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ORIGINAL PAPER

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Rapid identification of isometamidium-resistant stocks of *Trypanosoma b. brucei* by PCR–RFLP

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Abstract Analyses were made on the adenosine transporter-1 gene in *Trypanosoma brucei* (*TbAT1*), encoding a P2-like nucleoside transporter, from *T. brucei brucei* field stocks to investigate a possible link between the presence of mutations in this gene and isometamidium resistance. We have analysed the gene from 11 isometamidium-sensitive field stocks isolated from cattle in Uganda, two sensitive reference clones and two resistant reference clones. A sequence alignment showed that the isometamidium-sensitive *T. b. brucei* contained the wild-type sequence patterns. In contrast, the isometamidium-resistant *T. b. brucei* stocks showed the mutant-type sequence patterns with six point mutations that had previously been reported in a laboratory-derived arsenical-resistant *T. brucei* strain. To analyse the restriction fragment length polymorphism pattern of a fragment of *TbAT1* (nucleotides 430–1108), the 677-bp polymerase chain reaction products from eight of the isometamidium-sensitive and two of the isometamidium-resistant *T. b. brucei* were subjected to digestion with Sfa NI. The results revealed two different banding patterns: the digest resulted in fragment sizes of 566 and 111 bp in the case of *TbAT1* from isometamidium-sensitive stocks, whereas it produced fragment sizes of 435 and 242 bp in the case of *TbAT1* from isometamidium-resistant stocks. Thus, the isometamidium-sensitive and isometamidium-resistant *T. b. brucei* could be successfully distinguished by digestion with the restriction endonuclease Sfa NI.

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Introduction

African trypanosomes cause a serious and often fatal disease commonly called nagana in domestic livestock. In cattle, *Trypanosoma congolense*, *Trypanosoma vivax* and *Trypanosoma brucei* are the main species responsible for this disease. The control of trypanosomosis continues to rely principally on chemotherapy and chemoprophylaxis using the salts of three compounds: diminazene, an aromatic diamidine; homidium, a phenanthridine; and isometamidim, a phenanthridine-aromatic amidine (Leach and Roberts 1981). Unfortunately, the incidence of drug resistance by the parasites appears to be increasing (Peregrine 1994). So far, the resistance to one or more of the trypanocidal drugs in use has been reported from 13 sub-Saharan countries (Geerts and Holmes 1998) and, in some countries, multiple-drug resistance in *T. congolense* has been encountered (Clausen et al. 1992; Codjia et al. 1993; Afework et al. 2000). Trypanosome resistance to trypanocides increases costs, reduces the efficacy of production and depletes farmers of effective control tools (Donald 1994).

Isometamidium is the only recommended prophylactic drug that is widely used in the treatment of trypanosome infections in cattle and small ruminants across sub-Saharan Africa. Despite increasing reports of resistance to this drug, accurate data on the magnitude, distribution and frequency of emergency of isometamidium resistance are very limited (Geerts and Holmes 1998; Geerts et al. 2001). The common techniques currently in use to identify drug resistance in trypanosomes (tests in ruminants, tests in mice and in

vitro assays) suffer from a number of drawbacks. For example, requirements for large numbers of experimental animals, long periods in performing the tests and difficult adaptation of the parasites to grow in tissue culture or laboratory rodents (Geerts and Holmes 1998). Therefore, faster, more sensitive and more reliable methods are required. These would help avoid further increases in resistance levels and the waste of resources by continued use of ineffective drugs.

Trypanosomes lack the ability to synthesise purine bases de novo. They, therefore, depend on purine bases or nucleosides pre-formed by their mammalian hosts for survival (Hassan and Coombs 1988). Two classes of nucleoside transporters have been identified in *T. brucei*, the P1-type transporters that promote the uptake of adenosine and inosine and the P2-type transporters that mediate the uptake of adenosine and the purine base adenine. The P2 transporters also transport drugs such as pentamidine and melarsoprol (Carter and Fairlamb 1993; De Konig 2001). These investigators have linked the resistance to melaminophenyl arsenicals and diamidines in *T. brucei* to the loss/alteration of the P2 transporter activity. The gene that encodes for the P2 transporter, TbAT1, has been cloned, and studies have revealed several mutations in the gene in a laboratory-derived melarsoprol-resistant stock of *T. brucei*. When expressed in yeast, this mutated P2 transporter could no longer import melarsoprol and also isometamidium (Mäser et al. 1999). In these trypanosomes, the resistance resulted from a reduced net drug uptake. By exploiting two simultaneously occurring mutations that generate a restriction fragment length polymorphism (RFLP) for Sfa NI, Mäser et al. (1999) devised a means of distinguishing between the arsenical-sensitive *T. brucei* stock and its resistant laboratory derivative. This technique has been evaluated by using *T. b. gambiense* isolates from melarsoprol refractory patients in northwestern Uganda, a *T. b. rhodesiense* isolate from southeastern Uganda and a *T. b. gambiense* isolate from Angola (Matovu et al. 2001a). The present study was conducted with the aim of investigating possible links between the presence of mutations in *TbAT1* gene and isometamidium resistance in *T. b. brucei*, and assessing if mutations in the gene lead to RFLP, using known isometamidium-sensitive and isometamidium-resistant field and reference stocks.

Materials and methods

T. b. brucei field stocks

The *T. b. brucei* field stocks used (Table 1) were isolated in 1995 from naturally infected cattle from different regions of Mukono County, Uganda (Pötzsch 1999). By comparison to trypanosome reference clones, all Mukono stocks were sensitive to isometamidium chloride when tested in mice or in the long-term in vitro viability assay (Scheer 2001).

T. b. brucei reference stocks

CP 547 was isolated from a naturally infected cow in Jilib, Somalia in 1985 (Table 1). The isolate is resistant to diminazene, isometamidium, quinapyramine, melarsoprol, homidium and pentamidine (Zweygarth and Röttcher 1989). The clone CP 2469 was derived from a *T. brucei* stock 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). The clone ILTat 1.4

Table 1 Origin and isometamidium sensitivity of the *T. b. brucei* stocks used in the study

Trypanosome stocks	Code	Country	Year of isolation	Origin	Sensitivity to ISMM	References
Field stocks						
MBOT/UG/95 02 20	MBOT 02 20	Uganda	1995	Cattle	S	Scheer 2001
MBOT/UG/95 04 01	MBOT 04 01	Uganda	1995	Cattle	S	Scheer 2001
MBOT/UG/95 05 05	MBOT 05 05	Uganda	1995	Cattle	S	Scheer 2001
MBOT/UG/95 05 25	MBOT 05 25	Uganda	1995	Cattle	S	Scheer 2001
MBOT/UG/95 07 16	MBOT 07 06	Uganda	1995	Cattle	S	Scheer 2001
MBOT/UG/95 10 17	MBOT 10 17	Uganda	1995	Cattle	S	Scheer 2001
MBOT/UG/95 22 23	MBOT 22 23	Uganda	1995	Cattle	S	Scheer 2001
MBOT/UG/95 25 07	MBOT 25 07	Uganda	1995	Cattle	S	Scheer 2001
MBOT/UG/95 28 21	MBOT 28 21	Uganda	1995	Cattle	S	Scheer 2001
MBOT/UG/95 53 23	MBOT 53 23	Uganda	1995	Cattle	S	Scheer 2001
MBOT/UG/95 163 23	MBOT 163 23	Uganda	1995	Cattle	S	Scheer 2001
Reference stocks						
ILTaT 1.4	ILTaT 1.4	Kenya	1964	Steer	S	Miller and Turner 1981
STIB 345 RA	STIB 345	Kenya	1969	Glossina	S	Brun et al. 1979
CP 547	CP 547	Somalia	1985	Cattle	R	Zweygarth and Röttcher 1989
CP 2469	CP 2469	Somalia	1985	Cattle	R	Zweygarth and Röttcher 1989

ISMM Isometamidium chloride, *S* sensitive, *R* resistant

originated from the stock EATRO 795 isolated from a steer in Uhembo District in Kenya in 1964. The isolate was characterised as human serum sensitive in the blood incubation infectivity test and the human serum resistance test (Hawking 1976a,b). The clone STIB 345 RA was derived from the stock EATRO 1529 isolated in 1969 from a tsetse fly in Kiboko, Kenya. The isolate was reported to be sensitive to isometamidium (Hide et al. 2000). All the *T. brucei* study and reference stocks showed no detectable band for the human serum resistance-associated gene fragment (Radwanska et al. 2002) when analysed by polymerase chain reaction (PCR) (Afework 2005). This indicated that all the study stocks were *T. b. brucei*.

Purification of trypanosomes and DNA extraction

Bloodstream forms of trypanosomes were expanded in mice and were purified from the blood by di-ethyl-amino-ethyl chromatography (Lanham and Godfrey 1970), followed by repeated centrifugation and sediment washes with phosphate-buffered saline. DNA was extracted from purified trypanosomes according to the method described by Higuchi (1989). In brief, 250 µl of the purified trypanosome suspension was mixed with 250 µl lysis buffer (0.32 M sucrose, 0.01 M Tris, 0.005 M $MgCl_2$ and 1% Triton X-100, pH 7.5) and centrifuged at 13,000×*g* for 25 s. The pellet was washed three times with 500 µl lysis buffer. The final pellet was re-suspended in 250 µl of 1× PCR buffer. Then, 1.5 µl of proteinase-K (10 mg/ml) was added and mixed by vortexing. The samples were incubated at 56°C for 1 h followed by 95°C incubation for 10 min. The DNA concentration was estimated by means of a spectrophotometer at a wavelength of 260 nm, Gene Quant Calculator (Amersham Pharmacia Biotech, Freiburg, Germany); the samples were stored at −20°C until they were used for PCR amplification.

Genomic DNA amplification of the *TbAT1* gene fragments

From the DNA of each of the *T. b. brucei* stocks (Table 1), a 677-bp central fragment of the *TbAT1* gene (nucleotide 430–1,108) 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). PCR amplifications of DNA were carried out in 25-µl reaction volumes containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 0.1% Triton X-100, 2 mM $MgCl_2$, 200 µM each of the four dNTPs (dATP, dCTP, dGTP and dTTP), one unit of DNA polymerase, approximately 5 ng DNA and 1 µM 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 s. 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. Amplification was done using hot start Ampli Taq Gold polymerase (Applied Biosystems, GmbH, Darmstadt, Germany), and 30 PCR cycles were performed using the following conditions: 95°C, 15 min; 64°C, 2 min; and 72°C, 30 s. It was followed by a final extension of 10 min at 72°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.

DNA sequencing

The 677-bp PCR fragments of the *TbAT1* gene from the *T. b. brucei* stocks were used for the sequence analysis. For DNA sequencing, three independent amplification reactions were performed for each sample. The PCR products were pooled and purified using a commercial kit (highly pure PCR product purification kit, Roche Diagnostics GmbH, Penzberg, Germany). The DNA concentration was estimated by means of a spectrophotometer, the Gene Quant Calculator. The PCR product was outsourced for sequencing to SEQLAB (Göttingen, Germany). The gene was sequenced with forward and reverse primers. The 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 1,493-bp sequence data for the wild-type and mutant-type *TbAT1* (accessed from the GenBank with accession numbers AF152369 and AF152370, respectively). The sequences were analysed for base match, mismatch, insertion and deletion.

RFLP analysis with Sfa NI

To analyse the Sfa NI restriction profile of the *TbAT1* gene fragment, the genomic DNA of eight of the isometamidium-sensitive and two of the isometamidium-resistant *T. b. brucei* was amplified by PCR using the primers Sfa-s and Sfa-as as described before. The PCR products of three independent reactions were pooled and purified using a commercial purification kit (Roche Applied Science, Roche Diagnostic GmbH, Germany). The concentration of the PCR product was determined in a spectrophotometer using OD_{260}. Then, the purified 677-bp fragment of *TbAT1* (nucleotide 430–1,108) was subjected to Sfa NI digestion [New England Biolabs (NEB)]. Endonuclease digestions were performed in 20-µl reaction volumes; 17.5 µl of purified PCR products, 2 µl 10× NEB enzyme reaction buffer and 0.5 µl (0.5 units) of the enzyme were mixed in a final reaction volume of 20 µl. The mixture was incubated at 37°C for at least 3 h to achieve complete digestion. The digested samples were analysed along with the 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.

Results

Detection of a fragment of *TbAT1* gene in *T. b. brucei* stocks by PCR

The results of PCR analyses showed that the expected 677-bp fragments of the gene were successfully amplified from the genomic DNA of each of the *T. b. brucei* field and laboratory stocks (Fig. 1).

DNA sequencing

The results of the sequence analyses of the 677-bp central fragment of the *TbAT1* (nucleotides 430–1,108) (Fig. 2) indicated that all the *T. b. brucei* stocks in this study, which were phenotypically characterised as isometamidium-sensitive, contained the wild-type sequence patterns. In contrast, the isometamidium-resistant *T. b. brucei* stocks (CP 547 and CP 2469) showed sequence patterns that corresponded to the DNA sequence of the laboratory-derived melarsoprol-resistant STIB 777R stock. A summary of the nucleotide variations detected in the diagnostic positions of the 677-bp *TbAT1* gene sequence alignment among the different stocks is given in Table 2. Both nucleotide sequences from the isometamidium-resistant *T. b. brucei* showed the same set of six mutations detected in the laboratory-derived melarsoprol-resistant stock. These differences were as follows: substitution of C by T at nucleotide 471, G by A at nucleotide 532, G by A at nucleotide 542, A by G at nucleotide 716, A by G at nucleotide 857 and C by T at nucleotide 1008. Four of these nucleotide differences detected in the 677-bp gene fragments led to amino acid substitutions: $Ala^{178} \rightarrow Thr$ (A178T), $Gly^{181} \rightarrow Glu$ (Gl181E), $Asp^{239} \rightarrow Gly$ (D239G) and $Asn^{286} \rightarrow Ser$ (N286S). Furthermore, deletions of three nucleotides (TTC), which encode the amino acid phenylalanine, were detected at nucleotide positions 949, 950 and 951 of both of the resistant stocks.

The results of the analysis of the restriction sites of the 677-bp *TbAT1* gene fragments revealed that all isometamidium-sensitive *T. b. brucei* field stocks tested and both of the isometamidium-sensitive reference stocks (STIB 345 and ILTaT 1.4) possess similar Sfa NI restriction site, whereas the isometamidium-resistant reference *T. b. brucei* stocks (CP 547 and CP 2469) showed Sfa NI restriction sites different from those of the sensitive ones (Fig. 2). The point mutation that led to the substitution of the nucleotide G by A at nucleotide position 532 (G532A) in the sensitive stock abrogated the Sfa NI restriction site. In contrast, the mutation at 857 bp that led to the substitution of A by G (A857G) resulted in a new Sfa NI restriction site.

Sfa NI RFLP analysis

The results of the restriction digests of the 677-bp fragments of TbATl with the restriction endonuclease Sfa NI revealed two different banding patterns: The digest resulted in fragment sizes of 566 and 111 bp in the case of *TbAT1* from isometamidium-sensitive stocks and in fragment sizes of 435 and 242 bp in the case of *TbAT1* from isometamidium-resistant stocks (Fig. 3).

Discussion

Several studies have shown that the P2 adenosine transporter plays a role in the resistance of African trypanosomes to both melaminophenyl 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), which was proven 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

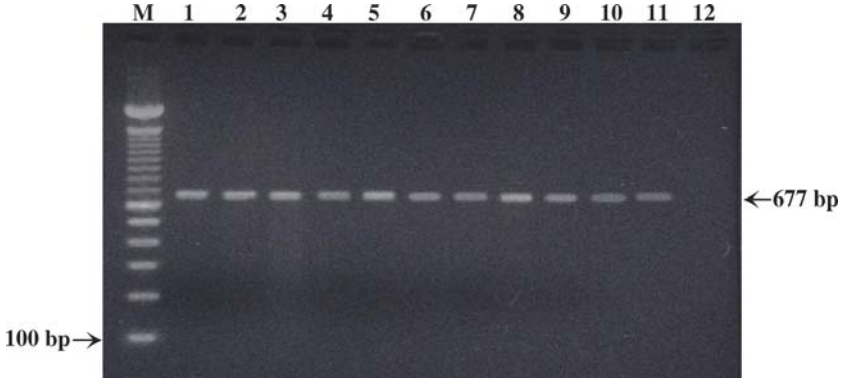

Fig. 1 PCR profile of a 677-bp fragment of *TbAT1* from the genomic DNA of *T. b. brucei*. *M*, 100-bp molecular size marker; *lane 1*, ILTat 1.4; *lanes 2–10*, MBOT 02 20, MBOT 04 05, MBOT 05 05, MBOT 05 25, MBOT 07 16, MBOT 10 17, MBOT 22 23, MBOT 25 07 and MBOT 28 21; *lane 11*, STIB 345; *lane 12*, negative control

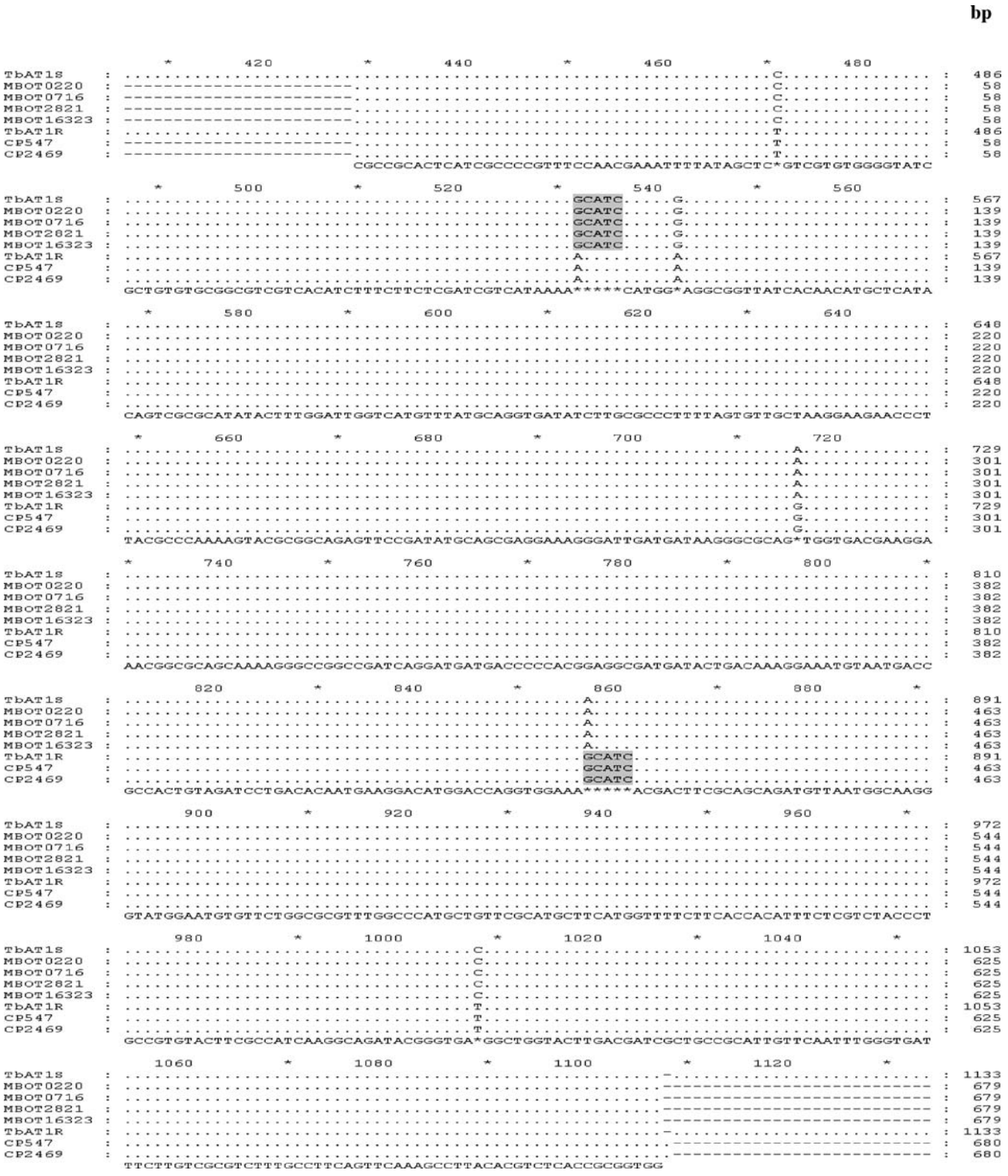

Fig. 2 Nucleotide sequence alignments of the *TbAT1* gene fragment in isometamidium-sensitive and isometamidium-resistant *T. b. brucei* stocks. Identical sequences are shown by *dots* (.) and differences by *letters* representing nucleotides. Sfa NI restriction sites are *shaded in dark*. *TbAT1S* and *TbAT1R* refer to the nucleotide sequences in the wild-type and mutant-type TbATl, respectively (GenBank accession numbers AF152369 and AF152370, respectively)

Table 2 Summary of the nucleotide variations detected in the 677-bp gene fragment (nucleotides 430–1,108) of the *TbAT1* gene between isometamidium-sensitive and isometamidium-resistant *T. b. brucei* stocks

Trypanosome stocks	Sensitivity to ISMM	Alignment positions					
		471	532[a,b]	542[a]	716[a]	857[a,b]	1,008
TbAT1[s]*	S	C	G	G	A	A	C
MBOT 02 20	S	C	G	G	A	A	C
MBOT 04 01	S	C	G	G	A	A	C
MBOT 05 05	S	C	G	G	A	A	C
MBOT 05 25	S	C	G	G	A	A	C
MBOT 07 16	S	C	G	G	A	A	C
MBOT 10 17	S	C	G	G	A	A	C
MBOT 22 23	S	C	G	G	A	A	C
MBOT 25 07	S	C	G	G	A	A	C
MBOT 28 21	S	C	G	G	A	A	C
MBOT 53 23	S	C	G	G	A	A	C
MBOT 163 23	S	C	G	G	A	A	C
STIB 345	S	C	G	G	A	A	C
ILTaT 1.4	S	C	G	G	A	A	C
TbAT1[r]*	R	T	A	A	G	G	T
CP 547	R	T	A	A	G	G	T
CP 2469	R	T	A	A	G	G	T

ISMM Isometamidium chloride, *S* sensitive, *R* resistant, *TbAT1[s]** melarsoprol-sensitive reference stocks (GenBank accession number AF152369), *TbAT1[r]** melarsoprol-resistant reference stocks (GenBank accession number AF152370), *A* adenine, *C* cytosine, *G* guanine, *T* thymine

[a]Nucleotide differences manifested at the amino acid level

[b]Position of point mutation that resulted in the Sfa NI RFLP

exclusively as veterinary trypanocide, both prophylactically and therapeutically (Kinabo and Bogan 1988). The 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 in *T. congolense* (Sutherland et al. 1991, 1992).

The current study tried to investigate if there is a link between the presence of mutations in the gene (*TbAT1*) and isometamidium resistance in *T. b. brucei*. A 677-bp fragment of *TbAT1* was successfully amplified from the genomic DNA of isometamidium-sensitive and isometamidium-resistant *T. b. brucei* stocks. The sequencing alignment analysis of the 677-bp central fragment of the *TbAT1* gene showed that all isometamidium-sensitive *T. b. brucei* reference and field stocks had a similar sequence pattern, which corresponded to the melarsoprol-sensitive wild-type *T. brucei* reference sequence (Genbank accession number AF 152369). On the other hand, the 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 northwestern Uganda with high treatment failure rates after melarsoprol therapy, in a drug-resistant *T. b. rhodesiense* isolate from southeastern Uganda and in a *T. b. gambiense* isolate from a relapsing patient from northern Angola (Matovu et al. 2001a). 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 arsenical 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. 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.

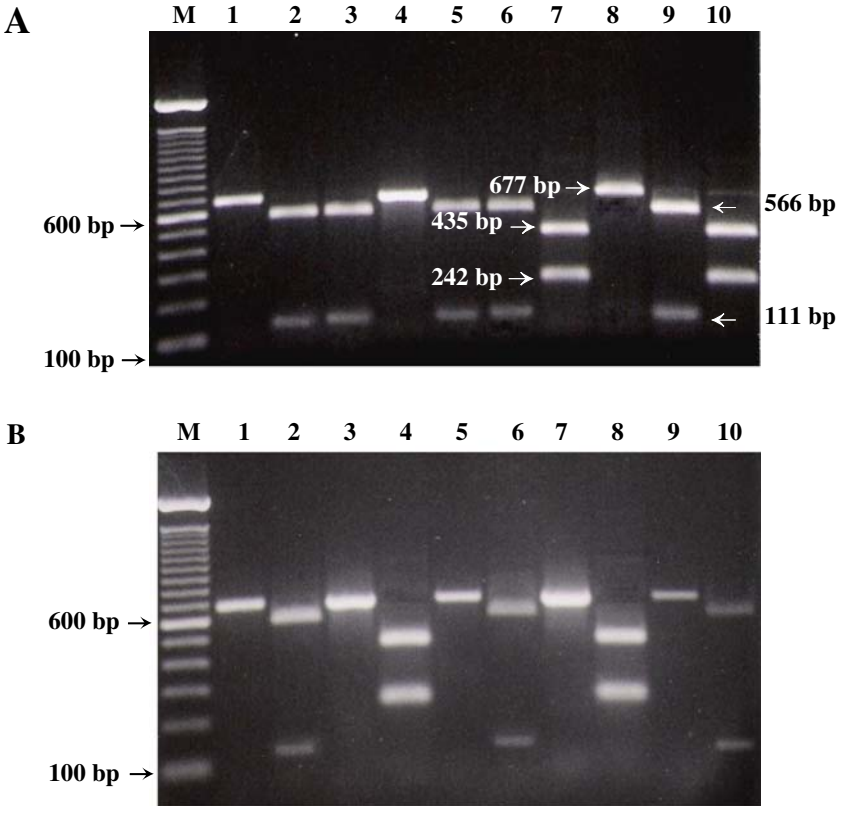

Fig. 3 Restriction profiles of a fragment of the *TbAT1* (nucleotides 430–1,108) from isometamidium-sensitive and isometamidium-resistant *T. b. brucei* stocks digested with Sfa NI. A diagnostic digest with Sfa NI produces fragment sizes of 566 and 111 bp in the case of *TbAT1* from isometamidium-sensitive stocks and 435 and 242 bp in the case of *TbAT1* from the resistant stocks. *M*, 100-bp molecular size marker. **a***Lane 1*, MBOT 163 23 undigested; *lane 2*, MBOT 163 23 digested; *lane 3*, MBOT 25 07, digested; *lane 4*, MBOT 02 20 undigested; *lane 5*, MBOT 02 20 digested; *lane 6*, MBOT 25 07 digested; *lane 7*, CP 547 digested; *lane 8*, CP 547 undigested; *lane 9*, MBOT 05 05 digested and *lane 10*, CP 2469 digested. **b***Lane 1*, ILTat 1.4 undigested; *lane 2*, ILTaT 1.4 digested; *lane 3*, CP 547, undigested; *lane 4*, CP 547 digested; *lane 5*, MBOT 04 01 undigested; *lane 6*, MBOT 04 01 digested; *lane 7*, CP 2469 undigested; *lane 8*, CP 2469 digested; *lane 9*, MBOT 10 17 undigested; *lane 10*, MBOT 10 17 digested

The polymorphism existing in the non-coding or untranslated regions could also result in changes in the three-dimensional structure.

Delespaux et al. (2005) have recently attempted to identify a marker gene for isometamidium resistance in *T. congolense.* They used amplified fragment length polymorphism 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-acid 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* stocks 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.

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 isometamidium-resistant *T. b. brucei* could be successfully distinguished by digestion with the restriction endonuclease Sfa NI. All phenotypically characterised isometamidium-sensitive *T. b. brucei* field isolates consequently 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 *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. 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 melarsoprol treatment. 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.

Despite reports of significant mortality of cattle after infections with *T. b. brucei* from Western Kenya and Uganda (Wellde et al. 1989), only a few attempts were made to study the spread of isometamidium-resistant *T. b. brucei* infections in the tsetse-infected areas of sub-Saharan Africa (Joshua 1988; Zweygarth and Röttcher 1989; Chitambo and Arakawa, 1991). *T. b. brucei* may become more pathogenic under stress conditions and in areas where other trypanosome species have been effectively reduced by chemotherapy (Kalu 1995). As 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. 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. 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 (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, as frequent treatment of cattle with isometamidium may 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 conclusion, this study showed that there may be a link between the presence of mutations in the nucleotide transporter gene (*TbAT1*) in *T. b. brucei* and isometamidium resistance. The point mutations in the *TbAT1* gene fragment in isometamidium-resistant *T. b. brucei* result in a change in Sfa NI restriction site. Thus, Sfa NI-RFLP, if validated with a large-scale screening of field isolates, may serve as a convenient diagnostic tool for rapid identification of isometamidium-resistant *T. b. brucei*.

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