# 4 MATERIAL AND METHODS

## 4.1 Origin and history of *T. congolense* and *T. brucei* stocks and clones

All the *T. congolense* and *T. brucei* stocks used in this study were isolated from naturally trypanosome-infected cattle in East and West Africa.

*T. congolense* stocks

The *T. congolense* field stocks (PA 73 and PA 77) were isolated from naturally infected cattle in Pawe district of northwestern Ethiopia (Table 2). The animals were parasitaemic 1-2 months post-treatment with 1 mg/kg bw isometamidium chloride (Afewerk et al., 2000). The isolates were passaged 3-4 times in mice and stored in liquid nitrogen at the Institute for Parasitology and International Animal Health, Free University of Berlin.

SA 267 and SA 268 were isolated in 1989 from naturally infected cattle from Kenedougou in southwestern Burkina Faso. These animals were not cured with 7 mg/kg bw diminazene, 1 mg/kg bw isometamidium and 1 mg/kg homidium. Chemotherapeutic trials in previously unexposed Zebu bulls and Sahelian goats infected with SA 268 demonstrated high levels of resistance to diminazene aceturate (7 mg/kg bw in cattle and 17.5 mg/kg bw in goats), isometamidium chloride (1 and 2 mg/kg bw i.v. in goats) and quinapyramine sulphate at 5 mg/kg bw in goats (Clausen et al., 1992).

The *T. congolense* stock SA 95 originated from an infected animal in the same area of Burkina Faso (Fama, Samorogoun) (Knoppe, 2002). In a drug sensitivity test, none of 5 experimental mice infected with this isolate were cured when treated with 1 mg/kg bw isometamidium, and only 2 out of 5 mice were cured when treated with 10 mg/kg bw isometamidium (Knoppe, 2002).

**Table 2:** Origin and year of isolation of isometamidium resistant *T. congolense* stocks

| Trypanosome stocks | Country | Year of isolation | Origin | Reference |
|---|---|---|---|---|
| MBOI/ET/97/PA 73 | Ethiopia | 1997 | Cattle | Afewerk et al., 2000 |
| MBOI/ET/97/PA 77 | Ethiopia | 1997 | Cattle | Afewerk et al., 2000 |
| MBOI/BK/89/SA 267 | Burkina Faso | 1989 | Cattle | Clausen et al., 1992 |
| MBOI/BK/89/SA 268 | Burkina Faso | 1989 | Cattle | Clausen et al., 1992 |
| MBOI/BK/98/SA 95 | Burkina Faso | 1998 | Cattle | Knoppe, 2002 |

*T. brucei* stocks

The *T. brucei* stocks used (Table 3) were isolated in 1995 from naturally infected cattle from different regions of Mukono County, Uganda (Pötzsch, 1999). Drug sensitivity studies both in mice and *in vitro* showed that none of the isolates were resistant to 1 mg/kg bw isometamidium, although some showed reduced sensitivity *in vitro* (Scheer, 2001).

**Table 3:** Origin and year of isolation of isometamidium sensitive *T. brucei* stocks from Uganda (Pötzsch, 1999; Scheer, 2002)

| *T. brucei* stocks | Region of isolation | Year of isolation | Origin |
|---|---|---|---|
| MBOT/UG/95 02 20 | Goma | 1995 | Cattle |
| MBOT/UG/95 04 01 | Goma | 1995 | Cattle |
| MBOT/UG/95 05 05 | Goma | 1995 | Cattle |
| MBOT/UG/95 05 25 | Goma | 1995 | Cattle |
| MBOT/UG/95 07 16 | Goma | 1995 | Cattle |
| MBOT/UG/95 10 17 | Goma | 1995 | Cattle |
| MBOT/UG/95 22 23 | Kauga | 1995 | Cattle |
| MBOT/UG/95 25 07 | Kauga | 1995 | Cattle |
| MBOT/UG/95 28 21 | Kyampisi | 1995 | Cattle |
| MBOT/UG/95 53 23 | Nakisunga | 1995 | Cattle |
| MBOT/UG/95 163 23 | Nakisunga | 1995 | Cattle |

## 4.2 Origin and history of *T. congolense* and *T. brucei* reference clones

*T. congolense* clones

**IL 2642** is a doubly-cloned population of *T. congolense*. It was derived from an isolate collected from a cow in Busoga, Uganda, in 1962 (Morrison et al., 1978). This population is known to be highly sensitive to isometamidium chloride in mice and cattle with $CD_{50}$ value of 0.007 mg/kg bw (Peregrine et al., 1988; Sones et al., 1988; Wilkes et al., 1997).

**IL 1180** is a doubly-cloned derivative of a *T. congolense* Savannha-type isolate collected from a lion in the Serengeti, Tanzania (Geigy and Kaufmann, 1973; Nantulya et al., 1984; Majiwa et al., 1985). In mice, the isometamidium chloride and diminazene aceturate 50% curative doses ($CD_{50}$s) are 0.018 and 2.3 mg/kg bw, respectively (Peregrine et al., 1991).

*T. b. brucei* clones

**CP 547** was isolated from a naturally infected cow in Jilib, Somalia in 1985. The isolate is resistant to diminazene, isometamidium, quinapyramin, melarsoprol, homidium and pentamidin (Zweygarth and Röttcher, 1989) and is sensitive to suramin and eflornithin (Zweygarth and Kaminsky, 1991). When a drug sensitivity test was undertaken in mice, none of the eight mice infected with this isolate could be cured with diminazene aceturate at a dosage level of 50 mg/kg bw (Zweygarth and Röttcher, 1989).

The clone **CP 2469** was isolated from a naturally infected cow in Hakaka, Soakow District, Somalia, in 1985. It is resistant to isometamidium and diminazene, but sensitive to suramin (Zweygarth and Kaminsky, 1991). In a drug sensitivity test conducted in mice, one of the eight mice infected with this isolates was cured when treated with 50 mg/kg bw diminazene aceturate (Kaminsky et al., 1989a).

The clone **ILTat 1.4** is an antigenic variant derived from EATRO 795. It was isolated from a steer in Uhembo District in Kenya in 1964 (Miller and Turner, 1981). The isolate was characterised as human serum-sensitive in the Blood Incubation Infectivity Test (BIIT) and the Human Serum Resistance Test (HSRT) (Hawking, 1976a,b). Using RFLP analysis and DNA probe hybridisation it was characterised as *´non-gambiense´* (Paindavoine et al., 1989).

**STIB 345 RA** was isolated in 1969 from a tsetse fly in Kiboko, Kenya. The isolate was designated as EATRO 1529 by the East African Trypanosomiasis Research Organisation and later it was designated as STIB 345 by the Swiss Tropical Institute, Basel. No case of sleeping sickness has been reported in Kiboko where it was isolated or in a surrounding radius of about 200km (Hide et al., 2000).

**MHOM/UG/92/IG 2602** is a *T. b. rhodesiense* stock that was isolated from a sleeping sickness patient who was refractory to trypanocidal treatment. The patient was from Kapyanga, Iganga Region of Uganda in 1990 (Dobschütz, 2002). In the current study, this isolate was used as a positive control in the PCR amplifications of the SRA (Serum Resistant Associated) gene in *T. brucei*.

**Table 4:** Summary of the history and drug sensitivity of the *T. congolense* and *T. b. brucei* reference clones

| Stocks/Clones | Country | Year of isolation | Origin | ISMM sensitivity in cattle | ISMM $CD_{50}$ (mg/kg bw) | DIM $CD_{50}$ (mg/kg bw) | References |
|---|---|---|---|---|---|---|---|
| *T. congolense* IL 2642 | Uganda | 1962 | Cattle | S | 0.007 | n.k. | Morrison et al., 1978; Wilkes et al., 1997 |
| *T. congolense* IL 1180 | Tanzania | 1971 | Lion | S | 0.018 | 2.3 | Geigy and Kaufmann, 1973; Peregrine et al., 1991 |
| *T. b. brucei* CP 547 | Somalia | 1985 | Cattle | R | n.k. | n.k. | Zweygarth and Röttcher, 1989 |
| *T. b. brucei* CP 2469 | Somalia | 1985 | Cattle | R | n.k. | n.k. | Kaminisky et al., 1989 |
| *T. b. brucei* ILTat 1.4 | Kenya | 1964 | Steer | S | n.k. | n.k. | Miller and Turner, 1981 |
| *T. b. brucei* STIB 345 | Kenya | 1969 | Glossina | S | n.k. | n.k. | Brun et al., 1979 |

n.k. not known; ISMM, isometamidium; DIM, diminazene; S, sensitive; R, resistant; $CD_{50}$, 50% curative dose in mic

## 4.3 Experimental animals

Mice were obtained from the breeding colony of the Institute for Parasitology and International Animal Health, Free University of Berlin. *Mastomys coucha*, 10 to 12 weeks of age, weighing 30 to 40 g, were used to propagate trypanosome field isolates, to derive isometamidium resistant trypanosome clones from reference sensitive clones and to undertake drug sensitivity tests in mice. Balb/C, 10 to 12 weeks of age, weighing 20 to 30 g, were used to generate clones from the *T. congolense* field isolates. Mice were maintained on a commercial ration and provided water *ad libitum*.

## 4.4 Propagation of *T. congolense* and *T. brucei* stocks in mice

Cryopreserved *T. congolense* stocks collected from Ethiopia and Burkina Faso and the reference clones were expanded in mice. Mice (*Mastomys coucha*) were inoculated intra-peritoneally with 0.5 ml of thawed and diluted parasites in culture medium. They were then monitored for the presence of parasitaemia by examining tail blood wet films every other day. The trypanosomes were then sub-passaged in Balb/C inbred mice two to three times prior to cloning. Similarly, *T. brucei* stocks collected from different regions in Mukono County in Uganda and the reference clones were expanded in mice (*Mastomys coucha*). When high parasitaemia was detected, the mice were exsanguinated and blood was collected from the heart and used for DNA extraction.

## 4.5 Cloning of trypanosome stocks

The cryopresrved *T. congolense* were expanded in 10-12 weeks old Balb/C inbred mice. Each of the isolates used for cloning were inoculated into two or three mice i.p. Mice were then monitored for parasitaemia by examining tail blood wet films every other day. When the parasitaemia was in the 'growing phase', blood was taken from the tail of the mice and used for cloning. Twenty-four hours before cloning started, mice were set up in groups of five, marked individually and injected with an immunosuppressive drug (cyclophosphamid at a dose of 300 mg/kg bw) i.p. This was repeated four times every two days after the mice were inoculated with the clones.

The cloning procedures were carried out in a high humidity atmosphere to delay evaporation. This was achieved by raising the relative humidity of the working place using a boiling water

bath. Briefly, about 4 drops of tail blood from the selected parasitaemic mouse was added to a 4-ml tube containing 1 ml of phosphate buffered saline glucose 6:4, pH 8.0, and 20% foetal calf serum (cloning buffer). The tube was centrifuged for 15 min at 50g to separate blood cells from trypanosomes. The supernatant was put into another tube and centrifuged once again for another 10 min at 1000g. The supernatant was withdrawn with a syringe and about 0.5ml of the sediment containing the trypanosomes was left in the tube. A drop from the sediment was put into some micro plate wells using a Pasteur pipette and the wells checked with a microscope (250x magnification) for the presence of trypanosomes. Dilutions were made with phosphate buffered saline glucose containing 20% foetal calf serum, based on the number of parasites per microscopic field, in such a way that approximately 1-2 parasites were present per drop. The drops used were of such sizes that they could entirely be included in a microscopic field for the presence of parasites to be easily searched by focusing alone. The outside wells of the micro-plate were filled with medium or warm water to prevent evaporation. When a single parasite was observed in a well, its presence and the absence of any other parasite(s) was confirmed by two other independent observers. Using a fibre-tip pen, the well containing the selected drop was marked while the plate was still under the microscope. Next, a drop of diluent was placed on the drop containing the trypanosome using a 1 ml syringe (which had been previously charged with 0.1 ml of diluent). Then, the whole drop was drawn into the syringe. The recipient mouse was inoculated immediately i.p. with as much as possible of the contents of the syringe. The needle was withdrawn and 0.3 ml or so of air drawn into the barrel of the syringe and a second i.p. inoculation was made into the mouse of this air together with the fluid contained in the 'dead space' of the syringe.

Examination of the mice began on day 2 post inoculation by the Haematocrit Centrifugation Technique (HCT), until parasitaemia was detected and thereafter by wet film examinations of tail blood. If no parasitaemia was detected for a period of 60 days, then the clone was considered not to have grown in the mouse and the mouse was withdrawn from the experiment. In cases of parasitaemia, sub-passages were made into two mice. When adequate parasitaemia was achieved, the clones were designated by a new name. Stabilates were prepared and stored in liquid nitrogen for isometamidium sensitivity investigations.

## 4.6 Induction of isometamidium resistance in *T. congolense* in experimentally infected mice

### *4.6.1 Mice*

Mice (*Mastomys coucha*), each weighing between 30 and 40 g, were obtained from the breeding colony of the Institute of Parasitology and International Animal Health, Free University of Berlin, and used in this experiment. Mice were allowed free access to both water and a pelleted ration.

### *4.6.2 Trypanosomes*

The isometamidium sensitive reference clone of *T. congolense* used to derive isogenic clones with different levels of resistance to isometamidium was IL 2642. The origin and history of this clone of parasite is described in section 4.2.

### *4.6.3 Treatment*

Isometamidium chloride (Trypamidium-Samorine® Merial, batch No. W 389961), freshly prepared in sterile distilled water, was used for i.p. treatment of mice. Dosages of isometamidium used in the study were calculated so that the required amount of drug was contained in a volume of 0.2 ml of sterile distilled water.

### *4.6.4 Study design*

In order to progressively increase the resistance of the trypanosome population, repeated sub-therapeutic treatments of infected mice with isometamidium chloride were carried out, as described for the induction of resistance to quinapyramine (Ndoutamia et al., 1993) and to isometamidium chloride (Peregrine et al., 1997). Briefly, the drug sensitive clone of *T. congolense* IL 2642 was initially expanded in 3-4 mice. The mice were then monitored every other day for parasitaemia by wet film examination of tail blood. When the level of parasitaemia in the mice was 50 or more trypanosomes per wet field preparation, the blood from the mice was pooled and suspended in phosphate buffered saline-glucose solution (pH 8.0) containing 10% foetal calf serum. Twenty-four hours prior to experimental infection, a

group of 10 mice were immunosuppressed by i.p. administration of 300 mg/kg bw cyclophosphmide monohydrate (Endoxan®). Each of the immunosuppressed mice was then infected i.p. with 1 x $10^5$ trypanosomes. Another group of ten immunocompetent mice (not treated with cyclophosphamide) were also infected with the same number of trypanosomes. Subsequently, the mice were monitored daily for parasitaemia. When most of the mice in either group developed parasitaemia and the level in at least one mouse was 50 or more trypanosomes per field, the mice were treated with a subcurative doses of isometamidium and then monitored daily for parasitaemia.

The first subcurative dose of isometamidium was 0.0035 mg/kg bw. When parasitaemia was detected in at least 50% of the isometamidium treated mice, the whole group was re-treated with a higher dose (e.g. 0.005 mg/kg bw). After treatment, the mice were again monitored daily. When relapse infections were detected, the parasitaemic mice were exsanguinated and the blood was pooled. Part of this blood was then frozen in liquid nitrogen (in case no mice relapsed after the next treatment) and part was used to infect the second group of 10 immunosupressed mice by i.p. administration of 300 mg/kg bw cyclophosphamide. Once the second group of mice became parasitaemic, they were treated with the second drug dose that the previous group of mice received. When the infections reappeared after this treatment, they were treated with a higher dose of the drug (e.g. 0.01 mg/kg bw). The infection was then passaged and the process was repeated until a predetermined degree of resistance was achieved. The same procedure was followed in the immunocompetent groups of mice. A schematic presentation of the dosages used and the sub-passages made is depicted in Figure 1. On achieving a degree of resistance, comparable to the level of resistance reported in the field, an attempt was made to stabilise the induced resistance by repeated treatments with isometamidium at the dose rate to which the highest resistance was expressed. Then, the final resistant population that arose was designated by a new name.

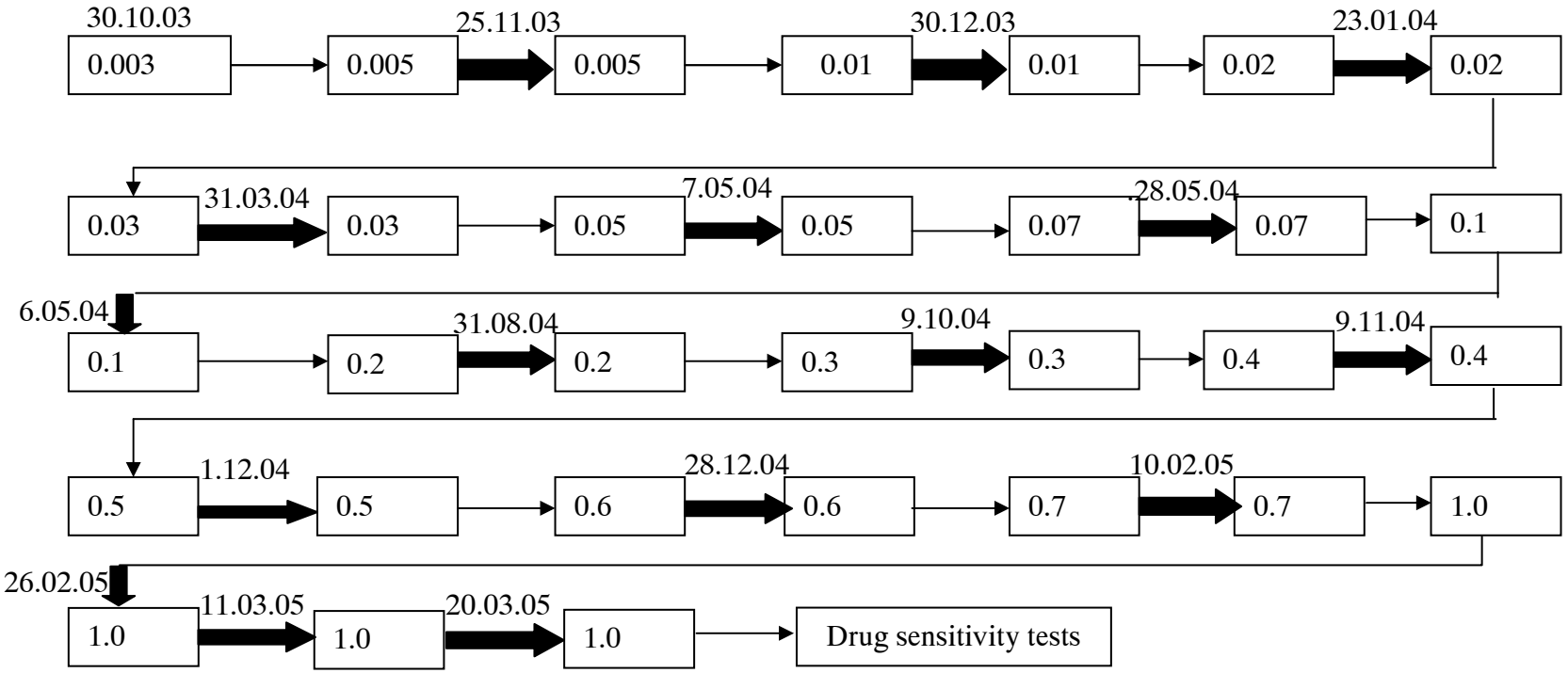


**Figure 1:** Induction of isometamidium-resistance in *T. congolense* in immunosuppressed mice. Drug dosages (mg/kg bw) are shown inside the boxes. The arrows indicate sequential treatments with the next dosage of the drug; the solid arrows indicate subpassage to the next experimental group of mice.

Parasites resistant to the highest dose rate of isometamidium (1.0 mg/kg bw) were passaged 3 times in two immunocompetent mice without drug treatment. After the 3 passages, isometamidium sensitivity tests were undertaken in normal immunocompetent mice according to a standard protocol (Eisler et al., 2001; Table 5), to compare the sensitivity of the newly derived population with the parent clone. To determine if selection for resistance to isometamidium resulted in cross-resistance to diminazene, the newly derived isometamidium-resistant *T. congolense* was characterised for their susceptibility to this trypanocidal drug. The laboratory-derived isometamidium-resistant isogenic clones of *T. congolense* were stored in liquid nitrogen in the cryobank at the Institute for Parasitology and International Animal Health, Free University of Berlin, for further parasitological and molecular investigations.

### 4.7 Standardised drug sensitivity tests in mice

The *in vivo* sensitivity of the developed clones to multiple dosages of isometamidium was assessed according to the protocol described in Eisler et al. (2001). A total of 4 clones derived from 2 different primary field isolates from Ethiopia (3 clones derived from PA 077 and 1 clone derived from PA 073) and 6 clones derived from 3 different primary field isolates from Burkina Faso (2 clones derived from SA 095, 3 clones from SA 267 and 1 clone from SA 268) were tested for their isometamidium sensitivity. Briefly, 6 groups of 6 mice each (1 control and 5 treatment groups) were used. Mice were each infected i.p. with 1 x $10^5$ trypanosomes. About 24 hours after inoculation, the first five groups of mice were treated i.p. with isometamidium chloride (Trypamidium-Samorine® Merial W 389961) at doses ranging from 0.01 to 20 mg/kg bw. The controls received the same amount of distilled water without drug i.p. Following treatments, parasitological examinations of the mice were conducted twice a week using phase-contrast or dark-ground microscopy (magnification x 250) of a wet smear of tail blood. When parasitaemias were observed in the control mice, the number of days to first detection of parasitaemia were recorded and the mice euthanised. The drug-treated groups were followed until a relapse occurred or until 60 days post-treatment. The $CD_{50}$ values were determined using the standard logit or probit analyses (Peregrine et al., 1991). The test protocol followed for the isometamidium sensitivity study in mice is shown in Table 5.

**Table 5:** Standardised protocol for isometamidium resistance study in mice (Eisler et al., 2001)

| | **Treatment Group** | **Control Group** |
|---|---|---|
| Number of groups of mice | 5 | 1 |
| Number of mice per group | 6 | 6 |
| Number of trypanosomes inoculated | $1 \times 10^5$ | $1 \times 10^5$ |
| Route of administration | Intraperitoneal (i.p.) | Intraperitoneal (i.p.) |
| Drug dosage rates (mg/kg bw) | 0.01, 0.1, 0.05, 3.0, 20 | Distilled water |
| Drug administration time | 24 h post-inoculation | 24 h post-inoculation |
| Drug administration route | i.p. | i.p. |
| Method of mice examination | Tail blood wet smear | Tail blood wet smear |
| Frequency of examination | Three times per week | Three times per week |
| Duration of follow-up | 60 days | 60 days |
| Interpretation of results | Probit analysis of number of mice cured at each dose | |

## 4.8 Molecular characterisation of the genetic identity of the *T. brucei* and *T. congolense* study clones and stocks

### *4.8.1 Purification of trypanosomes*

Bloodstream-forms of trypanosomes were expanded in mice and were purified from the blood by di-ethyl-amino-ethyl (DEAE) chromatography (Lanham and Godfrey, 1970), followed by repeated centrifugation and sediment washes with Phosphate Buffered Saline (PBS). Briefly, the blood from a parasitaemic mouse was suspended in 3 ml of Phosphate Buffered Saline Glucose (PSG) and then layered on a DEAE-52 column (Whatman, England) equilibrated in PSG, pH 8.0. The trypanosomes were eluted with PSG, pH 8.0. When the elution was complete, the trypanosomes were pelleted by centrifugation at 1,500g for 15 minutes at 4$^{o}$C. The trypanosomes were washed with 15 ml of PBS, centrifuged and were either used immediately or stored at -70$^{o}$C.

### *4.8.2 Extraction of genomic DNA from purified trypanosomes*

Two hundred and fifty microlitres of the purified trypanosome suspension was mixed with 250-$\mu$l lysis buffer (0.32 M Sucrose, 0.01 M Tris, 0.005 M $MgCl_2$, 1% Triton X-100, pH 7.5). The mixture was centrifuged at 13,000 g for 25 sec. The supernatant was poured out and the pellet washed with 500 $\mu$l lysis buffer. The centrifugation and washing steps were repeated two times. The final pellet was re-suspended in 250 $\mu$l of 1 x PCR buffer. Then, 1.5 $\mu$l of proteinase-K (10 mg/ml) was added and mixed by vortexing. The samples were incubated at 56ºC for 1 hour followed by 95ºC incubation for 10 minute to inactivate the proteinase-K. The DNA concentration was estimated by means of a spectrophotomer at wavelength of 260 nm, Gene Quant Calculator® (Amersham Pharmacia Biotech, Freiburg, Germany) and stored at –20ºC until used for PCR amplification.

### *4.8.3 DNA amplification*

#### *4.8.3.1 Trypanosoma brucei*

Nuclear repeat primers specific for *Trypanozoon*, TBR1 and TBR2 (Table 6), were used to amplify the *T. brucei* isolates by PCR. Subsequently, in order to distinguish the *T. b. brucei* from the human pathogenic *T. b. rhodesiense* the *T. brucei* stocks were screened for the presence or absence of SRA gene fragments (Radwanska et al., 2002).

Amplifications of DNA of *T. brucei* using the above mentioned primer pairs were carried out in 25 $\mu$l reaction volumes containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1 % Triton X-100, 2 mM $MgCl_2$, 200 $\mu$M each of the four dNTP´ s (dATP, dCTP, dGTP, dTTP), 1 unit of Ampli Taq Gold® polymerase (Applied Biosystems, GmbH, Darmstadt, Germany), approximately 5 ng DNA and 1 $\mu$M of each of the oligonucleotide primers. The reaction mixture was overlaid with one drop of mineral oil and centrifuged at 10, 000 g for 15 seconds. The tubes were transferred immediately into a Thermocycler Trio-Thermoblock™ (Whatman Biometra, Göttingen, Germany). As a negative control, the template was replaced by distilled water.

The PCR using the TBR1 and TBR2 primer pairs were performed by incubating the samples at three temperatures corresponding to three steps (denaturation, annealing and extension) in a cycle of amplification (at 95ºC for 10 minutes, followed by 94ºC for 60 s, 60ºC for 30 s and 72ºC for 30 s). This was repeated for 34 cycles. After a final extension step at 72ºC for 10 minutes, the samples were cooled and stored at 4ºC. The SRA gene-based PCR conditions were as follows: incubation for 15 min at $95^0$C, followed by 35 cycles of one minute at $94^0$C, one minute at $68^0$C, and one minute at $72^0$C, and a final extension for 10 minutes at $72^0$C. The samples were cooled and stored at 4ºC.

**Table 6:** Oligonucleotide primers for the specific DNA amplification of *T. brucei* and *T. congolense* stocks.

| **Primers** | **Specificity** | **Sequence (5' → 3`)** | **Product size (bp)** | **References** |
|---|---|---|---|---|
| TBR1 | *T. brucei* | CGA ATG AAT ATT AAA CAA TGC GCA GT | 177 | Moser et al. (1989a) |
| TBR2 | | AGA ACC ATT TAT TAG CTT TGT TGC | | |
| SRA A | *T. b. rhodesiense* | ATA GTG ACA AGA TGC GTA CTC AAC GC | 284 | Radwanska et al. (2002) |
| SRA B | | AAT GTG TTC GAG TAC TTC GGT CAC GCT | | |
| TCN 1 | *T. congolense* Savannha | TCG AGC GAG AAC GGG CAC TTT GCG A | 329 | Moser et al. (1989a) |
| TCN 2.38 | | GTT TTC GAT GAA AAT TAG GGA CAA ACA AAT CCC GCA CA | | |
| TCF 1 | *T.congolense* Forest | GGA CAC GCC AGA AGG TAC TT | 350 | Masiga et al. (1992) |
| TCF 2 | | GTT CTC GCA CCA AAT CCA AC | | |

*4.8.3.2 Trypanosoma congolense*

The oligonucleotide primers described for *T. congolense* Savannha (Moser et al., 1989) and Forest (Masiga et al., 1992) types were used for the amplification of the *T. congolense* study clones in order to determine the genetic identity of the clones. The PCR amplification was performed using the same conditions as described for *T. brucei*. Briefly, incubation at 95ºC for 10 minutes, followed by 94ºC for 60 s, 60ºC for 30 s and 72ºC for 30 s. This was repeated for 34 cycles. After a final extension step at 72ºC for 10 minutes, the samples were cooled and stored at 4ºC.

*3.8.4 Gel electrophoresis*

Two percent agarose gel containing 2.5-$\mu$l ethidium bromide in 1 x electrophoresis buffer was prepared. The electrophoresis chamber was filled with buffer solution (1 x electrophoresis buffer) until the top surface of the gel submerged by approximately 1 mm (about 400 ml). Two microlitres of the sample buffer were mixed with 10 $\mu$l PCR product and centrifuged at 10, 000 g for 20 sec. The samples (10 $\mu$l) and DNA ladder of 100 bp (10 $\mu$l) were placed into the agarose gel wells. The chamber was connected to a 67 Volt power supply and allowed to run for 45 to 60 minutes. The separated DNA products were detected using UV trans-illumination and the bands were photographed using a Polaroid® camera.

## 4.9 Characterisation of the TbAT1 gene in *T. b. brucei*

All the *T. brucei* study-isolates showing no detectable band for SRA gene when analysed by PCR using the primer sets SRA A and SRA B were considered to be *T. b. brucei*. Analysis was made on the TbAT1 gene that codes for the P2 adenosine transporter from *T. brucei brucei* field-isolates to investigate a possible link between the presence of mutation in this gene and isometamidium resistance.

### 4.9.1 Genomic DNA amplification of the TbAT1 gene fragments in *T. b. brucei*

From the DNA of each of the 11 isometamidium-sensitive *T. b. brucei* field stocks (Table 3) and 2 isometamidium-sensitive and 2 isometamidium-resistant reference clones (Table 4), a 677 bp central fragment of the TbAT1 gene (nucleotide 430-1108) was amplified by PCR using primers sfa-s (5´-CGC CGC ACT CAT CGC CCC GTT T-3´) and sfa-as (5´-CCA CCG CGG TGA GAC GTG TA-3´) (Mäser et al., 1999). Amplification was done using hot start Taq polymerase and thirty PCR cycles were performed using the following conditions: $95^0$C, 15 minutes; $64^0$C, 2 minutes and $72^0$C, 30 seconds, followed by a final extension of 10 minutes at $72^0$C. The PCR products were resolved in a 2% agarose gel and photographed using a Polaroid® camera. A 100 bp ladder was used as molecular size marker.

### 4.9.2 Sequence analysis of TbAT1 gene fragments in isometamidium-sensitive and -resistant *T. b. brucei*

The 677 bp fragments of the TbAT1 gene from isometamidium sensitive *T. b. brucei* field isolates and sensitive and resistant reference clones amplified as described in section 4.9.1 were used for the sequence analysis. For DNA sequencing, three independent amplification reactions were performed for each sample as described in section 4.9.1. The PCR products were pooled and purified using a commercial kit (Roche Applied Science, Roche Diagnostics GmbH, Penzberg, Germany). The DNA concentration was estimated by means of a spectrophotomer, the Gene Quant Calculator® (Amersham Pharmacia Biotech, Freiburg, Germany). The PCR product was outsourced for sequencing to SEQLAB (Göttingen, Germany). The gene was sequenced with forward and reverse primers. Sequences were recorded as chromatographic files. Forward and reverse sequences were compared for each gene fragment, and sequences were manually edited and aligned using Genedoc® software (Nicholas and Nicholas, 1997). The sequencing results obtained were aligned and compared to the 1493 bp sequence data from STIB 427, a melarsen cysteamine sensitive *T. brucei* strain, and STIB 777MCR, a melarsen cysteamine-resistant *T. brucei* clone kept in the GenBank (accessed from the database with accession numbers AF152369 and AF152370, respectively). The sequences were further analysed for base match, mismatch, insertion and deletion. The TbAT1 gene sequences of a total of 11 *T. b. brucei* field isolates characterised

phenotypically as isometamidium sensitive, 2 reference clones characterised as isometamidium sensitive and 2 clones characterised as resistant were analysed.

### 4.9.3 Sfa NI Restriction Fragment Length Polymorphism (RFLP) analysis of TbAT1 gene fragments in *T. b. brucei*

In order to analyse the Sfa NI restriction profile of the TbAT1 gene fragment, the genomic DNA of 8 of the isometamidium-sensitive and 2 of the isometamidium-resistant *T. b. brucei* was amplified by PCR using the primers Sfa-s (5'-CGCCGCACTCATCGCCCCGTTT-3') and Sfa-as (5-'CCACCGCGGTGAGACGTGTA-3'). PCR amplification was conducted as described in section 4.9.1. Before restriction site analysis of the TbAT1 gene fragment was carried out, the PCR products of three independent reactions were pooled and purified using a commercial purification kit (Roche Applied Science, Roche Diagnostic GmbH, Germany). Briefly this was performed as follows: five hundred microlitres of Binding Buffer (Guanidine-thiocyanate) was added to 100 $\mu$l PCR product and mixed. The mixture was transferred to the upper reservoir of a filter column and centrifuged at 13,000g for 1 minute. The flowthrough solution was discarded and the sample was washed twice with Washing Buffer. To elute the pure PCR product, 75 $\mu$l Elution Buffer or distilled $H_20$ was added onto the filter membrane and the column centrifuged at 13, 000 g for 1 minute. The concentration of the PCR product was determined in a spectrophotometer using $OD_{260}$. Then, the purified 677 bp fragment of TbAT1 (nucleotide 430-1108) was subjected to Sfa NI digestion (New England Biolabs). Endonuclease digestions were performed in 20 $\mu$l reaction volumes; 17.5 $\mu$l purified PCR products, 2 $\mu$l 10x NEB enzyme reaction buffer and 0.5 $\mu$l (0.5 units) of the enzyme were mixed in a final reaction volume of 20 $\mu$l. The mixture was incubated at 37ºC for at least 3 hours to achieve complete digestion. The digested samples were analysed along with undigested controls on a 2% agarose gel. A 100 bp ladder was used as molecular size marker. Later, the restriction profiles were photographed using a Polaroid® camera.

## 4.10 Characterisation of putative target sequences in *T. congolense* homologues to *T. brucei* adenosine transporter gene (TbAT1)

As a preliminary investigation, attempts were made to find out if the TbAT1 gene associated with arsenicals and diamidine resistances in *T. brucei* (Mäser et al., 1999) were present

among different isometamidium-sensitive and -resistant clones of *T. congolense*. Screening for homologues to the above putatively resistance-related *T. brucei* sequence was performed by multiple strategies.

### 4.10.1 Genomic DNA amplification of the TbAT1 in *T. congolense* isometamidium-sensitive and -resistant clones using the primers Sfa-s and Sfa-as (Mäser et al., 1999)

In order to detect the presence of the TBAT1 gene in *T. congolense*, trials were made to amplify a 677 base pair (bp) fragment of the purine transporter gene from the genomic DNAs of various isometamidium-sensitive and -resistant clones of *T. congolense*. The primers sfa-s (5´-CGC CGC ACT CAT CGC CCC GTT T-3´) and sfa-as (5´-CCA CCG CGG TGA GAC GTG TA-3´) as described in Mäser et al. (1999) were used. The genomic DNAs extracted from *T. b. brucei* reference clones (ILTat 1.4 and STIB 345) were used as positive controls. Amplification was done using hot start Taq polymerase and thirty PCR cycles were performed using the following conditions: $95^0$C, 15 minutes; $64^0$C, 2 minutes and $72^0$C, 30 seconds, followed by a final extension of 10 minutes at $72^0$C. The PCR products were resolved in a 2% agarose gel and photographed using a Polaroid® camera.

### 4.10.2 Investigation of TbAT1 homologous gene transcripts in *T. congolense* using flanking primers

The 1493 bp TbAT1 gene transcripts described in Mäser et al. (1999) were used to design four primers, two forward and two reverse. The primers were designed using the computer program Primer3 (htpp://frodo.wi.mit.edu) (Rozen and Skaletsky, 2000). Each of the forward primers was combined with each of the reverse primers, which formed four primer sets. The primer sets targeted different locations of the TbAT1 gene sequence. They were then used to search for homologous gene sequences in *T. congolense*. The four oligonucleotide primer sets are shown in Table 7. The procedure and conditions for amplification by PCR described in section 4.10.1 were followed.

**Table 7:** Oligonucleotide primer sets used to attempt amplification of TbAT1 gene fragments from *T. congolense* and the expected PCR product sizes

| No. | Pairs of primers | PCR product sizes (bp) |
|---|---|---|
| 1 | Sfa1-s: 5´- GGC GTC GTC ACA TCT TC TT - 3´<br>Sfa1-as: 5´- GGC AGG GTA GAC GAG AAA TG - 3´ | 479 |
| 2 | Sfa1-s: 5´- GGC GTC GTC ACA TCT TC TT - 3´<br>Sfa2-as: 5´- CAT TCC ATA CCC TTG CCA TT - 3´ | 405 |
| 3 | Sfa2-s: 5´- AGA ACC CTT ACG CCC AAA AG - 3´<br>Sfa1-as: 5´- GGC AGG GTA GAC GAG AAA TG - 3´ | 334 |
| 4 | Sfa2-s: 5´- AGA ACC CTT ACG CCC AAA AG - 3´<br>Sfa2-as: 5´- CAT TCC ATA CCC TTG CCA TT - 3´ | 260 |

### 4.10.3 Genomic DNA analysis of *T. congolense* for the presence of TbAT1 homologous sequences using degenerate primers

Degenerate primers are primers which have a number of options at several positions in the sequence so as to allow annealing to and amplification of a variety of related sequences. They were used to fish out conserved sequences of TbAT1 gene from the genome of *T. congolense*. Amino acid and nucleotide sequences of TbAT1 gene in *T. brucei* stored in the GenBank database (GenBank access number: AF152369 and AF152370) were used in a homology search that consulted a public domain genomic database. A 'blast' search in the National Center for Biotechnology Information (NCBI) database for matches with the 1493 bp adenosine transporter gene sequence yielded multiple sequence alignments that were homologous to the TbAT1 transporter genes. The sequence alignments in *T. b. evansi* and *T .b. equiperdum* were used. Different primer pairs were selected from highly conserved regions of the genes. Three pairs of degenerate primers were generated by performing a global DNA alignment of the gene sequences of *T. b. evansi* and *T. b. equiperdum* against the reference molecule, the TbAT1 nucleotide sequence in *T. b. brucei*. Three primer sets,

TBAT3-TBAT7, TBAT1-TBAT2 and TBAT1-TBAT7 were used to attempt the amplification of the respective genes in *T. congolense* (Table 8). One $\mu$M of each primer per reaction was used. Five $\mu$l of the genomic DNA were used per reaction as template. Gradient PCR was performed using a Hot-Start-Taq Polymerase. The PCR conditions for the gradient PCR were as follows: incubation at $95^0$C for 15 minutes, annealing temperatures ranged from $46^0$C to $64^0$C, $72^0$C for 30 seconds, followed by a final extension of 10 minutes at $72^0$C. The PCR was run for 30 cycles. The PCR products were resolved in a 2% agarose gel and photographed using a Polaroid® camera.

**Table 8:** Degenerate primer pairs used to search for TbAT1 homologous gene transcripts in *T. congolense*

| No. | Primer Sets | Expected PCR product sizes (bp) |
|---|---|---|
| 1 | TBAT1 Forward: 5´- GAR TAY TAY AAR TAN GCY CAR - 3´<br>TBAT2 Reverse: 5´- ARA RAA NAC CAT RAA RCA - 3´ | 867 |
| 2 | TBAT3 Forward: 5´- GAY CVT AAR TTY TGG AAR CAY ATG- 3´<br>TBAT7 Reverse: 5´- YTC NAC YTG MTC CAT MTC YTT CAT- 3´ | 698 |
| 3 | TBAT1 Forward: 5´- GAR TAY TAY AAR TAN GCY CAR - 3´<br>TBAT7 Reverse: 5´- YTC NAC YTG MTC CAT MTC YTT CAT - 3´ | 750 |

R = A/G (degeneracy = 2X), Y = C/T (degeneracy = 2X), N = A/C/T/G (degeneracy = 4X), V = A/C/G (degeneracy = 3X), M = A/C (degeneracy = 2X)

### 4.11 Data management and analysis

In the drug sensitivity studies, animals were regularly examined to check for relapse in infection for a period of 60 days. The day when each mouse was detected parasitaemic was recorded. Mice that did not develop parasitaemia for 60 days after treatment were considered cured, and for the purpose of analysis a value of 61 was given to each. The data were entered into Minitab statistical software (MINITAB$^{TM}$, MINITAB Release 13-20, 2000) and checked for normal distribution. In order to compare the means of relapses among groups, a one-way analysis of variance (ANOVA) was used. The number of days it took for a mouse to show relapse infection after treatment was taken as a response variable and the levels of doses of isometamidium used were taken as factors. P values of $< 0.05$ were taken as statistically significant (two-tailed).

In order to estimate the dosage of isometamidium that could cure 50% ($CD_{50}$) of the treated mice, probit analysis was conduced. Probit stands for probability integral transformation and a probit dose-response model permits a standard normal distribution of expected response to doses (EPA, 2002). For the probit analysis, infected mice were subjected to different levels of isometamidium treatment and the number of mice either cured or not (survival or failure) was recorded for each group. The dose of isometamidium used was taken as the 'stress factor' and the 'response variable' was whether or not a mouse was cured after being treated with isometamidium of a certain dosage. The distribution used was logistic and 1 mg/kg bw of isometamidium was taken as stress (stimulus). The probability of survival of infection as the dose of isometamidium changes was predicted. Comparisons of the $CD_{50}$ values of isometamidium among the different clones of trypanosomes tested were undertaken using 95% confidence intervals (CI). Similar procedures were followed to undertake probit analysis for diminazene aceturate. The computer software used to undertake the analyses was MINITAB$^{TM}$ statistical software (MINITAB Release 13-20, 2000).