09E-07	**25.03**	2.93E-05	**37.21**	1.17E-06	**6.88**	2.61E-08	**7.57**	4.12E-10	**0.88**

	cpw expt 3									
	Pentamidine	**RF**	Diminazene	**RF**	DB75	**RF**	Cymelarsan	**RF**	PAO	**RF**
WT s427	4.54E-09	**1.00**	6.58E-07	**1.00**	2.61E-07	**1.00**	4.25E-09	**1.00**	5.71E-10	**1.00**
KO	2.06E-09	**0.45**	2.79E-06	**4.24**	6.71E-07	**2.58**	6.85E-09	**1.61**	8.12E-10	**1.42**
KO-B48	3.97E-07	**87.41**	3.48E-06	**5.29**	4.78E-07	**1.83**	4.46E-08	**10.50**	5.95E-10	**1.04**
WT s427	4.04E-09	**1.00**	3.94E-07	**1.00**	6.29E-08	**1.00**	2.87E-09	**1.00**	2.69E-10	**1.00**
ak KO	1.90E-07	**46.99**	7.37E-06	**18.73**	1.82E-06	**28.93**	1.50E-08	**5.24**	3.20E-10	**1.19**
KO-ABR	4.12E-07	**102.06**	1.42E-05	**36.03**	5.44E-06	**86.46**	1.78E-08	**6.22**	9.13E-10	**3.39**

Swiss cell start density (4 x 10^4 cells/ml)

	ak expt 1									
	Pentamidine	RF	Diminazene	RF	DB75	RF	Cymelarsan	RF	PAO	RF
WT s427	2.24E-09	**1.00**	2.83E-07	**1.00**	2.06E-08	**1.00**	2.44E-09	**1.00**	2.36E-10	**1.00**
KO	2.23E-09	**0.99**	2.77E-06	**9.76**	6.45E-07	**31.28**	6.05E-09	**2.48**	1.80E-10	**0.76**
KO-B48	2.89E-07	**129.04**	9.86E-07	**3.48**	2.89E-07	**14.03**	3.42E-08	**14.03**	1.25E-10	**0.53**
WT s427	1.80E-09	**1.00**	2.70E-07	**1.00**	2.92E-08	**1.00**	2.55E-09	**1.00**	1.83E-10	**1.00**
KO	1.26E-08	**7.02**	2.00E-06	**7.41**	5.31E-07	**18.21**	1.35E-08	**5.30**	2.14E-10	**1.17**
KO-ABR	1.09E-07	**60.76**	6.95E-06	**25.76**	1.25E-06	**42.90**	2.13E-08	**8.33**	3.02E-10	**1.65**

	ak expt 2									
	Pentamidine	RF	Diminazene	RF	DB75	RF	Cymelarsan	RF	PAO	RF
WT s427	5.91E-09	**1.00**	1.50E-07	**1.00**	1.51E-08	**1.00**	2.45E-09	**1.00**	2.81E-10	**1.00**
KO	4.54E-09	**0.77**	1.29E-07	**0.86**	1.32E-08	**0.87**	2.60E-09	**1.06**	2.63E-10	**0.94**
KO-B48	2.30E-07	**38.93**	2.00E-07	**1.33**	1.04E-07	**6.88**	2.37E-08	**9.66**	2.53E-10	**0.90**
WT s427	1.48E-09	**1.00**	4.97E-08	**1.00**	8.22E-09	**1.00**	1.47E-09	**1.00**	2.08E-10	**1.00**
KO	2.51E-08	**16.97**	1.84E-06	**36.99**	3.68E-07	**44.78**	9.59E-09	**6.54**	2.95E-10	**1.42**
KO-ABR	3.96E-08	**26.76**	3.73E-06	**75.04**	3.73E-07	**45.34**	1.24E-08	**8.49**	3.74E-10	**1.79**

	ak expt 3									
	Pentamidine	RF	Diminazene	RF	DB75	RF	Cymelarsan	RF	PAO	RF
WT s427	2.38E-09	**1.00**	2.79E-07	**1.00**	4.26E-08	**1.00**	3.26E-09	**1.00**	2.48E-10	**1.00**
KO	3.66E-09	**1.54**	2.99E-07	**1.07**	1.64E-08	**0.39**	2.60E-09	**0.80**	3.07E-10	**1.24**
KO-B48	3.37E-07	**141.57**	1.67E-06	**5.98**	3.55E-07	**8.35**	4.18E-08	**12.84**	2.15E-10	**0.87**
WT s427	5.65E-09	**1.00**	3.10E-07	**1.00**	3.51E-08	**1.00**	3.61E-09	**1.00**	2.25E-10	**1.00**
KO	5.26E-08	**9.32**	3.86E-06	**12.46**	4.66E-07	**13.27**	1.52E-08	**4.22**	3.84E-10	**1.71**
KO-ABR	7.90E-08	**13.99**	7.35E-06	**23.74**	1.06E-06	**30.20**	1.71E-08	**4.75**	2.73E-10	**1.21**

Swiss cell start density (4 x 10^4 cells/ml)

	cpw expt 1									
	Pentamidine	RF	Diminazene	RF	DB75	RF	Cymelarsan	RF	PAO	RF
WT s427	5.58E-10	**1.00**	2.30E-07	**1.00**	1.40E-08	**1.00**	1.76E-09	**1.00**	8.54E-11	**1.00**
KO	5.12E-10	**0.92**	1.87E-06	**8.15**	3.50E-07	**25.03**	4.84E-09	**2.75**	5.20E-12	**0.06**
KO-B48	2.25E-07	**402.62**	1.19E-06	**5.18**	1.82E-07	**12.99**	2.97E-08	**16.91**	1.98E-10	**2.32**
WT s427	9.26E-10	**1.00**	2.93E-07	**1.00**	2.42E-08	**1.00**	2.44E-09	**1.00**	1.14E-10	**1.00**
KO	8.44E-09	**9.11**	3.58E-06	**12.24**	4.69E-07	**19.34**	1.14E-08	**4.67**	1.74E-10	**1.52**
KO-ABR	8.56E-08	**92.39**	7.13E-06	**24.36**	1.02E-06	**42.22**	1.77E-08	**7.27**	2.16E-10	**1.89**

	cpw expt 2									
	Pentamidine	RF	Diminazene	RF	DB75	RF	Cymelarsan	RF	PAO	RF
WT s427	2.75E-09	**1.00**	1.93E-07	**1.00**	3.56E-09	**1.00**	7.75E-10	**1.00**	2.21E-12	**1.00**
KO	1.96E-10	**0.07**	1.94E-07	**1.00**	1.07E-08	**3.00**	1.09E-09	**1.41**	1.22E-10	**55.08**
KO-B48	8.65E-08	**31.42**	1.56E-07	**0.81**	3.91E-08	**10.98**	1.95E-08	**25.20**	2.10E-10	**95.10**
WT s427	1.77E-11	**1.00**	6.40E-08	**1.00**	1.42E-09	**1.00**	6.31E-10	**1.00**	5.95E-14	**1.00**
KO	1.37E-08	**771.88**	1.61E-06	**25.09**	1.80E-07	**126.84**	6.10E-09	**9.66**	2.12E-11	**356.64**
KO-ABR	2.53E-08	**1429.70**	4.08E-06	**63.80**	2.68E-07	**189.55**	8.63E-09	**1.42**		

	cpw expt 3									
	Pentamidine	RF	Diminazene	RF	DB75	RF	Cymelarsan	RF	PAO	RF
WT s427	4.17E-10	**1.00**	2.72E-07	**1.00**	1.40E-08	**1.00**	1.59E-09	**1.00**	2.22E-10	**1.00**
KO	4.52E-10	**1.08**	2.53E-07	**0.93**	1.57E-08	**1.12**	1.63E-09	**1.02**	2.23E-10	**1.00**
KO-B48	3.08E-07	**739.04**	1.88E-06	**6.89**	1.93E-07	**13.78**	2.76E-08	**17.35**	1.33E-10	**0.60**
WT s427	5.32E-10	**1.00**	2.81E-07	**1.00**	1.56E-08	**1.00**	1.81E-09	**1.00**	2.23E-10	**1.00**
KO	3.92E-08	**73.59**	4.06E-06	**14.43**	3.39E-07	**21.71**	1.32E-08	**7.31**	2.76E-10	**1.24**
KO-ABR	3.78E-08	**70.98**	6.63E-06	**23.58**	5.49E-07	**35.12**	1.48E-08	**8.20**	1.94E-10	**0.87**

IC_{50} values obtained from Alamar blue triplicate experiments performed concurrently with Dr. C Ward for comparison.

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