Isolation, propagation and characterization of *Trypanosoma brucei gambiense* from Human African Trypanosomosis patients in south Sudan

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Dekan

To the memory of my father Justus Njogu Karuru.

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Summary

Sleeping sickness or Human African trypanosomosis (HAT) is a protozoal disease that is transmitted by tsetse fly vectors in Africa. Sleeping sickness due to *T. b. gambiense* is a major public health problem in countries in central and western Africa including Angola, Uganda, Democratic Republic of Congo (DRC) and Sudan. High rates of relapses (>20%) following melarsoprol treatment have been reported in many treatment centres including the MSF-F treatment centre at Ibba in south Sudan. The treatment failures could be due to individual (patients) variation in the drug pharmacokinetics, the patient's immune responses, or drug resistant parasites.

There is a growing interest in the elucidation of the reason(s) for relapses after melarsoprol treatment. Since the drug levels in blood or CSF do not differ between relapse and successfully treated patients, drug resistance has been suggested as a likely cause for melarsoprol treatment failures. There are no recently isolated parasites from high-relapse areas and hence detailed studies have been hindered. The objective of this PhD study was to isolate and characterize (phenotypically and genotypically) *T. b. gambiense* from HAT patients in the MSF-F treatment centre at Ibba.

In a first step, the protocols for the isolation of bloodstream forms of the parasite needed to be improved. As such, the suitability of the commercial cryomedium Triladyl® developed for bull semen was evaluated. We found that, the cryopreservation of *T. b. gambiense* in this medium led to a better survival of the trypanosomes than in the standard 10% glycerol. The samples (blood (50) and CSF (2)) from HAT patients were therefore cryopreserved using Triladyl® and stored at -150^{o}C in nitrogen vapour in a dry shipper. In the laboratory, attempts to propagate the isolated parasites in rodents were carried out. Of the 42 parasite positive isolates, 18 (43%) could be propagated in laboratory rodents (immunosuppressed *Mastomys natalensis* and SCID mice). Stabilates of these *T. b. gambiense* isolates are stored in two cryobanks at the Trypanosomiasis Research Centre (TRC) of the Kenya Agricultural Research Institute (KARI), Nairobi and the Swiss Tropical Institute (STI), Basel.

After the initial isolation of these *T. b. gambiense* isolates in immunosuppressed *M. natalensis* or SCID mice, further *in vivo* propagation could be done in various immunosuppressed rodent species (Swiss White mice, *M. natalensis,* C57/bl, C3H, and BALB/C). The highest

parasitaemia were achieved in C57/bl and BALB/C mice. The rodents however had to be immunosuppressed with cyclophosphamide at 300mg/kg prior to infection and repeated once a week at 200mg/kg. This scheme (cryopreservation and subsequent propagation) allows the isolation of *T. b. gambiense* from various endemic areas and therefore enhances monitoring of drug resistant trypanosomes.

The eighteen *T. b. gambiense* isolates were found to be sensitive to melarsoprol, melarsen oxide, and diminazene. The gene that codes for the P2 transporter, *TbATI*, was amplified by PCR and sequenced. The sequences were almost identical to the $TbAT1^{sensitive}$ reference, except for one point mutation, C1384T resulting in the amino acid change proline-462 to serine. None of the described $TbAT1^{resistant}$-type mutations were detected. In summary we found, in a sleeping sickness focus where melarsoprol had to be abandoned due to the high incidence of treatment failures, no evidence for drug resistant trypanosomes or for $TbAT1^{resistant}$-type alleles of the P2 transporter.

In conclusion, our findings cast doubts on the current suggestion that melarsoprol resistant parasites are the cause of the high rate of treatment failures reported. However, it is important to note that the number of isolates tested was still small and it is vital that more isolates, especially from relapse patients be tested

Zusammenfassung

Schlafkrankheit, auch humane afrikanische Trypanosomosis (HAT) genannt, ist eine Protozoenkrankheit, die in Afrika übertragen wird. Schlafkrankheit vom Typ *T.b. gambiense* ist ein grosses Problem im öffentlichen Gesundheitswesen von Ländern Zentral- und Westafrikas wie Angola, Uganda, Demokratische Republik Kongo (DRC) und Sudan. Hohe Rückfallraten (>20%) nach Melarsoprol Behandlung wurden in vielen Zentren beobachtet, darunter auch im MSF-F Zentrum in Ibba im Süden Sudans. Diese Rückfälle könnten auf Unterschiede in der Pharmakokinetik des Medikamentes in Patienten, auf die Immunantwort des jeweiligen Patienten oder auf resistente Parasiten zurückzuführen sein.

Es besteht ein grosses Interesse herauszufinden, was die Gründe für die hohe Rückfallrate nach Melarsoprol Behandlung sind. Da die Medikamentenspiegel im Blut oder CSF von Patienten, die geheilt wurden oder einen Rückfall erlitten, sich nicht als unterschiedlich herausstellten, wird Medikamentenresistenz mit grosser Wahrscheinlichkeit als der Grund für fehlgeschlagene Behandlungen betrachtet. Da es aber keine kürzlich isolierten Parasitenisolate aus dieser Gegend gibt, konnten bis anhin keine detailierten Studien durchgeführt werden. Das Ziel dieser PhD Arbeit war es, *T. b. gambiense* Isolate von HAT Patienten im MSF-F Behandlungszentrum in Ibba zu isolieren und zu charakterisieren (phänotypisch und genotypisch).

In einem ersten Schritt wurden die Protokolle zur Isolierung von Blutformen des Parasiten verbessert. Hierzu wurde untersucht, wie geeignet das für Stiersamen entwickelte, kommerziell erhältliche Kryomedium Triadyl® wirklich ist. Wir haben dabei festegestellt, dass die Kryopräservierung von *T. b. gambiense* in diesem Medium, verglichen mit dem standardisierten 10% Glycerin-Protokoll, zu einem besseren Überleben der Trypanosomen geführt hat. Die Proben (Blut (50) und CSF (2)) von HAT Patienten wurden darum in Triladyl® kryopräserviert und bei -150°C in flüssigem Stickstoff in einem „dry shipper“ gelagert. Im Labor wurden Propagationsversuche mit den isolierten Parasiten in Nagern durchgeführt. Von den 42 parasitierten Isolaten konnten 18 (43%) in Labornagern (immunosuprimierte *Mastomys natalensis* und SCID Mäuse) propagiert werden. Stabilate von diesen *T. b. gambiense* Isolaten werden in zwei Kryobanken am „Trypanosomiasis Research Centre (TRC) of KARI, Nairobi“ und dem „Swiss Tropical Institute“ (STI) gelagert.

Nach der Gewinnung von diesen *T. b. gambiense* Isolaten aus immunosupprimierten *M. natalensis* oder SCID Mäusen, konnten die Parasiten in verschiedenen immunosupprimierten Nagerspezies (Swiss White Mäusen, *M. natalensis*, C57/bl, C3H, und BALB/C) weiterpropagiert werden. Die höchsten Parasitämien wurden in C57/bl und BALB/C Mäusen erreicht. Die Nager mussten allerdings mit Cyclophosphamid immunosupprimiert werden (vor der Infektion mit 300mg/kg Cyclophosphamid, anschliessend einmal wöchentlich mit 200mg/kg). Dieses Schema (Kryopraeservation und anschliessende Propagation) wird die Isolation von *T. b. gambiense* aus verschiedenen endemischen Gebieten ermöglichen und darum das „monitoring" von resistenten Trypanosomen vereinfachen.

Die 18 propagierten *T. b. gambiense* Isolate wurden als sensitiv gegenüber Melarsoprol, Melarsenoxid, und Diminazene identifiziert. Das Gen kodierend füer den P2 Transporter, *TbATI*, wurde mittels PCR amplifiziert und sequenziert. Die Sequenzen waren fast gpeich wie die $TbAT1^{sensitive}$ Referenz, mit Ausnahme einer Punktmutation, C1384T, die in einer Aminosäureänderung von Prolin-462 zu Serin resultierte. Keine der beschriebenen $TbAT1^{resistant}$-Typ Mutationen konnten beobachtet werden. Zusammenfassend haben wir in einem Schlafkrankheits-Fokus, wo Melarsoprol Behandlungen aufgrund von hohem Auftreten von Behandlungsmisserfolgen aufgegeben werden mussten, keine Hinweise auf resistente Trypanosomen oder $TbAT1^{resistant}$-Typ Allele vom P2 Transporter gefunden.

Unsere Ergebnisse zweifeln darum die momentan vorherrschende Meinung, dass melarsoprol-resistente Parasiten der Grund für die hohe Rate an Behandlungsmisserfolgen sind, an. Es muss allerdings hevorgehoben werden, dass die Anzahl der von uns getesteten Isolate klein war und es wichtig wäre mehr Isolate, und vor allem Isolate von „relapse" Patienten, zu testen.

CHAPTER 1: Introduction

Prevalence of Human African Trypanosomosis

Sleeping sickness or Human African Trypanosomosis (HAT) is a protozoan disease that is transmitted only in Africa. It is an ancient disease with the first records dating to the 14th century. It is the third most important vector borne parasitic disease (Molyneux 1996). Although the figures for HAT are relatively small compared to other diseases, HAT is fatal if untreated and has the propensity to develop into epidemics making it a major public health problem.

The disease is only endemic in areas where tsetse flies of the genus *Glossina* (the only vector) are found. As such its occurrence is rather focal with ecological limits of tsetse fly distribution from approximately 14^{o}N (West Senegal) and 10^{o}N (East, Somali) to 20^{o}S at the northern fringes of the Kalahari and Namibian desert, depending on climate and vegetation (Molyneux 1996). The prevalence and distribution of the disease is as shown in Figure 1.

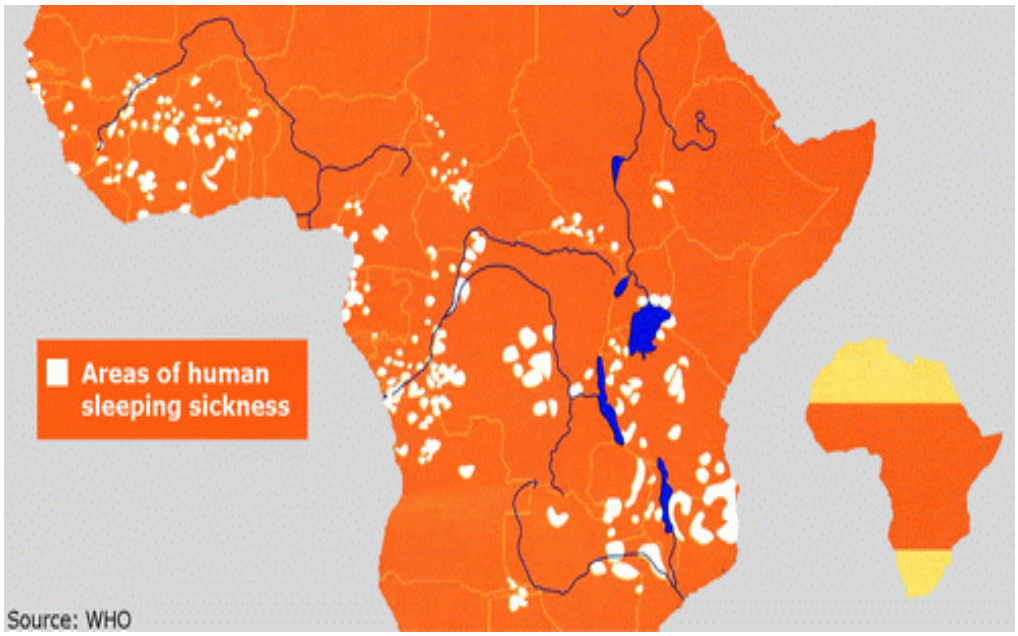

Figure 1: Distributions of sleeping sickness or Human African Trypanosomosis (Kuzoe 1993;WHO 1998a)

By the late 1950s, the incidence of sleeping sickness had been greatly reduced by mass campaigns including active case detection, treatment, chemoprophylaxis and vector control. However, sleeping sickness has reemerged as a major threat to health, with waves of new outbreaks and epidemics. Resurgence of the disease has been noted from large portions of Africa, with epidemic levels being reported in Democratic Republic of Congo (DRC), Angola, Sudan and Uganda (WHO 1998b). It is estimated that HAT threatens about 60 million people in 36 countries in sub-Saharan Africa. The prevalence of the disease is estimated at 300,000 to 500,000 with an annual mortality of approximately 50,000 (WH0 2004).

There are many causes of this resurgence but in particular, the spread of warfare in many endemic countries led to a breakdown of control programs. Other factors leading to new outbreaks are the emergence of more virulent parasite strains, changes in climate and vegetation, and movement of animals that are reservoirs of the disease.

Situation of HAT in Sudan

Sudan is a vast country (2.5 million km^2) with considerable variation in climate. Epidemics of sleeping sickness have been occurring in the South Sudan since the early 20^{th} century. The epidemics have mainly been reported in the southern and south western parts of the country bordering Uganda, DRC and Central African Republic (CAR). The foci include Raga, Yei, Kajokeji, Nimule, Tambura, and Yambio (Duku 1981).

In these foci, control has largely depended on international organizations and non governmental organizations (NGO), but the civil strife in particular (since 1959) disrupted the control programs. For instance, in the 1980s the Belgian-Sudanese control programme limited the incidence of HAT in the Sudan province of western Equatoria. When the fightings intensified in the 1990 this control programme collapsed. This resulted in absence of HAT control for more than a decade and led to resurgence of the disease with several foci reporting a prevalence exceeding 5%. Accurate data on the extent of the disease are limited. It is estimated that 10 million people are at risk of infection and thousands of persons are infected annually (Moore & Richer 2001).

As indicated earlier there are many active foci of HAT in south Sudan and control programs are mainly run by various NGOs: Malteser Germany in Yei county (ISCTRC 2005); International Medical Corps (IMC) in Tambura county, Médecins Sans Frontières (MSF) in Maridi, Mundri and Kajokeji Counties. There are only a few reports, restricted to a few of these foci and thus the actual situation of HAT in south Sudan is therefore not very clear.

In Tambura County a survey carried out between 1996 and 1997 indicated that the prevalence had doubled and the villages affected had increased from 54% to 100% since 1988. Another survey in 1997 estimated 5,000 HAT cases and a prevalence of 23%. High prevalence foci included Ezo (37%) in the southern part of Tambura county and Source Yubu (21.5%) (Moore *et al.* 1999). A recent study has shown that Maridi county has the highest prevalence (29%) in western Equatorial (Moore & Richer 2001). The prevalence of HAT is as shown in Figure 2.

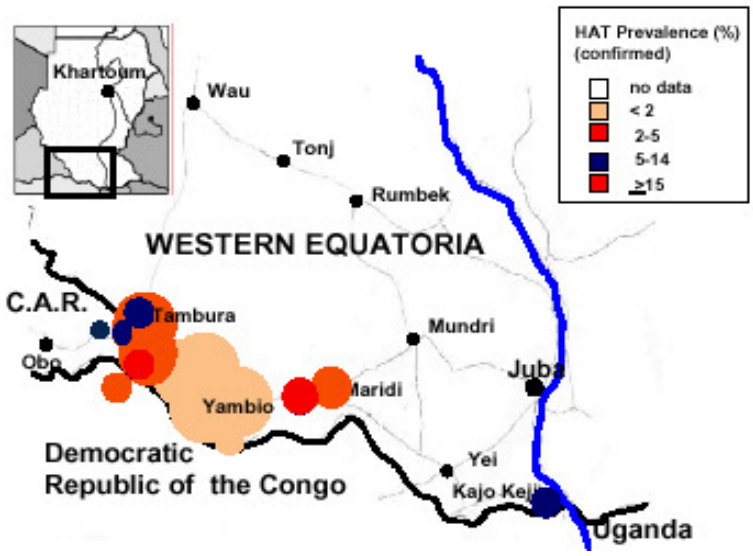

Figure 2: Prevalence of sleeping sickness in Southern Sudan, 1998 (Adapted from Moore & Richer (2001)).

The parasite

Taxonomy

Sleeping sickness was recognized in Africa for centuries but its cause was not discovered until about 1900. Sir Bruce identified the causative agent as a protozoan trypanosome that

was transmitted by the tsetse fly (Soltys (1953). The trypanosome causes disease in both man and his domestic animals.

Trypanosomes are classified under the sub-kingdom of protozoa, phylum Sarcomastigophora, order Kinetoplastida, family Trypanosomatidae and genus Trypanosoma. This genus has two groups, Stercoraria and Salivaria (Hoare 1970). Stercoraria contain genera in which the trypanosome completes its development in the hindgut and transmission is by fecal contamination. The species in Stercoraria include *T. cruzi* that causes Chagas disease in South America.

The salivarian group completes development in the salivary glands, and transmission is by inoculation of metacyclics with the saliva. The main genera in this group are: *Duttonella* (species: *Trypanosoma vivax*, and *T. uniforme*; *Nannomonas* (species: *T. congolense* and *T. simiae)*; *Pycnomonas* (species: *T. suis*) and *Trypanozoon (*species: *T. brucei brucei*, *T. b. rhodesiense*, *T. b. gambiense*, *T. evansi* and *T. equiperdum) (*Hoare 1970*). Duttonella, Nannomonas, Pycnomonas* and some species in *Trypanozoon (T. brucei brucei, T. evansi* and *T. equiperdum*) cause disease in animals.

The disease in man is caused by *T. b. rhodesiense and T. b. gambiense.* Trypanosomes that cause disease in animals are not infective to humans due to their sensitivity to human serum that hinders their survival in man. However, in individuals lacking the lytic factor, trypanosome infection is possible. Indeed a case of human trypanosomosis caused by *T. evansi* has recently been reported in India (Joshi *et al.* 2005).

Distribution of *T. b. gambiense* and *T. b. rhodesiense*

Trypanosoma b. rhodesiense is restricted to East Africa while *T. b. gambiense* is found in central and West Africa. The boundary between the distributions of the two parasites follows the Great Rift Valley. The separation is thought to have resulted from evolution of hominids (Welburn *et al.* 2001). Uganda is the only country where both diseases exist and recent reports indicate that there could be an overlap between *T. b. gambiense* and *T. b. rhodesiense* (Enyaru *et al*. 1999).

The two parasites are morphologically indistinguishable. Currently there are two tests based on molecular markers that distinguish *T. b. rhodesiense* from *T. b. gambiense:* The Serum Resistant Associated gene (*SRA*) is only found in *T. b. rhodesiense* and absent in *T. b. gambiense* (DeGreef *et al.* 1989); whereas *T. b. gambiense* specific glycoprotein gene (*TgsGP*), is present in *T. b. gambiense* but absent in *T. b. rhodesiense (Berberof et al. 2001).* In the event of suspected overlap, these test(s) can be used to distinguish the infecting parasite.

The trypanosome life cycle

Tsetse flies are the only vector of *Trypanosoma brucei.* There are about 20 species of tsetse flies but only a few transmit the disease. The flies have an average life span of between one to six months and live in warm, shady and humid areas. The parasites are ingested from an infected mammalian host by the blood-sucking insect vector. Once infected, the tsetse flies remain infective for life and a small number of infected tsetse flies can maintain endemic transmission cycles at relatively high levels.

During feeding, the tsetse fly takes up trypanosomes from the host. In the ectoperitrophic space between midgut epithelium and the peritrophic membrane, the stumpy trypomastigotes transform to procyclics. After rapid proliferation (10-12 days) the procyclics move to the proventriculus and subsequently migrate as epimastigotes via the hypopharynx to the salivary glands. The attached epimastigotes further differentiate and emerge as mammal-infective metacyclic trypanosomes after 13-15 days. The developmental life cycle is shown in Figure 3.

The infected fly will infect a mammalian host during feeding. Briefly, the biting tsetse fly deposits metacylic trypanosomes in the dermal tissues of the host. The metacylics rapidly transform into long slender bloodstream forms (BSF), which then multiply by binary fission and subsequently invade the lymphatic, blood system and later also the central nervous system. Besides these long slender forms, morphologically different, non-proliferating stumpy forms are observed at peak and declining parasitaemia in the blood of the host. It is these stumpy forms that are able to continue the life-cycle in the insect vector.

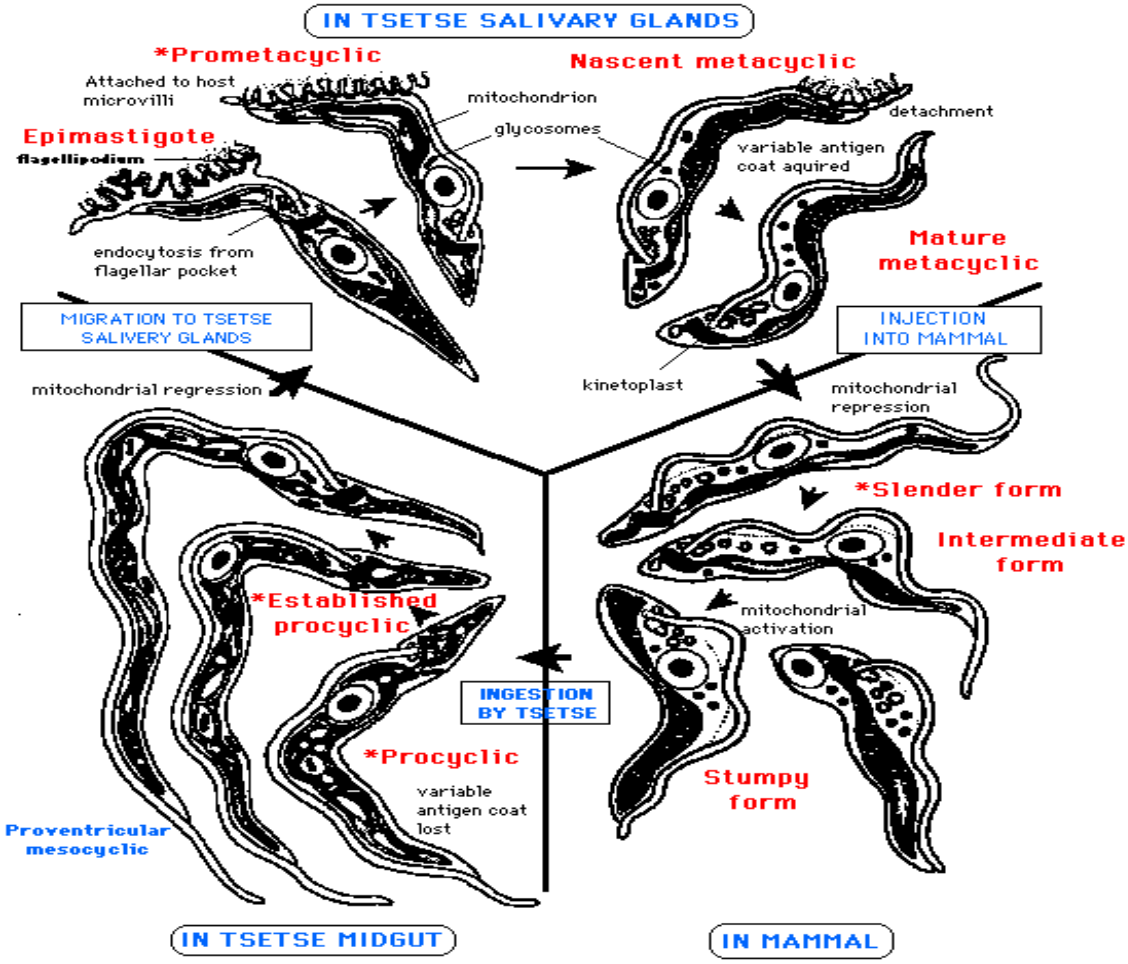

Figure 3*:* *Trypanosoma brucei* schematic representation of developmental cycle in a mammal and in the tsetse fly vector (Vickerman 1985)

Only a small proportion of tsetse flies which have taken up trypanosomes develop a mature infection and this takes about 3-4 weeks. It has been found that only teneral flies can develop a mature infection. The reasons are not very clear but the current suggestions is that the lack of lectin and poorly developed peritrophic membrane make the teneral flies susceptible to trypanosome infection (Lehane & Msangi 1991).

The disease

A few days after a tsetse bite, a painless nodular skin lesion (chancre) develops and lasts for about two weeks. At this initial stage, a satellite lymphadenopathy and fever with generalized malaise may be the only signs noted. As the trypanosomes invade the lymph, blood, bone marrow and tissue fluids, the patient experiences transient local oedema, sensational hyperaesthesia (Kerandels sign), intermittent fever accompanied by headaches, pains in the joints, splenomegaly and lymphadenopathy (Apted 1970). The disease progresses to the meningo-encephalitic stage (second stage), during which the parasites invade the cerebrospinal fluid (CSF) and brain tissues. This progressively leads to a variety of clinical manifestations, including headache, irritability, tremors, ataxia, convulsions, personality changes, daytime somnolence, pronounced wasting and coma (Dumas & Boa 1988).

Both infections by *T. b. rhodesiense* and *T. b. gambiense* follow this sequence of infection stages, but with marked differences in rate of progression. Generally *T. b. rhodesiense* is regarded as an acute infection, with progression to 2$^{nd.}$stage occurring in a matter of weeks. It leads to death (>80%) within six months of the onset of illness (Odiit *et al.* 1997). However, *T. b. gambiense* generally causes a chronic disease in which progression to 2$^{nd.}$stage may take several months or longer, and the 2nd stage may take several years (Barrett *et al.* 2003). Nevertheless there are reports of wide variation in disease severity in patients (Sternberg 2004). Generally, however, the Rhodesian disease is viewed as a compressed form because most symptoms as well as the neuropathological changes are the same in both forms (Kristensson *et al.* 2002)

The disease caused by *T. b. gambiense* is more widespread than *T. b. rhodesiense* (95% verses 5%). In addition, the HAT resurgences recently reported are mainly due to *T. b. gambiense*. Sleeping sickness due to *T. b. gambiense* is a major public health problem in DRC, Angola, Uganda and south Sudan (WHO 1998b).

Diagnosis of Gambian sleeping sickness

Humans constitute the epidemiologically important reservoir of *T. b. gambiense* and the cornerstone of control is dependent on case detection through population screening, followed by treatment of those diagnosed positive.

Many tests have been developed for diagnosis of HAT, including: latex agglutation test (Buscher *et al.* 1999); ELISA tests (Buscher *et al.* 1999;Nantulya 1988;Nantulya *et al.* 1992); immune trypanolysis test (Van Miervenne *et al*., 1995); polymerase chain reactions (Kanmogne *et al.* 1996;Kyambadde *et al.* 2000;Penchenier *et al.* 2000). Nevertheless, some of these tests are not in use, either because the test is still under evaluation or requires specialized facilities that are unavailable in laboratories in endemic countries. In the endemic countries, primary diagnosis is done serologically by use of the card agglutination test for trypanosomosis (CATT) (Magnus *et al.* 1978), and confirmation done by parasitological test. This is then followed by determination of the stage of disease.

Serological and parasitological diagnosis

A field CATT kit is available and the test is rapid and easy to perform, allowing screening of several hundred people in a day. This test has high sensitivity (Moore & Richer 2001) but false negatives have been reported in north western Uganda (Moore & Richer 2001) and in southern Sudan (MSF 2001). The test was developed from LiTat 1.3 antigen (Magnus *et al.* 1978) and false negatives may indicate the presence of another variant of trypanosomes in this region. Nevertheless, CATT reduces the workload of further tests to be done and considerably increases the detection rate. Patients that are CATT-positive undergo further parasitological test: microscopic examination of both the venous blood (using the haematocrit method (Woo 1970) and lymph node aspirate.

Stage determination

The stage of the disease is determined in accordance with the recommendations by WHO. Cerebrospinal fluid (CSF) is collected through a lumbar puncture and examined for the presence of trypanosomes, the number of white cells, and total protein concentration. If at least one of these parameters is abnormal the patient is considered to be at the 2nd stage disease. Detection of trypanosomes in CSF allows immediate classification of a patient in 2$^{nd.}$stage disease. In non-specialized laboratories trypanosomes are generally detected by direct examination of CSF, but sensitivity for detection can be increased by centrifugation of the CSF sample (Cattand *et al.* 1988;Woo 1970)

The CSF white cell count (WCC) is the most widely used parameter for stage determination. Patients with less than 5 cells/µl are classified as 1st stage while those with more than 5 cells/µl are classified as 2nd stage (WHO 1998). Although this criterion of staging is still common in most hospitals, studies (Doua *et al.* 1996;Miezan *et al.* 1994) have shown that the 5 cells criterion is insensitive and a number of patients in 1st stage of the disease will still be having up to 20 cells/µl in the CSF. Various methods for quantifying protein in the CSF are available including precipitation and colorimetric methods. The cut-offs are dependent on the particular test method.

However, diagnosis of HAT by these methods does not always give the true status of the infection and there is a continuous search for alternative diagnostic procedures. Ideally, the tests should be cheap, less invasive and easy to perform under field conditions.

Chemotherapy of Gambian sleeping sickness

Control of *T. b. gambiense* depends mainly on chemotherapy. Treatment of diagnosed patients relies on a small number of drugs: pentamidine (pentamidine isethionate BP, Pentacarinat®, Aventis, France), melarsoprol (Mel B, Arsobal®, Aventis, France), eflornithine (Ornidyl, Aventis, France) and nifurtimox (Lampit®, Bayer, Germany). The choice of drug for treatment further depends on the stage of the disease of the patient.

Pentamidine

The treatment of first stage *T. b. gambiense* disease relies mainly on pentamidine, a diamidine that was introduced in 1941. It is not clearly understood how pentamidine affects the parasites. Some of its effects include inhibition of trypanosomal S-adenosyl-L-methionine decarboxylase leading to reduced polyamine synthesis, competition with polyamines for binding to nucleic acids, particularly kinetoplast DNA (Berger *et al.* 1993;Williamson *et al.* 1975).

The recommended dosage is 4 mg/kg bwt administered daily for seven days by deep intramuscular injection. The side effects of pentamidine are considered minor and include; sterile abscesses or necroses at the injection site, nausea, vomiting, hypotension,

hypoglycemia, a feeling of faintness and tachycardia (Pepin & Milord 1994). The cure rate ranges from 93 to 98% and has not changed significantly over decades.

Suramin

Suramin was introduced in 1922. It is a sulphonated naphtylamine that is negatively charged at physiological pH. Suramin binds to plasma proteins and the plasma-protein-bound complex is taken up by trypanosomes by pinocytosis. It inhibits many dehydrogenases and kinases (Hawking 1978), thymidine kinase (Chello & Jaffe 1972) and dehydrofolate reductase (Jaffe *et al.* 1972). Suramin also inhibits the glycolytic enzymes (Willson *et al.* 1993), but only indirectly because the phospholipids bilayer prevents its uptake into the glycosome. Suramin is effective in the treatment against 1st stage infections of *T. b. gambiense* and *T. b. rhodesiense* but it is exclusively used in the Rhodesian disease. Usually, 5 injections of 2mg/kg are administered at intervals of 5-7 days. Cure rates of 100% have been reported (Apted 1980) but treatment failures have also been recorded (Wellde *et al.* 1989b).

Side effects, such as fever, photophobia, vomiting, flatulence, constipation and hyperaethesia have been observed. In rare cases fatal side effects including kidney damage and changes in blood picture may occur. Lethal idiosyncratic reactions (anaphylactic shock) have also been reported, especially when there is concomitant onchocerciasis (Van Nieuwenhove 2000).

At the first stage of the disease, treatment is cheaper and leads to minor adverse effects. Most patients are, however, presented for treatment when the CNS is already involved. At the second stage of the disease, the drugs used are melarsoprol, eflornithine or nifurtimox.

Eflornithine

Eflornithine was initially developed for cancer chemotherapy and in 1990, intravenous eflornithine was approved for treatment of the meningoencephalic stage of sleeping sickness. It is a selective irreversible inhibitor of ornithine decarboxylase, blocking polyamine biosynthesis. Polyamines are essential for growth and multiplication of the trypanosomes. As such eflornithine is cytostatic rather than cytotoxic and a competent immune system is essential for parasite clearance (Bacchi *et al.* 1980).

The current recommended dosage is 100 mg/kg body weight given intravenously every 6 hours for 14 days. The frequent side-effects observed are leucopenia, anaemia and diarrhoea

(Pepin & Milord 1994). In addition, abdominal pain, vomiting, dizziness, convulsions and thrombocytopenia are reported.
The major drawback of the intravenous eflornithine is related to its high cost of treatment and the inconveniences of administration, especially in understaffed rural hospitals of the disease-endemic countries. As such the current challenges are to evaluate the oral administration of DFMO (Na-Bangchang *et al.* 2004).

Nifurtimox

Nifurtimox was introduced in the 1960's for treatment of South American trypanosomosis (Chagas disease). The mode of action is not completely elucidated. It is suggested that free radicals and further superoxide and hydrogen peroxide are formed by cyclical reduction and oxidation of the nitro group of nifurtimox. Those free radicals bind to cellular components such as DNA, membrane lipids and proteins causing death of the parasite.

Nifurtimox is not registered for HAT but is used on compassionate basis. The treatment regimens used are very variable but the most widely used schedule is 15 mg/kg body weight/day in three oral doses for two weeks in adults and 20 mg/kg body weight/day in children. Nifurtimox has shown promise in the treatment of 2$^{nd.}$stage of *T. b. gambiense* infections in refractory patients alone and in combination with melarsoprol or eflornithine (Pepin *et al.* 1992b).

Common side effects of nifurtimox are anorexia, jaundice, nausea, vomiting, rash and fever. In addition, several cases of a reversible cerebellar syndrome incuding seizures, coma, and confusion were observed (Pepin *et al.* 1992b)

Melarsoprol

Melarsoprol is the first-line drug for treatment of second stage disease (Van Nieuwenhove 2000). It is a combination of the trivalent organic arsenical melarsen oxide (Mel OX) with the heavy metal chelator BAL (British Anti-Lewisite, dimercaprol). Melarsoprol's metabolite-Mel OX is the active drug (Keiser *et al.* 2000). Mel OX inhibits the polyamine synthetic pathway in the trypanosome and its primary target is trypanothione (Fairlamb *et al.* 1989;Keiser *et al.* 2000). Trypanothione is the major thiol-containing molecule in

trypanosomes and is essential for maintaining an intracellular reduced environment by detoxifying oxygen radicals. As such, the binding of Mel OX to trypanothione leads to oxidative stress in the trypanosomes.

The World Health Organisation (WHO) recommends four different schedules to be used, depending on the parasite and region (WHO, 1986). Further, differences in duration and mode of therapy have been observed depending on the country, local habits and the hospital involved. An alternative schedule was first proposed in 1995 (Burri *et al.* 1995) and was recommended in 2003 by the International Scientific Council of Trypanosomiasis Research and Control (ISCTRC) as the schedule of choice in *T. b. gambiense* endemic countries. In this schedule the patient receives 2.2 mg/kg bwt for ten consecutive days, intravenously (Burri *et al.* 2000;Schmid *et al.* 2004;Schmid *et al.* 2005). A shorter course of treatment is an important advancement since it is less burdensome for the patient, families, and health workers and is more cost-effective.

The most common adverse effects of melarsoprol treatment are fever, diarrhoea, pain in chest, necrosis at the site of injection due to leakage during administration. The worst is severe post-treatment reactive encephalopathy (PTRE) in about 5-10% with a fatal outcome of 1-5% of treated patients (Pepin & Milord 1994). Corticoids, in dosage of 1 mg/kg per day, have been used for prevention of fatal complications during treatment with melarsoprol on the assumption that the myocarditis and encephalopathy of sleeping sickness are largely of immunopathological origin. However, conclusive evidence of their value has not been demonstrated. In view of the high costs and doubtful evidence, routine use is not recommended

Melarsoprol treatment failures

Treatment with melarsoprol results in a normal failure rate of 3 - 9% attributed to patient-related factors (concomitant infections, nutritional status, etc). In the recent years higher treatment failure rates (> 20%) have been reported in the endemic areas of *T. b. gambiense* including, Uganda (Legros *et al.* 1999), Mbanza Kongo in northern Angola (Ruppol *et al.*, 1977) and southern Sudan (Legros *et al.* 1999;Moore & Richer 2001;Nieuwenhove *et al.* 1985). As a result of these high treatment failure rates, many SS treatment centers have

shifted from using melarsoprol to eflornithine as the first line treatment. The causes of these treatment failures are not yet clear.

Treatment failures may be due to various factors including; the patient's immune responses, individual (patients) variation in drug pharmacokinetics, or melarsoprol resistance in the infecting parasites.

Patient's immune status

Some of the drugs require a competent immune system in order to be fully effective. Diseases or conditions that lead to immunosuppression such as human immunodifiency virus (HIV) could therefore lead to low treatment success. Indeed, Milord *et al.* (1992) found that HAT patients co-infected with HIV responded less to eflornithine treatment than seronegative ones. The role of HIV in melarsoprol treatment failures is not established. Earlier studies have shown that HIV has no impact on epidemiology of HAT (Louis *et al.* 1991;Meda *et al.* 1997;Pepin *et al.* 1992a) because HIV was then relatively low in rural areas where HAT is endemic. Today, the situation has changed and the prevalence of HIV is high in rural areas and its role needs to be re-assessed. Other concomitant infections that cause immunosuppression such as malaria might also affect the prognosis of the disease. There are reports of co-infections of sleeping sickness and malaria (Schmid *et al.* 2004;Wellde *et al.* 1989c;Wellde *et al.* 1989a;Schmid *et al.* 2004) and sleeping sickness and Loasis (Enyaru 2002).

Pharmacokinetics of melarsoprol

Melarsoprol was introduced for the treatment of HAT before its pharmacokinetic profile was fully understood. It is only recently that its pharmacokinetics was established. Melarsoprol has an average half-life of 42 hours and average total clearance of 1 ml/min kg (Burri et al., 2001). Its concentration varies with time and from patient to patient. Immediately after treatment, the mean plasma levels are approximately 6 µg/ml (Burri & Keiser 2001) but drop rapidly to 1.3 µg/ml 24 hours later. However 120 hours later the levels drop even further to 220 ng/ml (Burri *et al.* 1993). The concentrations in the CSF are much lower, a maximum of 260 ng/ml, dropping to 64 ng/ml 24 hours after the last injection. In some patients the levels

were undetectable. The variation of the concentration with time in the CSF has not been done but it is postulated that the general trend would be a decrease with time.

To eliminate all trypanosomes, melarsoprol must be available in sufficient concentration in all body fluids and tissues. The sensitivities of trypanosome isolates also vary and sensitive strains appear to have the minimum inhibitory concentration (MIC) of around 1-30 ng/ml. Concentration of melarsoprol in the CSF is much lower (only 1-2%) than in plasma and CSF concentration is therefore critical in parasite clearance. Indeed it is believed that relapses result from parasites surviving in the CNS. As stated earlier the concentration of melarsoprol varies between patients. However, there was no difference in the concentrations in the CSF between relapse patients and cured patients (Brun *et al.* 2001;Burri & Keiser 2001). This may indicate that high treatment failure rate reported might not be due to low levels of melarsoprol in the CSF of relapse patients.

A lot of emphasis has been put on concentrations of melarsoprol in body fluids and rather less attention has been paid to its concentration in the tissues. Unfortunately, deep tissues are less accessible to the drugs and thus parasites localized here would be exposed to low concentrations. Indeed, *T. b. gambiense* is tissue-invasive and therefore has high affinity for deep tissue. This means that even a sensitive trypanosome isolate localized in the deep tissues may not be cleared. Very few studies have addressed this possibility and therefore there is need for more investigation.

Drug resistance in trypanosome populations

Melarsoprol is lipophilic and can diffuse across the trypanosome plasma membrane. In plasma however, melarsoprol is rapidly cleared (96% within 1 hour and 100% in 24 hours) to form active metabolites, including melarsen oxide. Melarsen oxide (Mel OX), a metabolite of melarsoprol, is the active principle (Keiser *et al.* 2000). Mel OX is hydrophilic and requires active uptake into the trypanosome. Studies by Carter and Fairlamb (1993) showed that the P2 nucleoside transporter mediates the uptake of Mel OX into the trypanosome Physiologically, the trypanosomatids use this transporter to scavenge adenosine from their hosts. These nucleoside transporters also mediate the uptake of diamidines (Barrett *et al.* 1995) and partly pentamidine (de Koning & Jarvis 2001). The common structural feature for

recognition is the amidine moieties in the melamine and benzamidine moieties in these compounds (Fairlamb *et al.* 1992) (Figure 4).

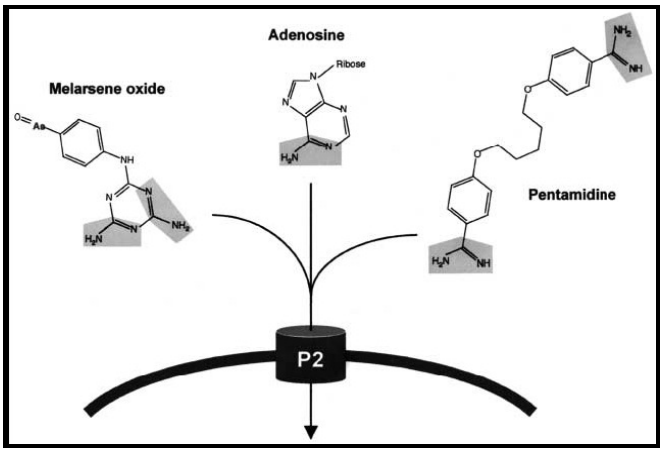

Figure 4: The structures of some of the molecules taken up via the P2 nucleoside transporter (Matovu *et al.* 2001c).

The P2 transporter gene *TbATI* has been expressed in the yeast *Saccharomyces cerevisiae* and was shown to promote the uptake of melaminophenyl arsenicals (Maser *et al.* 1999). Further, melarsprol resistance has been induced in a laboratory trypanosome strain and when *TbATI* was sequenced a ten nucleotide difference between wild-type *T. b. brucei* and its drug-resistant derivative was noted. Six of these mutations manifest at amino acid level. An easier way of identifying this set of mutation was developed by Maser *et al.* (1999) and two point mutations within the central fragment could be used to distinguish sensitive and resistant trypanosome populations. This restriction pattern has been detected in some 38/65 melarsoprol relapse *T. b. gambiense* isolates from N.W. Uganda (Matovu *et al.* 2001b) and is thought to be a possible marker for melarsoprol resistant trypanosomes.

These earlier studies focused on genetic markers of melarsoprol resistance and rather less attention has been paid to drug susceptibility testing. Only a few recently isolated populations have been tested (Brun *et al.* 2001;Matovu *et al.* 2001a), mainly because of the challenges in isolation and propagation of *T. b .gambiense* from patients.

Isolation of *T. b. gambiense* from patients

The isolation of *T. b. gambiense* from the patients with reportedly high relapse rate is a priority. As indicated earlier, high treatment failure rates have been reported in war-torn areas

(north western Uganda, Mbanza Kongo in northern Angola and southern Sudan), making isolation of the parasite logistically a difficult task. As indicacted by Brun *et al*., (2001) there are a number of approaches for isolating trypanosomes from an infected host (Figure 5).

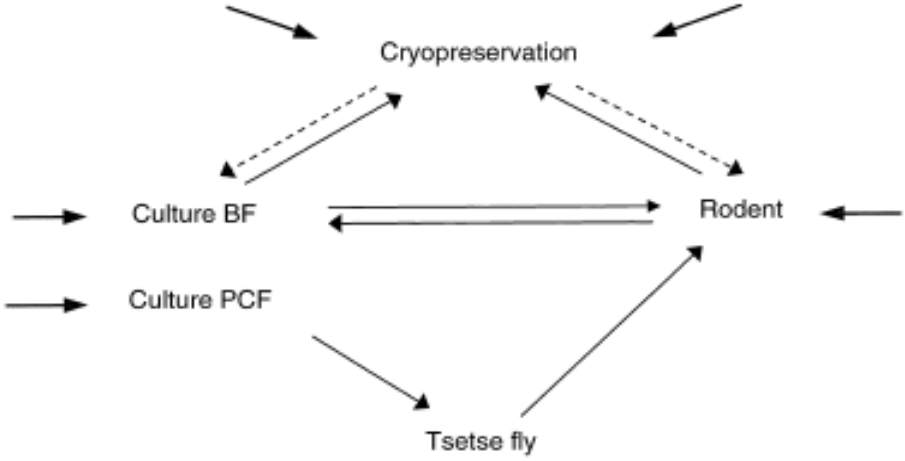

Figure 5: Possibilities to isolate and propagate *T. b. gambiense* from patient' blood or cerebrospinal fluid. BF: bloodstream form; PCF: procyclic forms; Solid arrow: established steps; dashed arrows: hardly achievable steps.(After Brun et al.(2001)).

Cryopreservation in the field and subsequent propagation of isolates in a susceptible rodent might be the easiest way to isolate bloodstream forms. To begin with, the protocols for cryopreservation and propagation need to be improved. Using this approach, reduced recovery or loss of *T. b. gambiense* has been reported (Brun *et al.* 2001;Matovu *et al.* 2001a). This highlights the need to improve the current cryopreservation protocols.

Rodent models for propagation

The low parasitaemia in patients combined with the limited susceptibility of rodents (Aerts *et al.* 1992;Matovu *et al.* 2001a) complicates further propagation of the isolated parasites. The multimammate rat *Mastomys natalensis* was found to be a better model for *T. b. gambiense* than rats or mice (Mehlitz 1978) and has been extensively used for propagation studies (Gibson *et al.* 1978;Matovu *et al.* 2001a;Mehlitz 1979;Zillmann & Mehlitz 1979). Less attention has been paid to propagating the parasites to high parasitaemia that are important for susceptibility testing (*in vitro* or *in vivo* drug assays). The parasitaemia could be improved by immunosuppression using irradiation or use of a chemical immunosuppressant. Irradiated

mice have been used for isolation, propagation and cloning of trypanosomes, but irradiation facilities are not always available. In addition, the slow growth rate of *T. b. gambiense* means that it is essential for immunosuppression to be repeated. The dosages as well as the number of doses that do not harm the rodents need to be assessed.

Drug susceptibility testing

Successful isolation of *T. b. gambiense* strains will allow both susceptibility and genotypic characterization. Various *in vitro* tests for determining the drug sensitivity of trypanosomes to trypanocides have been developed (Kaminsky & Brun 1993). None of the assays is superior to others in all respects. The assay applied depends on the specific aim of the study, on the trypanosome stocks, and on the equipment available. The (H^3) hypoxanthine incorporation assay does not require culture-adapted trypanosomes and has been shown to be suitable for *T. b. gambiense* studies (Brun *et al.* 1989;Brun & Kunz 1989). The test is able to distinguish sensitive from resistant isolates (Brun *et al.* 2001). *In vivo* drug tests are currently hindered by lack of a simple rodent model for *T. b. gambiense.*

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CHAPTER 2: Goals and objectives

Goal

The goal of the studies presented in this thesis was to determine the role of melarsoprol resistant trypanosomes in the high treatment failures rates in HAT patients in south Sudan.

Objectives

The project had the following objectives:

1. To improve the protocol for cryopreservation of bloodstream forms *T. b. gambiense* from HAT patients.
2. To isolate and establish a cyrobank of well characterized *T. b. gambiense* from south Sudan. The isolates would in future be available to World Health Organization (WHO) and other scientists.
3. To improve the propagation of *T. b. gambiense* field isolates in laboratory rodents.
4. To determine the sensitivities of the isolated trypanosomes to trypanocides melarsoprol, melarsen oxide, pentamidine, diminazene, DB 75 and nifurtimox.
5. To characterize the P2 nucleosides transporter gene (*TbATI)* in the isolated trypanosomes

CHAPTER 3: Cryopreservation of *Trypanosoma brucei gambiense* in a commercial cryomedium developed for bull semen

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Abstract

There have been major advances in the formulation of cryomedia for spermatozoa owing to their economic importance. In this study, the suitability of the commercial cryomedium Triladyl® developed for bull semen was evaluated for the cryopreservation of *Trypanosoma brucei gambiense*. Cryopreservation efficacy was determined by direct counting of motile trypanosomes and by viability assessment using *in vitro* and *in vivo* methods. Culture medium containing 10% glycerol was used as the control. Trypanosomes cryopreserved in Triladyl demonstrated a higher *in vitro* viability than those in culture medium with 10% glycerol. Similar results were obtained *in vivo* in immunosuppressed *Mastomys natalensis*. Trypanosomes cryopreserved in Triladyl showed better growth characteristics than those in culture medium with glycerol. It can be concluded that the use of Triladyl in the cryopreservation of *T. b. gambiense* leads to a better survival of the trypanosomes which could lead to an improved isolation of *T. b. gambiense* from sleeping sickness patients.

Key words: *Trypanosoma brucei gambiense;* cryopreservation; Triladyl; glycerol

Introduction

Characterisation of *Trypanosoma brucei gambiense* from human African trypanosomosis (HAT) patients, especially from those showing a relapse after treatment, is of high priority, e.g. to determine the drug sensitivity profile (Brun et al., 2001). The difficulty of obtaining isolated parasites, however, hinders such studies. Isolation of bloodstream forms has mainly been achieved by inoculating infected blood samples from patients into laboratory rodents. The low parasitaemia in patients and the limited susceptibility of rodents complicates the process (Dukes et al., 1989; Aerts et al., 1992; Matovu et al., 2001). The development of a kit for *in vitro* isolation (KIVI) of procyclic forms (Truc et al., 1992; Aerts et al., 1992) enhanced isoenzyme characterization and DNA characterization. The KIVI kit however, did not help to study drug sensitivity since the required assays depend on bloodstream forms.

Several possibilities for isolating *T. b. gambiense* from patients exist: isolation into *in vitro* culture, inoculation of susceptible animals, and cryopreservation of infected blood samples. Isolation of bloodstream forms into culture under field conditions is almost impossible because most cultures will be found to be contaminated by bacteria and fungi (Maser et al.,

2002). Inoculation into susceptible rodents is usually not feasible since the animals have to be transported to the patients far from a laboratory. Cryopreservation of infected blood samples in liquid nitrogen (-196^{o}C) in the field and subsequent propagation in susceptible rodents in the laboratory would provide an ideal way to isolate *T. b. gambiense* strains from patients.

The current methods for cryopreservation for trypanosomes were developed almost 50 years ago with minor improvements for field isolation by Dar et al. (1972). However, reduced recovery or loss of infectivity of *T. b. gambiense* following cryopreservation has been observed (Brun et al., 2001; Matovu et al., 2001; J. Enyaru. personal communication). Studies using media developed for cryopreservation of human spermatozoa indicated that these are superior to the standard media (J. Atouguia, personal communication). Cryomedia for human sperms are, however, very expensive. Triladyl, a medium developed for bull semen, is much more economical in use. In the present study we investigated the suitability of Triladyl for the cryopreservation of *T. b. gambiense* bloodstream forms.

Materials and methods

Media used for cryopreservation

Triladyl® is produced by MiniTüB GmbH & CO. KG (Tiefenbach, Germany). It contains 8% glycerol, 20% egg yolk, Tris buffer, citric acid, fructose and antibiotics (tylosin 5mg/l, gentamicin 25mg/l, spectinomycin 30mg/l, and lincomycin 15mg/l in distilled water). Triladyl was stored at 4^{o}C or frozen at -20°C. Control cells were frozen in culture medium (see below) containing 10% glycerol.

Trypanosome strains

Trypanosoma b. gambiense DAL 1402 was obtained from the cryobank of the "Project de Recherches Cliniques Sur la Trypanosomiase," in Daloa. It was isolated in1990 from a human patient in Cote d'Ivoire. *Trypanosoma b. gambiense* KETRI 2565 was obtained from the cryobank of the Trypanosomiasis Research Centre (TRC, formerly KETRI), Kenya. It was isolated in 1982 from a patient in Yambio, South Sudan.

Cultivation and cryopreservation

The trypanosome strain (*T. b. gambiense* DAL 1402) was cultivated in axenic culture in a mixture of RPMI 1640 and MEM with Earle's salts (1:1), supplemented with 5% heat-inactivated fetal bovine serum (FBS), 12% human serum from the local blood bank, 0.05mM bathocuproine sulphonate, 0.12mM mercaptoethanol, 1mM sodium pyruvate, 2mM L-glutamine, 1.5mM L-cysteine and 0.2mM hypoxanthine. Trypanosomes from axenic culture were centrifuged at 1200g for 10 minutes and re-suspended in fresh medium. The trypanosome concentration was determined by direct counting in a haemocytometer.

Tubes containing 900µl cryomedia were cooled in ice and 100 µl of trypanosome suspension were added. Various trypanosome concentrations ranging from 10 to 2 x 10^6 trypanosomes/ml were prepared. Aliquots (0.5ml) were transferred into cryo-ampoules (NUNC®, 1.8ml). The ampoules were cooled slowly at a rate of approximately -2^{o}C /minute in the vapour phase of a liquid nitrogen container and then immersed in liquid nitrogen at -196^{o}C. After at least five days of storage, the stabilates were thawed rapidly at 37^{o}C in a water bath to determine viability.

Upon thawing, the trypanosomes were centrifuged and re-suspended in culture medium. The recovery was assessed by counting the motile trypanosomes. Briefly, 10µl of thawed trypanosome suspension was transferred to a haemocytometer, motile trypanosomes counted, and the result expressed as a percentage of the original number of trypanosomes cryopreserved. When the initial concentration was less than 10^4 trypanosomes/ml, the trypanosomes could not be counted directly, but were seeded into wells of a 24-well culture plate and examined daily under a microscope. After four days of incubation, the concentration of trypanosomes was assessed by counting in a haemocytometer.

Infectivity of cryopreserved trypanosomes in immunosuppressed *M. n*atalensis

Mastomys natalensis from a breeding colony at the Trypanosomiasis Research Centre (TRC, formerly KETRI), Kenya, were immunosuppressed by administration of cyclophosphamide (200 mg/kg bw intraperitoneally (ip)) one day prior to infection. A donor *M. natalensis* was inoculated ip with 0.5ml of thawed *T. b. gambiense* KETRI 2565 suspension. The rodent was sacrificed 7 days post-infection when parasitaemia was 5-10 trypanosomes/field in a wet

smear of tail blood. Trypanosomes were separated from blood cells by differential centrifugation and the concentration of trypanosomes estimated in a haemocytometer.

Concentrations of 20, 200 and 2 x 10^4 trypanosomes/ml were prepared in control medium containing 10% glycerol or in Triladyl and cryopreserved as described above. After five days of storage, the stabilates were thawed and the contents of each ampoule inoculated ip into two immunosuppressed *M. natalensis*. A control group of four rodents for each inoculum dose received trypanosomes that were not cryopreserved. Parasitaemia was monitored from the third day on and every other day for 20 days by the haematocrit centrifugation technique and by examination of wet smears from tail blood (Herbert and Lumsden, 1976).

Optimum concentration of Triladyl for cryopreservation

Trypanosomes (2.7 x 10^5/ml) were cryopreserved at varying concentrations of Triladyl (20, 30, 40 50, 60, 70, 80 and 90% Triladyl in culture medium; see 2.3.). After five days in liquid nitrogen the stabilates were thawed and recovery assessed by counting the motile trypanosomes in a Neubauer haemocytometer. The percentage recovery of trypanosomes was calculated.

Results

Recovery and *in vitro* viability of trypanosomes after cryopreservation

Survival after cryopreservation was higher in Triladyl (73-50%) than in medium containing 10% glycerol (30%) (Figure 1). At low trypanosomes concentrations (<10^4) direct counting was not possible. Viability was assessed by culturing the thawed samples. The growth of trypanosomes frozen in medium containing 10% glycerol was lower than that of those frozen in Triladyl. In some instances, motile trypanosomes could be seen after cryopreservation but could not be propagated during subsequent *in vitro* cultivation. When 5 trypanosomes/ampoule (10 trypanosomes/ml) or fewer were cryopreserved, no motile trypanosomes could be observed either immediately after thawing or during the following 14 days of cultivation.

Infectivity of cryopreserved trypanosomes for *M. natalensis*

The inoculated number of trypanosomes influenced the parasitaemia pattern in immunosuppressed *M. natalensis* (prepatent period, duration to peak parasitaemia, and parasite density at peak parasitaemia). For example, a high inoculum led to a shorter prepatent period and a shorter duration to peak parasitaemia than a low inoculum. Differences in parasitaemia pattern were noted between the groups inoculated with cryopreserved and non-cryopreserved trypanosomes. At all concentrations tested, cryopreservation resulted in a pattern of parasitaemia corresponding to a smaller inoculum of viable cells: longer prepatent period, longer duration to peak parasitaemia and lower density at peak parasitaemia. This reduction in growth characteristics was more pronounced in samples frozen in the control medium than in those frozen in Triladyl (Table 1).

Optimum concentration of Triladyl for cryopreservation

Trypanosomes could be recovered after cryopreservation at all the concentrations of Triladyl tested. At low concentrations of Triladyl the percentage recovery was however less than 30%. At concentrations of Triladyl between 40 and 90%, recovery rates between 50 and 63% could be found. The differences in the percentage recovery between 40, 50, 60, 70, 80 and 90% Triladyl were not significant.

Discussion

The present study has demonstrated successful cryopreservation of trypanosomes in a medium used for cryopreservation of bull semen. A larger proportion of trypanosomes survived cryopreservation in Triladyl than in glycerol. In addition, cryopreservation in Triladyl resulted in higher *in vitro* viability and *in vivo* infectivity than those cryopreserved in 10% glycerol.

The survival rate of eukaryotic cells after cryopreservation depends on many factors such as the cryopreservation medium, volume of sample, freezing rate and thawing conditions (reviewed by Diamond 1995). Cryopreservation may also select for subpopulations of parasites with distinct characteristics in virulence or drug sensitivity. Therefore, a high survival rate is considered desirable for maintaining the population's heterogeneity and to

prevent a genetic drift. An ideal cryopreservation medium should hence recover a high proportion of the frozen trypanosomes. The results of this study indicate that Triladyl is better as compared to the control culture medium with 10% glycerol. Triladyl contains besides glycerol a second membrane protecting component namely egg yolk (20%). Egg yolk acts as a buffer but also coats the cells thereby protecting the lipoproteins in the cell membrane during freezing and thawing. The optimum concentration of Triladyl was found to be between 40% and 90%.

Apart from high survival rate, the subsequent viability of the preserved parasite is essential. It is important to remember that blood samples from some *T. b. gambiense* patients contain only 10-50 trypanosomes/ml of blood and in those cases, the high survival rate is crucial for the subsequent propagation. Viability can be determined by transferring the thawed trypanosomes to an *in vitro* culture system or injecting them to susceptible rodents. *In vitro* propagation of trypanosomes is frequently hindered by contamination (Maser et al., 2002) and advantages of cryomedia that contain antibiotics, such as Triladyl, have been shown in field isolation of trypanosomes (Dukes et al., 1989; Truc et al., 2004). Nevertheless, maintenance of bloodstream form requires a well equipped laboratory that may not be available in most field sites.

According to Brun et al. (2001), cryopreservation of infected blood samples and subsequent propagation in susceptible rodents is the easiest method to isolate *T. b. gambiense* from patients. In the current study we evaluated this procedure. Generally, *T. b. gambiense* cannot be propagated in normal mice and immunosuppressed *M. natalensis* were used. Our results indicate that parasites that were frozen in Triladyl developed more reproducibly and had better infectivity than those frozen in glycerol. Alternatively, severe combined immunodeficient (SCID) mice could be used for propagation. The SCID mice are highly susceptible to *T. b. gambiense* (Inoue et al., 1998).

This study showed that Triladyl is a better cryopreservation medium than standard culture medium with 10% glycerol resulting in a higher survival rate of the frozen trypanosomes. It remains to be demonstrated in the field that this new cryomedium leads to an improved isolation of *T. b. gambiense* from HAT patients in endemic areas.

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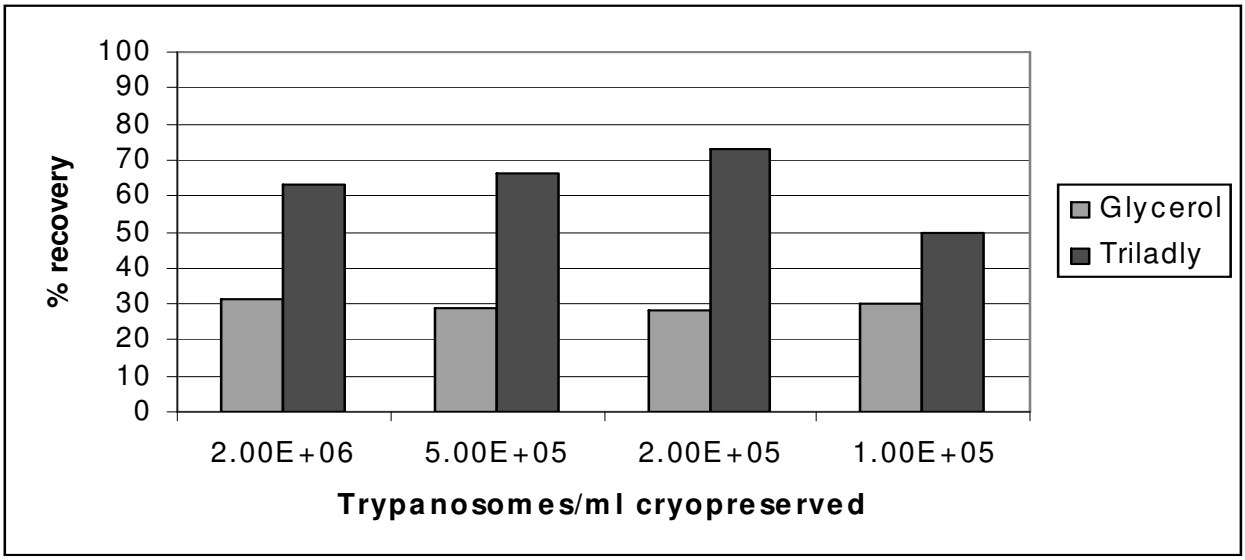

Figure 1: Survival rates of *T. b. gambiense* after cryopreservation in Triladyl and glycerol. Various concentrations of *T. b. gambiense* DAL 1402 were cryopreserved in Triladyl (90%) (■) and glycerol (10%) (□). After at least five days of storage, the samples were thawed and recovery assessed by counting the motile trypanosomes.

Number of trypanosomes frozen in 500µl	Cryomedium	Cryo-preservation	No of animals developing parasitaemia/ No injected	Pre-patent period, days	First peak parasitaemia, No. tryp/ml (day)
10		No	3/4	5	7.9×10^6(10)
	Triladyl	Yes	1/2	14	2.5×10^5(19)
	Glycerol	Yes	0/2	-.	-.
100		No	4/4	3	7.9×10^6 (9)
	Triladyl	Yes	1/2	7	2.5×10^6 (16)
	Glycerol	Yes	1/2	17	2.5×10^6 (19)
10000		No	4/4	3	3.1×10^7 (7)
	Triladyl	Yes	2/2	5	2.5×10^6 (7)
	Glycerol	Yes	1/2	7	2.0×10^6 (21)

Table 1: Infectivity of cryopreserved bloodstream forms of *T. b. gambiense* KETRI 2565 for *M. natalensis* frozen in different media. The trypanosomes were cryopreserved in 90% Triladyl or in control culture medium with 10% glycerol, and stored for five days in liquid nitrogen. Parasitaemia was monitored every other day for 20 days using the haematocrit centrifugation method and the method of Herbert and Lumsden (1976).

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CHAPTER 4: Sleeping sickness in Southern Sudan: A general outlook

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Working Paper

Introduction

Human African Trypanosomosis (HAT), also known as sleeping sickness, is caused by *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense*. The disease is endemic in 36 countries in sub-Saharan Africa. Re-emergence of epidemic sleeping sickness has been described by several authors (Cattand *et al.* 2001;Moore & Richer 2001;Van Nieuwenhove *et al.* 2001). *T. b. gambiense* epidemics have mainly been reported from the southern and south western parts of Sudan near the border of Uganda, the Democratic Republic of Congo (DRC) and the Central African Republic (CAR). Control of HAT in Sudan is largely dependent on programs initiated by international and Non-governmental organisations (NGO's). However, civil strife (since 1959), political and financial problems frequently disrupted the control program (Moore & Richer 2001). A recent resurgence of HAT within several foci has resulted in a prevalence exceeding 5%. Ibba in Maridi County in the province of Western Equatoria in southern Sudan was found to be the most active focus with a prevalence of 29% (Moore & Richer 2001). In 1997, Médecins Sans Frontièrs Holland (MSF-H) started a HAT control program in this county which was handed over to MSF-F (France) in 1999.

In the frame of a study with the 'Eastern Africa Network for Trypanosomosis' (EANETT) we visited the program of MSF-F in southern Sudan in March/April 2003. The aim was to isolate trypanosomes from HAT patients in order to determine genotypic and phenotypic characteristics. In this report, we discuss aspects affecting HAT control in southern Sudan, our experiences and problems encountered in the field.

Approval of study protocol was given by the World Health Organisation (WHO) and the Sudan People's Liberation Army (SPLA). Patients included in the study had to give a written or a thumb-printed consent by themselves or by their attendant(s). Information on the patient's history was obtained from the patients (or their attendants) or was extracted from hospital records. Questionnaire on age, sex, residence (sub-county, village), whether there were other relatives in hospital, concomitant diseases, and treatment regimes were collected. Direct observation on homesteads set up, vegetation and human activities were recorded as well.

The study site

MSF-France maintained a laboratory in the public hospital in Maridi and a hospital for second stage patients in Ibba. Since April 2002, a hospital for first stage patients was built in Kotobi, Mundri county. Most patients diagnosed were from Mundri, indicating that this county was more active than Maridi county (MSF-F personal communication; see Fig. 1) which might necessitate the expansion of the HAT hospital in Kotobi for the treatment of the second stage disease.

There were no tarmac roads and the earth roads had to be constructed by the local communities. The distance from Ibba to Kotobi for example is approximately 150 km, but the average time required by a four-wheel drive vehicle was about 5 hours (see Figure 2). Some villages in Mundri County were too far away and could not be accessed by MSF because of the lack of a proper road system.

The ecological characteristics of the two Counties appeared favourable for tsetse fly breeding and tsetse-human contact. The main vectors for *T. b. gambiense* are the riverine flies of the palpalis group. A tsetse control programme does not exist. The reasons may be found in financial, personnel and logistic limitations. The region around Ibba was mainly of equatorial climate with bush vegetation and rain forest, whereas the area visited in Kotobi presented rather a savannah-like climate, which was hotter and more humid than in Ibba. The homesteads of the residents were scattered and only partially cleared. The regional economy was mainly based on subsistence farming, where a significantly increased risk of infection was associated in earlier studies (Moore *et al.* 1999).

The inhabitants spent much of their time in the forests (farming, hunting, honey, and termite harvesting) and along the rivers (bathing, fetching water, fishing, washing). The type of human settlement might imply that communities were exposed to tsetse fly bites within the homesteads. Some of the patients lived in the same compound and were related. This area seems to be highly infested by tsetse flies and humans are exposed to tsetse flies everywhere. In Tambura county similar observations were made (Joja & Okoli 2001;Noireau *et al.* 1989) and exposure in the homesteads might explain the familial aggregation noted in this study.

Little is known about animal reservoirs of *T. b. gambiense* and their impact to epidemic foci (Herder *et al.* 2002) . It was demonstrated that domestic animals such as pigs, dogs, goats, and

chickens can act as reservoirs (Njiokou *et al.* 2004;Noireau *et al.* 1989). As an alternative source of blood, pigs in homesteads can even reduce the transmission of sleeping sickness to humans (Meda *et al.* 1993). Domestic animals at the visited sites included chicken and goats, and in Mundri cattle, were mainly owned by the nomadic tribes. Studies done to date show that animals might play an epidemiologically significant role as reservoir for *T. b. gambiense* trypanosomosis, but more studies need to be done (Herder *et al.* 2002;Noireau *et al.* 1989). A method to detect *T. b. gambiense* in various reservoir species with a high specificity is needed to conduct a large scale study on the distribution of *T. b. gambiense* in reservoir animals. CATT (card agglutination test for trypanosomosis) is not specific enough (Noireau *et al.* 1991) to detect *T. b. gambiense* from animal blood samples.

HAT screening in Western Equatorial

MSF-F aimed at reducing HAT through case detection and treatment, since humans are suggested to be the main reservoir of *T. b. gambiense*. The strategy to treat HAT patients as early as possible, lowers the disease incidence and human mortality (Moore & Richer 2001) . People were screened either passively in the hospital, or actively by a mobile team which moved from one village to another (See Figure 3). The screening was done in public or social places (such as markets or churches), reaching people during their daily activities. Since public transport is inexistent, people had to walk to the screening centres. However, not everyone reporting at screening sites agreed to be tested. In certain places, active screening was hampered by poor turn-up despite prior information and sensitisation campaigns to the communities. At one place, screening had to be terminated because of a minor fight among soldiers in the neighbouring village. Apart from these constraints, active screening requires a lot of resources (vehicles, personnel, equipment). This limitation determines the number of such campaigns that can be carried out in a given time period. Similar constraints might explain why most HAT patients treated at the NGO hospitals were identified via passive screening instead of active. It is inevitable that undiagnosed cases are still present within villages, presenting a danger for the healthy inhabitants.

In 2002, approximately 70% of the HAT patients treated in the Ibba hospital treated were in the 2nd stage of the disease (MSF-F, unpublished). This fact indicates that even after several years of active and passive screening by MSF-F, the number of second stage cases was still exceeding that of first stage cases. In other parts of southern Sudan, first stage cases made up

a total of 60% (Moore & Richer 2001). An effective control system should detect more first stage patients than second stage patients and this would make treatment safer, cheaper, and faster.

The diagnosis-scheme (recommended by WHO) included CATT-test (Magnus *et al.* 1978) and microscopic examination of lymph node aspirates and venous blood using the micro-haematocrit (mHCT) method. Lumbar puncture was carried out on all patients positive in any of the diagnostic tests to classify the stage of the disease. Staging was done by examining the cerebrospinal fluid (CSF) for trypanosomes (2^{nd} stage if positive) and by counting white blood cells (WBC) according to WHO classification (1^{st} stage $\leq$ 5cells/µl; 2^{nd} stage > 5cells/µl in CSF). Patients with second stage disease had WBC counts in CSF ranging from 5 to 1541 cells/µl.

As reported by Moore et al. (2001), the serological CATT diagnosis has a higher sensitivity than direct parasite detection methods. CATT is the primary method used for the diagnosis of both stages of *T. b. gambiense*. However, during this study it gave false negative results in two parasite-positive patients implying that patients with low, undetectable parasitemia might have been misdiagnosed. One of the two patients had been tested with CATT four times over 2 years and was found to be negative. At the time of this study, trypanosomes were detected only in the CSF and the patient was in coma, but still CATT-negative.

The CATT is using the LiTat 1.3 antigen (Magnus *et al.* 1978). The *T. b. gambiense* serotypes that do not express this antigen were thought to have a limited distribution. Similar false negative results with CATT diagnosis have been reported in north western Uganda (Enyaru *et al.* 1998;Matovu *et al.* 2003) and may be attributed to *T. b. gambiense* strains missing the LiTat 1.3 antigen gene.

Patients and treatment

Fifty-two HAT patients from Mundri (48), Maridi (2), Yambio (1), and Juba (1) were included in this study (Fig 1). The age of the patients ranged from 3 months to 62 years. Most patients (92%) were less than 40 years old. One patient was in first stage of the disease; all others in the second stage. Trypanosomes in CSF were detected in only 8 of the patients (Table 1). Melarsoprol has been the first-line drug for treatment of the 2^{nd} stage of *T. b. gambiense* infection since 1959. The high rates of treatment failures within a short period

(Moore & Richer 2001) and the high death rate following melarsoprol treatment (MSF, personal communication) led to a change of the treatment regime from melarsoprol to eflornithine (difluoromethylornithine) in 2001. This change resulted in a 3-fold increase in the number of patients seeking treatment and a decrease in drug related fatalities from 8% with melarsoprol to 1.6% with eflornithine (Chappuis *et al.* 2005). Figure 4 (c and d), shows the patients under treatment.

Treatment failures following eflornithine treatment were noted several times in the Ibba SS hospital, although eflornithine had been used for the last 2 years only. Seven patients (of the 52) relapsed after treatment: Three patients relapsed after pentamidine treatment (1st stage) and 4 after eflornithine treatment. Relapses after pentamidine treatment might have been due to misdiagnosed early 2nd stage disease. These patients were subsequently treated with eflornithine. The four eflornithine-relapse patients were re-treated with a combination of melarsoprol (1.2mg/kg/day iv for 10 days) and nifurtimox (15mg/kg/day divided into three doses for 10 days). Whether the treatment failures were due to drug resistant trypanosome is still under investigation (Brun *et al.* 2001). Treatment failures might be due to wrong drug application, individual differences in the pharmacokinetics of the drug, misclassification of the stage, interference with food (MSF-F personal communication), trypanosomes at body niches that are not accessible to the drug, or resistant trypanosome strains (WHO 1998).

The treated patients were not examined for trypanosomes immediately after completion of treatment. When they were checked 6 months to 2 years after treatment and classified as relapse patients, a re-infection could not be ruled out in a region of high endemicity. The WHO considers trypanosome-positive patients within 2 years after treatment as relapse cases.
Many HAT patients had other concurrent infections, such as malaria (21/52), filariasis (loa loa 23/52 and onchocerciasis 2/52), and gastro intestinal protozoa (*Giardia* and *Entamoemba histolytica* 2/52). Loa loa was left untreated, malaria was treated before, and the other infections were treated after completion of the HAT treatment regime.

High levels of treatment failures to common anti-malarials (such as chloroquine, sulfadoxine-primethamine, artemisinins) have also been noted. Therefore, Coartem® (Artemether and lumefantrine, Novartis, Switzerland) was the first line drug in the NGO hospitals. Malaria treatment started one day before the HAT therapy and was administered for 3 days. The patients having malaria and HAT were treated with Coartem® and eflornithine

simultaneously. Furthermore, little is known about the interaction between *Plasmodium falciparum* and *T. b. brucei,* and about the resulting immune responses.

The treatment failures noted with different drugs and in different diseases (malaria and HAT) might be due to host-related factors including individual variation in the drug pharmacokinetics and (the patients') immune responses. For an effective cure a competent immune system is necessary but malaria and HAT both lead to immunosuppression. Pepin et al.(1992) found that human immunodeficiency virus (HIV) co-infected HAT patients responded less to eflornithine treatment than seronegative individuals. Studies to establish the causes of treatment failure at patient's level as well as the drug sensitivity of infecting parasites should be a priority (Brun *et al.* 2001).

Lastly, a control of HAT by chemotherapy has been the major control strategy in southern Sudan since the disease was documented, but a combination with vector control might be the optimal strategy. The aim of this combined approach would be to lower the density of tsetse flies and the prevalence in the human population simultaneously, thus reducing the rate of transmission. This approach was successfully introduced in Tambura county (Joja & Okoli 2001).

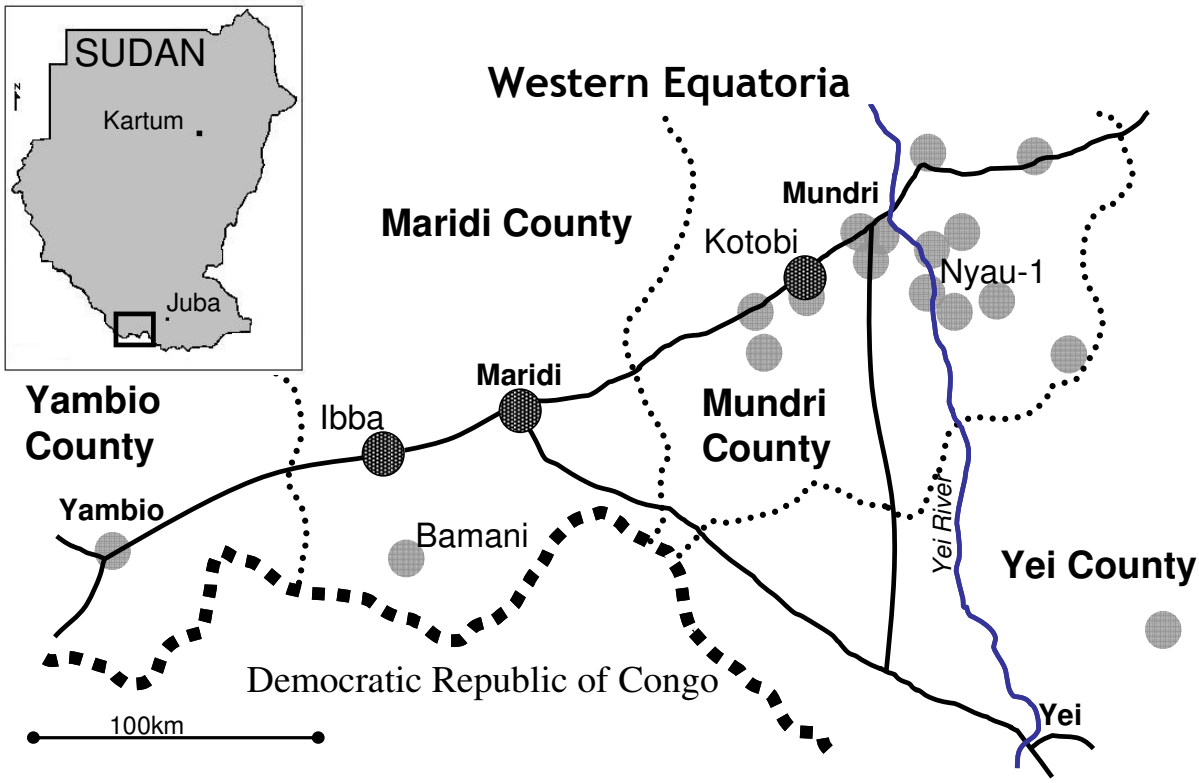

Fig 1: Map of Westen Equatoria; grey dots ( ) indicate the origin of the patients; MSF-hospitals/ laboratory are indicated by

Figure 2: The communication systems in south Sudan. There are two airstrips for the region in Yambio and Maridi county. The pictures shows; the airstrip at Maridi county after a heavy down pour (a) and a section of temporary roads used within the region (b)

Figure 3: Shows the active screening set-up (a & b) used by the mobile team. The set-up allowed all HAT diagnosis in the field. Also lumbar punctures were done in the field and not repeated in the hospitals. The laboratories in the hospitals (c) were adequately equipped for further diagnosis (malaria tests were done in the hospitals). The bomb shelters (d) were also available in the hospitals and staff quarters.

Figure 4: The two HAT treatment centres in Ibba (a) and Kotobi (b). On admission, each patient has to have one or two attendants (usually close relatives) to cook and take care. As such the number of people in the wards can be very high. The wards are general (the patients are not separated by age or sex) and a bed is allocated only to the patient. The wards are well organised and high hygiene measures maintained. (c) Shows a ward in Ibba centre with the second stage patients receiving eflorintihtine. (d) Shows a baby (less than 3 months) who was at second stage while the mother was at the first stage, a possible case of trans-placental transmission.

No.	Isolation no	S. C.[1]	Age[2]	Sex	Blood [3] tryps.	Blood [3] CATT	Lym[4]	CSF[5] tryps.	CSF[5] cells	treatment	other parasites
1	AAK0303047B	Mundri	11	male	neg.	n.d.	n.d.	neg	52	Nif./Mel	
2	**AAK0303048B**	Lozoh	20	male	+++	n.d	n.d.	neg	58	Nif./Mel	Loa Loa
3	AAK0303039B	Mundri	24	female	neg.	n.d.	Neg	neg	93	Nif./Mel	Loa Loa
4	AAI0304009B	Kediba	46	male	n.d.	n.d.	n.d.	pos	327	Nif./Mel	Loa Loa
5	AAK0303047B	Mundri	18	male	+	n.d	n.d.	neg	201	DFMO	Malaria
6	AAI0304020B	Lozoh	32	female	neg	n.d.	n.d.	neg	31	DFMO	malaria/ Loa Loa
7	AAK0303031B	Kozumbi	62	male	neg.	n.d.	n.d.	neg	35	DFMO	Malaria
8	AAK0303029B	Kotobi	<1	femal	n.d.	pos	n.d.	Neg	156	DFMO	
9	BB0303016B	Lozoh	6	male	+	pos	n.d.	Pos	128	DFMO	
10	**AAK0303043B**	Mundri	6	male	+++	neg	Pos	Neg	9	DFMO	Worms
11	**AAK0303045B**	Mundri	12	male	n.d.	pos	n.d.	Neg	8	DFMO	Malaria
12	AAK0303059B	Lozoh	9	female	n.d	pos	Neg	Neg	7	DFMO	malaria/*Giardia*/*Trichomonas*
13	**AAI0303037B**	Mundri	12	female	n.d.	pos	Pos	Neg	49	DFMO	malaria/*Giardia*/*Trichomonas*
14	**AAI0303030B**	Lozoh	12	female	n.d.	n.d.	Pos	Neg	8	DFMO	malaria/ *E. histolytica*
15	**AAK0303044B**	Mundri	12	female	+	n.d.	Pos	Neg	20	DFMO	malaria
16	**AAK0303054B**	Lazoh	25	male	++	pos	n.d.	Neg	22	DFMO	
17	AAK0303035B	Kotobi	40	female	n.d.	n.d.	Pos	Pos	403	DFMO	
18	**AAK0303032B**	Lozoh	22	female	+++	pos	n.d.	Pos	783	DFMO	
19	**AAK030310B**	Mundri	27	male	+++	pos	Pos	Neg	61	DFMO	worms/ *E. hystolytica*
20	BBK0304001B	Lazoh	38	male	+++	n.d.	Pos	Neg	8	DFMO	
21	AAK0303052B	Lozoh	31	female	+++	pos	n.d.	Neg	24	DFMO	malaria
22	**AAK0303042B**	Mundri	20	male	n.d.	n.d.	Pos	Neg	10	DFMO	malaria
23	AAK0303040B	Lozoh	16	female	++	pos	n.d.	Neg	15	DFMO	malaria
24	AAK0303046B	Mundri	37	female	+	n.d.	Pos	Neg	25	DFMO	malaria
25	**AAK0303030B**	Kotobi	21	female	++	pos	n.d.	Neg	4	Pent.	malaria
26	AAK0303027B	Kediba	41	female	+	pos	n.d.	Neg	20	DFMO	Loa Loa
27	AAK0303038B	Mundri	20	male	+	pos	n.d	Neg	7	DFMO	malaria
28	BBK0304011B	Kediba	34	male	+	pos	n.d.	Neg	31	DFMO	Loa Loa
29	**AAK0303051B**	Mundri	23	male	n.d.	pos	n.d.	Neg	50	DFMO	
30	AAKO303022B	Kediba	25	male	++	pos	n.d.	Neg	8	DFMO	malaria/ Loa Loa
31	**AAK0303028B**	Mundri	25	male	++	pos	n.d.	Neg	6	DFMO	Loa Loa
32	AAI0304006B	Lozoh	23	male	++	pos	Pos	Pos	1541	DFMO	
33	AAI0304007B	Lozoh	21	male	n.d.	pos	Neg	Neg	15	DFMO	Loa Loa
34	**AAI0304008B**	Lozoh	19	female	n.d.	pos	Pos	Neg	8	DFMO	Loa Loa
35	AAI0304010B	Mundri	18	female	++	n.d.	Pos	Neg	6	DFMO	malaria
36	AAI0304011B	Kediba	19	male	+	pos	Pos	Pos	167	DFMO	malaria/ Loa Loa
37	AAI0304012B	Lozoh	8	female	+	pos	Pos	Neg	16	DFMO	Loa Loa
38	AAI0304013B	Lozoh	15	female	+	pos	Pos	Neg	10	DFMO	malaria/ Loa Loa
39	AAI0304014B	Lozoh	27	female	+	pos	Pos	Neg	10	DFMO	Loa Loa
40	AAI0304015B	Lozoh	12	male	+	pos	Pos	Neg	104	DFMO	malaria
41	AAI0304016B	Lozoh	37	female	neg	pos	Pos	Neg	10	DFMO	malaria
42	AAI0304017B	Lozoh	62	male	neg	pos	Pos	Neg	49	DFMO	Loa Loa
43	AAI0304018B	Tatigiri	32	female	+	pos	Pos	Pos	413	DFMO	Loa Loa
44	**AAI0304019B**	Lozoh	10	male	++	pos	Pos	Neg	17	DFMO	Loa Loa
45	**AAI0304021B**	Lozoh	23	female	++	pos	Pos	Neg	45	DFMO	Loa Loa
46	**AAI0304022B**	Lozoh	9	female	+++	pos	Pos	Neg	8	DFMO	Loa Loa
47	AAI0304023B	Lozoh	32	female	neg	pos	Pos	Neg	11	DFMO	Loa Loa
48	AAI0304024B	Lozoh	20	male	n.d.	pos	Pos	Pos	625	DFMO	malaria/ Loa Loa
49	AAI0304025B	Ibba	32	female	neg	n.d.	Pos	Pos	779	DFMO	Loa Loa
50	AAI0304026B	Yambio	25	female	n.d.	neg	n.d.	Pos	309	DFMO	
51	AAI0304027B	Ibba	28	female	n.d.	n.d.	Pos	Neg	11	DFMO	Loa Loa
52	AA0304028B	Mundri	51	male	n.d.	Pos	n.d.	n.d.	-	DFMO	

Table 1: Isolates of all patients examined. Patients 1 to 7 are relapse cases. Grey-marked isolation numbers indicate successful propagation in laboratory rodents followed by cryopreservation. DFMO = eflornithine; [1] Sub County; [2] age in years; [3] blood examination by hematocrit centrifugation technique (= tryps.) to estimate parasitemia (+ as 1-5, ++ as 6-15 and +++ as >15 trypanosomes in 8 capillaries) and by card agglutination test (CATT) [4] Lymph node aspirate; [5] cerebrospinal fluid (CSF) examination for trypanosomes (tryps.) and white blood cells (cells).

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CHAPTER 5: Blood-borne infections and haematological profiles in patients admitted at a sleeping sickness hospital in southern Sudan

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Working paper

Abstract

The occurrence of blood-borne diseases and the associated haematological changes were investigated in 50 patients admitted at a sleeping sickness (SS) hospital in southern Sudan. The majority of the sleeping sickness patients had co-infection with loiasis (36%), malaria (30%) or both malaria/Loaisis (10%). Only 24% of the patients had a mono-infection. Anaemia was observed in the majority (79%) of the patients. The mean white blood cell count (WBC) in the patients was 10,652/µl (range: 2,760-51,320/µl). Only a minority (22%) of the patients showed leucocytosis but the rest showed either normal (66%) or below normal counts (12%). Most patients showing leucocytopenia had SS infection only, while the majority of those showing leucocytosis were co-infected with malaria. On the other hand most patients with normal WBC counts had loasis co-infection. Differential white cell counts conducted in 23 patients revealed that most of them had lymphocytosis (83%) and eosinophilia (96%) and neutropaenia (61%). This study has shown that co-infections of malaria and loaisis are common in sleeping sickness patients in southern Sudan. The resulting haematological changes are severe. Apart from the SS and malaria control it might be necessary to also introduce control programs against nematode infections and particularly loasis in this region. This combined control of the three diseases would decrease morbidity and co-morbidity due to multiple parasitic diseases.

Key words: Multiple infections, haematology, sleeping sickness, malaria, loiasis, south Sudan

Introduction

Sleeping sickness (SS), malaria and loiasis are common in Africa (WHO, 1996). The prevalence of sleeping sickness is estimated at 300,000 to 500,000 with an annual mortality of approximately 50,000 (WH0 2004). Sleeping sickness is caused by either *Trypanosoma brucei rhodesiense*, which is prevalent in eastern Africa, or *T. b. gambiense*, in west and central Africa. On the other hand, malaria is responsible for 1-3 million deaths and 300 million infections each year, concerning mainly children under the age of five years (Breman 2001). The vast majority of malaria infections are caused by *Plasmodium falciparum*, although *P. vivax, P. ovale*, and *P. malariae* are also prevalent. Lastly, Loasis, which is caused by *Loa Loa,* is endemic in forest and swamp areas of west and central Africa where it

inflicts 3 million individuals (Fain 1981). Prevalences of up to 35% have been reported in some of the countries (Boussinesq & Gardon 1997). An important common feature of the above mentioned diseases is that they are prevalent particularly among the poorer segments of rural communities. And since the climate in these regions is ideal for a variety of vectors, it therefore follows that mixed infections are inevitable in Africa. In addition, the different parasites reside in the same body compartments leading to similar clinical signs including high fever, weakness, headache, joint pains and neurological disorders.

However, most studies have examined these infections as separate entities. Rather less attention has been paid to multiple infections (Cox 2001). During a study which was aiming at isolating *T. b. gambiense* from patients in southern Sudan we noticed a high rate of co-infection with malaria and loaisis both detectable in blood. Here we present the haematological profiles and differential cell counts in these patients.

Materials and methods

Study area and patients

A cross-sectional study was conducted on 50 sleeping sickness patients admitted at the Ibba hospital, which is located in Maridi County, western Equatorial province of southern Sudan between March and April 2003. The area comprises of an equatorial rainforest, with thick vegetation and a humid climate, providing a good habitat for the breeding of vectors of many tropical diseases. The study focused on SS patients admitted at the hospital. The patients (or their parents or guardians in case of children) were asked to give written consent.

A questionnaire including questions on age, sex and self-reported symptoms of the disease was used. Other diagnosis on stool samples for detection of intestinal helminths and protozoa, as well as urine samples for detection of *Trichomonas* was undertaken for symptomatic patients. These results are not subject of the present analysis.

Sample collection

A sample of 2-3 ml of venous blood from each patient was collected into heparinised vacutainer tubes. Finger prick blood and cerebrospinal fluid (CSF) (lumbar puncture) was also collected.

Parasitological assessment

a) Sleeping sickness

Briefly, diagnosis of SS was done by microscropic examination of venous blood using the haematocrit centrifugation technique and the card agglutination test for trypanosomiasis (CATT) (Magnus *et al.* 1978). Lymph node aspirates and CSF were also examined for the presence of trypanosomes, and the number of white cells in the CSF was determined using a Neubauer haemocytometer. Patients without trypanosomes and less than 5 cells/mm^3 in CSF were classified as 1st stage while those with trypanosomes and/or more than 5 cells/mm^3 were classified as 2nd stage.

b) Malaria and Loa loa

Thick and thin blood smears were prepared from all patients and examined under the microscope for malaria and Loa loa parasites (Chadee *et al.* 2003). The presence of Loa loa was further determined using haematocrit centrifugation technique for better detection of microfilaria in the buffy coat.

c) Haematology

The packed cell volume (PCV), haemoglobin (HB) concentration and total white blood cell (WBC) counts were determined from venous blood. Haemoglobin concentration was determined using a haemoglobinometer while the total WBC was determined by the manual counting method in a haemocytometer (Cheesbrough, 2001). Twenty-three of the 50 patients were randomly selected and blood smears prepared for differential white cell counts.

Since the mean haematological values of the local population in south Sudan have not been established, the normal haematological values were based on reference ranges as detailed by (Dacie 2001).

Statistical analyses

Data was entered in Microsoft Excel and transferred into Epi dats 2.1. Analysis was done by use of the statical software package STATA (version 7.0, stata). Chi Square analysis was used to determine difference between anaemia occurrence in female and males.

Results

Patient description

Fifty patients infected (positive by any of the techniques used) with SS patients (23 males and 27 females) were included in the study. The mean age of the patients was 24 (0.25 – 62) years. The patients' numbers of white cells in the CSF ranged from 5 to1541 cells/µl. However, only eight of these second stage patients had trypanosomes in CSF. The patients reported various disease symptoms including headache (48/50), pruritus (41/50) and arthragia/ myalgia (47/50), insomnia (13/50) and mental disturbance (10/50). The number of symptoms reported varied and an average of 3.2 symptoms was reported per patient.

The majority of the sleeping sickness patients had a co-infection with either loiasis (36%), malaria (30%) or both malaria and loaisis (10%) (Table 1). Only 24% of the patients had a mono-infection of SS. Co-infection was more common in female (85%) than in the male patients (65%). Other cases of parastic infections noted were onchocerciasis (2) giardiasis (2), trichomonas (2), helminthosis (2) and amoebiasis (2).

Erythrocytes

The erythrocyte changes were assessed in 49 patients (data for one patient was inadvertently lost) and both methods of determination of anaemia-PCV and haemoglobin concentration gave similar findings (P>0.05-T-test). Anaemia was observed in most (80%) of the patients (6/11, 18/20 and 15/18 of the children, adult female, and adult male respectively) (Table 2). Anaemia was more common in female than in male patients (Chi Square (X2)=8.09, degree of freedom (df)=1 P=0.004).

Leucocytes

The leucocyte count (WBC) varied between the patients and a mean of 10, 652 cells/µl (range 2,760-51,320/µl) WBC was noted. Based on the total WBC counts, the patients were categorized into three groups; below normal, normal, and above normal counts. The normal values have been previously defined by Dacie (2001) and vary with age and sex of an individual. Only a minority (22%) of the patients showed leucocytosis. However, the rest of the patients showed either normal (72%) or below normal counts (12%). Further analysis

within the three categories of WBC counts indicated that most patients (66%) showing leucocytopenia had SS infection only while majority (55%) of those showing leucocytosis were co-infected with malaria. On the other hand 52% with normal counts had loasis co-infection (Table 3).

The effect of the infections on differential white cell counts were undertaken with 23 selected patients. Most patients had high lymphocyte (19/23) and eosinophil (22/23) counts, while neutropaenia was observed in 14/23 patients (Table 4). Monocytosis was observed mainly in patients with SS only and all patients with SS/loasis co-infection had distinct eosinophilia.

Discussion

The present study demonstrated the occurrence of mixed blood-borne infections in patients from a hospital in southern Sudan. The majority (76%) of the SS patients had co-infections of malaria or Loasis. There are a few mentions of co-infections of sleeping sickness and malaria (Schmid *et al.* 2004;Wellde *et al.* 1989b;Wellde *et al.* 1989a) and Loasis (Enyaru et al., 2002). A few studies also report on co-infection of malaria and filariasis (Chadee *et al.* 2003) or other helminthes (Egwunyenga *et al.* 2001;Raso *et al.* 2004).

Underlying reasons for the occurrence of multiple blood borne infections may be the favourable humid climate of southern Sudan that offers an environment that is conducive to a variety of vectors, including mosquitoes (*Anopheles* spp), tsetse flies (*Glossina* spp.) and deer flies (*Chrysops* spp.), which transmit plasmodium, trypanosomes, and Loa loa, respectively. In addition, the high prevalence of the diseases has been compounded by the longstanding (over 20 years) civil war in the area, which disrupted disease control programmes leading to a resurgence of the diseases (MSF 2001). In the present study, female patients had more often multiple infections than males. This may be attributed to occupational activities by females, such as drawing of water and collection of firewood that expose them to more contact with the vectors (Moore *et al.* 1999).

Anaemia was observed in most of the patients but only two patients had severe anaemia. Indeed, it is known that in highly endemic malaria and SS areas, such as southern Sudan, it is almost inevitable that the infections will be associated with anaemia (Beales 1997;Egwunyenga *et al.* 2001). For both malaria and SS, the pathogenesis of anaemia is

complex and multifactorial, and mainly results from a combination of increased destruction and reduced production of red blood cells (Abdalla 1990;Emeribe & Anosa 1991). As reported in other studies, the anaemia was more common in females than in males. This could be compounded by the fact that females had more co-infections, and biologically, they have lower erythrocytic values than males (Egwunyenga *et al.* 2001). Red blood cell dynamics could also be affected by a patients' nutritional status, and by other infections which were not the subject of our study.

The immune responses during an infection determine host resistance to the invading pathogen and ideally, elevated WBC levels are a good prognostic sign. In this study leucocytosis was observed in only a few of the patients and was mostly noted in patients with malaria co-infection. Indeed, other studies have shown that leucocytosis is a common feature in malaria patients (Richards *et al.* 1998). However, most patients, especially those with co-infection of loaisis, showed an inadequate immune respons since they had either normal WBC counts or leucocytopaenia. Other investigations (Anosa & Kaneko 1983;Emeribe & Anosa 1991) have reported leucocytopaenia in late stage of SS. In addition to the leucocytopenia, most patients showed lymphocytosis. The latter is the main white cell reaction in trypanosomosis and malaria infections (Emeribe & Anosa 1991;Stephens 1986) and is geared towards elimination of the parasites. However, leucocytopenia indicates a depressed or exhausted immune system and, in this study, was mainly due to neutropenia. This feature is common in both malaria (Abdalla 1988) and chronic livestock trypanosomosis (Naylor 1971;Valli *et al.* 1979). Neutrophils play very significant role in parasite clearance and neutropenia could lead to increased susceptibility to other infections (Greenwood *et al.* 1973).

Malaria and trypanosomiais lead to parasite-induced immunodepression. Since *T. b. gambiense* causes a chronic disease and leads to long-term immunodepression, a superimposed malaria infection may not only take advantage of the environment but also lead to enhancement of trypanosome parasitemia. In fact, concerted immuno-depression from the two parasites has been implicated in dramatic enhancement of parasitaemia in malaria and trypanosomiasis co-infections. However, the outcome of multiple interactions is not necessarily predictable and can be influenced by factors such as stage of infection, age, and sex of the host (Cox 2001).

The situation here is further complicated by co-infection with loa loa. In helminth (loa loa) infections the immune response is Th_2 driven, but Th_1 in protozoan infections (malaria or trypanosomiasis). The interaction between trypanosome and helminths is that the strong Th_1 response known to occur in trypanosomiasis infection abrogates the Th_2 responses, which are necessary for immunity to helminths. Indeed a *T. brucei* and *T. congolense* infections in animals were able to counter the Th_2 response leading to increased establishment and fecundity of nematode worms (Chiejina *et al.* 2003). Experimental studies have also shown agonistic and antagonistic interactions in trypanosome-heminthes co-infections that appeared to depend on which of the parasites was inoculated first (Fakae *et al.* 1994;Griffin *et al.* 1981).

There are two points worth further discussion. The interaction of the parasites in multiple infections further complicates diagnosis and treatment. First loa loa noted in most patients hinders microscopic observation of trypanosomes in the buffy coat junction (Enyaru et al., 2002). Secondly, the occurrence of multiple infections raises an important issue relating to the early diagnosis and treatment of the gambiense form of SS, as its symptoms do not appear rapidly when compared to malaria. However in both diseases, the clinical signs in the early stage (high fever, weakness and headache, joint pains) and late stage (neurological disease) are to an extent similar. It is possible that an early trypanosome detection could be achieved when the patients present themselves at the hospital with malaria symptoms. Lastly, the order of treatment in a patient with multiple infections is critical. Like in other SS treatment centres, malaria was treated, followed by SS. Onchocerciasis was treated after a patient was cured of SS, mainly because antifilarial chemotherapy has in some cases resulted in severe adverse side effects, including encephalopathy (Blum *et al.* 2001;Gardon *et al.* 1997) and could complicate the management of the neurological form of SS. Loasis was not treated and it is necessary to develop methods for its control.

To summarise, this study has pointed to a high incidence of mixed malaria, SS and loasis infections in southern Sudan, with complex and severe haematological changes in the patients. Detailed studies are required in order to clearly understand the host-parasite interactions during such co-infections.

	Gender		Age(years			
	Male	**Female**	**0 -9**	**10-19**	**20-39**	**>40**
Mono-infection (n=12)	8	4	3	1	7	1
Malaria co-infection (n=15)	5	10	1	7	6	1
Loiasis co-infection (n=18)	7	11	2	2	11	3
Malaria and loiasis poly infection (n=5)	3	2	0	2	2	1
Total (n=50)	23	27	6	12	26	6

Table 1: Sleeping sickness, malaria and loiasis cases among different age groups and sexes admitted in Ibba hospital in southern Sudan

	Anaemia status		**Haematological parameters**	
		number	**Mean PCV (range)**	**Mean HB (range)**
Males				
>12 years	Anaemic	15	30.0 (21-37)	10.1 (7-12.2)
	Normal	3	41 (38-45)	13.3 (13-14)
<12 years	Anaemic	1	28	n.d.
	Normal	3	38.3(37-40)	12.3(12.2-12.6)
Females				
>12 years	Anaemic	18	29.6 (20-34)	9.9 (7-13)
	Normal	2	39 (36-42)	13 (12-14)
<12 years	Anaemic	5	29.4 (24-32)	9.7 (8.6-10)
	Normal	2	42 (40-44)	14 (13-15)

Table 2: Erythrocyte changes in patients among different age groups and sexes attending Ibba Hospital, southern Sudan

Category of WBC counts	Infection status			
	SS only (n=12)	SS & malaria (n=15)	SS & loa loa (n=18)	SS, malaria & Loa loa (n=5)
Below normal (n=6)	66	16.5	16.5	0
Normal (n=33)	18	21	52	9
Above normal (n=11)	18	55	10	18

Table 3: Leucocyte levels in patients having the various infection combinations

Infection Status	Category	Lymph.	Neutroph.	Eosinoph.	Monocyt.	Basophil.
SS alone (n=8)	Normal	12.5	37.5	12.5	12.5	50
	Low	12.5	67.5	0	0	12.5
	High	75	0	87.5	87.5	37.5
	MCC	5182	2222	2741	351	190
SS + malaria (n=8)	Normal	0	25	12.5	62.5	87.5
	Low	0	62.5	0	0	0
	High	100	12.5	87.5	37.5	12.5
	MCC	7443	2380	1976	577	229
SS + Loiasis (n=7)	Normal	20	25	0	100	100
	Low	20	75	0	0	0
	High	60	0	100	0	0
	MCC	2525	1900	1564	235	20

MCC = mean cell counts (/µl), SS = sleeping sickness

Table 4: Percentage of patients with different levels of leucocytes

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CHAPTER 6: Isolation and propagation of *Trypanosoma brucei gambiense* from sleeping sickness patients in South Sudan

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Abstract

This study aimed at isolating *Trypanosoma brucei gambiense* from human African trypanosomiasis (HAT) patients under field conditions. It was carried out in South Sudan in collaboration with Médecins sans Frontières-France (MSF-F). Fifty-three HAT patients identified during active screening surveys were recruited, most of whom were in 2nd stage disease (51/52). Blood and cerebrospinal fluid samples collected from the patients were cryopreserved using Triladyl® as cryomedium. The samples were stored at -150°C in liquid nitrogen vapour in a dry shipper. 18 patient stabilates could be propagated in laboratory rodents (immunosuppressed *Mastomys natalensis* and SCID mice), but only 5 of them caused a patent parasitaemia in Swiss white mice. Parasitaemia was highest in SCID mice. In *vivo* viability appeared to be dependent on the parasitaemia in the patient. Further subpassages in *M. natalensis* increased the virulence of the trypanosomes and all 18 isolates recovered from *M. natalensis* or SCID mice became infective to other immunosuppressed mouse models. A comparison of Swiss white mice, *M. natalensis,* C57/bl and Balb/C, all immunosuppressed, demonstrated that all rodent breeds were susceptible after the 2nd subpassage and developed a parasitaemia higher than 10^6/ml by day 5 post-infection. The highest parasitaemias were achieved in C57/bl and Balb/C. This indicated that propagation of *T. b. gambiense* isolates after initial isolation in immunosuppressed *M. natalensis* or SCID mice could be done in a range of immunosuppressed rodents.

Key words: *Trypanosoma brucei gambiense*, propagation, cryopreservation, isolation, *Mastomys natalensis*, SCID mice, Sudan, Sleeping sickness

Introduction

Sleeping sickness or human African trypanosomiasis (HAT) is endemic in 36 countries in sub-Saharan Africa. It is estimated that 60 million people are at risk of infection, 500,000 people are already infected and approximately 50,000 die every year (WHO Report, 2004). The disease is caused by *Trypanosoma brucei rhodesiense* and *T. b. gambiense*. The former causes an acute disease and is mainly found in east Africa, while *T. b. gambiense* generally causes a chronic disease in western and central Africa. In Sudan, HAT due to *T. b. gambiense* is highly endemic with an estimated 1 to 2 million people at risk of infection, and thousands of people infected.

Control of the disease relies mainly on chemotherapy and, to a limited extent, on vector control. The drugs used in Sudan are pentamidine for 1st stage and melarsoprol and eflornithine for 2nd stage disease. Melarsoprol, an arsenical drug, is normally the first line drug for treatment of 2nd stage disease. In recent years, high rates of melarsoprol treatment failure (>20%) have been reported in *T. b. gambiense* endemic areas in Uganda, Angola and Sudan (Legros et al., 1999; Burri and Keiser, 2001).

In one area of South Sudan, Maridi county (Western Equatoria), the rate of melarsoprol treatment failure was found to be high (Moore and Richer, 2001). Additionally, a high fatality rate following melarsoprol treatment was noted (MSF Report, 2002). This has led to a change of treatment policy, and since September 2001 eflornithine became the first-line drug for 2nd stage disease in the Ibba hospital (MSF Report, 2002).

The role of resistant trypanosomes in treatment failures is not well understood mainly because of a lack of *T. b. gambiense* isolates from various endemic countries. One reason for this is the difficulty to isolate this trypanosome species and to propagate it in laboratory rodents (Brun et al., 2001). The purpose of this study was to isolate *T. b. gambiense* from patients in Ibba sleeping sickness hospital. At the same time, the study investigated ways of improving the field isolation of *T. b. gambiense* in endemic areas which are hard to access. Specifically, we evaluated a new cryomedium (Maina et al., submitted) and later studied the propagation of the isolated parasites in various mouse breeds.

Materials and Methods

Isolation of trypanosomes from patients

Cryopreservation medium

Triladyl® (MiniTüB GmbH & CO. KG; Tiefenbach, Germany), a medium for bull semen cryopreservation, was used for the cryopreservation of the blood and CSF samples from HAT patients (Maina et al., in press). Triladyl® contains 8% glycerol, 20% egg yolk, Tris buffer, citric acid, fructose, and antibiotics (tylosin 5mg/l, gentamicin 25mg/l, spectinomycin 30mg/l, and lincomycin 15mg/l). It was stored at 4°C or at -20°C.

Liquid nitrogen

Three nitrogen tanks (Taylor-Wharton) were used: one tank type 34HC (capacity 34 litres) and two dry shippers, CX100 (capacity 4 litres). The storage tank was kept in the MSF-F laboratory at Maridi and was used to refill the dry shippers. The dry shippers were taken to the hospitals for specimen collection.

Patients

Active screening of HAT patients was done by MSF-F in Mundri and Maridi counties, Western Equatoria, South Sudan (Figure 1). Identified 1st stage patients were transferred to Kotobi, Mundri county, and 2nd stage patients to Ibba, Maridi county. In the hospital, the patients were informed of the objectives and protocols of the study. To be included in this study the patients (or their guardians) had to give their consent (written/ thumb print).

Only patients with confirmed *T. b. gambiense* infection were enrolled. Diagnosis was either done serologically (Magnus et al., 1978) or parasitologically (trypanosomes detected either in lymph node aspirate, venous blood or cerebrospinal fluid (CSF)). In the CSF the number of lymphocytes was determined. Patients with no trypanosomes and less than 5 cells/mm^3 in CSF were classified as 1st stage. Those with trypanosomes and/or more than 5 cells/mm^3 in CSF were classified as 2nd stage. Patients who had been previously treated for HAT less than 12 months ago were considered relapses or treatment failures.

The World Health Organization (WHO) and the Ministry of Health of Sudan People's Liberation Army (SPLA) approved the study protocol.

Cryopreservation of patient stabilates

Samples of 2-3 ml of venous blood from patients were collected into heparinised vacutainer tubes. Samples of 2 ml of CSF were also collected from two self-reporting patients attending the hospital. Trypanosomes in the samples were concentrated by centrifugation at 10,000 RPM for 10 minutes and the pellet re-suspended in Triladyl® at a 1:1 ratio for blood and 1:10 for CSF. Aliquots of 500µl were then transferred into NUNC® ampoules. Four to five stabilates were prepared per patient and stored in a liquid nitrogen dry shipper in the vapour phase at -150°C. The dry shippers containing the stabilates were transported to the Trypanosomiasis Research Centre (TRC) in Kenya within a month.

Propagation of *T. b. gambiense* in laboratory rodents

Laboratory animals

Mastomys natalensis and Swiss white mice were bred at TRC. C57/bl and Balb/C mice were purchased from the International Livestock Research Institute (ILRI) and the Kenya Medical Research Institute (KEMRI), both in Nairobi, Kenya. Severe combined immunodeficient (SCID) (C.B-17/Icr-scid/scid), C3H (strain code 025), and FVB (strain code 207) mice were purchased from the Charles River Laboratories, Inc., Germany. NMRI mice were purchased from RCC Ltd., Itingen, Switzerland. The animals were housed under conventional conditions, and fed on commercial pellets and water ad libitum.

All animals (except for SCID mice) were immunosuppressed by administration of cyclophosphamide at 300mg/kg body weight intraperitoneally prior to infection and re-treated every 10 days with 200mg/kg.

Propagation of stabilates from HAT patients in M. natalensis and SCID mice

The presence and viability of trypanosomes in the patient's stabilate was determined by inoculating the stabilates in to one SCID mouse and two immunosuppressed *M. natalensis.* The stabilates were thawed rapidly in a water bath at 37^{o}C and immediately injected intraperitoneally (ip) into the laboratory rodents (one stabilate into three rodents). Parasitaemia was monitored every other day for 60 days by examination of tail blood using the haematocrit centrifugation technique (HCT) and/or by the method of Herbert and Lumsden (1976). Parasitaemic animals were euthanised using CO_2, and blood was collected by cardiac puncture. The infected blood (passage 1) was cryopreserved in Triladyl® and frozen in the vapour phase of a liquid nitrogen container at -2^{o}C/minute and finally stored in liquid nitrogen. Successful stabilates were given an isolation code different from the original patient stabilate.

Rodents that remained aparasitemic for 60 days after inoculation of cryopreserved material were euthanised and blood collected by cardiac puncture. For each rodent, eight capillaries were prepared and examined for trypanosomes using the HCT method. If no patent infection could be found, another two *M. natalensis* were injected with another stabilate.

Propagation of stabilates from HAT patients in Swiss white mice

The eighteen stabilates which could be propagated either in immunosuppressed *M. natalensis* or in SCID mice were used to evaluate the susceptibility of Swiss white mice for *T. b.*

gambiense after immunosuppression. An original stabilate from the patients was thawed and aliquots injected in to one Swiss white mouse and one *M. natalensis*, both immunosuppressed.

Genotypic characterization

Trypanosomes (passage 1) were purified using an anion exchange column (Lanham and Godfrey, 1970) and DNA was extracted using the Puregene DNA extraction kit (Gentra Systems, Minneapolis, USA) as previously described by Matovu et al. (2001b).

Amplification of the serum resistance-associated (*SRA*) gene and the *T. b. gambiense* specific glycoprotein (*TgsGP*) was done as previously described by Radwanska et al. (2002a and b). However, for both genes a nested PCR was done. For SRA, the two sets of primers used were SRA-out-s (5-CCTGATAAAACAAGTATCGGCAGCAA-3') and SRA-out-as (5'-CGGTGACCAATTCATCTGCTGCTGTT-3') and SRA-inner-s (5'-ATA GTG ACA TGC GTA CTC AAC GC-3'), SRA-inner-as (5'-AAT GTG TTC GAG TAC TTC GGT CAC GCT-3'). For the *T. b. gambiense* specific glycoprotein (TgsGP) gene the primer pairs were: TbsGP-outer-s (5'-GCGTATGCGATACCGCAGTAA-3') and TbsGP-outer-as (5'-GCTTCAACCGCCGCTGCTTCTA-3'); TbsGP-s (5'-GCTGCTGTGTTCGGAGAGC-3') and TbsGP-as (5'-GCCATCGTGCTTGCCGCTC-3').

For control PCRs, the actin gene was amplified using primers act-s (5'-CCGAGTCACACAACGT-3') and act-as (5'-CCACCTGCATAACATTG-3'). The amplifications were done in 50µl reaction containing PCR buffer (Qiagen), 200mM of the four dNTPs, 1uM of each primer, and Taq polymerase in a PTC 200 Peltier (MJ Research) thermocycler under the following conditions: initial denaturation at 94°C for 3 minutes; 30 cycles of 94°C for 45 seconds, 62°C (55°C for actin) for 45 sec and 72°C for 120 sec; a final extension for 10 min, followed by rapid cooling at 4°C. 8µl of the PCR product were analyzed by electrophoresis in a 1.25% agarose gel. The gels were stained with ethidium bromide (0.2µg/ml) and visualized under UV.

For sequencing, two independent PCR reactions were pooled, purified using QIAquick columns (Qiagen, Basel, Switzerland), and sequenced with the Big Dye Terminator kit (Applied Biosystems). The products were run at the Computational and Molecular Population Genetic Lab (CMGP, Zoology Department, University of Bern) on the ABI-sequencer.

Susceptibility of various strains of mice to *T. b. gambiense* isolates

The susceptibility of various strains of mice to the *T. b. gambiense* isolates was determined in two independent experiments. In one test, the susceptibility of immunosupressed Swiss white mice to the 18 isolates (passage 1) was determined. Each isolate was tested in two mice and at an inoculum of $4x10^5$ trypanosomes.

In the second experiment, the growth pattern of five randomly selected isolates was determined in C57/bl, Balb/C, Swiss white, NMRI, C3H, FVB and *M. natalensis*. Prior, the parasites (from the primary propagation) were passaged twice in *M. natalensis* and then in the respective rodent strain. Trypanosomes were harvested from a donor rodent and inoculated into four rodents. Each animal was inoculated with $4x10^5$ trypanosomes. Thawing of stabilates, inoculation, and parasitaemia determination were carried out as described earlier.

Results

Patients

Fifty-three confirmed HAT patients were enrolled in the study. 49 were from Mundri, two from Maridi, one from Yambio, and one from Juba county. The mean age of the patients was 24 years (range 0.25- 62) with >90% being below 40 years. Most patients (51/53) were in the 2^{nd} stage of the disease, with CSF lymphocyte numbers ranging from 5 to 1541/mm^3. Seven of the patients had previously been treated (7-12 months before): four with eflornithine (at 400mg/kg/day for fourteen days) and three with pentamidine (4mg/kg/day for seven days).
Blood samples were collected from 50 out of 52 patients: 42 patients were parasite positive in the blood and eight were aparasitaemic. Samples of CSF were collected from two patients attending the hospital for passive screening. For all other patients a lumbar puncture had already been done in the field during active screening, and for ethical reasons a second lumbar puncture was not performed in the hospital.

Primary propagation of stabilates from patients

Details of the patients of whom trypanosomes could be isolated are shown in Table 1. Fifty blood and two CSF samples were inoculated into both *M. natalensis* and SCID mice. Of the blood samples, 16 caused patent infections in either rodent species while the two CSF samples did not lead to a parasitaemia in the rodents. For the 34 patients of whom the blood stabilates could not be propagated in the first attempt, a second stabilate was inoculated in two

immunosuppressed *Mastomys natalensis*. However, only two more isolates (K03030 and K03032) could be obtained in this second propagation attempt. A slightly higher recovery was obtained in *M. natalensis* (14/18) as compared to SCID mice (12/18). Only 8/18 stabilates could be propagated in both rodents. The stabilates had a similar pre-patent period in SCID mice (10.25 ± 4.26 days) and in *M. natalensis* (11.25 ± 6.3 days). In *M. natalensis* however, only 3/14 stabilates had parasitaemias of 10^6/ml or higher, whereas in the SCID mice 9/12 stabilates reached that parasitaemia. Patient stabilates that were successfully inoculated in rodents were designated with the name '*Mundri*' (the name of the county in South Sudan where all the patients came from) and a consecutive number; furthermore they are termed isolates. The details of the viable isolates are shown in Table 2.

Determination of the suitability of Swiss white mice for patient stabilates

Only five (I03030, I03037, K03048, K03045, and K03051) of the eighteen patient stabilates which could be propagated in either immunosuppressed *M. natalensis* and/ or in SCID mice caused patent infection in immunosuppressed Swiss white mice (Table 3). Parasitaemias in Swiss white mice were low (apart from K03048) and could only be detected by the HCT method. With one stabilate (K03051) parasitaemia was sporadic and inconsistent. The parasitaemia was generally much higher in *M. natalensis* and SCID mice than in Swiss white mice.

Genotypic characterization

T. brucei subspecies-specific genotyping was performed by PCR using the serum resistance-asscoiated gene *SRA* as a marker for *T. b. rhodesiense* (Radwanska et al., 2002a) and the *T. b. gambiense*-specific glycoprotein gene *TbgGP* for *T. b. gambiense* (Berberof et al., 2001). The marker genes were amplified from genomic DNA by nested PCR and analyzed on agarose gels (Fig. 2). All isolates tested were positive for *TbgGP* and negative for *SRA*. To verify that the correct gene was amplified, PCR products were directly sequenced. Apart from a few silent mutations the obtained sequence (GenBank accession **DQ224158**) was identical to that published for *TgsGP* (GenBank accession **AJ277951**), proving that the isolated trypanosomes were indeed *T. b. gambiense*.

Susceptibility of mice to secondary propagation of *T. b. gambiense*

All the 18 isolates (*Mundri* 1 to 18, first passage) inoculated into immunosuppressed Swiss white mice caused patent infection, with a pre-patent period of 4 days. Most of the isolates (11/18) caused low and inconsistent parasitaemia (less than 10^4/ml), but the remaining isolates (*Mundri* 02, 03, 04, 06, 08, 14, and 17) grew to high parasitaemia with levels over 10^6 trypanosomes/ml (data not shown).

The growth patterns of five randomly selected isolates (passage 4) were compared in immunosuppressed C57/bl, Balb/C, Swiss white and *M. natalensis* (Table 4). The parasites were infective for all rodents used, with a similar prepatent period (3 days) and a parasitaemia higher than 10^6 trypanosomes/ml by the 5^{th} day. There was variation in the growth pattern between the isolates and between rodent strains. For all five isolates, the highest parasitaemia (over 5 x 10^7 trypanosomes/ml) was noted in C57/bl mice. Overall, C57/bl and Balb/C sustained higher parasitaemias (over 10^6/ml) for several days than Swiss white or *M. natalensis*. In all isolates the parasitaemias dropped to undetectable levels by day 16 post infection, and only a few deaths (4/80) were noted during the 60 day observation period. In NMRI, C3H and FVB mice parasitaemia was always very low and far below the parasitaemia in C57/bl and Balb/C (data not shown).

Discussion

This is the first report of successful isolation of *T. b. gambiense* from patients in South Sudan. 18 viable isolates could be cryopreserved under field conditions using the new cryomedium Triladyl® (Maina et al., in press). In previous studies, low recovery rate or complete loss of *T. b. gambiense* populations following cryopreservation in medium containing 10% glycerol have been reported (Brun et al., 2001; Matovu et al., 2001a). The achieved recovery rate of 43% is very satisfactory and may be attributed to the superior cryopreservation medium Triladyl®.

Viability of the isolates in rodents also depended on the degree of parasitaemia in the patients. Twenty-nine (about 78%) of the 37 tested patients had trypanosomes in the blood but parasitaemia was low (only detectable by HCT) and varied between the patients. There was a direct correlation between parasitaemia in the patient and viability in rodents, i.e. the recovery rate was higher in the category isolated from patients with detectable parasitaemia as compared to patients with undetectable parasitaemia. Earlier reports also noted that the

limited infectivity of *T. b. gambiense* for laboratory rodents led to low isolation success (Dukes et al., 1989; Aerts et al., 1992), especially from patients with low parasitaemia.

In the current study we used the African rodent *M. natalensis* (immunosuppressed) for primary propagation of the patient stabilates on a comparative basis to SCID mice which have no functional T and B lymphocytes (Bosma et al., 1983). *M. natalensis* was reported to be a better model for *T. b. gambiense* than rats and mice (Mehlitz, 1978) and have been extensively used for propagation studies (Gibson et al., 1978; Zillmann and Mehlitz, 1979; Dukes *et al*. 1989; Aerts et al., 1992; Matovu et al., 2001a). Inoue et al (1998) first described the good susceptibility of SCID mice for *T. b. gambiense* isolates. The parasitaemias attained were higher in SCID mice than in immunosuppressed *M. natalensis.* That finding could be confirmed by our study. The use of SCID mice is however restricted by the high costs of the mice. On the other hand, breeding colonies of *M. natalensis* were successfully established at institutes in East Africa.

Normal in- or outbred laboratory mice have generally not been found to be susceptible for *T. b. gambiense* isolates, though they can easily be infected with *T. b. rhodesiense* isolates. However we found that after immunosuppression, different breeds of normal laboratory mice could be infected with the 2nd or 3rd passage of our *Mundri T. b. gambiense* isolates. Even stabilates prepared from patients were infective for immunosuppressed Swiss white mice, though with a lower success rate (5/18) as compared to *M. natalensis* (14/18) or SCID mice (12/18). However, after a second passage, all 18 isolates were infective for immunosuppressed Swiss white mice, and seven of them resulted in a parasitaemia >10^6/ml. In other mouse strains, (C57/bl, Balb/C, NMRI, C3H and FVB) the parasitaemia was very low, except for the three isolates K03028, K03048, K03043 which reached parasitaemias >10^5/ml in C57/bl and Balb/C by day 14 post-infection. Passaging the isolates at least three times increased their virulence resulting in a parasitaemia >10^7/ml. Earlier reports indicated that C57/bl mice are able to control a trypanosome infection (Hertz et al., 1998), but immunosuppression of C57/bl resulted in a higher parasitaemia than in untreated mice (Black et al., 1983).

In conclusion, we recommend that infected blood samples from *T. b. gambiense* patients should be cryopreserved in Triladyl® medium. Primary propagation and the subsequent subpassage should be done in immunosuppressed *M. natalensis* or in SCID mice. Further

subpassages can then be done in immunosuppressed laboratory mice (e.g. C57/bl or Balb/C). Available mouse breeds should be screened after immunosuppression with cyclophosphamide at 300 mg/kg prior to infection and repeated once/week at 200 mg/kg. This propagation scheme is efficient and also economical. It will allow the characterization of *T. b. gambiense* from various hot spots of Central Africa, hence enhancing the monitoring of drug resistant trypanosomes. These new *T. b. gambiense* isolates are a great resource for further scientific investigations. Their characterization, especially for drug sensitivity, will assist in elucidating the cause of the high melarsoprol treatment failure rates reported in the area.

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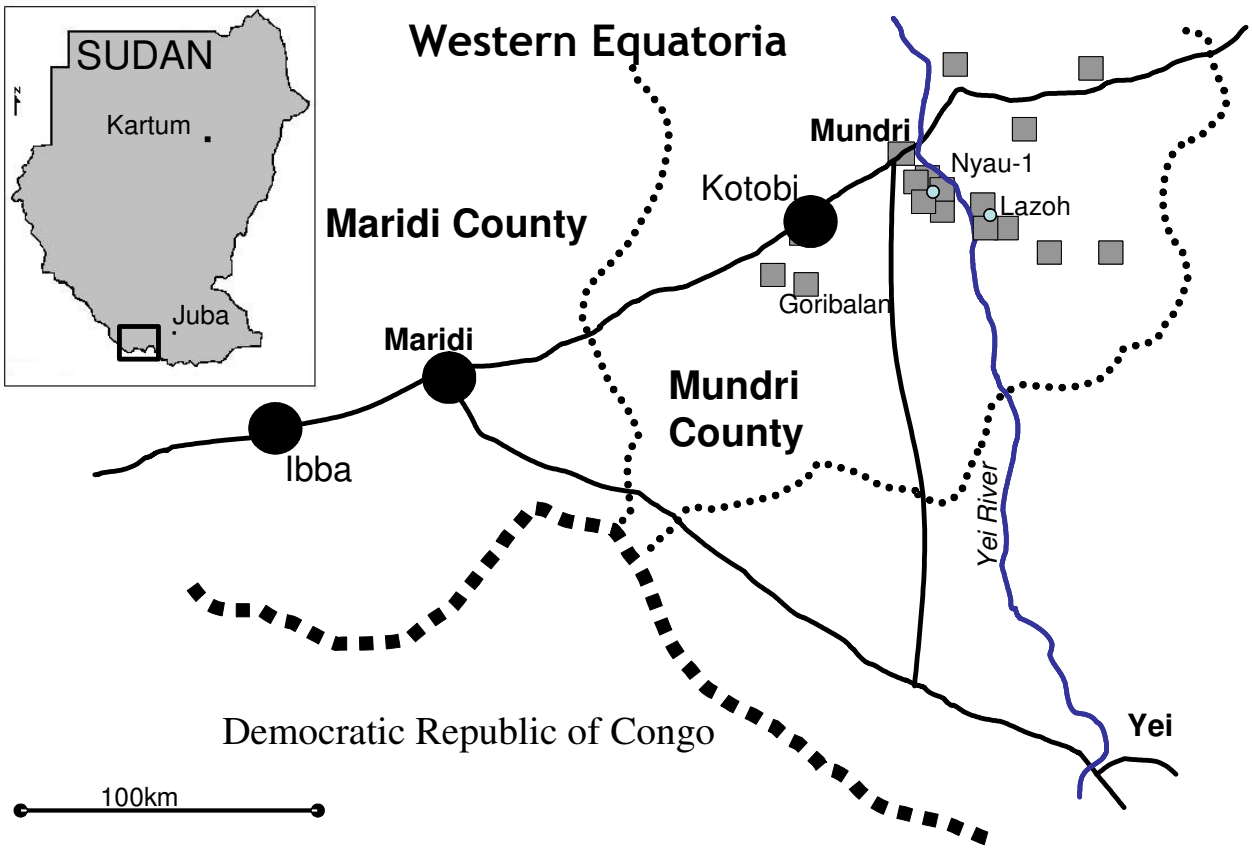

Figure 1: Study site in Western equatorial in South Sudan. Grey squares the origin of the patients while black circles indicate the MSF-laboratories.

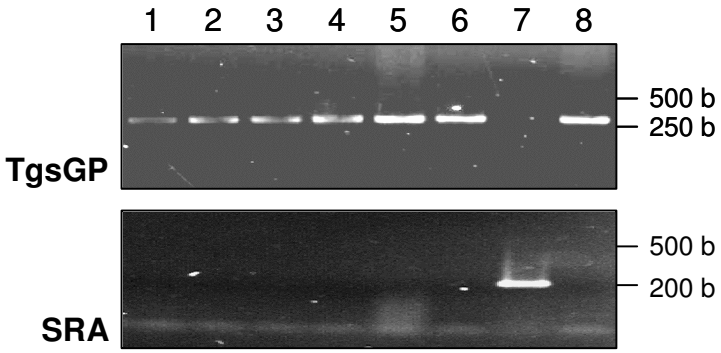

Figure 2: Genotypic characterization: Amplification of the *T. b. gambiense*-specific glycoprotein (*TgsGP*, top) and the serum resistance-associated gene (*SRA*, bottom) in trypanosomes isolated from HAT patients. As all 18 stabilates gave the same result only 6 of them are shown here (lanes 1-6). *T. b. rhodesiense* STIB 704 (lane 7) and *T. b. gambiense* STIB 754 (lane 8) were used as controls.

Patient stabilate	**Bomas** (village)	**Age** (yrs)	**Sex** Male:M Female:F	**Proof of trypanosomes in:**			**CSF[d]** cells/mm3
				Blood[a]	**Lymph[b]**	**CSF[c]**	
				(not done (n.d.); positive +; negative -)			
I04008	Midi	19	F	n.d.	+	-	8
I03037	Amandi	12	F	n.d.	+	-	49
K03028	Nyau 1	25	M	++	n.d.	-	6
K03048	Buangyi	20	M	+++	n.d.	-	58
K03051	Nyau 1	23	M	n.d.	n.d.	-	50
K03030	Goribalan	21	F	++	n.d.	-	4
K03042	Nyau 1	20	M	n.d.	+	-	10
I04019	Lazoh	10	M	++	+	-	17
K03043	Wiri Lui	6	M	+++	+	-	9
I04015	Singo	12	M	+	+	-	104
K03045	Nyau 1	12	M	n.d.	n.d.	-	8
I04022	Lazoh	9	F	+++	+	-	8
K03010	Mundri	27	M	+++	+	-	61
I04021	Gingo	23	F	++	+	-	45
I03030	Lazoh	12	F	n.d.	+	-	8
K03044	Nyau 1	12	F	+	+	-	20
K03032	Singirigwa	22	F	+++	n.d.	+	783
K03054	Moto	25	M	++	n.d.	-	22

Table 1: T. b. gambiense isolates from Mundri County which could be propagated in laboratory rodents. The blood samples were cryopreserved in Triladyl® and stored in nitrogen vapour in dry shippers. All patients had new infections and were in 2nd stage disease, apart from one patient in 1st stage (stabilate K03030) and another patient (stabilate K03048) who had been treated 12 months earlier with eflornithine.

[a]The haematocrit centrifugation technique was used to estimate parasitaemia in the blood and graded as + (1-5), ++ (6-15) and +++ (more than 15 trypanosomes visible). [b] lymph node aspirate; [c] cerebrospinal fluid; [d] counted lymphocytes in the cerebrospinal fluid.

Patient stabilate	**Isolate No.**	**SCID mice** (Passage 1)		***M. natalensis*** (Passage 1)		
		Pre-patent period	Parasitaemia at freezing (day post infection)	No. with patent infection	Pre-patent period	Parasitaemia at freezing (day post infection)
I04008	Mundri 01	7	1.6×10^7 (7.2)	2/2	12	+++ (14)
I03037	Mundri 02	7	1.6×10^7 (7)	½	8	+++ (10)
K03028	Mundri 03	11	1.6×10^7 (12)	2/2	4	+++ (6)
K03048	Mundri 04	4	1.6×10^7 (6)	2/2	4	2.5×10^4 (5)
K03051	Mundri 05	7	0.8×10^7 (14)	½	23	++ (26)
K03030	Mundri 06	11	1×10^6 (21)	¼	9	++ (11)
K03042	Mundri 07	14	++ (14)	2/2	10	++ (12)
I04019	Mundri 08	13	+ (13)	2/2	21	3.2×10^7 (25)
K03043	Mundri 09	5	3.2×10^7 (9)	0/4	-	-
I04015	Mundri 10	11	1.6×10^7 (11)	0/4	-	-
K03045	Mundri 11	17	1.0×10^6 (22)	0/4	-	-
I04022	Mundri 12	16	2.5×10^4 (24)	0/4		-
K03010	Mundri 13	-	-	2/2	3.5	0.8×10^7 (5)
I04021	Mundri 14	-	-	½	18	2×10^6 (21)
I03030	Mundri 15	-	-	2/2	6	2.5×10^4 (7)
K03044	Mundri 16	-	-	½	11	+++ (13)
K03032	Mundri 17	-	-	¼	17	+++ (18)
K03054	Mundri 18	-	-	½	11	+ (13)

Table 2: Infectivity of T. b .gambiense isolates from Mundri County for immunosuppressed Mastomys natalensis and SCID mice. Primary propagation was done in two immunosuppressed *M. natalensis* and one SCID mouse for each isolate. Parasitaemia was monitored starting day 3 post-infection and every other day for 60 days. When trypanosomes were visible by wet film, parasitaemia was estimated by the method of Herbert and Lumsden (1976) and graded as antilog trypanosomes/ml. At low levels of parasitaemia, the haematocrit centrifugation technique was used. Parasitaemia was graded as + (1-5), ++ (6-15) and +++ (more than 15 trypanosomes visible).

Patient stabilate	***M. natalensis***			**Swiss white mice**		
	Pre-patent period days	Highest parasitaemia antilog	Days to peak parasitaemia	Pre-patent period days	Highest parasitaemia Antilog	Days to peak parasitaemia
I03037	17	6.3	30	28	+++	28
K03048	5	7.8	17	3	8.4	17
K03051	7	6.3	17	12	+	12
K03045	21	6.3	24	5	+	5[a]
I03030	-	n/a	n/a	33	++	38

Table 3: Primary propagation of blood stabilates from patients in immunosuppressed Swiss white mice compared to immunosuppressed *M. natalensis*

Five of the 18 stabilates which could successfully be propagated in SCID mice or immunosuppressed *M. natalensis* (Table 2) could also be propagated in immunosuppressed Swiss white mice. One Swiss white mouse and one *Mastomys natalensis* for comparison were inoculated with a primary stabilate from patients. Parasitaemia was monitored every other day for 40 days. Parasitaemia was estimated as described in Table 2.

[a] Parasites were only detected on day five post infection, thereafter, the mouse was negative through to day 21 when it died

Isolate No. (patient stabilate)	**Rodent breed**	**Mean Pre-patent period** (in days) (number of mice developing infection)	**Period over 10^6/ml** (mean duration in days)	**Highest parasitaemia** (Days post infection)
Mundri 02 (I03037)	C57/black	3 (3/4)	4-16 (12.5)	2.5 x10^8 (7)
	Balb/C	3 (4/4)	4-14 (4.25)	1.6 x 10^7 (8.5)
	Swiss white	3 (3/4)	5-7 (2)	0.8 x 10^7 (6)
	M. natalensis	3 (3/4)	6-8 (2)	3.2 x 10^6 (7)
Mundri 05 (K03051)	C57/black	3 (3/4)	5-9 (4.66)	5.0 x10^7 (7.66)
	Balb/C	-	-	-
	Swiss white	3 (4/4)	5-16 (4.5)	1.3 x10^7 (8.25)
	M. natalensis	3 (4/4)	6-7 (1.5)	4.6 x10^6 (6)
Mundri 09 (K03043)	C57/black	3 (4/4)	5-8 (3.75)	7.4 x 10^7 (6.75)
	Balb/C	3 (4/4)	5-9 (4.75)	6.4 x 10^7 (7.25)
	Swiss white	3 (4/4)	5-7 (3)	1.4 x 10^6 (6.5)
	M. natalensis	3 (3/4)	5-7 (3)	0.8 x 10^7 (6)
Mundri 13 (K03010)	C57/black	3 (4/4)	5-14(6.25)	1.5 x 10^8 (7.5)
	Balb/C	3 (4/4)	5-14 (6.26)	8.9 x 10^7 (7.5)
	Swiss white	3 (4/4)	5-7 (1.75)	3.2 x10^6 (6.25)
	M. natalensis	3 (2/4)	5-7 (2.5)	1.1 x10^7 (5)
Mundri 17 (K03032)	C57/black	3 (3/4)	5-18 (13)	5.0 x 10^8 (14)
	Balb/C	3 (3/4)	6-9 (3.33)	5.0 x 10^7 (7.33)
	Swiss white	3 (4/4)	6-7 (2)	5.0 x 10^6 (6.5)
	M. natalensis	3 (4/4)	5-7 (3)	6.4 x 10^7 (7)

Data shown are means. Group size = 4.

Table 4: Comparison of the growth characteristics of *T. b. gambiense* isolates in different rodents. The animals were immunosuppressed with cyclophosphamide 300mg/kg prior to infection. Each animal was inoculated with 4 x 10^4 trypanosomes (passage 3) intraperitoneally. Parasitaemia was monitored every other day by examination of tail blood for 20 days as described in Table 2.

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CHAPTER 7: Genotypic and phenotypic characterization of *T. b. gambiense* isolates from Ibba, South Sudan, an area of high melarsoprol treatment failure rate

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Abstract

As a consequence of drug resistance problems, melarsprol was replaced by eflorinthine as first line drug in many treatment centres in south Sudan. Resistance of trypanosomes to melarsoprol is ascribed to reduced uptake of the drug via the P2 nucleoside transporter. The aim of this study was to look for evidence of drug resistance in *T. b. gambiense* isolates from sleeping sickness patients in Ibba, South Sudan, an area of high melarsoprol failure rate. Eighteen *T. b. gambiense* stocks were phenotypically and genotypically characterized. *In vitro*, all isolates were sensitive to melarsoprol, melarsen oxide, and diminazene. Infected mice were cured with a four day treatment of 2.5 mg/kg bwt melarsoprol, confirming that the isolates were sensitive. The gene that codes for the P2 transporter, *TbATI*, was amplified by PCR and sequenced. The sequences were almost identical to the $TbAT1^{sensitive}$ reference, except for one point mutation, C1384T resulting in the amino acid change proline-462 to serine. None of the described $TbAT1^{resistant}$-type mutations were detected. In a *T. b. gambiense* sleeping sickness focus where melarsoprol had to be abandoned due to the high incidence of treatment failures, no evidence for drug resistant trypanosomes or for $TbAT1^{resistant}$-type alleles of the P2 transporter could be found. These findings indicate that factors other than drug resistance contribute to melarsoprol treatment failures.

Key words: *T. b. gambiense,* Sudan, Melarsoprol, *TbAT1* gene, Drug sensitivity

Introduction

Human African Trypanosomosis (HAT) caused by *Trypanosoma brucei gambiense* is widespread in western and central Africa - eastwards from Senegal to Zaire, southwestern Sudan and northwestern . It is estimated that 60 million people are at risk of infection, 500,000 people are already infected and approximately 50,000 die every year (WHO 2004). Control of the disease depends on chemotherapy but only a small number of drugs are available. The choice of drug further depends on the stage of the disease. The early stage relies on pentamidine and the late stage, when trypanosomes have invaded the CNS, on melarsoprol, eflornithine and nifurtimox.

Melarsoprol, an arsenical, is the first-line drug for treatment of the 2nd. stage disease. Treatment failure rates of 3% to 9% are normal and are attributed to patient-related factors (concomitant infections, nutritional status etc). However, in recent years high rates of melarsoprol treatment failures (>20%) have been reported in *T. b. gambiense* endemic areas (Burri & Keiser 2001;Legros *et al.* 1999;Ruppol & Burke 1977) and there has been a growing interest in the cause(s). Since drug levels in blood or CSF were not found to be different between relapse and successfully treated patients (Burri & Keiser 2001), drug resistance has been suggested as a likely cause for melarsoprol treatment failures. Melarsoprol resistance has been induced in *T. b. brucei* laboratory strains and was shown to be caused by loss of the P2 adenosine transporter that mediates also uptake of melarsoprol and diamidines (Carter and Fairlamb 1993, Carter *et al*., 1995). Subsequently it was shown that the transporter is encoded by the *TbAT1* gene (Maser *et al.* 1999;Matovu *et al.* 2003) and recent studies indicated a possible link between particular *TbAT1* alleles and occurrence of relapses (Maser *et al.* 1999;Matovu *et al.* 2001b). However, all studies dealing with field isolates have focused on genetic markers of resistance rather than drug sensitivity testing, probably because of the challenges in isolation and propagation of *T. b. gambiense*. Thus, it has not been clarified whether melarsoprol treatment failures are actually caused by drug-resistant trypanosomes (Brun *et al.* 2001;Matovu *et al.* 2001a).

We recently isolated 18 *T. b. gambiense* stocks from Ibba centre, Maridi county, western Equatorial, south Sudan where high treatment failure rates had been reported (Moore & Richer 2001). The trypanosome isolates were propagated and the sensitivities to melarsoprol, melarsen oxide, pentamidine, diminazene, DB 75 and nifurtimox determined *in vitro.* The sensitivity of nine of these isolates to melarsoprol was further tested *in vivo*. Then, the *TbATI* gene was amplified, sequenced and compared with published sequences of melarsprol sensitive and resistant isolates.

Materials and methods

Trypanosomes

Eighteen *T. b. gambiense* stocks isolated in 2003 from HAT patients attending the MSF-F Ibba hospital in Maridi, western Equatorial, south Sudan, were used (Maina *et al*., submitted). *Trypanosoma b. gambiense* STIB 930 (a derivative of TH1/78E (031) which was isolated from a patient in Cote d'-Ivoire in 1978 was used as the reference sensitive strain.

***In vitro* drug sensitivity assay**

The medium used was a mixture of RPMI 1640 and MEM (1:1), supplemented with 5% heat-inactivated fetal bovine serum (FBS), 12% inactivated human serum, 0.05 mM bathocuproine sulphonate, 0.12 mM 2-mercaptoethanol, 1 mM sodium pyruvate, 2 mM L-glutamine and 1.5 mM L-cysteine. Trypanosome isolates were propagated in laboratory rodents and harvested by cardiac puncture. Separation of the trypanosomes was as described by Lanham and Godfrey (1970). Trypanosomes (2 x 10^6 ml^{-1}) were incubated in 96-well microtiter plates at 37^{o}C in a humidified atmosphere containing 5% CO_2 with serial dilutions of the trypanocidal drugs (in duplicate) for 24 hours. Tritiated hypoxanthine was then added to each well giving a final concentration of 1 µCi/well and the plates were incubated for a further 16 hours. Trypanosomes were then harvested on a multimash 2000 cell harvester (Dynatech) where the parasites were lysed hypotonically and macromolecules trapped on filter paper. The filters were dried in a microwave at 85°C and radioactive incorporation measured by liquid scintillation counting. The number of counts per minute for trypanosomes incubated in different drug concentration was expressed as a percentage of control cultures. IC_{50} were calculated by linear interpolation (Huber & Koella 1993). The test was repeated at least three times. Data are presented as means and standard deviation (SD).

In vivo drug sensitivity determination in mice

Swiss White mice weighing 20-25 g were used. Briefly, the mice were immunosuppressed with 300 mg/kg bwt cyclophosphamide one day prior to infection and again on days 14 and 28 post infection. Nine of the 18 isolates were tested. Briefly, for each stabilate, eight mice were infected with *T. b. gambiense* stocks intraperitoneally with 5 x 10^4 trypanosomes. One group of four mice was treated with 2.5 mg/kg bwt melarsoprol daily for four days (day 4-7) while the other group served as an untreated control. Parasitaemia was determined by examination of tail blood using the HCT method (Woo 1970) as follows; on day four (before treatment), on day seven (immediately after end of treatment), thereafter twice a week for the first two weeks and then once a week until day 60. Mice with no detectable parasitaemia at day 60 were considered cured.

PCR

DNA was extracted from bloodstream form (BSF) trypanosome isolates (passaged once in a laboratory rodent) using the Puregene DNA extraction Kit (Gentra Systems, Minneapolis, USA) (Matovu *et al.* 2001b).

Full length *TbAT1* was amplified from genomic DNA by hot start PCR with primers ant-s (5'-GCCCGGATCCGCTATTATTAGAACAGTTTCTGTAC-3') and ant-as (5'-GCCCCTCGAGCCGCATGGAGTAAGTCTGA-3'). This initial PCR step was followed by second round of nested PCR using the primer pair ATLF (5-GAAAGCTTAATCAGAAGGATGCTCGGGTTTGACTACA-3) and ATLR (5-GAGGATCCTGAACAGTATTCGTATGACGATTAGTGCTAC-3). For control PCR, the actin gene was amplified using primers act-s (5-CCGAGTCACACAACGT-3) and act-as (5-CCACCTGCATAACATTG-3). The amplification was done in 50 µl reaction volume containing 1 x PCR buffer (Qiagen), 200 mM of each dNTP, 1 µM of each primer, and 1 unit of Taq polymerase in a PTC 200 Peltier (MJ Research) thermocycler under the following conditions: initial denaturation at 94 ^{O}C for 3 minutes; 30 cycles of 94 ^{O}C for 45 seconds, 55 ^{O}C for 45 seconds and 72 ^{O}C for 120 seconds; a final extension at 72 ^{O}C for 10 min, followed by rapid cooling at 4 ^{o}C. The products were analyzed by gel electrophoresis through 1.25% agarose gel stained with ethidium bromide (0.2µg/ml) and visualized under UV light.

Cloning of PCR products

For two isolates (I04015 and KO3048) the *TbATI* gene was cloned. The PCR products were precipitated (10% volume of 3 M NaCl and 2.5 volumes of ethanol), re-dissolved in H_2O, and ligated into the vector pGEM ® T-easy (Easy Vector System, Promega USA). The plasmids were isolated from transformed *E. coli* DH5α and purified using the QIAprep Spin Miniprep Kit. Inserts were sequenced with the standard primers T7 and SP6.

Direct sequencing of PCR products

The *TbAT1* gene of ten of the isolates was sequenced. Two independent PCR reactions were pooled and purified (QIAquick kit, Qiagen, Basel, Switerzland). For each pool, four sequencing reactions were done in a PTC 200 Peltier (MJ Research) thermocycler under the following conditions: 40 cycles of 96^{O}C for 50 seconds, 96^{O}C for 10 seconds, 50^{O}C for 10 seconds; 60^{O}C 4 minutes. The excess dye was removed (DyeEx™ 2.0 spin columns, Qiagen), sample dried and run at the CMG sequencing facility of the Zoology Department, University of Bern.

Results

In vitro sensitivity of isolates

The sensitivities to melarsoprol, melarsen oxide, pentamidine, diminazene, DB 75 and nifurtimox of 16 *T. b. gambiense* isolates, were tested *in vitro* (for two isolates parasitaemia achieved was too low for the assays). Sensitivity of the isolates to melarsoprol (5 ± 1.47nM) and melarsen oxide (3.9 ± 0.9nM) were significantly similar. The sensitivities to diamidines; pentamidine, diminazene and DB 75 were within a similar range. In addition, the sensitivities to all the drugs tested were similar to those of a reference sensitive isolate *T. b. gambiense* STIB 930 (Table 1). Thus none of the isolates showed reduced sensitivity to the drugs tested.

In vivo drug sensitivity determination in mice

In order to test the *in vivo* susceptibility of the *T. b. gambiense* isolates to melarsoprol, immunosuppressed Swiss White mice, eight per stabilate, were infected. The animals were parasitaemic by the fourth day post-infection. All melarsoprol treated mice were cured. Trypanosomes were not detectable from day seven (immediately after the end of treatment) and the animals remained aparasitaemic during the whole experimental period of 60 days. In addition, none of the treated animals died during the experimental period.

The control groups remained parasitaemic with the parasitaemia pattern varying between the isolates. The parasitaemia was sporadic and inconsistent for most of the isolates. The *T. b. gambiense* isolates caused a chronic disease and some mice died during the experimental period (Table 2).

Characterization of *TbAT1*

As determined by PCR, the *TbAT1* gene was present in all the *T. b. gambiense* isolates. The PCR products had the expected size of about 1400 bp. The PCR products of 10 of the isolates were directly sequenced and compared to the published reference sequences; *TbAT1* $^{\text{sensitive}}$ (GenBank accession number AF152369) and *TbAT1* $^{\text{Resistant}}$ (accession number AF1552370) (Maser *et al.* 1999)(Figure 2). The *TbAT1* sequences of the 10 isolates were almost identical to the *TbAT1* $^{\text{sensitive}}$ reference sequence. However, at position 1384 a new mutation was noted in all the isolates, C1384T. In addition, two new mutations were noted in isolate I03022, A83G and C1086T. The mutation C1086T is silent while A83G results in amino acid change asparagine-28 to serine. C1384T mutates proline-462 to serine. The amplified genes lacked

any of the nine mutations described from melarsoprol resistant isolates. Direct sequencing of pooled PCR products from independent reactions has the advantage that mutations introduced by Taq polymerase are not seen in the electrophoretogram. However, since the *T. b. gambiense* isolates were not cloned, direct sequencing of PCR products will not detect *TbAT1* alleles present only in a minority of the trypanosomes. We also determined the sequence of cloned PCR products and found three more mutations: a trinucleotide deletion of codon 26 and two silent mutations, C-1086 to T and A-1168 to G (Table 3).

Discussion

The MSF-F hospital in Ibba was the only second-stage sleeping sickness (SS) treatment centre in western equatorial south Sudan, serving both Maridi and Mundri counties. In this hospital, high melarsoprol failure rates (20%) have been reported (Moore & Richer 2001). In addition, a high death rate following melarsoprol treatment was also being recorded (MSF 2001). The efficacy of melarsoprol was questioned leading to a change in the treatment approach. In September 2001, DFMO became the first-line drug for the 2nd stage disease. However, this was done without determining the cause of the melarsoprol treatment failures. In line with current priorities (Brun *et al.* 2001;WHO 1999) this study aimed at elucidating the cause(s) of the high rates of treatment failure in that area.

Decreased sensitivity in the trypanosomes population is suspected to be an important contributor to treatment failure (Matovu *et al.* 2001a). However, sensitivity determination was done using only a few recently isolated parasites (Brun *et al.* 2001;Matovu *et al.* 2001a) mainly because *T. b. gambiense* is difficult to isolate and propagate (*in vitro* or in rodent hosts) (Brun *et al.* 2001). Here we used eighteen *T. b. gambiense* stocks that were recently isolated from HAT patients attending the Ibba SS hospital. *In vitro* drug sensitivity tests indicated that the isolates are sensitive to melarsoprol and melarsen oxide. IC_{50} values to melarsoprol were in the same range as those of older *T. b. gambiense* isolates from Cote d'Ivore (Brun *et al.* 2001). *In vivo* the isolates were sensitive to 2.5 mg/kg bwt melarsoprol administered for four consecutive days.

The use of melarsoprol for *in vitro* determination of arsenical sensitivity has been questioned mainly because in plasma, melarsoprol is rapidly metabolized to form active metabolites including melarsen oxide. Melarsen oxide is the active drug not melarsoprol (Keiser *et al.*

2000). Nevertheless most laboratories continue to use melarsoprol in tests since it is readily available as compared to melarsen oxide. In this study therefore, we assessed the sensitivities of the *T. b. gambiense* isolates to these two compounds. There was no difference in the IC_{50} values obtained with melarsoprol and with melarsen oxide, indicating that melarsoprol can be used for determination of arsenic sensitivity *in vitro*. The isolates from the HAT patients were also sensitive to the trypanocides in current use (pentamidine and nifurtimox) and to DB 75 the active principle in DB 289, a promising diamidine for oral treatment (Jannin & Cattand 2004).

These sensitivity tests were conducted on parasites that had been sub-passaged between four and seven times in rodents. It is an inherent problem with *T. b. gambiense* that drug sensitivities cannot be determined with trypanosomes isolated freshly from an infected patient, as these cells do not grow *in vitro* and have a very low virulence to rodents (Aerts *et al.* 1992;Dukes *et al.* 1989;Matovu *et al.* 2001a). Sub-passages in rodents are therefore inevitable; in this study we kept them at a minimum in order to preserve the nature of the isolates and prevent selection.

Resistance to melarsoprol has been suggested to be due to reduced drug uptake. The P2 nucleoside transporter mediates the uptake of melaminophenyl arsenicals (Carter & Fairlamb 1993) and diamidines (Barrett *et al.* 1995;Carter *et al.* 1995). The transporter is encoded by the *TbAT1* gene and recent studies indicated a possible link between particular *TbAT1* alleles and occurrence of relapses (Maser *et al.* 1999;Matovu *et al.* 2001b). All the *T. b. gambiense* isolates analyzed here contained a full-size *TbAT1* open reading frame. To date only in one isolate, *T. b. gambiense* K001 from Angola, has the *TbAT1* gene been found deleted (Matovu *et al.* 2001b), indicating that deletion of *TbAT1* may not occur frequently. The amplified genes lacked the ten mutations noted earlier in resistant isolates and were almost identical to the *TbAT1* reference sequence from melarsoprol sensitive *T. b. brucei*. All sequences had one new coding mutation C1384T (resulting in P462S); a few sequences carried additional mutations (Table 2). All these mutations have not been reported before. Though their functional implications, if any, still need to be investigated, there is at present no evidence for $TbAT1^{resistant}$-type alleles in the *T. b. gambiense* isolates characterized here.

It is important to note that none of the eighteen patients had previously been treated with melarsoprol and hence there is no clinical evidence of resistance (melarsoprol stopped to be

used as the first-line drug in September 2001). Nevertheless the parasites were isolated from an area of very high rate of melarsoprol refractoriness (Moore & Richer 2001) and resistance to melarsoprol was shown to be stable even in the absence of drug pressure (Kaminsky *et al.* 1989;Scott *et al.* 1996).

In conclusion, all *T. b. gambiense* isolated from patients in south Sudan were sensitive to melarsoprol. At this point, we cannot formally exclude a bias against eventual melarsoprol-resistant trypanosomes during isolation. Nevertheless, our results indicate that factors other than drug resistance may contribute to relapses from melarsoprol therapy. Recently isolated *T. b. gambiense* from Cote d`-Ivoire, Cameroon and DR Congo were also found to be sensitive to melarsoprol (R. Brun personal communication). Similarly in isolates from NW Uganda, there was little evidence of resistance in *T. b. gambiense* to melarsoprol (Matovu *et al.* 2001a). These findings cast doubts on the current suggestion that melarsoprol resistant parasites are the cause of the high rate of treatment failures. Host factors and other parasite-derived- factors, such as affinity of trypanosomes to extravascular sites that are less accessible to melarsoprol, also need to be investigated in order to explain the high melarsoprol relapse rates in certain sleeping sickness foci. A combination of factors may be responsible for the phenomena of melarsoprol treatment failures in *T. b. gambiense* patients (Brun *et al.* 2001).

	Melarsoprol	Melarsen oxide	Pentamidine	Diminazene	DB 75	Nifutimox
STIB 930*	**10.3 ± 6.7**	**6.5 ± 5.6**	**6.74 ± 1.6**	**8.98 ± 2.3**	**12.9 ± 0.8**	**1748 ± 189**
K03048	5.9	4.2	23.2	12.9	3.6	469.9
K03043	2.1	3.3	8.3	5.6	9.4	1117.3
K03030	4.7	2.3	11.7	4.7	4.4	1385.3
K03028	5.7	2.4	6.1	2.1	13.3	2631.4
K03042	3.8	2.5	6.3	16.1	18.4	417.7
I04008	4.9	3.8	5.8	9.3	2.1	525.6
K03051	3.9	4.0	17.0	8.8	13.9	341.1
I04012	5.9	6.4	3.2	11.6	8.9	978.0
I04019	8.5	2.7	14.3	10.1	17.8	1148.6
I03030	2.5	1.3	7.4	7.1	13.5	396.8
K03045	7.0	3.1	3.2	10.1	1.9	nd
I03037	2.8	2.0	10.3	12.7	nd	nd
K03010	3.3	6.3	8.9	14.5	nd	nd
K03054	1.3	3.0	Nd	6.3	nd	nd
K03032	10.8	8.2	Nd	0.7	nd	nd
104021	8.8	7.3	Nd	1.9	nd	nd
Means for all isolates	**5 ± 1.47**	**3.9 ± 0.9**	**10.98 ± 0.9**	**8.68 ± 2.3**	**8.39 ± 0.9**	**928.4 ± 102**

Table 1: *In vitro* sensitivities of *T. b. gambiense* isolates from south Sudan. The results are from a hypoxanthine assay and are given as means of IC_{50} (nM) from four independent assays (mean ± 2SD are shown for the reference strain and for the isolates combined). **T. b. gambiense* STIB 930 a melarsoprol sensitive isolate was used as the reference.

Isolate No.	Parasitaemia											No of animals that died during the experimental period (Days post infection)
Days post infection	**4**	**8**	**12**	**15**	**19**	**26**	**33**	**40**	**47**	**52**	**60**	
K03048	++	++	+++	+++	+++	+++	+++	++	++	n	+++	2 (23,35)
K03043	++	++	++	+	+	++	n	n	n	++	N	1(28)
K03042	++	+	n	n	n	+	++	++	n	+	++	0
I04008	+	+	+	+	++	++	++	n	n	+	++	3(47,47, 47)
K03051	+++	+++	+++	++	+++							3 (4, 24, 24, 24)
I03037	+++	++	++	++	+++	+++	+++	++	+++			4(34, 47, 49, 53)
K03010	+++	++	+	++	++	n	++	++	++	+++	+++	3(54, 55, 59)
K03032	++	+	+	++	++	+	+	+	+	++	++	1(58)
104021	+	n	+	+	++	+++	+++	+++	+++	++	+++	0

Table 2: Infection pattern of nine human *T. b. gambiense* isolates in immunosuppressed and untreated Swiss White mice. Haematocrit centrifugation technique was used to estimate parasitaemia - graded as; n- negative, + (1-5), ++ (6-15) and +++ (more than 15). All the trypanosome isolates had a pre-patent period of at most four days. Melarsoprol treated (2.5mg/ml for four consecutive days) mice were aparasitaemic from day 8 to the end of the experimental period and none died during the 60 day period (data not shown).

Position in gene		76-78	83	1086	1168	1384
	Isolate					
	TbATI ^{s}	**GTG**	**A**	**C**	**A**	**C**
	K03043	GTG	A	C	A	T**
	K03030	GTG	A	C	A	T**
	K03028	GTG	A	C	A	T**
	I03037	GTG	A	C	A	T**
	K03051	GTG	A	C	A	T**
	I04008	GTG	A	C	A	T**
	104022	GTG	G**	T***	A	T**
	K03045	GTG	A	C	A	T**
	I04015	GTG	A	C	A	T**
	I04015c	GTG	A	T***	G***	T**
	K03048	GTG	A	C	A	T**
	K03048c	---*	A	C	A	T**

Table 3: **Comparison of the *T. b. gambiense* isolates amplified *TbATI* gene with *TbATI* ^{sensitive} (GenBank accession number AF152369) (Maser *et al.* 1999)**. A few mutations were noted; a coding mutation C1390T in all the isolates, another coding mutation (A83G) and a silent mutation (C1086T) in I04022. The cloned gene from 104015 and K03048 mutation at C1390T was also noted. Additional mutations were noted in cloned genes. The mutations resulted in *amino acid loss, **coding mutation, ***silent mutation.

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CHAPTER 8: General Discussion

This thesis was conducted under the auspices of the Eastern Africa Network for Trypanosomosis (EANETT). The network was founded in 1999 with a mission to improve control of animal and human African trypanosomosis in Eastern Africa. Member countries include Uganda, Kenya, Sudan, Tanzania, Malawi and Switzerland. Between the member institutions in these countries, the network aims at establishing collaboration in research, training and control of trypanosomosis, links for exchange of information and technologies. The study reported here is an example of the links developed by the network which enabled the work to be carried out in three of the member countries Sudan, Kenya and Switzerland.

This thesis addresses one of the objectives of the network -to identify the reasons for melarsoprol treatment failures in *T. b. gambiense* sleeping sickness patients and thus enable the development of strategies for the treatment of refractory cases. In the EANETT member countries, high treatment failure rates have been reported in North West Uganda and southern Sudan. In Uganda, this problem was being addressed by scientists in Livestock Research Institute (LIRI) (Matovu *et al.* 2001b;Matovu *et al.* 2001a) but no corresponding studies had been carried out in south Sudan. The current study focused on one of the areas in south Sudan, Ibba in Maridi county, where high treatment failure rates have also been reported.

In the Maridi county, Médecins Sans Frontières France (MSF-F) runs a HAT control program that depends on case detection (passive and active surveillance) and treatment of the diagnosed patients at the Ibba MSF-F hospital. This was the only treatment centre for the second-stage disease serving both Maridi and Mundri counties. The high failures rates (20%) (Moore & Richer 2001) and high mortality after melarsoprol treatment led to poor recruitment of patients to this centre (MSF report, 2001). The efficacy of melarsoprol was questioned and it was replaced by eflornithine as the first-line drug for the second stage disease. However, this change was however done without determining the cause of the treatment failures but melarsoprol resistance has been suggested as a likely explanation. In line with current priorities (Brun *et al.* 2001;WHO 1998) our goal was to elucidate the role of drug resistant trypanosomes in the high rates of treatment failure. However, there were no recently isolated *T. b. gambiense* available and the first challenge was therefore to isolate parasites from the patients in this area.

Isolation of *T. b. gambiense* from HAT pateints

Prior to the field studies, laboratory studies geared at improving the isolation protocols were carried out. We first had to determine the duration the liquid nitrogen in the tank and in the dry shippers would last. Scientific studies in areas experiencing conflict have several limitations and thus execution of even the best study protocols may not be successfully carried out on the ground. This part of the study was done in collaboration with MSF-F. The organization has been working in the area since 1999. At the time of the study, MSF-F conducted HAT screening (passive and active) in the two Mundri and Maridi counties, and was running two treatment centres; first and second stage treatment centre at Kotobi in Mundri and at Ibba in Maridi County, respectively. As such the community had a lot of confidence in the organization. Some of the benefits of working under such an organization will be discussed briefly.

a) Assurance of security

The area has had civil war since 1952 and was under the control of the Sudan People Liberation Movement/Army (SPLM/A). In the market places and in other public places the ex-soldiers were never shy to show off their armory that ranged from modern guns, grenades to traditional machetes, bows and arrows. MSF-F remained in a state of general alert and maintained high security guidelines at all times. Bomb shelters and runaway bags were the first items visitors were introduced to. This was essential since attacks on MSF premises occasionally occurred and at such times all the foreign staff would be evacuated. Such interruption of the control activities led to the delay of the field studies for more than six months.

b) Enormous resources are required for HAT investigation in the field

Enormous resources (personnel and finances) are needed to undertake field work on HAT. The advantages of working within the established MSF-F program therefore cannot be underestimated. The longstanding civil war has led to destruction of the infrastructure and, worse, hindered the rebuilding. The only reliable mode of transportation within excursion into and out of the region was limited to air on the NGO's cargo aircrafts. The area also experience heavy equatorial downpours and during such times, landing or take off on the earth airstrips are greatly a precarious affair. This becomes limiting since essential supplies

such as liquid nitrogen required for trypanosome storage were not available in south Sudan. Secondly there were no permanent roads and even on the best roads the maximum speed was only about 30 km/hour. Lack of public transport in the region meant that the MSF-F mobile team had i) to go into the villages to screen the communities, ii) transfer those diagnosed positive to the treatment centres iii) then transfer them back home after treatment. In summary, the field study had to coincide with the dry season, active screening surveys, flight in/out of Maridi and the area had to be relatively safe.

Implication of main findings and suggestions for further research

a) Cryopreservation and primary propagation of *T. b. gambiense*

There are several possibilities to isolate bloodstream forms of *T. b. gambiense* from patients (Brun *et al.* 2001). However, cryopreservation of infected blood samples in liquid nitrogen and subsequent propagation in susceptible rodents has been suggested as the most promising approach and hence this method was used. Samples of blood from 50 HAT patients were cryopreserved in Triladyl®, stored in vapour nitrogen and airlifted to the Trypanosomiasis Research Centre (TRC) of KARI in Kenya.

Successful isolation of trypanosomes depends on propagation of the stabilates in laboratory rodents. Previous studies have shown that propagation of *T. b. gambiense* is complicated by the low parasitaemia in patients and the limited susceptibility of common rodents (Aerts *et al.* 1992;Matovu *et al.* 2001a). In this study the susceptibility was increased by using immunosuppressed *Mastomys natalensis* and Severe Combined Immunodeficient (SCID) mice for primary propagation. Eighteen *T. b. gambiense* cryosamples were, as a result, successfully propagated. These isolates are stored in two cryobanks at the TRC-KARI, Nairobi, and the Swiss Tropical Institute (STI), Basel. This is the first report of successful isolation of a large number of *T. b. gambiense* isolates from HAT patients. The isolates are an important resource especially for pre-clinical screening studies of up-coming trypanocidal compounds.

Previous studies have reported low recovery rates or complete loss of *T. b. gambiense* populations following cryopreservation in medium containing 10% glycerol (Brun *et al.* 2001;Matovu *et al.* 2001a). One main contribution of the present study is the improvement in

cryopreservation of *T. b. gambiense* (Chapter 3). The high recovery rate attained in this study may be attributed to the superior cryopreservation medium. Various teams have adopted the use of Triladyl cryomedium in *T. b. gambiense* isolation and higher isolation success has been achieved (Pyana *et al.* 2005).

The SCID mice are highly susceptible to *T. b. gambiense* (Inoue *et al.* 1998) and in this study we found that the SCID mice are more susceptible than immunosuppressed *M. natalensis*. However, the SCID mice are expensive (40USD/mouse) and are not readily available in Africa. Secondly, because they are immunodeficient, they are prone to various infections and therefore require specific housing and maintenance conditions. As such they may not be the appropriate choice in some endemic countries in Africa. In such situations immunosuppressed *M. natalensis* may be used.

In the present study, a colony of *M. natalensis* was established at KARI-TRC. The baseline biological reference values, reproductive performance as well as the optimum dosage regime of immunosuppression using cyclophosphamide, have also been established (Kagira *et al.* 2005). Breeders from this colony may be used to initiate colonies in *T. b. gambiense* endemic countries. Indeed one of the EANETT objectives is to establish colonies in member countries including Sudan, Uganda and Tanzania as was agreed at the EANETT workshop on standardization in 2005.

b) Propagation of *T. b. gambiense* in other species of mice

Normal in-bred or out-bred laboratory mice are generally thought not to be susceptible to *T. b. gambiense* isolates. In this study, however, we found that immunosuppression of mice species with cyclophosphamide (300 mg/kg prior to infection and repeated once/week at 200 mg/kg) increases their susceptibility to *T. b. gambiense.* Indeed, samples directly isolated from the patients were infective also for immunosuppressed Swiss White mice. In addition, at second passage, propagation of the isolates could be done in various species of mice but all species had to be immunosuppressed. Recently, it was described that *Grammomys surdaster,* which lives in mountains in Central Africa, is a good model for primary isolation of *T. b. gambiense* (Buscher *et al.* 2005*)*. The *G. surdaster* are more nervous than laboratory rodents and experience is needed in handling them. The age appropriate for experimentation (15 weeks old) is also higher than that of laboratory mice (21 days).

This study recommends that primary propagation and the subsequent subpassage should be done in immunosuppressed *M. natalensis* or in SCID mice. Further subpassages can then be done in immunosuppressed laboratory mice (e.g. C57/bl or Balb/C). However, the choice should depend on what mice species is available and on the facilities in the specific laboratories. Available mice breeds should be screened after immunosuppression with cyclophosphamide at 300 mg/kg prior to infection and repeated once a week at 200 mg/kg. This propagation will allow the characterization of *T. b. gambiense* from various hot spots, hence enhancing the monitoring of drug resistant trypanosomes.

c) Melarsoprol resistance in the *T. b. gambiense* isolates

In the Ibba treatment centre, high treatment failure rates (Legros *et al.* 1999;Moore & Richer 2001) and high mortality (MSF 2001) following melarsoprol treatment were considered serious. One of the major objectives of this PhD study was to determine whether the parasites-*T. b. gambiense* are melarsoprol resistant. This was addressed in two ways; determining the sensitivity of parasites to the drug and characterizing the *TbATI* gene which is thought to be involved in drug resistance.

i) Sensitivity tests

At the time of parasite isolation, the treatment approach for the second stage disease had changed and eflornithine had replaced melarsoprol as the first-line drug. Currently, melarsoprol is only used in eflornithine relapse patients and therefore melarsprol relapse patients are few. Ideally, resistance studies should be carried out on parasites isolated from relapse patients. In view of the fact that resistance to melarsoprol is stable even in the absence of drug pressure (Kaminsky *et al.* 1989;Scott *et al.* 1996); it would be expected that the melarsoprol resistant populations would still be circulating among the patients. In this study, none of the eighteen patients from whom the *T. b. gambiense* were isolated had previously been treated with melarsoprol.

There are various *in vitro* tests for determining the sensitivity of trypanosomes to trypanocides and to new compounds as reviewed by Kaminsky & Brun (1993). For sensitivity testing of field isolates the assay chosen should; i) not require pre-adapted trypanosomes ii) be able to distinguish between sensitive and resistant isolates iii) allow appropriate exposure of the parasites to the compound/trypanocidal drug . In this study we used the hypoxanthine incorporation assay since it meets these criterions (Brun *et al.* 1989;Brun & Kunz 1989;Brun

et al. 2001). Sensitivity tests indicated that the 18 *T. b. gambiense* isolates were sensitive to arsenicals (melarsoprol and Mel OX).

These results are in agreement with previous studies. Recently isolated *T. b. gambiense* from Côte d'Ivoire, Cameroon and DRC were also found to be sensitive to melarsoprol (R. Brun personal communication). Similarly, earlier studies have not identified any difference in susceptibility between relapse and first treatment cases (Brun *et al.* 2001;Matovu *et al.* 2001a). In addition, the isolates were sensitive to the trypanocides in current use (pentamidines and nifurtimox) as well as to DB 75, the active principle of DB 289, a promising drug for the 1st stage disease.

The other contribution of the current study is on the use of melarsoprol for *in vitro* determination of arsenical sensitivity. The use of melarsoprol for *in vitro* determination of arsenical sensitivity has been questioned mainly because in plasma, melarsoprol is rapidly metabolized (96% within 1 hour) to form active metabolites including melarsen oxide. Mel OX is the active drug not melarsoprol (Keiser *et al.* 2000). Nevertheless most laboratories continue to use melarsoprol in tests since it is readily available as compared to Mel OX. This study has demonstrated that there is no difference in the IC_{50} values obtained with melarsoprol and with Mel OX, indicating that melarsoprol can be used for determination of arsenical sensitivity *in vitro.*

ii) Nature of the *TbATI* gene in the isolates

Melarsprol resistance has been induced in laboratory strains and was shown to be caused by loss of the P2 adenosine transporter that also mediates uptake of melarsoprol and diaminidines (Carter & Fairlamb 1993;Carter *et al.* 1995). The transporter is encoded by the *TbAT1* gene (Maser *et al.* 1999;Matovu *et al.* 2003). The amplified *TbATI* gene of these Sudanese isolates were highly similar to that of the melarsoprol sensitive isolate (Maser *et al.* 1999). This further confirms the susceptibility test that the isolates are sensitive to melarsoprol.

Recent studies indicate that *TbATI* may not be the only gene involved in melarsoprol resistance. Maser and Kaminsky (1998) identified the ABC transporters and over-expression of *T. brucei* MRPA has been shown to result in resistance to melarsoprol (Alibu *et al.* 2006). The gene is predicted to mediate efflux of melarsoprol. Since these isolates were sensitive to

melarsoprol it was not necessary to characterise expression of *TbMRPA*. In isolates showing melarsoprol resistance it would be important to determine its role.

Markers for mapping melarsoprol resistance

The question of the reliable marker for melarsoprol resistance is still unanswered. Recent studies indicate that *TbATI* and *TbMRPA* genes might be reliable genetic markers. However, the contribution of each of these genes in the field, in particular to melarsoprol treatment failures, is yet to be determined. Indeed, genetic markers for resistance provide a fast and easy assessment of the problem and it is not a wonder that most of the studies dealing with field isolates have focused on genetic markers of resistance. Recently, detection of arsenical drug resistance in *T. brucei* with a simple fluorescence test has been developed (Stewart *et al.* 2005). These tests should be able to distinguish between melarsoprol resistant and sensitive phenotypes in the field. In addition, validation test should be carried out in various *T. b. gambiense* endemic areas.

Before these tests are validated, the susceptibility tests should be viewed as the gold standard for determining melarsoprol resistance. The findings to date cast doubts on the suggestion that melarsoprol resistant parasites are the cause of the high rate of treatment failures reported. However, it is important to note that the number of isolates tested so far is still small and it is vital that isolation of *T. b. gambiense* from melarsoprol relapse patients continues. Under the EANETT the protocols for sensitivity testing were standardized at the 7th EANETT workshop and their use in various laboratories may provide results that are comparable (EANETT 2005).

Other possible causes of the melarsoprol treatment failure

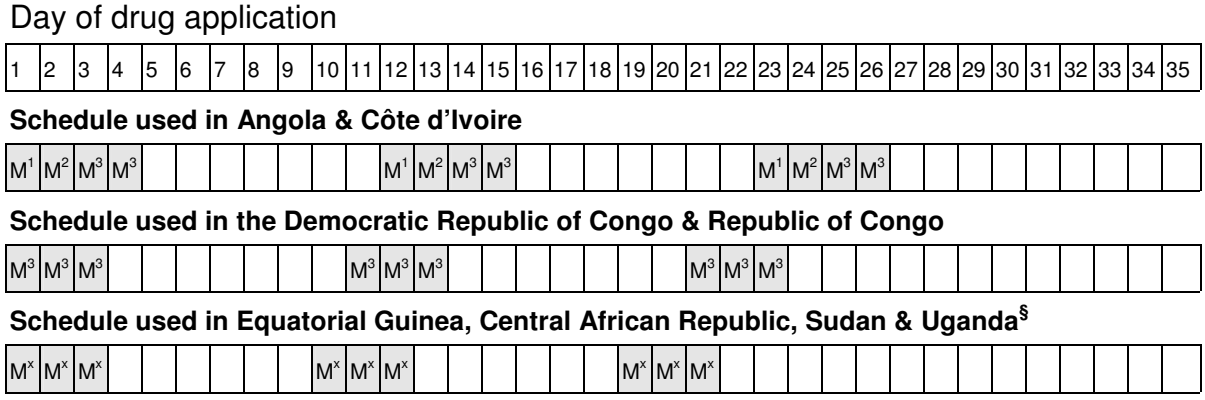
The current study did not identify the reason(s) for the high rates of treatment failure following melarsoprol treatment. As pointed out earlier by Brun *et al*. (2001), a combination of factors may be responsible for the phenomenon of melarsoprol treatment failure in HAT patients. Two factors, the parasite distribution in tissues and the melarsoprol dosage regime, are worthy further discussion.

a) Parasite distribution in tissues or organs

The other parasite factor that may affect the efficacy of melarsoprol is the parasite distribution in the tissue or organs where the drug levels are sub-therapeutic. Such organs will serve as areas where the parasites proliferate irrespective of the drug levels in blood and may well serve as the sources of parasites for re-invasion after cessation of drug levels. In a recent study in mice, relapse populations did not show any difference in sensitivity to selected drugs. Unfortunately, investigations on the distribution of trypanosomes in these extra-vascular sites are not straight forward but there are indications that the spleen may be one site (Bernhard 2006). Indeed *T. b. gambiense* is tissue invasive and the affinity of the parasite to extra-vascular sites other than the CNS which are inaccessible to melarsoprol may be responsible for the relapses. Whether the different strains have varying affinities for the extravascular tissue is not clear and further studies are necessary.

b) Melarsoprol dosage schedules, a possible cause of treatment failure

Melarsoprol was introduced for treatment of HAT before its pharmacokinetic parameters were fully understood and therefore the recommended schedules were empirically developed. The recommended schedules depend on the region as shown in figure 1.



M^1=Melarsoprol 1.2 mg/kg bw; M^2=Melarsoprol 2.4 mg/kg bw; M^3=Melarsoprol 3.6 mg/kg bw (max. 5 ml)
M^x=1 series of 1.8, 2.16, 2.52 mg/kg bw , 1 series of 2.52, 2.88, 3.25 mg/kg bw, 1 series of 3.6, 3.6, 3.6 mg/kg bw (max. 5 ml)

Figure 1: The older melarsoprol dosage schedules for treatment of *T. b. gambiense* patients as recommended by the WHO (WHO, 1986).

Further, differences in duration and mode of therapy have been observed depending on the country, local habits and the hospital involved. The different schedules would result in varying pharmacodynamics and therefore different responses in the patients. In fact, the high

frequency of relapses appears to be associated with the incremental dosage regimen of melarsoprol (Cross *et al.* 2006;Legros *et al.* 1999;Pepin & Mpia 2005;Schmid *et al.* 2004b;Stanghellini & Josenando 2001). In the Ibba hospital the incremental dosage regime (regime c) was also used. Under this regime the first dosage (1.2 and 1.8mg/kg) is too low and hence the low total dosage may explain the relapse. Ideally the parasites should be exposed to trypanocidal levels during the three or four days of the series. However, when the first dosage is low, it means that the parasites are exposed to sub-therapeutic levels of melarsoprol and hence the series of dosage are in reality too short to eliminate all parasites.

Current schedule of melarsoprol and the implication

Various studies (Burri *et al.* 1993;Burri *et al.* 1994;Keiser *et al.* 2000) shed light on the pharmacokinetics of melarsoprol and enhanced the development of the 10 days treatment schedule. Trials of the schedule have been carried out in various endemic countries (Burri *et al.* 2000;Schmid *et al.* 2004a;Schmid *et al.* 2005b). This schedule was recommended in 2003 by the International Scientific Council of Trypanosomiasis Research and Control (ISCTRC) as the schedule of choice in all *T. b. gambiense* endemic countries. In this new schedule the patient receives 2.16 mg/kg bwt for ten consecutive days, intravenously.

This results in a sustained level of melarsoprol in the plasma, thus trypanosomes are exposed to a constant melarsoprol concentration for 10 days. Standardized treatment means that relapses will be more comparable. In addition, a shorter regime is less burdensome for the patient, families, and health workers and is more cost-effective (Schmid *et al.* 2005a).

The recent findings (this PhD thesis and other studies) indicate that melarsoprol resitance might not be the cause of the high treatment failures reported. With this in mind and development of the new 10 treatment schedules, it would be reasonable to recommend melarsoprol use for the second stage disease. However, melarsoprol has another major limitation. It is highly toxic, with 5-10% of the treated patients developing acute encephalopathy and leading to fatality in approximately 50% of cases (WHO 1998). Concomitant infection with malaria is thought to increase the risk of encephalopathy (Pepin & Milord 1994). The other risk factors for encephalopathy include HIV (Blum *et al.* 2001), alcohol (Blum *et al.* 2001;Pepin & Milord 1994) and strenuous physical activity (Blum *et al.*

2001). Some of these risk factors cannot be entirely eliminated and thus high mortality will still limit the use of melarsoprol.

Way forward for HAT chemotherapy

a) Eflornithine

Eflornithine is currently the drug of choice for the second stage disease in many treatment centres. Eflornithine treatment is associated with a low (2-3%) case-fatality (Milord *et al.* 1992;Pepin *et al.* 2000) as compared to melarsoprol. Recently, the case-fatality rates and adverse effects of eflornithine (400 mg/kg per day for 14 days) and melarsoprol (2.2 mg/kg for 10 days) were compared in Kiri, Kajo-keji County. Eflornithine was shown to be safer than melarsoprol for the second stage disease. The case fatality rate is the lowest reported for second stage HAT. This safety has been confirmed in other treatment centres in Yei, Mundri and Maridi Counties (Chappuis *et al.* 2005). These findings further justify the preference of eflornithine over melarsoprol as the drug of choice for the treatment of the second stage disease of *T. b. gambiense.*

Limitations of eflornithine in HAT treatment

i) Intravenous eflornithine

The major drawback of the intravenous eflornithine is related to its high cost of treatment and the inconveniences of administration, especially in understaffed rural hospitals of the endemic countries. About 56 i.v. infusions (100mg/kg every 6 hours for 14 days) are administered, the approximate cost of these infusion and hospitalisation is at least USD 270 (Van Nieuwenhove 2000). This cost is still very high and the current challenge is oral administration of eflornithine (Na-Bangchang *et al.* 2004).

ii) Immunodepression in the patients

The other drawback of the current treatment regime is the use of eflornithine as monotheraphy. Eflornithine is trypanostatic, has a short half-life and thus has questionable efficacy in immunodepressed patients. Indeed, Milord *et al*. (1992) found that HAT patients co-infected with human immunodeficiency virus (HIV) responded poorly to eflornithine treatment. Earlier studies showed that HIV had no impact on epidemiology of HAT (Louis *et al.* 1991;Pepin *et al.* 1992b). Then, HIV was then relatively low in rural areas where HAT is

endemic. Today, the situation has changed and the prevalence of HIV is high even in rural areas and its role needs to be re-assessed.

Apart from HIV, the effects of other concomitant infections that lead to immunodepression also need to be considered. For instance, a high occurrence of malaria in HAT patients has been reported (Schmid *et al.* 2004a;Wellde *et al.* 1989b;Wellde *et al.* 1989a). Individually, malaria and trypanosomiasis lead to parasite–induced immunodepression, and concerted immuno-depression from the two parasites may lead to an even more severe immuno-depression in the host.

Filariasis is another parasitic disease that is prevalent in central and western Africa and frequently reported in HAT patients (Enyaru et al., 2002). The strong Th_1 known to occur in trypanosomosis infection is likely to counteract Th_2 responses, which are necessary for immunity to helminths (Cox 2001;Dwinger *et al.* 1994). Thus it would be expected that in patients with multiple infection the immune system may not mount adequate response to all infections. It would be important for the treatment centres to also treat these other infections as this might improve the prognosis of the patients.

It is imperative that the efficacy of eflornithine be protected by use in combination therapy rather than monotherapy (Moore 2005). One possible combination is eflornithine and melarsoprol. Experiments have shown synergism between eflornithine and melarsoprol (Jennings 1988b;Jennings 1988a;Jennings 1990). The few clinical studies carried out also indicate its effectiveness against melarsoprol resistant Gambian trypanosomosis (Mpia & Pepin 2002;Simarro & Asumu 1996).

Alone and in combination with melarsoprol or eflornithine, nifurtimox has shown promise in the treatment of late stage of *T. b. gambiense* infections in melarsoprol refractory patients (Pepin *et al.* 1992a) Nifurtimox is being used for treatment of South American trypanosomosis (Chagas disease) but not registered for HAT. The ‘Drugs for Neglected Diseases initiative’ and WHO Special Programme on Research and Training in Tropical Diseases are currently assessing the utility and potential registration of nifurtimox for HAT.

The recent five year donation of eflornithine, melarsoprol, nifurtimox, and pentamidine from Aventis to the WHO has made the drugs widely available for use. However, even though the

production of these drugs is temporarily assured, major efforts to improve the delivery of the drugs to the treatment centres are required**.**

b) Prospects for development of new drugs for HAT

The chemotherapy of HAT is greatly constrained by the limited number of drugs available. The situation is further complicated by the fact that in the last decades very few advances have been made. Sleeping sickness is transmitted mainly in rural Africa and thus has no profitable market that would encourage drug development (Nwaka & Ridley 2003).

Drugs can be developed by random screening, using the knowledge from traditional medicine or rationally. There has been a lot of research on natural products/traditional medicines (Hoet *et al.* 2004;Freiburghaus *et al.* 1996a;Freiburghaus *et al.* 1996b;Freiburghaus *et al.* 1997;Kubata *et al.* 2005) and the current challenge is to translate these insights into chemical insights that can form a basis for innovative drug discovery. Since 1999, the picture for drug development for neglected diseases has changed. Numerous partnerships between the public and private sectors have emerged to address this problem(Croft 2005).

Remarkable advances have been achieved by an international consortium that is funded by the Bill and Melinda Gates Foundation. The consortium consists of various institutions including the STI, which is responsible for *in vivo* and *in vitro* evaluation of compounds. The consortium has identified a diamidine prodrug DB289 that is promising for oral treatment of early-stage disease (Jannin & Cattand 2004). The consortium continues the search for clinical candidates for the late stage disease.

The other important initiative in trypanosomiasis is the 'Drugs for Neglected Diseases *Initiative*' (DND*i*). It's a global initiative to develop new affordable medicines for neglected diseases. The initiative catalyses and coordinates research, and manage drug development projects. It out sources most R&D activities - from discovery through predevelopment to development, including clinical trials - while maintaining leadership over the process (Pecoul 2004).

The current efforts in drug Research and Development may in the future provide new, effective, safe, and affordable drugs for the treatment of sleeping sickness.

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APPENDIX

STUDY OF REASONS FOR FAILURES AFTER MELARSOPROL TREATMENT IN SOUTH SUDAN

Study undertaken by the Kenya Trypanosomiasis Research Institute, Kikuyu, and the Swiss Tropical Institute, Basel, Switzerland, in the framework of the Eastern Africa Network for Trypanosomosis (EANETT)

Responsible for the study: Prof. Reto Brun[1]

Scientists carrying out the study: Naomi Wangari Maina[2] & Michael Oblere[1]

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[2]Kenya Trypanosomiasis Research Institute (KETRI), P. O. Box 362, Kikuyu, Kenya

Consent Form

Section I – general information

INFORMED CONSENT FOR: _____

What is your disease?

You are suffering from sleeping sickness, a serious disease which untreated might progress and lead to death.

What is the objective of the study?

Sometimes treatment with melarsoprol is failing. It is not clear why this is happening. One possibility is that the parasites which cause disease are resistant to the drug. It is therefore of paramount importance to have parasite populations available for studies of resistance in the laboratory.

What am I contributing to?

Your participation may help to find the reasons for melarsoprol treatment failures. This will lead to better treatment strategies and help to cure more patients.

What should I do to participate in the study?

In the course of your standard examination blood and cerebrospinal fluid have to be taken. Small amounts of your blood and spinal fluid will be frozen for subsequent studies.

Can I refuse to participate in the research?

Yes, you can refuse at any time to participate. If you refuse there will be no negative consequence. You receive treatment as appropriate.

Will confidentiality be kept?

Only the research team will have access to personal information. The samples will only be used for parasites only no further analysis on the samples will be done. Your anonymity and confidentiality will be safeguarded in any publication of the results of this research through use of pseudonyms.

Section II – Signatures

I agree to participate in the study to elucidate the reasons for failures after melarsoprol treatment, which involves collection of blood and cerebrospinal fluid.

Name and signature of hospital staff who read and explained the above text:

Name: _____ Signature: _____

Name and signature of participating patient or guardian:

Name: _____ Thumb print/Signature: _____

Witness (hospital staff, only applicable if the subject and/or his guardian are illiterate):

Name: _____ Thumb print/Signature: _____

Place and date: _____ _____

NB: The patients' retained Section I of this consent form. Further information was also provided, the pictorial brochures on sleeping sickness was given to each patient to take home. For the patients who gave consent, the questionnaire was filled out. Most of the patients/guardians could not read or write. As such the scientists assisted by an interpreter, usually a hospital assistant, asked the questions and filled the forms.

Questionnaire

You have been purposively selected to participate in this study. In this part of the study, you will be asked a few questions and further information may be extracted from your hospital records. Please answer the questions to the best of your knowledge. Your participation may help to find the reasons for melarsoprol treatment failures. This will lead to better treatment strategies and help to cure more patients (Most of the patients/guardians could not read or write. As such the scientists assisted by an interpreter (hospital assistant) asked the questions and filled the forms)

(Fill in the information asked ⌊_⌋⌊_⌋⌊_⌋⌊_⌋, tick appropriate box ☒

Patient No.	⌊_⌋		
Patient full name	⌊_⌋		
Date of admission	⌊_⌋⌊_⌋ (d ⌊_⌋⌊_⌋ m m ⌊_⌋⌊_⌋ y y)	Date of discharge	⌊_⌋⌊_⌋ (d ⌊_⌋⌊_⌋ m m ⌊_⌋⌊_⌋ y y)
Present residence	County ⌊_⌋ Payams ⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋ Bomas ⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋		
Probable place of infection	County ⌊_⌋ Payamas ⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋ Bomas ⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋⌊_⌋		
Age	⌊_⌋⌊_⌋ (Years)	Sex Male ☐ (m) Female ☐ (f)	
Weight	⌊_⌋⌊_⌋ (kg)	Height	⌊_⌋⌊_⌋⌊_⌋ (cm)
How long ago were first signs observed		⌊_⌋⌊_⌋ (Months) ⌊_⌋⌊_⌋ (Years)	
Previous treatment for trypanosomiasis yes ☐ (y) no ☐ (n) How many treatments have you had? ……			

Details of previous treatments for Trypanosomosis (if applicable)

Treatment number	**Diagnosis** Parasites in blood- (b CSF- (c White cell counts in CSF	**Medication** Arsobal® (a) Pentamidine (p) Nifurtimox (n) DFMO (D) Not known (n)	**Where** Private center (p) Public center (u)	**Was the treatment completed** Yes (y) No (n) Do not know (d)	**How long ago** 1 month (m) 6 months (s) 1 year (y) 2 years or more (2y)	Remarks (if hospital record are available)
1						
2						

NB : FOR MORE THAN THREE TREATMETENT ADDITONAL ROWS WOULD BE UDED.

PRESENT TREATMENT FOR TRYPANOSOMOSIS

Name of responsible for treatment	⌊_⌋

PRESENT TREATMENT: DAYS OF DRUG APPLICATION

(Tick box ☒ for each day when melarsoprol was applied, mark box with ❍ for each day when Nifurtimox and with D for each day DFMO was applied)

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25

If treatment deviates from normal schedule give justification in observations section (keywords only)

LABORATORY EXAMINATIONS AT ADMISSION / DISCHARGE

	Before treatment			After treatment		
Date of examination	⌊_⌋⌊_⌋⌊_⌋ ⌊_⌋⌊_⌋ ⌊_⌋⌊_⌋			⌊_⌋⌊_⌋⌊_⌋ ⌊_⌋⌊_⌋ ⌊_⌋⌊_⌋		
Consciousness	Fully conscious Drowsy ☐ (d) Comatose		☐ (p) ☐ (c)	Fully conscious Drowsy Comatose		☐ (p) ☐ (d) ☐ (c)
Lymphadenopathy (Swollen neck lymph nodes)	Absent Palpable		☐ (a) ☐ (p)	(indicate only if > 1 cm)		
Serology (If other than CATT used specify in field)	CATT	pos neg other n.d.	☐ (p) ☐ (n) ☐ (o) ☐ (b)	(If other test specify) ⌊_⌊_⌊_⌊_⌊_⌊_⌊_⌊_⌊_⌊_⌋		
Trypanosomes in lymph nodes	Microscopy	pos neg n.d.	☐ (p) ☐ (n) ☐ (b)	Microscopy	pos neg n.d.	☐ (p) ☐ (n) ☐ (b)
Trypanosomes in blood	Microscopy (Blood smear)	pos neg n.d.	☐ (p) ☐ (n) ☐ (b)	Microscopy (Blood smear)	pos neg n.d.	☐ (p) ☐ (n) ☐ (b)
Trypanosomes in CSF Double centrifugation of CSF used	Microscopy	pos neg n.d. yes no	☐ (p) ☐ (n) ☐ (b) ☐ (y) ☐ (n)	Microscopy	pos neg n.d. yes no	☐ (p) ☐ (n) ☐ (b) ☐ (y) ☐ (n)
White blood cells in CSF (no / mm^3) (Counting chamber)	Microscopy	⌊_⌊_⌊_⌋		Microscopy	⌊_⌊_⌊_⌋	
Malaria	Microscopy	pos neg n.d.	☐ (p) ☐ (n) ☐ (b)			
Filariae (incl. *Mansonella perstans*)	Microscopy	pos neg n.d.	☐ (p) ☐ (n) ☐ (b)	(Specify species if possible) ⌊_⌊_⌊_⌊_⌊_⌊_⌊_⌊_⌊_⌊_⌋		

CLINICAL EXAMINATIONS AT ADMISSION / DISCHARGE

	Before Treatment	After Treatment
Nutritional status (Circumference of upper arm, cm)	⌊⌊.⌋	⌊⌊.⌋
Fever (°C)	⌊⌊.⌋	⌊⌊.⌋
Headache	Yes ☐ (y) no ☐ (n)	yes ☐ (y) no ☐ (n)
Pruritus	yes ☐ (y) no ☐ (n)	yes ☐ (y) no ☐ (n)
Daytime sleep	normal ☐ (y) not ☐ (n)	normal ☐ (y) not ☐(n)
Nighttime sleep	normal ☐ (y) not ☐ (n)	normal ☐ (y) not ☐ (n)
Tremor	yes ☐ (y) no ☐ (n)	yes ☐ (y) no ☐ (n)
Speech impairment	yes ☐ (y) no ☐ (n)	yes ☐ (y) no ☐ (n)

	Before Treatment	After Treatment
Abnormal movements	yes ☐ (y) no ☐ (n)	yes ☐ (y) no ☐ (n)
Walking disability	yes ☐ (y) no ☐ (n)	yes ☐ (y) no ☐ (n)
General motor weakness	yes ☐ (y) no ☐ (n)	yes ☐ (y) no ☐ (n)
Unusual behavior	yes ☐ (y) no ☐ (n)	yes ☐ (y) no ☐ (n)
Inactivity	yes ☐ (y) no☐ (n)	yes ☐ (y) no ☐ n)
Aggressivity	yes ☐ (y) no ☐ (n)	yes ☐ (y) no ☐ (n)
Disturbed appetite (bulimia / anorexia)	yes ☐ (y) no ☐ (n)	yes ☐ (y) no ☐ (n)

Check yes if any deviation from normal pattern are observed

OBSERVATIONS AND OTHER DIAGNOSIS

(For additional information and comments only; all entries must be written clearly readable)

Date	Remark	Responsible for remark

OTHER DRUGS USED (Indicate only drugs additional to regular treatment in this center)	
Additional drugs used	yes ☐ (y) no ☐ (n)
1.	
2.	
3.	

Animal care and use

All protocols involving laboratory animals were approved by the KETRI Institutional Animal Care and Use Committee (IACUC).

Abbreviations

BSF	Bloodstream forms
CAR	Central Africa Republic
CATT	Card agglutination Test for Trypanosomiasis
CNS	Central nervous system
CSF	Cerebrospinal fluid
DFMO	DL-a-difluoromethylornithine
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
DRC	Democratic Republic of Congo
EANETT	Eastern Africa Network for Trypanosomosis
HAT	Human African Trypanosomosis
HB	Haemoglobin
HCT	Haematocrit centrifugation technique
HIV	Human Immunodefiency Virus
IC_{50}	Drug concentration inhibiting parasite growth by 50%
IMC	International Medical Corps
ISTRC	International Scientific Council of Trypanosomiasis Research and Control
KARI	Kenya Agricultural Research Institute
Mel B	Melarsoprol
Mel OX	Melarsen oxide
MEM	Minimum essential medium
MIC	Minimum inhibitory concentration
MSF	Médecines Sans Frontièrs
NGO	Non-Governmental Organizations
PCV	Packed cell volume
SPLM/A	Sudan's People Liberation Movement/Army
SRA	Serum Resistance Associated gene
SS	Sleeping Sickness
STI	Swiss Tropical Institute
TgsGP	*T. b. gambiense* specific glycoprotein
TRC	Trypanosomiasis Research Centre
WBC	Total white cell
WCC	White cell count
WHO	World Health Organization

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Curriculum Vitae

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Academic qualifications

1972-1978
Primary School at Siron, Nyahururu, Kenya
1979-1981
Secondary School in Naivasha Girls, Kenya
1982-1984
High school in Moi Forces Academy, Nairobi Kenya
1986-1989
Bachelor of Science, degree at the University of Nairobi, Kenya
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Masters of Science (Pharmacology) at University of Strathclyde, Glasgow, UK.
Project: Role of Monophosphoryl Lipid A on nitric oxide production in *leishmania* infected macrophages. Supervisor Dr. Christina Cater and Jim Alexander.
Practical attachment course, at the London School of Hygiene and Tropical Medicine (LSHTM), under Dr. S. L. Croft.
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PhD Studies in Medical Parasitology, under the supervision of Prof. Reto Brun at Department of Medical Parasitology and infection Biology, Swiss Tropical Institute (STI), Dr. Joseph Mathu Ndung'u, of Biotechnology Research Centre of KARI, Kenya and Prof. Pascal Mäser, of Institute of Cell Biology, Baltzerstrasse 4, 3012 Bern, Switzerland

Employment

1. At Jomo Kenyatta University of Agriculture Technology, from January 2006 as Lecturer in the Biochemistry Department.

2. At Kenya Trypanosomiasis Research Institute (KETRI) now TRC-KARI, from1989 to December 2005. In various positions and undertaking a variety of research activities as follows;

- Senior Research Officer: 2004 - Dec 2005
- Research Officer I: 1998 – 2004
- Research Officer II: 1993 - 1998
- Assistant Research Officer: 1989 - 1991

Presentations made at meetings and seminars during the PhD studies.

a) Annual workshops for the Eastern Africa Network for Trypanosomiasis.

Naomi Maina, Pascal Maser, Joseph Mathu Ndungu and Reto Brun (2004). Role of drug resistant trypanosomes in the high treatment Failure rates in South sudan. 7th, workshop. Mombasa Kenya.

Naomi Maina, Pascal Maser, Joseph Mathu Ndungu and Reto Brun (2004). Molecular characterization of trypanosomes isolated from HAT patients in southern Sudan. 6th, workshop. Khartoum, Sudan.

Naomi W. N. Maina, Christina Kunz and Reto Brun (2002).Cryopreservation of trypanosome brucei gambeinse in a cryoprotection medium developed for Bull semen. 4th workshop. Tororo, Uganda.

Naomi W. N. Maina, Michael Oberle, Charles Oteino, Christina Kunz, Mathu Ndungu and Reto Brun (2003). *Trypanosoma brucei gambiense* isolates from South Sudan: Propagation and characterization for Drug sensitivity and SRA gene. 5th workshop. Bagamoyo, Tanzania

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