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Assessment of the occurrence of trypanocidal drug resistance in trypanosomes of naturally infected cattle in the Adamaoua region of Cameroon using the standard mouse test and molecular tools

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ABSTRACT

From May to November 2005, a study was carried out to assess the occurrence of trypanocidal drug resistance (DR) in trypanosomes of naturally infected cattle of the Adamaoua region of Cameroon. Two distinct Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) procedures were used together with an Allele specific-PCR (AS-PCR) and the standardized single-dose mouse test. Using the mouse test, 3 of the 13 *Trypanosoma brucei* isolates and all 14 tested *Trypanosoma congolense* isolates were resistant to ISM. However, only 11 of the 25 *T. congolense* isolates were diagnosed as resistant to ISM using the *Mbo*II-PCR-RFLP. Resistance to DA was identified in 1 of the 13 *T. brucei* isolates and all 11 *T. congolense* isolates which were tested with the mouse test. Using the AS-PCR or *Bcl*I-PCR-RFLP, 3 of the 13 *T. brucei* isolates and all 25 *T. congolense* isolates respectively were found resistant.

The data presented in this study prove that DR is widespread in the Adamaoua Department of Cameroon. The problem appears to be more serious in *T. congolense* than in *T. brucei*. Appropriate measures need to be taken in order to control bovine trypanosomosis in this area.

1. Introduction

Resistance to the commonly used animal trypanocides has emerged in sub-Saharan Africa and interferes with effective veterinary management of trypanosomosis (Geerts and Holmes, 1998; Delespaux and de Koning, 2007). Although the number of case reports of DR is increasing, very few systematic surveys have been carried out to assess its true prevalence. An important prerequisite to undertaking such studies is the availability of reliable and simple tests for DR. The commonly used techniques (*in vivo*, *in vitro* assays and field tests) suffer from a certain number of drawbacks amongst which the requirement of large number of experimental animals, the long duration of the tests and the difficult adaptation of trypanosomes to tissue culture or laboratory rodents. Recently, however, molecular tools have been developed for a more rapid and convenient diagnosis of DR eventually allowing epidemiological surveys at a larger scale (Delespaux et al., 2005, 2006; Nerima et al., 2007). These molecular tools, however, still need further validation before being used as stand-alone diagnostic methods.

Recently, DR was reported for the first time in Cameroon using a field test in trypanosomes isolated from a cattle herd in the village of Kontcha (Adamaoua Department) (Mamoudou et al., 2006). Therefore, this study was undertaken in order to assess the occurrence of DR in the Adamaoua Department using both the single dose mouse test as a validated reference method for DR screening and PCR-RFLPs still under validation.

2. Materials and methods

2.1. Trypanosome isolates

Between May and November 2005, 221 blood samples were collected from 10 to 15 randomly selected animals in 17 cattle herds in certain areas of the Adamaoua Department with a known high prevalence of trypanosomosis (Mamoudou et al., 2006). At the time of sampling, background epidemiological data, including sex, age, weight, disease records and drug use were collected. Blood samples of 0.5 ml from cattle found positive for *Trypanosoma congolense* or *Trypanosoma brucei* using the buffy coat technique (Murray et al., 1977) were inoculated intraperitoneally into mice aged 5–8 weeks, weighing on average 30 g each. When parasitaemia reached a minimum of 7.1 on the Herbert and Lumsden scale (Herbert and

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Lumsden, 1976), mice were euthanized and stabilates were prepared using 25% of a DSMO (20%) solution as a cryopreservative for long-term storage in liquid nitrogen.

2.2. Single-dose test in mice

Twenty trypanosome isolates were tested using the single-dose test in mice according to the protocol described by Eisler et al. (2001). OF1 mice of 8–10 weeks old, weighing 25–30 g, housed in a fly-proof stable were maintained on a commercial pellet ration and water ad libitum. Briefly, each trypanosome isolate was inoculated into three groups of six mice each. Twenty-four hours after inoculation with 10^5 trypanosomes, the mice were treated. The first group of six mice was treated with 1 mg/kg bw isometamidium chloride (Trypamidium-Samorin®, Merial France A445971; ISM), the second group with 20 mg/kg bw diminazene aceturate (Berenil®, Hoechst AG, Germany 01W005; DA) and the control group was injected with distilled water. Mice were then monitored twice a week for a period of 2 months for the presence of trypanosomes through the examination of wet smears of tail blood. All mice found parasitaemic were euthanized and removed from the experiment. An isolate was considered as resistant when more than one out of the six treated animals became positive within the observation period.

2.3. DNA extraction

Cryostabilates of trypanosomes (DMSO 20% as cryopreservative) were reactivated by intraperitoneal injection in mouse. At the first peak of parasitaemia, the mouse was euthanized, and the blood collected with anticoagulant. The DNA was then extracted using a routine Phenol Chloroform Isoamyl alcohol method.

2.4. DNA amplification

Standard PCR amplifications were carried out in 25-μl reaction mixtures containing a 5 μl DNA sample (at 10-ng μl^{-1} in case of DNA reference 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, 0.5 U Taq polymerase enzyme (Goldstar®, Eurogentec). While negative control samples were constituted of pure water (5 μl), positive ones contained 5 μl DNA of *T. theileri* for the diagnosis of trypanosome species and the same quantities of reference *T. congolense* DNA samples (IL1180R & IL1180P) for the test of resistance to ISM. 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 60 s at 94 °C, 90 s at 58 °C and 120 s at 72 °C.

2.5. Primers

Primers used for species identification were described by Geysen et al. (2003), for ISM resistance in *T. congolense* by Delespaux et al. (2005), for DA resistance in *T. congolense* by Delespaux et al. (2006) and for DA resistance in *T. brucei* by Nerima et al. (2007).

2.6. PCR-RFLP and allele specific PCR (AS-PCR)

The PCR-restriction fragment length polymorphism (PCR-RFLP) technique, using the 18 s subunit of the ribosomal DNA (18 SsurDNA), was used to confirm that the *T. congolense* strains belonged to the Savannah subgroup (Geysen et al., 2003). The *T. brucei* strains were characterized at the species level according to Geysen et al. (2003) and Delespaux et al. (2003). *Mbo*II-PCR-RFLP reactions for the diagnosis of ISM-resistance in *T. congolense*, *Bcl*I-PCR-RFLP reactions for the detection of resistance to DA in *T. congolense* and AS-PCR for the detection of resistance to DA in *T. brucei* were performed as described by Delespaux et al. (2005), Delespaux et al. (2006) and Nerima et al. (2007), respectively.

Table 1
Detailed results of the mouse test and AS-PCR for *T. brucei* isolates from cattle from the Adamaoua region in Cameroon

No.	Species	Code	Mouse test (ISM)	Mouse test (DA)	AS-Specific PCR (DA)
1	*T. brucei*	Mybo	S	S	S
2	*T. brucei*	Sarma	R	R	R
3	*T. brucei*	LSTI	S	S	S
4	*T. brucei*	GMZ/GADZ	S	S	S
5	*T. brucei*	Pawti	S	S	S
6	*T. brucei*	Becti	S	S	S
7	*T. brucei*	Salsa	S	S	S
8	*T. brucei*	Zali	R	S	S
9	*T. brucei*	GADZ2	S	S	R
10	*T. brucei*	Gwti	S	S	R
11	*T. brucei*	Faro	R	S	S
12	*T. brucei*	Zwili	R	S	S
13	*T. brucei*	Wilde	R	S	S

S: sensitive; R: resistant.

3. Results

Out of 221 collected samples, 46 (20.81%) were found positive for trypanosomes. Three of these 46 samples were diagnosed as mixed *brucei–congolense* infections and were not further characterized and three *T. congolense* did not multiply in mice. The remaining 40 isolates (13 *T. brucei* and 27 *T. congolense*) were characterized for ISM and DA resistance either by the single dose mouse test, by PCR-RFLP, by AS-PCR or by a combination of *in vivo* and molecular techniques (detailed results in Tables 1 and 2). Table 3 shows that 38.46% of the 13 tested *T. brucei* isolates and 100% of the 14 tested *T. congolense* isolates were resistant to ISM using the mouse test. Using the *Mbo*II-PCR-RFLP, only 44% of the 25 *T. congolense* isolates were diagnosed as resistant to ISM. In total 70% of the 27 *T. brucei and T. congolense* isolates were resistant to ISM in the mouse test.

Table 4 shows that 7.69% (1/13) of the *T. brucei* isolates and 100% (11/11) of the *T. congolense* isolates were resistant to DA using the mouse test. Using the molecular tests, 23.08% (3/13) of the *T. brucei* isolates and 100% (25/25) of the *T. congolense* isolates were resistant.

Among the *T. brucei* isolates, only one isolate was found resistant to both drugs in the mouse test whereas all *T. congolense* isolates ($n = 11$), which were examined in the mouse test using both drugs, were identified as resistant to both. Using the molecular methods, however, only 44% of the 25 *T. congolense* isolates were found resistant to both drugs.

4. Discussion

The data presented in this study prove that DR is widespread in the Adamaoua Department of Cameroon. Results show that the situation of DR in *T. congolense* is extremely worrying as 100% of the tested strains were characterized as resistant to both drugs when using the mouse test. In such a situation, the efficacy of the sanative pair as the commonly accepted method for delaying the development of DR is seriously threatened. Further characterization of the trypanosome isolates resistant to both drugs should clarify whether or not the sanative pair can still be used. Field isolates might consist of a pan-mictic population of trypanosomes resistant to a single drug or a homogenous population of multi-

Table 2
Detailed results of the mouse test and both PCR-RFLPs for the *T. congolense* isolates from cattle from the Adamaoua region in Cameroon

No.	Species	Code	Mouse test (ISM)	Mouse test (DA)	*Mbo*II-PCR-RFLP (ISM)	*Bcl*I-PCR-RFLP (DA)
1	*T. congolense*	Galim	R	R	S	R
2	*T. congolense*	Bile	ND	ND	R	R
3	*T. congolense*	Guemf	R	R	S	R
4	*T. congolense*	Garba	ND	ND	S	R
5	*T. congolense*	Djem	R	R	S	R
6	*T. congolense*	Kont RS	R	R	S	R
7	*T. congolense*	Alme	R	R	S	R
8	*T. congolense*	Lompt	R	R	R	R
9	*T. congolense*	Sadek	R	ND	R	R
10	*T. congolense*	Guasgue	R	R	R	R
11	*T. congolense*	Sabon	R	R	S	R
12	*T. congolense*	Mbabo	ND	ND	S	R
13	*T. congolense*	Lare	ND	ND	S	R
14	*T. congolense*	Wogdo	R	ND	S	R
15	*T. congolense*	TIPSAN	ND	ND	R	R
16	*T. congolense*	LIKOK	R	R	R	R
17	*T. congolense*	BEKA	R	R	ND	ND
18	*T. congolense*	JABE	R	R	ND	ND
19	*T. congolense*	BAGARMI	ND	ND	R	R
20	*T. congolense*	Duel	R	ND	S	R
21	*T. congolense*	LEWA	ND	ND	S	R
22	*T. congolense*	HANLOWA	ND	ND	R	R
23	*T. congolense*	TELO	ND	ND	R	R
24	*T. congolense*	DIBI	ND	ND	R	R
25	*T. congolense*	POUSS	ND	ND	S	R
26	*T. congolense*	POLI	ND	ND	S	R
27	*T. congolense*	PETE	ND	ND	R	R

S: sensitive; R: resistant; ND: not done.

Table 3
Detection of resistance to ISM in *T. brucei* and *T. congolense* isolated from cattle from the Adamaoua region in Cameroon using the mouse test and/or *Mbo*II-RFLP

	Isolates examined in mouse test		Isolates examined using molecular test	
	No. tested	No. (%) resistant	No. tested	No. (%) resistant
T. brucei	13	5 (38.46)	ND[a]	
T. congolense	14	14 (100)	25	11 (44)

[a] Not done because there is no appropriate test available.

resistant trypanosomes or a mixture of both together. In each of these situations different measures will have to be taken to avoid further worsening of the situation. The cloning and the individual characterization of trypanosomes isolated from the field samples will be necessary to bring more insight in that matter.

The comparison between the single dose mouse test and molecular methods has to be done very cautiously. Drug sensitivity test in mice and molecular tools provide information on the resistance phenotype and genotype, respectively. Both have their advantages and drawbacks. It is well known that – although there is a quite good correlation of the mouse test with the test in ruminants – the curative dose in mice cannot be extrapolated to cattle (Geerts and Holmes, 1998). The mouse test can be used as a single dose test for epidemiological purposes (study of area-wide occurrence of DR) allowing spatial and temporal comparisons or as a multi-dose test, which measures the degree of resistance in individual trypanosome isolates (Eisler et al., 2001). Similarly, the molecular tests can also be used either for epidemiological or for diagnostic purposes. In this study we used the molecular techniques as epidemiological tools to assess the importance of the problem of DR in the Adamaoua region. The use of genetic markers allows a much more rapid screening of a large number of trypanosome isolates than the *in vivo* single dose mouse test, which is very laborious and time consuming (2 months) and which needs 18 mice for each isolate to be tested for DA and ISM. It is obvious that the presence of resistance genes may not always be associated with a drug resistance phenotype. On the other hand, not all drug resistance phenotypes are detected using molecular tools.

All *T. congolense* strains were found resistant to ISM in the mouse test but only 44% using the *Mbo*II-PCR-RFLP. This seems to confirm previous results of Delespaux et al. (2005) indicating the existence of different pathways of resistance against ISM. Indeed, the *Mbo*II-PCR-RFLP is based on single genetic marker and allows, by definition, the diagnosis of a single mutation in a single gene, missing for this reason, genetic modifications in other genes, i.e. alternative pathways of ISM-resistance (Delespaux et al., 2005).

Table 4
Detection of resistance to DA in *T. brucei* and in *T. congolense* isolates using the mouse test and the appropriate molecular test

	Isolates examined in mouse test		Isolates examined using molecular tests[a]	
	No. tested	No. (%) resistant	No. tested	No. (%) resistant
T. brucei	13	1 (7.69)	13	3 (23.08)
T. congolense	11	11 (100)	25	25 (100)

All isolates are originating from cattle from the Adamaoua region in Cameroon.
[a] AS-PCR for *T. brucei* and *Bcl*I-PCR-RFLP for *T. congolense*.

Although this technique was able to identify the majority of ISM resistant trypanosome isolates originating from Eastern Africa, this is obviously not the case for Cameroonian isolates, which indicates that strategies adopted by trypanosomes of the Central African region to develop resistance to ISM might be different. The data clearly show a lack of sensitivity of the molecular tool for the detection of resistance to ISM in *T. congolense* in this particular geographical area. The molecular tools for the detection of resistance to DA, however, were shown to be quite effective. The AS-PCR seems to be an excellent diagnostic tool for the detection of resistance to DA in *T. brucei* and appears to be cheaper and quicker than a PCR-RFLP based test, which confirms the results obtained by Nerima et al. (2007) using *T. b. gambiense*. The excellent concordance between the *Bcl*I-PCR-RFLP, the AS-PCR method and the mouse test for the diagnosis of resistance to DA confirms the specificity of the uptake mechanism of DA by the P2-like purine transporter as nearly all resistant phenotypes of *T. congolense* in mouse present the Val 306 Ile mutation described by Delespaux et al. (2006) and nearly all sensitive phenotypes of *T. brucei* lacked the *Sfa*NI mutation described by Mäser et al. (1999). The fact that the discordance in results between the two methods occurs in *T. brucei* isolates diagnosed as resistant by the molecular method but sensitive by the mouse test indicates the higher sensitivity of the former tool.

5. Conclusion

Trypanocidal DR appears to be widespread in the Adamaoua Department of Cameroon. Apparently, the problem is more serious in *T. congolense* than in *T. brucei*, which is unfortunate because the former is more pathogenic to cattle than the latter. Appropriate measures such as the intensification of vector control activities and restriction of the use of trypanocides to clinical cases as described by Holmes et al. (2004) will need to be taken in order to control bovine trypanosomosis in this area. Apparently, the *Mbo*II-PCR-RFLP method for the detection of resistance to ISM is not sensitive enough to replace the mouse test in the study area. Efforts should be made to develop new molecular tools for the diagnosis of alternative pathways of resistance against ISM. The AS-PCR and the *Bcl*I-PCR-RFLP, however, proved to be quick and sensitive tools for the diagnosis of emerging resistance to DA in *T. brucei* and *T. congolense*, respectively.

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