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Drug Resistance Updates 11 (2008) 205–209
DRUG RESISTANCE Updates
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Five-fold increase in *Trypanosoma congolense* isolates resistant to diminazene aceturate over a seven-year period in Eastern Zambia

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Abstract

Two groups of *Trypanosoma congolense* isolates collected from cattle in 1996 ($n = 39$) and 2003 ($n = 38$) in the Eastern Province of Zambia were analyzed by *Bcl*I-*PCR-RFLP* to assess the evolution of diminazene aceturate (DA) resistance over a period of seven years. The results show a significant increase of DA resistance in this relatively short period of time. In 1996, among the 39 isolates, 61.5% were found sensitive, 12.8% resistant and 25.7% had a mixed *Bcl*I-*PCR-RFLP* profile. In 2004, among the 38 isolates, 10.5% were found sensitive, 63.2% were resistant and 26.3% showed a mixed *Bcl*I-*PCR-RFLP* profile. In vivo tests in mice showed that isolates with a sensitive or mixed RFLP profile were sensitive to DA whereas isolates with a resistant RFLP profile were resistant. Since there are no indications that the drug pressure has increased between 1996 and 2003, it is suggested that genetic exchange of resistance genes might explain the increased frequency of resistance to DA.

Keywords: *Trypanosoma congolense*; Drug resistance; Molecular tools; Diminazene; PCR-RFLP; Zambia

1. Introduction

About 9 million km^2 of sub-Saharan Africa, representing about one-third of the total land, is infested with tsetse flies (*Glossina* spp.), which are the main vectors for *Trypanosoma* (Mattioli et al., 2004). Within this region, some 46–62 million head of cattle and other livestock species are at risk of trypanosomosis, which represents a major constraint on ruminant livestock production (Swallow, 2000). In addition, dozens of human trypanosomosis (sleeping sickness) cases occur in the region annually. Trypanocidal drug resistance (TDR) has become a worrying burden in sub-Saharan Africa and was reported in 17 countries (Delespaux et al., 2008). Although TDR is reported widely (mainly case reports, plus a few area-wide studies), no information is available on the temporal development of resistance in trypanosomes. Indeed, the ease with which TDR spreads in the field remains an open question. This is mainly due to the logistical difficulties and high costs of longitudinal surveys in a specific site where trypanocidal drugs are used. Furthermore, the in vivo testing of a large number of field isolates for drug sensitivity requires an even larger number of laboratory animals.

The availability of a reliable and sensitive molecular tool that allows the rapid screening for diminazene aceturate (DA) resistance (Delespaux et al., 2006) and the availability of a large number of *T. congolense* strains isolated from cattle in 1996 and 2003 in the same geographic area of Eastern Zambia made it possible to determine the spread of DA resistance in a geographically confined area during this period.

2. Materials and methods

2.1. Study area

All trypanosome isolates were collected from cattle kept in the Petauke and Katete Districts of the Eastern Province of Zambia. They were collected at 19 sampling sites scattered over the area. Details of each of the isolates are presented in Table 1. The study area is infested with tsetse flies of the

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doi:10.1016/j.drup.2008.10.002

Table 1
Codes and individual results of the BclI-PCR-RFLP of the 77 isolates of the study.

Code	RFLP profile	Sampling	Code	RFLP profile	Sampling
TRT1[a]	Sensitive	1996	Chipopela324C1[b]	Sensitive	2003
TRT10[a]	Sensitive	1996	Chipopela38	Sensitive	2003
TRT11[a]	Sensitive	1996	Chitawe162	Sensitive	2003
TRT12[a]	Sensitive	1996	Chitawe172	Sensitive	2003
TRT13	Sensitive	1996	Alick326C1[b]	Resistant	2003
TRT20[a]	Sensitive	1996	Alick339C6[b]	Resistant	2003
TRT29	Sensitive	1996	Alick355	Resistant	2003
TRT31[a]	Sensitive	1996	Alick589	Resistant	2003
TRT35	Sensitive	1996	Alick69	Resistant	2003
TRT37	Sensitive	1996	Alick91	Resistant	2003
TRT38[a]	Sensitive	1996	Alick92	Resistant	2003
TRT39	Sensitive	1996	Alick95C1[b]	Resistant	2003
TRT40[a]	Sensitive	1996	Chipopela313[c]	Resistant	2003
TRT42[a]	Sensitive	1996	Chipopela37	Resistant	2003
TRT43[a]	Sensitive	1996	Chitawe163	Resistant	2003
TRT44[a]	Sensitive	1996	Chitawe556	Resistant	2003
TRT45[a]	Sensitive	1996	Kapeya27	Resistant	2003
TRT46[a]	Sensitive	1996	Kapeya272	Resistant	2003
TRT47[a]	Sensitive	1996	Kapeya357	Resistant	2003
TRT5[a]	Sensitive	1996	Kasanda	Resistant	2003
TRT54[a]	Sensitive	1996	Kasanda1	Resistant	2003
TRT56[a]	Sensitive	1996	Kasanda20	Resistant	2003
TRT57[a]	Sensitive	1996	Kasanda22	Resistant	2003
TRT8[a]	Sensitive	1996	Katepela1081	Resistant	2003
TRT2[2]	Resistant	1996	Mphita4018	Resistant	2003
TRT21[d]	Resistant	1996	MsoroM19	Resistant	2003
TRT24	Resistant	1996	MsoroM7	Resistant	2003
TRT49	Resistant	1996	Seya147	Resistant	2003
TRT61	Resistant	1996	Alick351	Mixed	2003
J4J4	Mixed	1996	Kapeya116	Mixed	2003
TRT15	Mixed	1996	Kapeya12	Mixed	2003
TRT16	Mixed	1996	Lombo3020	Mixed	2003
TRT17C1[b,a]	Mixed	1996	Seya129	Mixed	2003
TRT25	Mixed	1996	Seya148	Mixed	2003
TRT3[a]	Mixed	1996	Seya389	Mixed	2003
TRT32	Mixed	1996	Yobo2007	Mixed	2003
TRT33	Mixed	1996	Yobo2028	Mixed	2003
TRT48[a]	Mixed	1996	Yobo2038	Mixed	2003
TRT51	Mixed	1996			

[a] Tested in mice: more than 80% relapses at 1 mg/kg DA, less than 20% relapses at 20 mg/kg DA, 0% relapses at 40 mg/kg.
[b] Cloned trypanosomes.
[c] Tested in mice: more than 20% relapses at 20 mg/kg.
[d] Tested in mice: less than 20% relapses at 20 mg/kg, more than 20% relapses at 5 mg/kg.

species *Glossina morsitans morsitans* that takes the majority of its bloodmeals from cattle (Van den Bossche and Staak, 1997). *Trypanosoma congolense* is the dominating trypanosome species (Simukoko et al., 2007). The prevalence of trypanosomosis in cattle is about 30% and the disease is controlled using trypanocidal drugs, mainly diminazene aceturate (Van den Bossche et al., 2000). In 1996, the prevalence of DA and ISM resistance in the area was evaluated by in vivo tests to be 11.3% and 34% respectively (Sinyangwe et al., 2004).

2.2. Trypanosome isolates

Two groups of *T. congolense* savannah type isolates were used in this study. The first group ($n = 39$) consisted of isolates collected in 1996 (Sinyangwe et al., 2004), the isolates from the second group ($n = 38$) were collected in 2003 (Masumu et al., 2006).

Trypanosomes were isolated by intraperitoneal injection of 0.5 ml of blood of cattle, found positive for *T. congolense* using the buffy coat technique (Murray et al., 1977), into mice aged 5–8 weeks and weighing on average 30 g each. After the parasitaemia had reached a minimum of 7.1 on the Herbert and Lumsden (1976) scale, mice were euthanized and stabilates were prepared using 25% of a DMSO (20%) solution as a cryopreservative for long-term storage in liquid nitrogen.

Five of those isolates (TRT17C1, Alick95C1, Alick 324C1, Alick326C1, Alick339C6) were cloned (Smith et al., 1982) to compare the *BclI-PCR-RFLP* profiles of isolates

(possible mixture of sensitive and resistant trypanosomes) and clonal populations.

2.3. Sensitivity testing in mice

For each isolate, 5×10^5 trypanosomes were inoculated into 20 adult OF1 mice weighing on average 30 g each. Twenty-four hours after infection, each of the 4 groups of 5 mice was treated intraperitoneally with 0.2 ml of a DA solution at the following doses: 1.0 mg/kg, 20 mg/kg, 40 mg/kg for group 1, 2 and 3 respectively. The fourth group was injected with water as a no-treatment control. Following treatment, wet films of tail blood was monitored twice weekly for 60 days for the presence of trypanosomes. For the TRT2 and TRT21 isolates, the experiment was repeated following the same procedure but with a no-treatment control group and a treated group at a dose of 5.0 mg/kg. An isolate was considered as resistant to a specific dose (i.e. 1, 5, 20 or 40 mg/kg) when more than 20% of the mice relapsed (Eisler et al., 2001).

2.4. DNA extraction

The cryostabilates of trypanosomes 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 a routine phenol–chloroform–isoamyl alcohol method (Sambrook et al., 1989). To confirm that the *T. congolense* strains belonged to the Savannah subgroup, the PCR-restriction fragment length polymorphism (PCR-RFLP) technique was used on the small subunit of the ribosomal DNA (Ssu-rDNA) (Geysen et al., 2003; Delespaux et al., 2003).

2.5. DNA amplification

Standard PCR amplifications were carried out in 25 μl reaction mixtures containing 5 μl DNA sample (at 10 ng/μl in the 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 pmol of each primer and 0.5 U *Taq* polymerase enzyme (Goldstar, Eurogentec). The reaction mixture was overlaid with 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 59 °C and 45 s at 72 °C. A 5 μl volume of each sample was electrophoresed in a 2% agarose gel for 30 min (100 V) and stained with ethidium bromide for 30 min.

2.6. Primers

Primers used for the diagnosis of DA resistance in *T. congolense* were described by Delespaux et al. (2006). The forward primer Ade2F sequence was ATAATCAAAGCTGCCATGGATGAAG, the reverse primer Ade2R sequence being GATGACTAACAATATGCGGGCAAAG.

2.7. BclI-PCR-RFLP

The *Bcl*I-PCR-RFLP reactions for the detection of resistance to DA were performed as described by Delespaux et al. (2006).

2.8. Statistical analysis

The Fischer exact test (Weisstein, 2008) was used to analyse the two categorical variables: drug sensitivity with three observed categories (sensitive, resistant, mixed) and time of sampling with two observed years (1996 and 2003).

3. Results and discussion

Fig. 1 shows the three different *Bcl*I-*PCR-RFLP* profiles which were obtained, i.e. sensitive, resistant and mixed. Mixed profiles may be the result of either a mixture of trypanosomes consisting of homozygous sensitive, homozygous resistant and/or heterozygous subpopulations, or from a whole parasite population that is heterozygous for the point mutation in the *TcoAT1* gene conferring DA-resistance (Delespaux et al., 2006). To confirm the hypothesis that heterozygous strains were present in the trypanosome population and have a mixed RFLP profile, the profile of five cloned strains was compared. The *Bcl*I-*PCR-RFLP* showed that the cloned strains Alick95C1, Alick326C1 and Alick339C6 presented resistant RFLP profiles and can thus be classified as homozygous resistant. The cloned strain Chipopela324C1, on the other hand, presented a sensitive RFLP profile and can thus be classified as homozygous sensitive. Finally, the cloned strain TRT17C1 presented a mixed RFLP profile and can thus be considered as heterozygous for the G to A point mutation in the P2-type purine transporter gene TcoAT1,

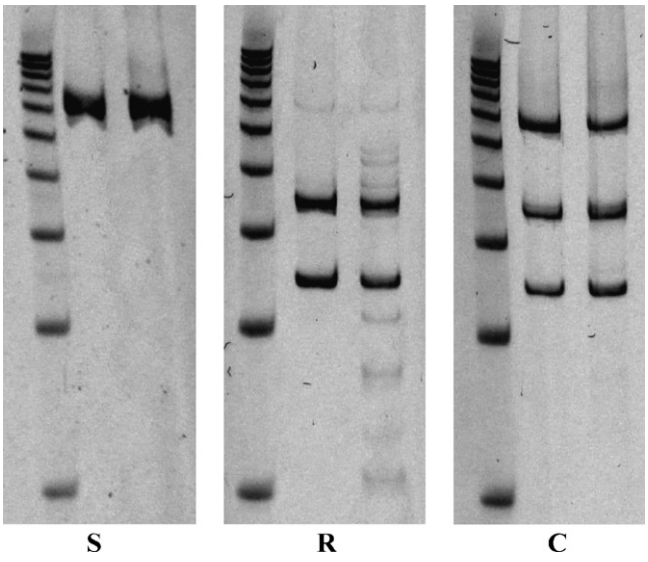

Fig. 1. Example of the BclI-PCR-RFLP sensitive (S), resistant (R) and mixed (M) profiles.

Table 2
Number of *T. congolense* isolates collected in the Eastern Province of Zambia in 1996 and 2003 and presenting a sensitive, resistant or mixed BclI-PCR-RFLP profile.

Year of isolation	Number of strains with *Bcl*I-PCR-RFLP profile			Total
	Sensitive (%)	Resistant (%)	Mixed (%)	
1996	24 (61.5)	5 (12.8)	10 (25.7)	39 (100)
2003	4 (10.5)	24 (63.2)	10 (26.3)	38 (100)

Table 3
Results of the mouse test for the evaluation of the sensitivity of *T. congolense* isolates to DA at different doses.

Number of *T. congolense* isolates	RFLP profile	Number of relapsing mice for different doses of DA			
		1 mg/kg	5 mg/kg	20 mg/kg	40 mg/kg
22	S or M	5/5	ND	≤1/5	0/5
2	R	5/5	≥1/5	≤1/5	0/5
1	R	ND	ND	=6/6	ND

S: Sensitive; M: mixed; R: resistant; ND: not done.

which confirms the presence of heterozygous individuals in the trypanosome populations.

Table 2 summarises the *Bcl*I-*PCR-RFLP* profiles of all the isolates. The data show that the number of DA sensitive isolates has decreased from 61.5% in 1996 to 10.5% in 2003, whereas the DA resistant isolates increased from 12.8 to 63.2 % over the same time period. The association between the date of sampling and the DA-sensitivity of the isolates is highly significant ($p = 6.014 \times 10^{-7}$) representing a huge increase of the proportion of resistant isolates in the trypanosomes isolated in 2004.

Out of the 77 isolates, 25 were tested in mice. The results (Table 3) show that 22 isolates with either a sensitive or mixed *Bcl*I-*PCR-RFLP* profile can be considered as sensitive to DA because there were less than 20% relapses at a dose of 20 mg/kg DA. Two isolates with a resistant *Bcl*I-*PCR-RFLP* profile had less than 20% relapses at 20 mg/kg DA, but more than 20% relapses at a dose of 5 mg/kg. This confirms earlier findings that the molecular test might be more sensitive in detecting DA resistance than the mouse test (Delespaux et al., 2006). One isolate representing a resistant *Bcl*I-*PCR-RFLP* profile showed more than 20% relapses (100%) at 20 mg/kg. The results of the tests in mice do not allow differentiating between recessiveness or incomplete dominance as no difference was observed between homozygous sensitive and heterozygous trypanosomes at the doses used, but complete dominance can be excluded. A full range of DA doses between 1 and 20 mg/kg might allow for the differentiation between the DA sensitivity of homozygous sensitive and heterozygous trypanosome strains. Since these results confirm earlier findings (Delespaux et al., 2006) that there is a good correlation (88.5–91.7%) between the *Bcl*I-*PCR-RFLP* test and the mouse test, the *Bcl*I-*PCR-RFLP* test can be considered as an effective, quick and accurate molecular tool for monitoring DA-resistance. The usefulness of such a tool was already demonstrated in a large-scale study on trypanocidal drug resistance in Cameroon (Mamoudou et al., 2008).

Considering the magnitude of the observed increase in DA-resistance in the study area over a seven-year period, it seems unlikely that the spread in the trypanosome population of the point mutation within the *TcoAT1* gene could be the result of point mutations that occur independently due to increased drug pressure. Genetic exchange has been demonstrated in *Trypanosoma brucei* (Jenni et al., 1986; Tait and Turner, 1990; Gibson and Whittington, 1993; Gibson and Stevens, 1999; Tait et al., 2007). Although genetic exchange has not been demonstrated in *T. congolense*, it is highly likely that it occurs similarly to *T. brucei*. It may explain the observed rapid development of DA-resistance in the trypanosome population of the study area. This is especially so since trypanocidal drug use, with a treatment frequency of 1.25 DA treatments/animal/year (Van den Bossche et al., 2000), and thus trypanocidal drug pressure is not very high and has not changed over the years.

The results of this study clearly demonstrate that DA-resistance is developing very fast in the Eastern Province. The factors contributing to this rapid development need further investigation and will contribute substantially to a better understanding of the epidemiology of trypanocidal drug resistance. Since a high prevalence of resistance to ISM has already been demonstrated in the study area (Sinyangwe et al., 2004), the findings of the present study underline the need for alternative methods to control the disease such as vector control using, for example, insecticide-impregnated targets or insecticide-treated cattle.

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