6 DISCUSSION

6.1 Cloning of trypanosome stocks

In the current study, attempts were made to clone stocks of *T. congolense* from Ethiopia and Burkina Faso with different isometamidium sensitivity phenotypes, in order to use the clones derived for detailed parasitological and molecular drug sensitivity studies. An overall cloning success rate of 20.3% was achieved, which ranged between 16.67% and 25.00% for the individual stocks. Success in establishing infections from single organisms varies widely. In general, cloning-success in *T. brucei* is higher as compared to *T. congolense*. Barry et al. (1979) reported cloning success rates of 70% and 50% in *T. b. rhodesiense* and *T. b. brucei*, respectively. Mutugi et al. (1995) achieved cloning success rates of 60% using suramin sensitive stocks of *T. evansi*, whereas they did achieve cloning success rates of only 1.7 and 8.3% using suramin-resistant clones of *T. evansi*. These results suggested that it is easier to clone trypanosome stocks which are sensitive to a drug than those that are resistant to the action of the drug. Cloning success is determined by the ability of a single parasite to withstand the host's defence mechanisms and establish an infection. Comparison of titration results with simultaneous organism-counts suggests that at some periods of the infection only small proportions of organisms may be infective (Lumsden et al., 1973). Also differences occur between different antigenic types. McNeillage and Herbert (1968) indicated that with highly virulent antigenic types one out of two organisms inoculated singly grew in the recipient animals. However, in the case of a less virulent type under identical conditions, only one in twenty-three organisms was established. Several authors (Mutugi et al., 1995) have observed a loss of virulence and/or a loss of fitness in drug-resistant trypanosomes. However, another study using four populations of *T. congolense*, ranging from extremely sensitive to strongly resistant to isometamidium, found no difference in virulence between them (ILRI, 1996). Only the most resistant showed reduced viability, taking longer time to establish parasitaemia in mice than the other three. The loss of fitness in other drug-resistant microbes is a well-known, but not invariable, phenomenon and is probably also present in trypanosomes (Geerts and Holmes, 1998). The stocks of *T. congolense* in the current study from which the clones were derived are highly resistant to both isometamidium and diminazene. Therefore, the low cloning success rates observed in the current studies may partly be related to the lower viability and fitness of the drug-resistant trypanosomes

compared to the sensitive ones, and as a result of which, a single resistant trypanosome is unable to survive in a mouse as readily as a sensitive one. Well-designed experiments using significant numbers of resistant and sensitive isolates of trypanosomes should provide valuable data on this issue.

6.2 Isometamidium sensitivities of clones of *T. congolense* from East and West Africa

The isometamidium sensitivities of *T. congolense* clones derived from stocks from Ethiopia and Burkina Faso were assessed to multiple dosages of isometamidium in mice, according to the protocol described by Eisler et al. (2001). *T. congolense* reference clones of a known isometamidium sensitivity phenotype (IL 2642 and IL 1180) were used as isometamidium-sensitive controls and were tested in mice parallel to the study clones. The results demonstrated that all the *T. congolense* clones derived from different stocks in Ethiopia and Burkina Faso expressed high levels of resistance to isometamidium with reference to *T. congolense* IL 2642 and IL 1180. The clones from Burkina Faso expressed significantly ($p<0.05$) higher levels of resistance in mice than the clones from Ethiopia. Analyses of variance (ANOVA) of the mean relapse intervals showed that, for most of the clones tested, there were clear relationships between the time of relapses and the doses of isometamidium used, in as much that mice treated with lower doses relapsed after a shorter time than mice treated with higher doses ($p=0.001$). IIemobade (1979) indicated that the drug resistance phenomenon in trypanosome population is dose-dependent. Rowlands et al. (1993) showed in a trial to compare the therapeutic efficacy of diminazene aceturate that the proportion of animals that relapsed following treatment decreased with higher dosages. Afewerk et al. (2000) also reported similar observations.

The CD_{50} values for the study clones from Ethiopia ranged between 9.86 and 13.37 mg/kg bw, whereas, the clones from Burkina Faso had CD_{50} values ranging from 19.8 to > 20.0 mg/kg bw. These values are higher than the CD_{50} values of clones of *T. congolense* derived from a known isometamidium-resistant stock reported earlier (Peregrine et al., 1991). Peregrine et al. (1991) derived nine clones from a drug-resistant *T. congolense* stock (IL 2856) which originated from Burkina Faso and characterised them in mice for their sensitivity to isometamidium and diminazene. All the clones expressed a high level of resistance to isometamidium with CD_{50} values ranging from 1.5 to 5.1 mg/kg bw. The isometamidium CD_{50} values of the *T. congolense* resistant clones IL 3000 and IL 3338 in mice were 2.9 and

20 mg/kg bw, respectively (Wilkes et al., 1997). Compared to these reference clones, all the clones from Ethiopia and Burkina Faso expressed high levels of resistance to isometamidium in mice. Earlier studies on the stocks of *T. congolense* from Ethiopia (Afewerk et al., 2000) and from Burkina Faso (Clausen et al., 1992; Knoppe, 2002), from which the current study clones were derived, showed that the stocks expressed high level of resistance in both mice and cattle. Several authors have shown that there is a good correlation of isometamidium sensitivities between the results obtained, using resistance tests in mice and those using tests in ruminants (Hawking, 1963; Sones et al., 1988; Peregrine et al., 1991; Ainanshe et al., 1992; Codjia et al., 1993). However, although a broad agreement exists between both tests, the curative doses in cattle cannot be extrapolated from the results obtained in mice (Sones et al., 1988). This was substantiated by Ainanshe et al. (1992), who isolated a *T. congolense* isolate (AF1) with a CD_{80} in mice of 20 mg/kg bw isometamidium, comparable to the *T. congolense* (K 2880) described by Sones et al. (1988). None of the three cattle inoculated with the former isolate and treated at 2 mg/kg bw isometamidium were cured, whereas the same dosage was able to cure two cattle inoculated with the later isolate. Furthermore, Kone (1999) examined two Zambian *T. congolense* isolates (TRT 6 and 17) both with a CD_{80} in mice of 1 mg/kg bw isometamidium. He inoculated two cattle with each of these isolates and treated them with 0.5 mg/kg bw isometamidium. Both animals inoculated with TRT 6 were cured whereas only one of the two cattle inoculated with TRT 17 was cured. Therefore, the results obtained from mice test should be interpreted with some caution. Given that there is only an approximate relationship between the drug sensitivities between stabilates in mice and cattle, it may be necessary to ascertain whether treatment with manufacturers' recommended drug dosage is also unsuccessful in cattle infected with the same isolate (Eisler et al., 2001).

These studies demonstrated that there was no significant ($p>0.05$) variation in expression of resistance to isometamidium amongst three of the clones derived from a single stock (PA 77) from Ethiopia. Similarly, two of the clones derived from a stock of Burkina Faso (SA 95) expressed a similar ($p>0.05$) level of resistance to isometamidium. Although the isometamidium CD_{50} values for the stock SA 267 and three of the clones derived from it could not be determined, these populations seem to express a similar level of resistance to isometamidium (CD_{50} values > 20 mg/kg bw). These results suggest that trypanosomes of a similar background may not differ significantly in expression of resistance to isometamidium. However, further tests using a large number of isolates and clones as conducted by Peregrine et al. (1991) are required to determine if resistance to isometamidium in *T. congolense* is a

stable phenotype among clones derived from a stock. Peregrine et al. (1991) derived 9 clones from the *T. congolense* stock IL 2856 and characterised them for their sensitivity to isometamidium. The isometamidium CD_{50} for IL 2856 was 3.6 mg/kg, whereas the CD_{50} values for the clones derived from this stock ranged between 1.5 and 5.1 mg/kg. Eight of the clones expressed a significantly lower level of resistance to isometamidium than the stock from which they were derived. In addition, among the 9 clones there was significant variation in expression of resistance to isometamidium.

Finally, these studies showed that the standardised multi-dose mouse test (Eisler et al., 2001) can provide detailed information on isometamidium sensitivities of stocks and clones from different regions of Africa, based on their CD_{50} values. It also enables to undertake probability survival analysis and approximately determine the dosage of isometamidium sufficient to bring about complete cure of infections in mice. Further studies are needed to find out if the methods used to quantify resistance in mice are sensitive enough to detect alterations in expression of resistance.

6.3 Induction of isometamidium resistance in *T. congolense*

The studies here have demonstrated that it is possible to increase the level of resistance of a cloned *T. congolense* population to isometamidium in immunosuppressed mice by repeated treatment with sub-curative doses of isometamidium. The level of resistance of the IL 2642 clone to isometamidium was increased at least 159-fold (from a CD_{50} of 0.0086 to 1.37 mg/kg bw) over a period of 16 months. Furthermore, experiments to characterise the cross-resistance phenotype of the isometamidium-resistant derivative in mice demonstrated that induction of resistance to isometamidium was associated with some level of cross-resistance to diminazene aceturate. The 159-fold increase in resistance to isometamidium was associated with a 2.2-fold (from a CD_{50} of 8.2 to 18.02 mg/kg bw) increase in resistance to diminazene aceturate, when tested in mice. Drug resistance in trypanosomes is often considered to arise from under-dosage in the field (Leach and Roberts, 1981). The results of the current studies confirm that administering drugs below effective levels can produce drug resistance. The exposure of parasites to sub-therapeutic drug concentrations has been considered the most important factor for the development of resistance (Whiteside, 1960). Sub-therapeutic drug concentrations exert a strong selective pressure for the emergency of resistant clones that pre-exist in the trypanosome population (Geerts and Holmes, 1998). Holmes et al. (2004)

therefore maintain that drug resistance in trypanosomes is likely to occur under large-scale drug use, by using inadequate dosing and by using correct dosing with drugs that are only slowly eliminated from the body.

Conversely, similar doses of drug and similar protocols in normal immunocompetent mice failed to lead to the development of isometamidium resistance in the IL 2642 clone. All the immunocompetent mice infected with the same clone of parasite were cured when treated with the lowest doses of isometamidium used in the current study, and it became evident that generation of resistance could not be quickly or easily done. This shows that the host immune system also plays an important role in the development of drug resistance in trypanosomes. Immunosuppression of the host may considerably reduce the efficacy of trypanocidal drugs and can lead to the rapid development of drug resistance. This has been exemplified by treatment of trypanosomosis with α-DFMO which is principally trypanostatic and requires a competent immune system to eliminate the arrested trypanosome population (De Gee et al., 1983). The results in the current study are in line with earlier studies. Osman et al. (1992) induced a high level of resistance in trypanosomes in immunosuppressed mice infected with *T. evansi* through progressive treatment of the mice with sub-curative doses of trypanocidals. In contrast, attempts made by the same authors to develop Mel Cy and diminazene resistance in immunocompetent mice were not successful. Mutugi et al. (1994) investigated factors which influence induction of resistance to suramin in two *T. evansi* stocks, using cloned and uncloned trypanosomes in immunosuppressed or immunocompetent mice. 3000-fold resistance was achieved in cloned trypanosomes in immunosuppressed mice. Later, Pospichal et al. (1994) produced a population of *T. b. brucei* with reduced sensitivity to melarsenoxide cysteamine (Mel Cy) in immunosuppressed mice using sub-curative drug treatment, which showed various levels of cross-resistance to melasoprol, diminazene and pentamidin. Fang et al. (1994) reported that the CD_{100} values of immunosuppressed mice infected by suramin-resistant clones were significantly higher than those of intact mice infected with the same clones, showing that the efficacy of suramin is immunodependent. It is likely, then, that a normal effective dose fails to produce a cure in immunocompromised hosts and results in the emergency of drug-resistant strains. Thus, in the presence of immunosuppression due to trypanosomosis or other parasitic diseases, the exposure time of trypanosomes to the drug is increased, which will favour selection for resistance. The emergency of drug-resistant strains in immunocompromised hosts could have serious implications particularly in trypanosomosis, a disease known to adversely affect the immune system of its hosts (Goodwin, 1970).

Moreover, simultaneous infections of trypanosome-infected cattle in trypanosome endemic areas of sub-Saharan Africa with other hemoparasites (*Babesia* spp., *Theileria* spp., *Anaplasma* spp.), which induce various degrees of immunosuppression, are common (Nyeko, et al., 1990). This may have an important implication for the development of isometamidium resistance in the field. Other predisposing causes may be environmental, e.g., nutritional status and stress, also rendering the host unable to play its supportive role in the elimination of infections (Matovu et al., 2002b).

On the other hand, the level of resistance by a clone of *T. congolense* to quinapyramine could successfully be increased about 42-fold (from a CD_{50} dose of 0.23 mg/kg bw for the parental clone to >9.6 mg/kg bw for the resistant derivative) by repeated sub-curative treatment of infected non-irradiated mice over a period of 208 days (Ndoutamia et al., 1993). This resistance was associated with a 6-fold increase in resistance to isometamidium (from a CD_{50} of 0.018 to 0.10 mg/kg bw), a 28-fold increase in resistance to homidium (from a CD_{50} of 0.37 to 10.35 mg/kg bw), and a 5.5-fold increase in resistance to diminazene (from a CD_{50} of 2.3 to 12.7 mg/kg bw) in infected mice. In goats infected with the resistant clone, one of five goats treated with 3 mg/kg bw quinapyramine sulphate, none of five goats treated with 0.25 mg/kg bw isometamidium chloride, two of five goats treated with 1 mg/kg bw homidium chloride, and three of five goats treated with 3.5 mg/kg bw diminazene aceturate subsequently relapsed, showing that except for isometamidium, the clone expressed multiple drug resistance even in the definitive host (Ndoutamia et al., 1993). Similarly, Peregrine et al. (1997) have successfully induced 94-fold resistance to isometamidium in a clone of *T. congolense* by repeated sub-curative treatment of infected non-irradiated mice over 11 months from a CD_{50} of 0.018 mg/kg bw for the parental *T. congolense* IL 1180 to a CD_{50} of 1.7 mg/kg bw for the resistant *T. congolense* IL 3343 clone. This clone also exhibited multiple drug resistance with a high level of resistance to homidium chloride (33-fold increase in CD_{50} from 0.37 to 12.1 mg/kg bw) but lower levels of resistance to diminazene aceturate (3.4-fold increase in CD_{50} from 2.3 to 7.8 mg/kg bw) and quinapyramine sulphate (4.2-fold increase in CD_{50} from 0.23 to 0.97 mg/kg bw). Similarly, a low level of cross-resistance to diminazene was recorded in the current study. Afework et al. (2000) reported a high level of resistance expressed to diminazene and isometamidium by *T. congolense* clonal populations derived from stocks isolated in northwestern Ethiopia. Whiteside et al. (1960) recommended the use of diminazene as a ‘sanative’ combination with isometamidium in the field to avoid the development of resistance to isometamidium. However, reports of cross-resistance between

isometamidium and diminazene demonstrated in clonal populations in the current and previous studies indicate that the use of these compounds as 'sanative pairs' in the long term may be jeopardised. Similar studies need to be carried out with large number of *T. congolense* field isolates before any generalization can be made. Recent studies on *T. b. brucei* demonstrated that both isometamidium (Mäser et al., 1999) and diminazene (De Koning et al., 2004) are transported by similar transporter genes, which suggests that both drugs may possibly have similar routes of entry and similar mechanisms of action. If the same is true with *T. congolense*, then the use of these two compounds as 'sanative' pairs might not be biologically justified.

The high level of resistance developed in immunosuppressed mice in the current study was found to be stable and persistent when the clones were subsequently tested in immunocompetent mice, after sub-passages in immunocompetent mice in the absence of drug treatment. This is in agreement with the report by Osman et al. (1992) who demonstrated that the high level of drug resistance developed in immunosuppressed mice was still present when the clones were tested in normal immunocompetent mice. Mutugi et al. (1994) later showed that suramin-resistance in *T. evansi* was stable after 10 passages in mice in the absence of suramin. Mulugeta et al. (1997) indicated that drug resistance in trypanosomes, once induced, appears to be stable and irreversible.

Finally, this study demonstrated that a series of *T. congolense* clones with varying degrees of isometamidium resistance could be generated in animal models. To our knowledge these are only the second sets of isometamidium-resistant clones of *T. congolense* derived in the laboratory. The clones can be used to study the genetic, biochemical and molecular basis of isometamidium resistance and to investigate the mode of action of drugs. They may also be used to investigate how drug resistance phenotypes arise in the field. This may provide information on the mechanism of isometamidium resistance in *T. congolense*, which might then suggest chemotherapeutic strategies to overcome drug resistance in the field. These drug resistant trypanosomes may also provide a useful tool in experimental chemotherapy and have potential applications in the primary screening of candidates of new trypanocides. All have potentially far-reaching impact on the control of resistance. Nevertheless, since changes that are not linked to the drug resistance phenotype may arise at any step during the derivation of a drug resistant population of trypanosomes from a sensitive one, it is important to include all the intermediate clones when undergoing such investigations.

6.4 Molecular characterisation of isometamidium-sensitive and -resistant trypanosomes

Several studies have shown that the P2 adenosine transporter plays a role in the resistance of African trypanosomes to both melaminpohenyl arsenicals and diamidines (Carter et al., 1995; Ross and Barns, 1996). A *T. b. brucei* gene, TbAT1, was found to encode an adenine-sensitive adenosine transporter when expressed in yeast (Mäser et al., 1999; Matovu, 2001a), which was proofed by the gene deletion study conducted later (Matovu et al., 2003). This later study demonstrated the total absence of P2-type transport in TbAT1-null bloodstream-form trypanosomes and indicated that loss of TbAT1 reduced the sensitivity of trypanosomes to melaminophenyl arsenicals.

Isometamidium chloride, a conjugate of the homidium (ethidium) and part of the diminazene molecule, is used exclusively as veterinary trypanocide, both prophylactically and therapeutically (Kinabo and Bogan, 1988). Resistance to isometamidium is a serious problem in many parts of sub-Saharan Africa. Isometamidium transport and resistance has been largely studied in *T. congolense* which, together with *T. brucei* and *T. vivax*, is the main cause of trypanosomosis in African livestock. Several authors have reported reduced uptake of isometamidium associated with resistance. Sutherland et al. (1991, 1992) reported an inverse relationship between drug uptake and resistance to isometamidium in *T. congolense*. In *T. b. brucei*, the P2 transporter may be responsible for part of the isometamidium uptake, as the drug was found to inhibit P2-mediated adenosine uptake (Mäser et al., 1999). However, apart from the indications that isometamidium is a substrate of the P2-transporter in a study conducted in a yeast expression system, no further work had been conducted using field isolates to investigate the involvement of the gene encoding the P2-transporter (TbAT1) in isometamidium resistance. The current study tried to investigate if there is a link between the presence of mutation in the gene (TbAT1) and isometamidium resistance in *T. b. brucei*.

In the current study, a 677 bp fragment of TbAT1 was successfully amplified from the genomic DNA of isometamidium-sensitive and -resistant *T. brucei brucei* stocks. Additional fragments of the gene were amplified using oligonucleotide primer sets designed from the TbAT1 gene sequences described by Mäser et al. (1999). A total of 14 *T. brucei brucei* stocks have been analysed. The results demonstrated that fragments of the size expected could be amplified from the DNA of both isometamidium sensitive field stocks and the resistant

reference stocks. Thus, the gene could be detected in all of the *T. b. brucei* stocks tested in the present study.

Although several methods are currently in use to detect mutations in genes, these methods do not identify the precise nature of the mutation (De Souza, 2000). The exact location and nature of the mutation can be defined by sequencing of the DNA fragment in which the mutation is thought to occur. Direct sequencing refers to the sequence analysis of the DNA without prior sub-cloning into a vector (Sanger et al., 1977). The sequencing alignment analysis of the 677 bp central fragment of the TbAT1 gene in the current study showed that 11 of the *T. b. brucei* field stocks tested had a similar sequence pattern, which corresponded to the melarsoprol-sensitive wild-type *T. brucei* reference sequence (Genbank accession number AF 152369). All of the 11 stocks in the current study were characterized for their isometamidium sensitivity using *in vitro* and *in vivo* (in mice) methods (Scheer, 2001), who reported that the stocks were sensitive to isometamidium at the dose rate of 1 mg/kg bw when tested in mice. In contrast, the resistant reference stocks (CP 547 and CP 2469) did show high level of resistances, such that mice treated at dose rates of 3 mg/kg bw were not cured. Interestingly, the isometamidium-sensitive *T. b. brucei* stocks, which showed similar sequence patterns, had originated from three different geographical regions of Uganda. Thus, phenotypically isometamidium-sensitive *T. b. brucei* originating from different regions appeared to have similar sequence patterns of the TbAT1 gene fragments. However, complete sequence of the gene is required before it can be concluded that the gene sequences are identical.

On the other hand, screening of the 677 bp fragment of TbAT1 from the isometamidium resistant *T. b. brucei* proved the presence of six point mutations. Four of the nucleotide differences led to amino acid substitutions. All the point mutations detected had been described in the sequence of the laboratory-derived melarsoprol-resistant stock STIB 777R (Mäser et al., 1999). The same sets of mutations had been detected in the TbAT1 gene of *T. b. gambiense* isolated from a focus in north-western Uganda with high treatment failure rates following melarsoprol therapy, in a drug-resistant *T. b. rhodesiense* isolate from south-eastern Uganda and in a *T. b. gambiense* isolate from a relapsing patient from northern Angola (Matovu et al., 2001a). The current study demonstrates the presence of an identical set of mutants within the isometamidium-resistant *T. b. brucei* to the ones reported for arsenical resistance in *T. b. rhodesiense* and *T. b. gambiense* (Matovu et al., 2001a), and in STIB 777R

(Mäser et al., 1999), which was selected in mice for Cymelarsan resistance (Pospichal et al., 1994). Thus, in trypanosomes of different subspecies and from geographically distant locations the same sets of mutations have been observed. If these results can be supported by further studies using known isometamidium-resistant field isolates of *T. b. brucei*, it may be possible that a similar transporter gene to that implicated for arsenicals resistance in *T. b. rhodesiense* and *T. b. gambiense* is responsible for isometamidium resistance in *T. b. brucei*. If that is the case, it will have great implications for the development of arsenical resistance in human sleeping sickness. Frequent treatment of cattle with isometamidium in tsetse-infested areas of Africa may select for isometamidium-resistant *T. b. rhodesiense* and *T. b. gambiense*, which may lead to cross-resistance to arsenicals. Furthermore, deletions in three consequent nucleotides, which resulted in an amino acid (phenylalanine) deletion, have been detected in both of the isometamidium-resistant *T. b. brucei* clones. The differences in amino acid sequences observed may have arisen in the course of the development of resistance to isometamidium. On the other hand, although TbAT1 is a single copy gene, trypanosomes are diploids containing one copy of the gene in each of the two alleles. Therefore, cloning of the gene from the DNA of isometamidium-sensitive and -resistant *T. b. brucei* and analysing several clones for sequence variations is required in order to confirm the presence of the polymorphism. Sequence polymorphisms, particularly those found within the coding region could result in changes in the function of the protein or an alteration in the affinity of the protein for its target. This would most likely be the case if the alteration resulted in changes at the amino acid level. The polymorphism existing in the non-coding or untranslated regions could also result in changes in the three-dimensional structure.

Restriction fragment length polymorphism analysis (RFLP) is a technique in which species may be differentiated by analysis of patterns derived from cleavage of their DNA. The method can be exploited to detect differences in specific regions of genes or gene transcripts (Botstein et al., 1980). In the current study, the 677 bp gene sequences of TbAT1 were analysed for differences in endonuclease restriction sites, and polymorphism has been detected for the Sfa NI restriction enzyme. Thus, the isometamidium-sensitive and -resistant *T. b. brucei* could be successfully distinguished by digestion with the restriction endonuclease Sfa NI. Consequently, all of the 11 *T. b. brucei* field isolates, which are phenotypically characterised as isometamidium-sensitive (Scheer, 2001), had similar Sfa NI restriction sites. Identical Sfa NI restriction sites were observed in the genes of the reference isometamidium-sensitive laboratory clones of *T. b. brucei* (STIB 345 and ILTAT 1.4). In contrast, the

isometamidium-resistant reference *T. b. brucei* stocks tested (CP 547 and CP 2469) showed restriction sites further downstream. The point mutation at 532 bp (mutation from nucleotide G to A) that occurred in the resistant stock abrogated the Sfa NI restriction site, whereas the mutation at 857 bp (mutation from nucleotide A to G) resulted in a new Sfa NI restriction site. This mutation in the isometamidium resistant *T. b. brucei* stock resulted in the shift of Sfa NI restriction sites some 323 bp further downstream. The restriction profiles observed in the current study are in accordance with earlier reports (Mäser et al., 1999; Matovu et al., 2001a). Mäser et al. (1999) have shown that in the laboratory-derived melarsoprol-resistant stock STIB 777R, one Sfa NI site within TbAT1 is abrogated, while a new such site is generated 323-bp further downstream by an independent mutation. They have subsequently observed a similar RFLP pattern from a patient refractory to melasoprol treatment. Thus, they suggested that amplification of a fragment of the purine transporter gene by PCR followed by Sfa NI digestion might serve as a convenient means for rapid identification of TbAT1^{r}-type drug-resistant trypanosomes. Furthermore, Matovu et al. (2001b) observed the same pattern in a previously described diminazene and isometamidium resistant *T. b. rhodesiense* isolated from southeast Uganda (Matovu et al., 1997). However, a large-scale screening of field isolates is required to determine the correlation of specific TbAT1 alleles with treatment failures and its value in diagnosis of drug resistance. If Sfa NI RFLP is found to be sensitive enough for the detection of mutated TbAT1, it might be developed into a good epidemiological tool for the early detection of drug-resistant strains of *T. brucei*, which would be valuable for successful chemotherapy and control of the diseases.

Although some reports exist on isometamidium resistance in *T. b. brucei* in cattle (Joshua, 1988; Zweygarth and Röttcher, 1989; Chitambo and Arakawa, 1991), because of the less pathogenic nature of this species of the parasite to cattle compared to *T. congolense* and *T. vivax* (Taylor and Authié, 2004), only few attempts were made to study the spread of isometamidium-resistant *T. b. brucei* infections in the tsetse infected areas of sub-Saharan Africa. However, a notable exception was recorded from Western Kenya and Uganda, where *T. b. brucei* was found in the CNS of native cattle and was associated with significant mortality (Wellde et al., 1989). *T. b. brucei* may also become more pathogenic under stress conditions and in areas where other trypanosome species have been effectively reduced by chemotherapy (Kalu, 1995). Since all economically important animal-infective trypanosome species co-exist in the field, simultaneous infections of cattle with more than one species of trypanosomes are frequent. Furthermore, *T. b. brucei* is highly virulent in horses, donkeys and

camels, causing acute or subacute diseases leading to death (Taylor and Authié, 2004). Thus, frequent curative and prophylactic treatments aimed at *T. congolense* and *T. vivax*, as has been observed in many parts of sub-Saharan Africa, may lead to repeated exposure of the *T. b. brucei* to the drugs, which ultimately selects for resistant populations. Transmission by tsetse flies does not appear to affect the drug sensitivity of trypanosomes and drug-resistant strains remain resistant after passage through tsetse flies (Moloo and Kutuza, 1990; Peregrine et al., 1991). The long-term stability of resistance of *T. congolense* to diminazene, isometamidium and homidium over a period of 4 years in the Ghibe valley of Ethiopia (Mulugeta et al., 1997) suggests the magnitude of the problem, once drug resistance is established in an animal population. Assefa and Abebe (2001) noted that resistant populations established in an area can be disseminated to alternative hosts, for example, from cattle to donkeys or donkeys to cattle, in a given locality. The selected resistant populations can easily spread over a larger area of sub-Saharan Africa through tsetse flies and uncontrolled animal movements. Moreover, domestic animals are important reservoirs of human infective trypanosomes (Mehlitz et al., 1982). Kalu et al. (1995) reported that human infective *Trypanozoon* isolated from cattle in Nigeria were resistant to diminazene and isometamidium. Matovu et al. (1997) isolated *T. b. rhodesiense* from cattle in southeast Uganda, which were resistant to diminazene and isometamidium in mice. There is also an indication that similar transporter genes are involved in resistance to melarsoprol (Mäser et al., 1999), diminazene (De Koning et al., 2004) and isometamidium (Matovu et al., 2001b) by *T. brucei*. This might have important implications in the control of sleeping sickness, since frequent treatment of cattle with isometamidium my lead to repeated exposure of human infective trypanosomes in the reservoir hosts, which will select for isometamidium resistance and hence cross-resistance to melarsoprol and diamidines. Therefore, understanding the molecular mechanism of isometamidium resistance in *T. b. brucei* and identification of the gene(s) involved in the resistance phenotype are important steps in the early identification of marker genes and early detection of resistance in the field, which might have important connotations in its own right and in the control of sleeping sickness as well.

In the current studies, attempts made using different strategies to amplify the TbAT1 gene fragment from the genomic DNA of isometamidium-sensitive and -resistant *T. congolense* did fail to yield the expected PCR product. However, it cannot be concluded that the gene does not exist in this species of the parasite; the gene can be present with very low homology. Therefore, further studies are required to identify the gene in the cDNA library and analyses

are needed to ascertain its likely involvement in isometamidium resistance. Application of methods that do not require prior knowledge of the function and location of the gene responsible for a specific phenotype may also prove to be a suitable approach to identify a gene(s) involved in isometamidium resistance in *T. congolense*.

The genetic basis of isometamidium resistance in *T. congolense* is less clear. Gene amplification under conditions of drug pressure has been demonstrated in trypanosomes, but there is no evidence that this occurs as a mechanism of drug resistance in trypanosomes (Ross and Sutherland, 1997). Several authors have reported reduced uptake of isometamidium associated with resistance in *T. congolense* (Sutherland et al., 1991, 1992, 1992a). Subsequently, it has been shown that the uptake of isometamidium chloride by both sensitive and resistant clones of *T. congolense* was through an energy-dependent, specific, receptor-mediated transport system on the parasite surface (Sutherland et al., 1992). Sutherland and Holmes (1993) proposed that an alteration or replacement of a specific receptor in isometamidium chloride resistant *T. congolense* results in an increased efflux of the drug and that this increased efflux at least partially mediates the reduction in sensitivity to the compound. Recently, Mulugeta et al. (1997) showed that the maximal uptake rates (V_{max}) of isometamidium in resistant *T. congolense* were significantly lower than in sensitive populations. It remains to be shown whether this is caused by a decreased number of protein transporters of isometamidium in the plasma membrane and/or by changes in the balance between influx and efflux. More recently, Wilkes et al. (1997) have demonstrated changes in the mitochondrial electrical potential in isometamidium-resistant *T. congolense*. However, little attempts were made to identify and clone the gene(s) involved in isometamidium resistance in *T. congolense* (Majiwa et al., 2000). The recently cloned TbAT1 gene, which encodes P2 type transport activities, was found to mediate transport of isometamidium chloride (Mäser et al., 1999) and diminazene aceturate (De Koning et al., 2004) in *T. b. brucei*. Detailed works on the involvements of similar transporter genes in isometamidium resistance in *T. congolense* are required. Delespaux et al. (2005) have recently attempted to identify a marker gene for isometamidium resistance in *T. congolense.* They used Amplified Fragment Length Polymorphism (AFLP) to compare two isogenic clones of *T. congolense*, one of which has a CD_{50}, which is 94-fold higher than the other. They identified a predicted gene coding for an 854 amino acide protein, which has homology with an ATP-binding cassette and an ABC-type multidrug/protein/lipid transporter system. The gene in the resistant strain has a triplet insertion coding for an extra lysine. Using PCR-RFLP, the insertion was

analysed in genomes of 35 *T. congolense* isolated from different geographical locations. The insertion was present in most of the *T. congolense*, which were characterised as isometamidium-resistant, but absent in the sensitive isolates. Five of the isometamidium resistant *T. congolense* strains tested showed an RFLP profile similar to that observed among the sensitive strains. This may suggest that more than one mechanism of resistance to isometamidium exists in *T. congolense*. Thus, this marker needs to be further validated using a large number of isolates with different isometamidium-sensitivity phenotypes from different geographical origins.

Although trypanocidal resistance is well documented in *T. congolense*, little is known about the genome and as a result only little sequence information is available Therefore, in order to identify genetic markers for the detection of drug resistance in *T. congolense*, further studies are required using methods which require no prior sequence information and probes. The stocks and clones of *T. congolense* characterised for isometamidium sensitivity in the current and in previous studies would be suitable materials to undertake such detailed studies.