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SSCP analysis of the P2 purine transporter TcoAT1 gene of *Trypanosoma congolense* leads to a simple PCR-RFLP test allowing the rapid identification of diminazene resistant stocks

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Abstract

Analyses were made on a *Trypanosoma congolense* contig coding a putative P2-like nucleoside transporter (the contig was named in this study TcoAT1). The sequence includes a start and stop codon and presents a high similarity with the gene TbAT1 of *T. brucei* (Smallest Sum Probability 2.8e−136). To investigate a possible link between point mutations and diminazene aceturate (DA) resistance in mice, the TcoAT1 putative genes of 26 *T. congolense* strains, characterised for DA sensitivity in the single dose mouse test, were screened by means of the Single Strand Conformation Polymorphism technique (SSCP). Results showed that the SSCP profiles of 23 out of 26 (88.5%) *T. congolense* strains were confirmed by the sensitivity test in mice with the commonly accepted criterion for sensitivity to diminazene being a CD80 of 20 mg/kg in the mouse test. The remaining *T. congolense* strains showed a resistant SSCP profile and relapsed in mice after treatment at doses lower than 20 mg/kg indicating that the SSCP is more sensitive than the single dose mouse test for the detection of resistance to diminazene. However, none of the strains used in this study showed a sensitive SSCP profile while they were resistant in the single dose mouse test. The sequencing of the TcoAT1 gene of two sensitive, two intermediate and two resistant strains allowed the set up of a PCR-RFLP test for the discrimination between sensitive and resistant strains confirming the SSCP results for the 26 strains of this study.

Keywords: *Trypanosoma congolense*; *Trypanosoma brucei*; Drug resistance; Diminazene; Purine transport; SSCP; PCR-RFLP

1. Introduction

There are numerous reports of resistance to the veterinary diamidine drug diminazene aceturate (DA, Berenil®) in various *Trypanosoma* species from many different parts of Africa (e.g. Ainanshe et al., 1992; Chitambo and Arakawa, 1992; Peregrine and Mamman, 1993; Geerts et al., 2001; Sinyangwe et al., 2004; Anene et al., 2006). However, development of resistance in trypanosomes still seems to be limited to areas where the disease is highly endemic and where there is a high frequency of drug use. A rapid, specific and reliable diagnostic method to determine the presence or establishment of resistance is of great importance to determine the most appropriate trypanosomiasis control strategies in a particular area. Existing methods to determine the susceptibility of trypanosomes to trypanocides do not

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allow quick large-scale screenings of isolates due to high costs and the required labor intensity. PCR-based methods would therefore bring enormous improvements in the management of the disease. A PCR-based method for the diagnosis of isometamidium (ISM) resistance, another diamidine drug, was described by Delespaux et al. (2005). Although various mechanisms may be responsible for the development of resistance to ISM in trypanosomes, this method only detects one mechanism which is speculated to be a modification of a putative transporter by addition of one lysine in the resistant phenotypes. The role of the P2 type purine transporter in the transport of diamidine drugs and the effects of inhibition, knocking down or silencing of the gene have been described extensively in the literature (Carter and Fairlamb, 1993; Barrett et al., 1995; Carter et al., 1995; de Koning and Jarvis, 1999; Mäser et al., 1999; Matovu et al., 2003; de Koning et al., 2004, 2005; Witola et al., 2004). A PCR-RFLP technique focused on the TbAT1 gene was recently proposed by Afework et al. (2006) to diagnose ISM resistance in *T. brucei*. In our study, a *T. congolense* putative gene (TcoAT1) presenting a high similarity with the adenosine transporter 1 gene (TbAT1) of *T. brucei* (Smallest Sum Probability 2.8e−136) and coding a putative P2-like nucleoside transporter was screened by SSCP for point mutations possibly linked to changes in sensitivity to DA.

Table 1
Origins of the 26 *Trypanosoma congolense* and the 7 *T. brucei* strains used in the study

Country	Host	Isolate code	Provided by	Species
Tanzania	Lion	IL1180	ILRI[a]	*T. congolense*
Zambia	Cattle	TRT8	ITM	*T. congolense*
Zambia	Cattle	TRT10	ITM[b]	*T. congolense*
Zambia	Cattle	TRT11	ITM	*T. congolense*
Zambia	Cattle	TRT12	ITM	*T. congolense*
Zambia	Cattle	TRT20	ITM	*T. congolense*
Zambia	Cattle	TRT43	ITM	*T. congolense*
Zambia	Cattle	TRT44	ITM	*T. congolense*
Zambia	Cattle	TRT45	ITM	*T. congolense*
Zambia	Cattle	TRT46	ITM	*T. congolense*
Zambia	Cattle	TRT54	ITM	*T. congolense*
Zambia	Cattle	TRT56	ITM	*T. congolense*
Cameroon	Cattle	KONT124	ITM	*T. congolense*
Cameroon	Cattle	KONT129	ITM	*T. congolense*
Cameroon	Cattle	KONT133	ITM	*T. congolense*
Cameroon	Cattle	KONT151	ITM	*T. congolense*
Ethiopia	Cattle	PA87	FUB[c]	*T. congolense*
Zambia	Cattle	TRT2	ITM	*T. congolense*
Zambia	Cattle	TRT47	ITM	*T. congolense*
Zambia	Cattle	TRT21	ITM	*T. congolense*
Zambia	Cattle	TRT31	ITM	*T. congolense*
Zambia	Cattle	Yobo 2038	ITM	*T. congolense*
Zambia	Cattle	Chipopela 313	ITM	*T. congolense*
Burkina Faso	Cattle	SBA 1640	FUB	*T. congolense*
Burkina Faso	Cattle	SBA 1642	FUB	*T. congolense*
Burkina Faso	Cattle	FMA98	CIRDES[d]	*T. congolense*
Cameroon	Cattle	GADZ	ITM	*T. brucei*
Cameroon	Cattle	LSTI	ITM	*T. brucei*
Cameroon	Cattle	ZALI	ITM	*T. brucei*
Cameroon	Cattle	GWTI	ITM	*T. brucei*
Cameroon	Cattle	BEKTI	ITM	*T. brucei*
Cameroon	Cattle	MYBO	ITM	*T. brucei*
Cameroon	Cattle	SARMA	ITM	*T. brucei*

All *T. congolense* strains belong to the Savannah subgroup.
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2. Materials and methods

2.1. Trypanosome strains

Twenty-six *T. congolense* isolates from cattle in Burkina Faso, Cameroon, Ethiopia, Tanzania or Eastern Province of Zambia and previously characterised for DA-sensitivity or resistance by the single dose mouse test were used. Each isolate was inoculated into 12 mice; 6 were used as controls and 6 were treated with 20 mg/kg of DA intraperitoneally. The groups were followed up for 60 days as described by Eisler et al. (2001).

Seven *T. brucei* isolates from cattle in Cameroon were added to check for a possible extrapolation of the results obtained in *T. congolense* to *T. brucei*. Details of the origin the 33 strains used in this study are shown in Table 1.

2.2. DNA extraction

Cryostabilates of trypanosomes (DMSO 20% as cryopreservative) were reactivated by intraperitoneal injection in mice. At the first peak of parasitaemia, the mice were euthanized, and the blood collected with anticoagulant. The DNA was then extracted using the QIAamp® DNA Blood Midi Kit.

The PCR-restriction fragment length polymorphism (PCR-RFLP) technique, using the small subunit of the ribosomal DNA (Ssu-rDNA), was used to confirm that the *T. congolense* strains belonged to the Savannah subgroup and the *T. brucei* strains were characterised at the species level (Geysen et al., 2003; Delespaux et al., 2003).

2.3. Primers

Three sets of primers were designed for the amplification of the 1419 bp putative gene using the Primer3 (Rozen and Skaletsky, 2000) computer programme (http://frodo.wi.mit.edu/) and the contig sequence congo 1447b12.p1k_6 (GeneDB—Welcome Trust Sanger Institute). The first forward primer (Ade1F GATACATTTGGTGAATCGATAGCG) was selected 45 bp upstream of the start codon of the TcoAT1 gene, the first reverse primer (Ade1R CTTCATCCATGGCAGCTTTGATTAT) started at 522 bp after the start codon, the second forward primer (Ade2F ATAATCAAAGCTGCCATGGATGAAG) started at 522 bp after the start codon, the second reverse primer (Ade2R GATGACTAACAATATGCGGGCAAAG) at 1145 bp after the start codon, the third forward primer (Ade3F CTTTGCCCGCATATTGTTAGTCATC) at 1145 bp after the start codon and the third reverse primer (Ade3R CCCAATCGTAACACCTCCTGTTATT) 238 bp downstream the stop codon. The expected sizes of the three PCR products were 616 bp (Ade1 amplicon), 648 bp (Ade2 amplicon) and 518 bp (Ade3 amplicon) for the first, the second and the third, respectively.

2.4. DNA amplification

Standard PCR amplifications were carried out in 25 μl reaction mixtures containing 5 μl DNA sample (at 10 ng μl^{-1} in case of reference DNA samples), 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 200 μM of each dNTP, 20 pmoles of each primer and 0.5 U *Taq* polymerase enzyme (Goldstar, Eurogentec). The reaction mixture was overlaid by 50 μl fine neutral mineral oil (Sigma) and placed on a heating block of a programmable thermocycler (PTC-100 TM, M.J. Research Inc.). After a denaturation step of 4 min at 94 °C each of the 40 cycles consisted of 30 s at 94 °C, 45 s at 58 °C and 45 s at 72 °C. A 5 μl volume of each sample was electrophoresed in a 1% agarose gel for 30 min (100 V) and stained with ethidium bromide for 30 min.

2.5. Restriction of the PCR products

Restriction enzymes were chosen to cut the amplicons in fragments of an adequate size to allow SSCP study (optimal between 100 and 300 bp). Digestions were performed for the first amplicon with *Alu*I and *Hin*fI in the appropriate NEBuffer (New England BioLabs) for the second amplicon with *Mbo*II and *Hph*I and for the third amplicon with *Alu*I and *Alw*I, according to the manufacturer's specifications, using 10 units μg^{-1} DNA (0.6 U μl^{-1} PCR product) on 6 μl of amplified DNA in 15 μl total volume. The reaction was left overnight in a water bath at the specified temperature.

2.6. Electrophoresis and staining

Eight microliters of D2 buffer (95% formamide, 10 mM NaOH, 0.05% bromophenol blue, 0.05% xylene cyanol) was mixed with 4 μl of digested PCR product then heated for 5 min at 95 °C, cooled on ice and left for at least 10 min at −18 °C. Six microliters of this mixture was added on an ELCHROM GMA gel® (Elchrom Scientific AG) and the electrophoresis run according to the manufacturer's specifications at 8 °C. Gels were stained with SYBR green in accordance with the manufacturer's specifications.

Table 2
SSCP results on the Ade2 amplicon (*T. congolense*) with *Hph*I restriction compared to the PCR-RFLP test and the sensitivity tests in mice at 20 and 5 mg/kg diminazene aceturate (DA)

Isolate	SSCP profile	PCR-RFLP	Sensitivity to DA (20 mg/kg)	Sensitivity to DA (5 mg/kg)
IL1180	Sensitive	Sensitive	Sensitive[a]	ND[b]
TRT8	Sensitive	Sensitive	Sensitive	ND
TRT10	Sensitive	Sensitive	Sensitive	ND
TRT11	Sensitive	Sensitive	Sensitive	ND
TRT12	Sensitive	Sensitive	Sensitive	ND
TRT20	Sensitive	Sensitive	Sensitive	ND
TRT43	Sensitive	Sensitive	Sensitive	ND
TRT44	Sensitive	Sensitive	Sensitive	ND
TRT45	Sensitive	Sensitive	Sensitive	ND
TRT46	Sensitive	Sensitive	Sensitive	ND
TRT47	Sensitive	Sensitive	Sensitive	ND
TRT54	Sensitive	Sensitive	Sensitive	ND
TRT56	Sensitive	Sensitive	Sensitive	ND
TRT31	Sensitive	Sensitive	Sensitive	ND
KONT124	Resistant	Resistant	Resistant[c]	ND
KONT129	Resistant	Resistant	Resistant	ND
KONT133	Resistant	Resistant	Resistant	ND
KONT151	Resistant	Resistant	Resistant	ND
PA87	Resistant	Resistant	Resistant	ND
SBA1640	Resistant	Resistant	Resistant	ND
SBA1642	Resistant	Resistant	Resistant	ND
FMA98	Resistant	Resistant	Resistant	ND
Chipopela 313	Resistant	Resistant	Resistant	ND
TRT2	Resistant	Resistant	Sensitive (1/5)[d]	ND
TRT21	Resistant	Resistant	Sensitive (0/5)	(2/6)
Yobo 2038	Resistant	Resistant	Sensitive (0/5)	(6/6)

[a] Less than 20% relapses in the DA treated group at the dose of 20 mg/kg.
[b] Not done.
[c] More than 20% relapses in the DA treated group at the dose of 20 mg/kg.
[d] Number of relapses.

2.7. Cloning and sequencing of the Ade2 amplicons

The Ade2 amplicons of six *T. congolense* strains (two sensitive IL1180 and TRT8, two intermediate TRT2 and TRT21 and two resistant KONT 124 and KONT 151) (see Table 2) were sequenced. The PCR products of 648 bp were cloned using the Topo-cloning® kit (Invitrogen, Carlsbad, CA, USA), exactly as described by the manufacturer. The recombinant plasmids containing the desired inserts were purified and their inserts sequenced using the Model 377-XL Sequencer (PE-Applied Biosystems, Eurogentec®, Belgium).

2.8. RFLP of the Ade2 amplicons

Based on the sequences obtained from the six Ade2 amplicons, the restriction enzyme *Bcl*I (recognition sequence TĜATCA) was selected to further investigate the GTC–ATC codon shift between the sensitive and resistant strains. The theoretical digest of the 648 bp Ade2 amplicon gave 354–256–38 bp and 610–38 bp fragments for the resistant and sensitive strains, respectively.

3. Results

3.1. SSCP profiles

Fig. 1 shows the SSCP profiles of the Ade2 amplicons digested with *Hph*I that allowed discrimination between sensitive and resistant strains of *T. congolense*. Other combinations did not reveal any polymorphism related to drug resistance and are therefore not shown. SSCP analysis of seven *T. brucei* isolates allowed identifying three of them as putatively sensitive and four as putatively resistant. Fig. 2 shows the SSCP profiles of the Ade2 amplicons of some of the *T. brucei* isolates digested with *Hph*I.

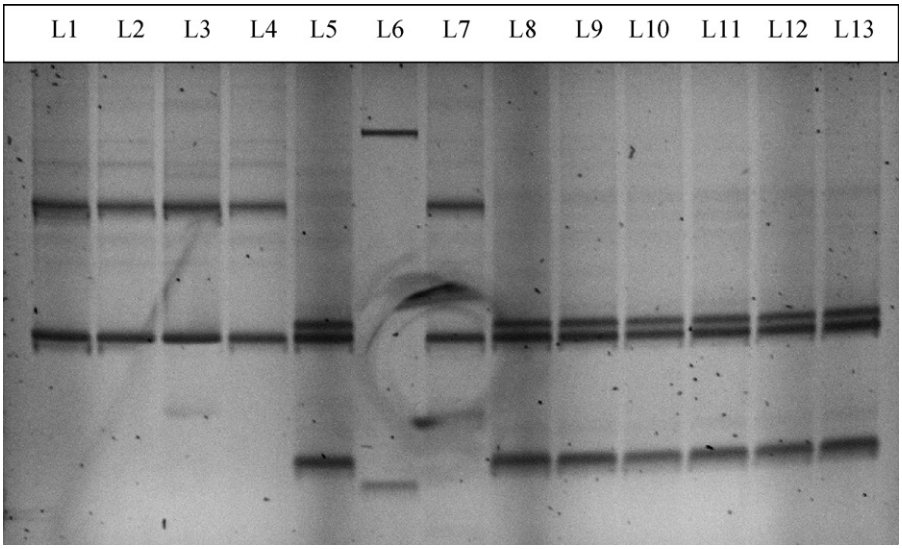

Fig. 1. SSCP profiles of *Trypanosoma congolense*: L1–L4 sensitive strains, L5 resistant strain, L6 ladder, L7 sensitive strain, L8–L13 resistant strains.

3.2. Comparison of SSCP profiles and results of the single dose mouse test

Results shown in Table 2 indicate that for 23 of the 26 *T. congolense* strains the outcome of the SSCP test was identical to the outcome of the single dose mouse resistance test. However, the SSCP test identified as resistant some strains (TRT21 and YOBO 2038) that relapsed at a DA dose lower than the commonly accepted discriminatory dose of 20 mg/kg or that presented one relapse among the group of five mice treated at 20 mg/kg (TRT2).

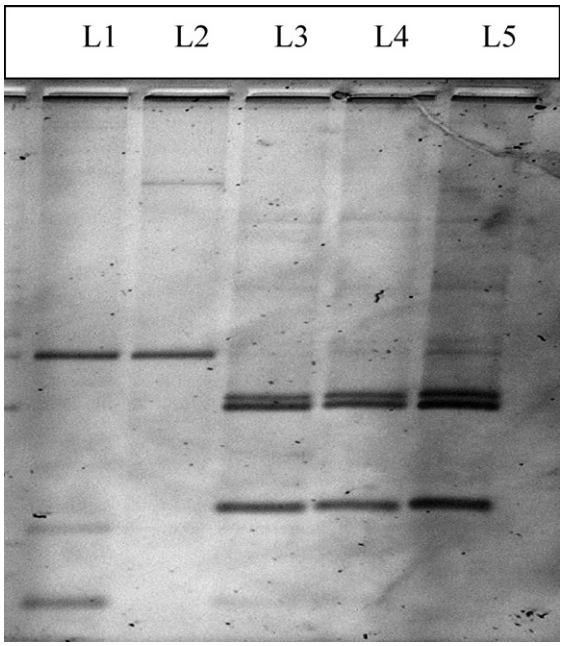

Fig. 2. SSCP profiles of five *Trypanosoma brucei* strains with L1, L2 as sensitive-like profiles, L3–L5 as resistant-like profiles.

3.3. Sequencing of the six Ade2 amplicons and PCR-RFLP results

The clustal alignment of the six Ade2 amplicons sequences (translated into the corresponding amino acids) revealed a conserved Val 306 Ile permutation in the intermediate and resistant phenotype when compared to the sensitive one (see Table 2 for drug sensitivity details). This amino acid permutation is caused by the shift between a GTC (sensitive phenotype) and a ATC codon (resistant phenotype). This codon shift was confirmed by PCR-RFLP in the 20 other strains of this study and correlated perfectly with the SSCP results.

4. Discussion

Mediated uptake of DA by *T. b. brucei* was first demonstrated by Girgis-Takla and James (1974) but a similar conclusion of mediated uptake for the related diamidine stilbamidine had previously been drawn by Hawking (1944). However, it was only after Carter and Fairlamb (1993) discovered that a P2 aminopurine transporter was responsible for the uptake of melarsoprol (an arsenical diamidine) that it was concluded that the same transporter mediated the transport of diamidines as well.

Barrett et al. (1995) found that induction of DA resistance in *T. equiperdum* coincided with loss of P2 activity and Carter et al. (1995) first demonstrated the uptake of P2-mediated [^{3}H]-pentamidine by *T. b. brucei*. The dependence of DA on P2 transporters was evidenced by the drug resistance profile of the *tbat1*$^{-/-}$ knockout line, which was highly resistant to DA (Matovu et al., 2003). Similarly, RNAi knockdown of the AT1 gene in *T. evansi* caused high levels of in vitro resistance to DA but not to suramin nor to antrycide. A low level of resistance to ISM was also observed (Witola et al., 2004).

In this study, a *T. congolense* putative gene (TcoAT1) presenting a high similarity with the adenosine transporter 1 gene (TbAT1) of *T. brucei* and coding for a P2-like nucleoside transporter (Smallest Sum Probability 2.8e−136) was screened by SSCP for point mutations possibly linked to changes in sensitivity to DA. The consistent correlation, in 88.5% of the strains, between the SSCP-profiles and the single dose mouse tests was very satisfactory considering the fact that the technique did not identify false sensitive profiles. The fact that some relapses did occur after treatment at lower doses than the commonly accepted discriminatory dose of 20 mg/kg seems to indicate that the SSCP technique is more sensitive to detect resistance or emerging resistance than the mouse test. The perfect correlation between the SSCP profiles and the PCR-RFLP test allows the use of the latter method that is easier and cheaper than the SSCP.

The recently proposed PCR-RFLP technique for the diagnosis of ISM resistance in *T. brucei* (Afework et al., 2006), is based on the same fragment of the gene (430–1108 bp after the start codon and 522–1108 bp in the present study). The sequencing of the Ade2 amplicon of this study indicates that it is not the same set of mutations that confers resistance to DA in *T. congolense* and to DA and ISM in *T. brucei*. In *T. brucei* a set of six point mutations are observed in the DA and melarsoprol resistant phenotypes (Mäser et al., 1999). In *T. congolense* a single point mutation (G to A) is conferring resistance and appears as a Val 306 Ile amino acid permutation. Contrary to what is observed in *T. brucei* where ISM and DA seem to share the same mechanism of resistance (Afework et al., 2006), in *T. congolense* those mechanisms appear to be distinct (Delespaux et al., 2005) and this observation is well confirmed by what is observed in the field. Indeed, cross-resistance between DA and ISM in *T. congolense* is not frequently observed (Sinyangwe et al., 2004) and the use of the sanative pair, which is strongly recommended by the Program Against African Trypanosomiasis (PAAT) (Geerts and Holmes, 1998), is still an effective method to decrease the incidence of ISM or DA single drug resistance.

The polymorphism observed in the SSCP of the *T. brucei* strains is encouraging (Fig. 2). However mouse tests are required to confirm the correlation between SSCP-profiles and in vivo resistance. If this correlation is confirmed, the technique could provide a powerful tool to diagnose diminazene and melarsoprol resistance in human infective trypanosomes.

5. Conclusion

The PCR-RFLP technique described here is a quick and sensitive tool for the diagnosis of existing or emerging trypanocide resistance to diminazene. The observed high sensitivity of the technique compared to the commonly used mouse test is perhaps not surprising and suggests that molecular methods allow for the identification of resistant genotypes that still have sensitive phenotypes or ‘emerging resistance’. After validation using a higher number of trypanosome strains from different geographical areas, the test presented in this study could be a valuable tool for determining the presence or emergence of DA in *T. congolense* and possibly of DA and melarsoprol resistance in *T. brucei*. Such information is important in developing appropriate trypanosomiasis control strategies.

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