Trypanocidal failure suggested by PCR results in cattle field samples

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Abstract

The aim of this study was to assess, whether polymerase chain reaction (PCR) allows sensitive screening of treatment failure suspicions in areas, where drug resistance against African animal trypanosomosis (AAT) appears to be a problem. PCR was used to detect trypanosome infections prior to, 14 and 28 days after controlled treatment of 738 cattle from 10 villages in Kénédougou, Burkina Faso with isometamidium chloride and diminazene aceturate. Using three sets of primers, PCR was three–four times more sensitive and better at species identification, than standard microscopic examination. The better sensitivity and species specificity of PCR have important advantages for drug resistance studies in the field.

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1. Introduction

Resistance to one or more of the three trypanocidal drugs (diminazene, homidium, isometamidium) currently used for the treatment of trypanosome infections in cattle is known to be present in at least 13 sub-Saharan countries (Geerts and Holmes, 1998). In eight of these countries, multiple drug resistant trypanosome populations have been reported (Geerts and Holmes, 1998). The control of trypanosomosis in livestock will largely depend on the use of these compounds, because it is unlikely that new trypanocides will be developed and released in the near future. Information about the extent and impact of drug resistance is therefore urgently needed for the planning and implementation of disease management in affected areas.

Several methods have been developed during recent years for the detection of drug resistance in trypanosomes. Most of the in vivo and in vitro assays for this purpose are better suited for the determination of drug resistance of a small number of isolates, than for large-scale screening. The single dose mouse test
protocol recently introduced by Eisler et al. (2001) allows investigation of a reasonably large number of isolates. However, there are several disadvantages, most Trypanosoma vivax isolates, and also some Trypanosoma congolense isolates, do not grow in mice; although a mouse test may give a broad indication of the sensitivity of a strain, it cannot be used to predict curative doses for cattle and care should be exercised in extrapolating the results of a mouse test to cattle (Sones et al., 1988).

Assessing the success of trypanocidal drug treatments in the field is the most relevant indicator for decision makers. Standard parasitological methods used in the field, such as the phase-contrast buffy coat technique (BCT) (Murray et al., 1977), suffer from relatively poor sensitivity (Paris et al., 1982) and ability to identify mixed infections and distinguish between trypanosome species. Previous studies have shown PCR to be a specific and sensitive method for the diagnosis of trypanosome infections in experimentally and naturally infected cattle (Masake et al., 1997, 2002; Clausen et al., 1998; Solano et al., 1999; Desguenès and Diósvila, 2002). PCR has been used to monitor the efficacy of diminazene aceturate treatment in experimental infections, cattle infected with Trypanosoma brucei (Clausen et al., 1999), and sheep with T. congolense savannah, T. congolense forest and T. vivax (Bengaly et al., 2001). Under natural challenge, PCR and DNA probe hybridization were used to confirm the effectiveness of isometamidium chloride prophylaxis in cattle infected with T. brucei and T. vivax populations in Mukono County, Uganda (Clausen et al., 2001). During the first two months post-treatment, none of the aparasitaemic blood samples was positive with PCR. The first DNA signals appeared 3 months after treatment, corresponding to the prophylactic activity of isometamidium. It was concluded that these parasites were not resistant to isometamidium.

The objective of this study was to assess the potential of PCR as a method to detect drug failures in cattle in an area where trypanocidal drugs are widely used and resistance of trypanosome populations to isometamidium and diminazene has been reported (Pinder and Authié, 1984; Clausen et al., 1992; McDermott et al., 2003). The relative performance and the ability of PCR to identify single and mixed infections by different trypanosome species were compared to BCT.

2. Material and methods

2.1. Field study

This study used samples from field research in village cattle in the southern districts of Kénédougou, Burkina Faso, which aimed to assess trypanosome drug resistance after prophylactic block treatment with isometamidium chloride, and curative treatment of persistent infections with diminazene aceturate (McDermott et al., 2003). Ten villages, nine of which had a high initial trypanosome prevalence (>10%), were selected. In each of the 10 sites, 25–130 cattle were randomly selected and ear-tagged (n = 738). The BCT (Murray et al., 1977) was used to detect and identify trypanosome species. After blood sampling (November 1998), all cattle, irrespective of parasitological status, were treated with isometamidium chloride at 1.0 mg/kg bw. Thereafter, all cattle were examined for trypanosome infections every 2 weeks for 3 months using the BCT. Parasitologically positive cattle were treated with diminazene aceturate at 3.5 mg/kg bw.

2.2. Field samples for PCR analysis—collection, storage and selection

Blood from each animal was collected from the jugular vein into EDTA-coated 10 ml vacutainer glass tubes (Venoject®, Terumo) during each visit. Samples were put in cryo tubes (1 ml of each) and kept on ice until they were taken to CIRDES, where they were stored at −18 °C until DNA extraction. A subset of 180 pre-treatment blood samples, comprising of all (90) BCT-positive blood samples plus another 90 randomly selected samples from BCT-negative cattle, were analysed by PCR using oligonucleotide primers specific for T. brucei, T. vivax and T. congolense savannah (Fig. 1). Samples identified by BCT as T. congolense positive but negative by PCR, were re-tested using primers specific for T. congolense forest.

Post-treatment blood samples from 177 cattle of the above mentioned subset were collected a fortnight after the isometamidium block treatment. Another 34 post-treatment blood samples from the animals of the subset, which were BCT-positive a fortnight after the isometamidium block treatment, were
collected a fortnight after additional diminazene treatment (28 days after the isometamidium-block treatment). All post-treatment samples were examined for \textit{T. brucei}, \textit{T. vivax} and \textit{T. congolense savannah} by PCR.

### 2.3. Reference trypanosome DNA

The analytical specificity and sensitivity of PCR amplification were assessed using purified trypanosome DNA of \textit{T. brucei} ILTat 1.4 originally isolated from a steer in Uhembo, Kenya (Miller and Turner, 1981); \textit{T. vivax} IL1392 Zaria Y 486 originating from a steer in Zaria, Nigeria (Leeflang et al., 1976); \textit{T. congolense} savannah SAMOROGOUAN/82/CRTA/53 isolated in 1982 from a zebu in Samorogouan, Burkina Faso (Pinder and Authié, 1984) and \textit{T. congolense} forest DINDERESSO/80/CRTA/3 originating from a dog in Dinderesso, Burkina Faso (Pinder and Authié, 1984).

### 2.4. DNA extraction

**2.4.1. DNA from blood samples**

According to the DNA extraction protocol from Higuchi (1989) 250 \( \mu \)l of EDTA blood was mixed with 250 \( \mu \)l lysis buffer (0.32 M sucrose, 0.01 M Tris, 0.005 M MgCl\(_2\), 1% Triton X-100, pH 7.5), centrifuged at 12,000 \( \times \) g for 25 s, and the pellet formed was washed three times with lysis buffer. The final pellet was re-suspended in 250 \( \mu \)l PCR buffer (10 mM Tris–HCl, 50 mM KCl, 0.1% Triton X-100, pH 8.3) containing 200 \( \mu \)g proteinase K/ml, incubated at 56 °C for 3 h and at 95 °C for 10 min.

**2.4.2. DNA from purified trypanosomes**

A 100 \( \mu \)l trypanosome cell lysate was loaded onto a QIAamp spin column (Qiagen, Hilden, Germany). The DNA was adsorbed onto the QIAamp silica membrane and washed in two centrifugation steps before being eluted with 200 \( \mu \)l distilled water. The DNA yield and purity was determined by absorbance readings at 260 and 280 nm using a Gene Quant RNA/DNA calculator (Pharmacia Biotech, Freiburg, Germany).

### 2.5. DNA amplification

Polymerase chain reaction was carried out in 25 \( \mu \)l reaction volumes containing 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 0.1% Triton X-100, 3.0 mM MgCl\(_2\), 200 \( \mu \)M each of the four dNTPs, 1 unit of Taq Gold polymerase (Perkin-Elmer, Weiterstadt, Germany) and 1 \( \mu \)M of each of the primers. Oligonucleotide primer pairs specific for \textit{T. brucei} spp., \textit{T. vivax}, \textit{T. congolense} savannah (and \textit{T. congolense} forest) were used (Table 1). The reaction mixtures were placed in a Personal-Cycler (Biometra, Göttingen, Germany) at 95 °C for 10 min, followed by 94 °C for 60 s, 60 °C for 30 s and 72 °C for 30 s. This was repeated for 34 cycles. After a final extension step at 72 °C for 10 min the samples were cooled and stored at 4 °C. Ten microlitres of each PCR product were electrophoresed in a 2% agarose gel containing 0.5 \( \mu \)g/ml ethidium bromide.

### 2.6. DNA probe hybridisation of PCR products

All PCR products were hybridised with the respective DNA probes for \textit{T. vivax} and \textit{T. congolense} savannah. The \textit{T. brucei} specific probe was only used with PCR products from villages, where this species was prevalent according to PCR results.

The DNA probes were prepared as follows: \textit{T. vivax} was expanded in mice and separated from infected blood by DEAE-cellulose (Lanham and Godfrey, 1970), whereas procyclic forms of \textit{T. congolense} savannah and \textit{T. brucei} were initiated and expanded in axenic cultures (Mehlitz and Tietjen, 1988) and washed three times with PBS. All trypanosome sus-
Table 1
Oligonucleotide primers for the specific DNA amplification of different trypanosome species and subtypes; TBR 1.45, TBR 2.47 and TCN 2.38 have been elongated with several bases (in italics) in order to increase their melting temperature ($T_m$).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Specificity</th>
<th>Sequence of the primer (5′→3′)</th>
<th>$T_m$ (°C)</th>
<th>Product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TV 80.24</td>
<td>T. vivax</td>
<td>CAGTGCCTGCGCTCGTACACGGAC</td>
<td>80.7</td>
<td>266</td>
<td>Clausen et al. (1998)</td>
</tr>
<tr>
<td>TV 322.24</td>
<td>T. brucei</td>
<td>GCACGCCACATAGCCGGGGAACAG</td>
<td>78.9</td>
<td></td>
<td>Based on Moser et al. (1989)</td>
</tr>
<tr>
<td>TBR 1.45</td>
<td>T. brucei</td>
<td>TACCGTATTATATACACACG</td>
<td>76.8</td>
<td>173</td>
<td>Based on Moser et al. (1989)</td>
</tr>
<tr>
<td>TBR 2.47</td>
<td>T. brucei</td>
<td>ACAGCTATTATATACACACG</td>
<td>76.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCN 1</td>
<td>T. congolense</td>
<td>TCAGCGAGAACGGGCACTTTGC</td>
<td>80.7</td>
<td>339</td>
<td>Based on Moser et al. (1989)</td>
</tr>
<tr>
<td>TCN 2.38</td>
<td>Savannah</td>
<td>TGTTTTGAGGAAATGGGAC</td>
<td>79.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCF 1</td>
<td>T. congolense</td>
<td>GGAACCTGCCACAAATTGCTT</td>
<td>63.0</td>
<td>350</td>
<td>Msiga et al. (1992)</td>
</tr>
<tr>
<td>TCF 2</td>
<td>Forest</td>
<td>GTTCCTGCAACAAATGGAAC</td>
<td>64.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Pensions were submitted to DNA extraction and PCR as described before. The generated PCR product was purified by use of the QiAquick PCR-purification kit® (Qiagen, Hilden, Germany) and the DNA yield was determined as previously described. Probe labelling and hybridisation were based on enhanced chemiluminescence using the ECL direct nucleic acid labelling and detection kit® (Amersham, UK) according to the recommendations of the manufacturer.

3. Results

3.1. Pre-treatment samples

3.1.1. BCT

Before isometamidium-block treatment, the overall prevalence was 12.2% (±2.4), with 90 parasitologically positive out of a total of 738 examined cattle. Single infections with T. congolense, T. vivax and T. brucei were identified in 70.0% (63/90), 23.3% (21/90) and 1.1% (1/90) of the cases, respectively. Mixed infections were identified in 5.6% (5/90) of the positive cases, of which three consisted of T. vivax/T. congolense, and two involved T. brucei/T. vivax and T. brucei/T. congolense, respectively.

3.1.2. PCR

Each set of primers showed absolute specific amplifications compared to DNA from reference stocks. Each set of primers exhibited high analytical specificity, generating specific products at a dilution of 100 fg DNA per 25 μl reaction volume.

Of the 90 BCT positive pre-treatment samples, 83 (92.2%) were identified by PCR using three sets of primers (for T. brucei, T. vivax and T. congolense savannah) (Fig. 2), and 85 (94.4%) were identified altogether, when the previously not recognized blood samples were analysed with an additional fourth primer set (for T. congolense forest). Out of the 90 BCT-negative samples 34 (37.8%) were PCR positive using the sets of primers for T. brucei, T. vivax and T. congolense savannah. According to these results, the overall PCR-prevalence for the total of 738 cattle was estimated as 44.4% (±8.8) as compared to a BCT prevalence of 12.2% (±4.4). Specific DNA products of T. congolense savannah, T. vivax and T. brucei were amplified in 54.7% (64/117), 17.9% (21/117) and 1.7% (2/117) of the samples, respectively. Mixed infections were identified in 30 cases (25.6%), which consisted of 4 triple and 26 double infections. Of these 14 were combinations of T. congolense savannah/T. brucei and 12 were combinations of T. congolense savannah/T. vivax.

3.1.3. DNA probe hybridisation

All of the amplification products were confirmed by DNA probe hybridisation. Another eight hybridisation signals occurred with the PCR negative samples: 11.1% (763) of the PCR negative samples for T. congolense savannah and 1.6% (1/63) of PCR negative samples for T. brucei gave a positive reaction.
3.2. Post-treatment samples—14 days after isometamidium treatment

3.2.1. BCT

Two weeks after isometamidium treatment, parasitaemia was detected in 35 (19.8%) of the 177 cattle. Thirty-four of the BCT-positives came from the 89 BCT-positive cattle at the time of treatment and the other came from one of the 88 BCT-negative cattle (Fig. 3). *T. congolense* was the most abundant species, with 80.0% (28/35) of cases, whereas *T. vivax* and *T. brucei* infections were identified in 14.3% (5/35) and 2.9% (1/35) of cases, respectively. One (2.9%) mixed infection with *T. brucei*/*T. vivax* was also detected.

3.2.2. PCR

Polymerase chain reaction succeeded in amplifying specific products in 75 (42.4%) of the 177 post-treatment samples, of which 73 (97.3%) were PCR-positive before isometamidium treatment (Fig. 4). All parasitologically positive cases with one exception, were confirmed by the PCR (34/35), a detection rate of 97.1%. In two villages, trypanosomes were detected after isometamidium treatment by PCR but not by BCT. Polymerase chain reaction revealed 92.0% (69/75) *T. congolense* savannah, 5.3% (4/75) *T. vivax* and 2.7% (2/75) mixed infections. *T. brucei* was not detected.

3.2.3. DNA probe hybridisation

The DNA probes confirmed the specificity of the PCR products. Moreover, eight comparatively weak signals appeared with PCR-negative samples: 6.9% (7/102) with PCR-negative samples for *T. congolense* savannah and 1.0% (1/102) with PCR-negative sample for *T. vivax*.

3.3. Post-treatment samples—14 days after additional diminazene treatment

3.3.1. BCT

Blood samples from 34 out of the 35 BCT-positives of the 177 cattle (Fig. 3), which had been treated with diminazene a fortnight after the isometamidium treatment, were collected 14 days later. Trypanosomes were present in 5 (14.7%) of the samples; all were identified as *T. congolense*.

3.3.2. PCR

Out of these 34 samples, 21 (61.8%) were PCR-positive. All BCT-positive cases were confirmed.
BCT results: pre isometamidium treatment samples

2% (2) missing 1% (1) positive
97% (87) negatives
50% (90) positives

BCT results: post isometamidium treatment samples

1% (1) missing
61% (55) negatives
38% (34) positives

Fig. 3. Post-isometamidium treatment BCT results of 90 pre-treatment BCT-positive and 90 pre-treatment BCT-negative blood samples.

T. congolense was the most abundant species. Apart from one infection with T. vivax (4.8%), all remaining infections (20/21) were due to T. congolense.

PCR results: pre isometamidium treatment samples

2% (1) missing 3% (2) positive
95% (60) negatives
62% (73) positives

PCR results: post isometamidium treatment samples

2% (2) missing
36% (42) negatives
65% (117) positives

Fig. 4. Post-isometamidium treatment PCR results of 117 pre-treatment PCR-positive and 63 pre-treatment PCR-negative blood samples using the primer pairs specific for Trypanosoma brucei, T. vivax and T. congolense savannah.

3.3.3. DNA probe hybridisation

All PCR results were confirmed by DNA hybridisation with specific probes for T. congolense savannah and T. vivax.
4. Discussion

The results from this study show that PCR can provide important additional information in assessing the resistance to trypanocidal drugs of trypanosomes infecting cattle in the field. While the presence of isometamidium- and diminazene-resistant trypanosome populations in cattle in the province of Kénédougou, Burkina Faso, had already been confirmed by BCT, PCR proved to be more sensitive, indicating that drug-resistant trypanosome populations were more common and widespread than suspected (McDermott et al., 2003). It is likely that drug-resistance with regard to AA T could be missed in low-prevalence areas (or areas, where the degree of resistance in trypanosome populations is low), when only BCT is used for screening of treatment failures.

Polymerase chain reaction was more sensitive than BCT in the diagnosis of trypanosome infections in cattle before trypanocidal treatment and of persistent infections after trypanocidal treatment. Polymerase chain reaction results from 90 BCT-positives and 90-BCT negative cattle before isometamidium treatment, showed that a small percentage of BCT-positive samples could not be identified by PCR and that a significant proportion of BCT-negative samples were positive with PCR. These results are consistent with previous reports (Solano et al., 1999; Clausen et al., 1998, 2001).

The failure to detect PCR products in BCT-positive samples could be due to a number of reasons. Technical problems, such as inhibition of Taq polymerase, excessive amounts of DNase or a loss of DNA during extraction can lead to negative PCR results (Masake et al., 1997; Solano et al., 1996, 1999; Reifenberg et al., 1997). Another crucial issue is the choice of primer sets. The identification of two samples with the primers for *T. congolense* forest shows the importance of knowing what species of trypanosomes might be present in a given area. Because of resource constraints, only three primer sets, considered most likely to detect the pathogenic trypanosomes in cattle present in the study area were used. Depending on the main hosts and vectors in an area, a restricted range of sets of primers may make PCR diagnosis relatively more or less sensitive compared to BCT. Additionally, the existence of unknown or not characterized trypanosome species or subspecies has been suggested by several authors (Majiwa et al., 1994; Morlais et al., 1998; Solano et al., 1999). On the other hand, the most pathogenic as well as economically important trypanosome species and subtypes can be screened exclusively by PCR using corresponding sets of primers and furthermore, the parasitologically not distinguishable subtypes of *T. congolense* can be differentiated by PCR. This is particularly valuable with regard to their different pathogenicity. Cattle experimentally infected with *T. congolense* savannah showed severe clinical disease, which led inevitably to death, whereas self-curing and asymptomatic syndromes were caused by *T. congolense* forest and Kilifi-type respectively as demonstrated by Bengaly et al. (2002).

The much better ability of PCR, compared to BCT, to differentiate infections by trypanosome species and to identify mixed infections is also important in the assessment of trypanocidal drug resistance. In this study, BCT was comparatively inferior to PCR at detecting *T. brucei* (six times) and mixed infections (four times). Most *T. brucei* infections were associated with mixed infections. The higher sensitivity of the PCR to reveal mixed infections has already been demonstrated by several authors (McNamara et al., 1995; Reifenberg et al., 1997; Morlais et al., 1998; Solano et al., 1996, 1999).

In Kénédougou Province, resistance has previously been reported for *T. congolense*, first to isometamidium in mice (Pinder and Authié, 1984) and then to both isometamidium and diminazene in cattle and goats (Clausen et al., 1992). Yet, a survey conducted prior to the study in 1998 demonstrated the extensive use of isometamidium and diminazene in this area (Ouedraogo, 2002). Since resistance to isometamidium seemed to be widespread and established for a long time, isometamidium was applied at the higher dose of 1 mg/kg bw in the present study. Given that *T. brucei* has not been identified in parasitologically positive cattle in a preceding cross sectional study (1998–1999), diminazene was applied at the dose of 3.5 mg/kg bw (McDermott et al., 2003).

In the present study, isometamidium and diminazene failure has been detected for *T. congolense* and to some extent for *T. vivax* by BCT as well as by PCR. Two *T. brucei* infections (with one mixed infection with *T. vivax*) were detected by BCT 2 weeks after isometamidium treatment but these were both identified as *T. congolense* savannah by PCR and are
assumed to be errors in microscopic identification. But the identification of drug resistance and treatment failure in *T. brucei* infections in both animals and humans deserves very careful investigation, in which PCR can play an important role. In our experience, drug resistance in *T. brucei* populations in cattle is rare. However, *T. brucei* populations have the ability to invade the CNS where they are largely inaccessible to drugs and are less likely to be diagnosed by PCR on blood samples. In cases where *T. brucei* is present, it is therefore suggested to supplement PCR studies with the collection of isolates and testing of their drug sensitivity in mice as described by Eisler et al. (2001).

Given the fact that administration of drugs was done by project staff, we assume that all trypanosomes detected post-treatment were somewhat resistant to trypanocides. Besides, the mean packed cell volume (PCV) of parasitologically positive cattle was lower compared to parasitologically negative cattle throughout the whole study period of three months, although the mean values of parasitologically positive cattle started to improve 6 weeks after the initial isometamidium block treatment (Woitag, 2003). Resistance of *T. congolense* against isometamidium and diminazene was also confirmed by testing of trypanosome isolates from this study in mice (Diarras, 2001; Knope, 2002) and by testing of isometamidium levels in serum using an ELISA test (Diarra, 2001) as described by Eisler et al. (1997). Combining PCR with the isometamidium-ELISA assay could be used to screen for isometamidium-resistant trypanosomes in routinely treated cattle, rather than requiring controlled treatments as undertaken here. For the assessment of infections after diminazene treatment, trypanosome DNA can be amplified in blood samples from cattle up to 4 days after a sterilizing diminazene treatment by PCR (Claussen et al., 1999). In order to avoid false positive results, therapeutic success should not be evaluated within 4 days after treatment. However, the test should be performed as soon as possible after this time because of the short prophylactic activity of diminazene.

All of the PCR amplification products were identified with specific DNA probes. The 100-fold sensitivity increase of the PCR with specific DNA probes as suspected earlier by Moser et al. (1989) could not be confirmed. Compared to other methods, PCR seems to be the most sensitive method for monitoring the efficacy of drug treatment of *T. congolense* and *T. vivax*-infections in cattle under field conditions, although it may not be reliable enough to reveal resistant *T. brucei*-infections.

Given that new drugs are unlikely to be developed and that resistance to common trypanocides has been widely and increasingly recognized, the identification and management of drug resistance will be an increasingly important concern in the treatment and control of animal trypanosomosis. This study has demonstrated that the increased sensitivity and better ability to differentiate different trypanosome species are key advantages for using PCR as a research tool in the assessment of drug resistance in trypanosome populations. PCR used in controlled field treatment studies or combined with drug detection assays can more sensitively detect the emergence of drug resistance in an area. This additional sensitivity is of great practical importance in research circumstances where standard parasitological tests are of limited sensitivity. These include areas with a high prevalence of *T. brucei* and mixed trypanosome infections and in areas with a high proportion of trypanotolerant livestock species, which have the ability to control trypanosome parasitaemias.

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