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Diagnostic evaluation of PCR on dried blood samples from goats experimentally infected with *Trypanosoma brucei brucei*

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Abstract

In seven goats experimentally infected with a pleomorphic clone of *Trypanosoma brucei brucei*, parasitaemia was monitored weekly for 6 weeks by wet blood film and microhaematocrit buffy coat examination. Dried blood samples on filter paper were concomitantly collected and tested by PCR using three different primer sets, putatively specific for *Trypanozoon*, *T. vivax* and *T. congolense*. With the originally designed ORPHON5J *Trypanozoon* primers, PCR tests became positive after 1 week (six animals) or 2 weeks (one animal) of infection and remained consistently positive until the end of the experiments, thus yielding an overall positivity rate of 97%, as compared with 74% for all parasitological tests together. The *T. vivax* and *T. congolense* primers yielded no positive PCR results.

Keywords: Trypanosoma brucei; Goat; PCR; Diagnosis

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1. Introduction

Parasitological diagnosis of African trypanosomiasis in man or domestic animals is commonly hampered by low levels of parasitaemia. Whereas antibody detection tests are useful for screening purposes but do not distinguish between past and present infections, antigen detection tests are not yet sufficiently reliable.

Detection of trypanosomal DNA by the polymerase chain reaction (PCR) opens new perspectives, but only few publications hitherto have been dealing with practical methods for examination of blood samples and most of the test protocols are rather laborious (Wuyts et al., 1995; Desquesnes and Tresse, 1996; Kanmogne et al., 1996; Penchenier et al., 1996; Masake et al., 1997). For large-scale surveys, a technique applicable to dried blood samples on filter paper seems most convenient. Samples can be eluted in the presence of Chelex® 100 according to a procedure described by Walsh et al. (1991). Recently this technique has been evaluated with relative success by Katakura et al. (1997) for diagnosis of cattle trypanosomiasis and by de Almeida et al. (1997) to detect parasite DNA in goats infected with *T. vivax*. Reported here are similar PCR assays in goats infected with *T. brucei*, another trypanosome species affecting man and domestic animals. Aiming at maximal test specificity for trypanosomes of the subgenus *Trypanozoon*, a newly designed ORPHON5J primer set was used. It is targeting a DNA sequence situated at the 5′ junction of the spliced leader sequence orphon element (Nelson et al., 1984; Parsons et al., 1986). For control purposes, PCR tests were also run in parallel with *T. vivax* and *T. congolense* primer sets.

2. Materials and methods

2.1. Experimental infection

Seven healthy female Saanen goats, 12–18 months old and kept in a fly proof stable near Antwerp, were subcutaneously inoculated with 10^4 trypanosomes of a pleomorphic clone of *T. b. brucei*, variable antigen type AnTat 1.1 E, derived from the Ugandan stock cryostabilate EATRO 1125 (Van Meirvenne et al., 1975). Within 4 weeks of infection, all the animals developed a severe illness characterized by recurrent fever, lack of appetite and anaemia. One goat died during the fourth week of infection.

Heparinized venous blood samples collected from each animal 1 week before and weekly after infection were examined for the presence of trypanosomes. Except for the third week after infection, dried blood samples on filter paper were concomitantly collected for PCR assays.

2.2. Trypanosome detection tests

All post-infection blood samples were examined for the presence of trypanosomes by two versions of the microhaematocrit buffy coat (BC) and two versions of the

wet blood film (WBF) technique. For buffy coat examination, six capillary tubes (internal diameter of 1 mm) were filled with blood and centrifuged in a micro-haematocrit centrifuge for 12 min. One capillary was cut and a wet BC preparation examined under the phase contrast microscope (10×40), in the same manner as originally described for dark ground microscopy (Murray et al., 1977). In case of a negative result, a second capillary tube was examined in the same way. Another four capillary tubes were mounted in a viewing slide and all the BC-plasma interface layers directly examined under the microscope with a $10\times$ objective (Woo, 1971). Conventional WBF: 5 μl of blood was put onto a microscope slide, mounted with a coverslip (24×24) and the entire preparation examined under the phase contrast microscope (10×40, 1000 microscopic fields at least), counting all the trypanosomes encountered. SDS-haemolysed WBF (Van Meirvenne et al., 1989): bringing all reagents to room temperature, 10 μl of blood was put onto a slide, mixed with 10 μl of a 1% sodiumdodecylsulfate solution in isotonic Tris-1% glucose buffer, pH 8, covered with a coverslip (24×24) and examined as the conventional WBF (10×25).

2.3. Polymerase chain reaction (PCR)

2.3.1. Trypanosome pellets

To serve as reference materials for the PCR assays, the following cryopreserved trypanosome clone populations were grown in mice: *T. b. brucei* AnTat 5.3 stabilate ITMAS 271181, *T. b. rhodesiense* AnTat 25.1 ITMAS 130381A, *T. b. gambiense* AnTat 8.1 ITMAS 250679D, *T. evansi* RoTat 1.2 ITMAS 161089, *T. vivax* ILRAD 700 ITMAS 060989, *T. congolense* LION 231 ITMAS 170282. The *T. theileri* cryostabilate ITMAS 190696, derived from a Belgian cow, was grown in a cell free culture medium (Aerts et al., 1992).

Trypanosomes isolated from mouse blood by the DEAE anion exchange column technique or from culture medium by centrifugation were suspended in milli-Q water and frozen at -20°C. Three 20 μl aliquots of the thawed pellet suspensions, containing, respectively 50, 2500 and 50000 trypanosomes, were directly submitted to PCR as further described for blood spot extracts.

2.3.2. Blood samples

About 30 μl heparinized blood was spotted onto filter paper (Whatman 4) and allowed to air dry. The individual samples were separately packed in plastic bags with silica gel and kept at -20°C for up to 2 months before testing.

DNA was extracted following a modification of the procedure described by Walsh et al. (1991). From each blood spot, two circular 6 mm confettis (~ 10 μl of blood in total) were cut out with a chromium-plated punche which was intermittently sterilized above a Bunsen flame for ~ 2 s. Both confettis were eluted for 30 min with 1 ml distilled water, in an eppendorf tube with screw cap. After ~ 15 min, the tube was inverted a few times. Following centrifugation in a fixed angle rotor (10 min at $7800 \times g$) 850 μl supernatant was carefully pipetted off. To the undisturbed pellet and confettis, 200 μl of a freshly prepared Chelex® 100 (BioRad)

1% suspension in distilled water was added. The mixture was stirred-up by rubbing the tube over the meshes of a tube rack, incubated at 56°C for 30 min, boiled in a water bath for 8 min and eventually vortexed for 2 min at maximum speed. After a final centrifugation step (5 min at 7800 × *g*) 20 μl of supernatant was submitted to PCR.

2.3.3. Primers

The sequence of the originally designed, putatively *Trypanozoon* specific ORPHON5J primers is as follows; The 23-mer upper oligo (ORPHON5J-u): 5′-GAT CCC TCT CCA CCA ATC GAC CG-3′, the 21-mer lower oligo (ORPHON5J-l): 5′-AAC TGC CCC GAC CTC CGC AGT-3′.

The TVW primer set, which targets *T. vivax* repetitive satellite sequences, has already been described by Masiga et al. (1996) and has been recently evaluated for PCR application in goats experimentally infected with *T. vivax* (de Almeida et al., 1997).

The GOL primer set is a modification of TCN-1, 2 (Moser et al., 1989). These primers bind to a repetitive nuclear sequence of the *T. congolense* genome. Their sequence is as follows:

The 24-mer upper oligo (GOL-u): 5′-GAG AAC GGG CAC TTT GCG ATT TTC-3′.

The 24-mer lower oligo (GOL-l): 5′-GAC AAA CAA ATC CCG CAC AAC CAT-3′

2.3.4. DNA amplification and detection

Amplification was done in 500 μl eppendorf tubes. Each tube received 30 μl of a PCR cocktail consisting of: 25 μl 2 × Goldstar PCR-mix (EuroGenTec) enriched with 400 μM of each of the four deoxynucleotides and 3.0 mM $MgCl_2$, 0.5 μl 1 U/μl Goldstar DNA polymerase (EuroGenTec), a single primer set and distilled water (q. suff.). The amount of each ORPHON5J, TVW and GOL primer was, respectively 20, 5 and 30 pmol. PCR cocktails were overlaid with ∼50 μl mineral oil (Sigma). Finally, 20 μl of blood extract or pellet suspension was pipetted through the oil layer.

For each of the three primer sets used, the amplification programmes were as follows: ORPHON5J: (50 cycles) 30 s at 94°C, 90 s at 68°C; (1 cycle) 120 s at 72°C; TVW: (40 cycles) 30 s at 94°C, 60 s at 60°C and 30 s at 72°C; (1 cycle) 120 s at 72°C; GOL: (40 cycles) 30 s at 94°C, 45 s at 50°C, and 30 s at 72°C; (1 cycle) 120 s at 72°C. To avoid non specific binding of the primers, the reaction tubes were put in the thermal cycler (Omnigene, Hybaid) after the denaturation temperature (94°C) had been reached.

PCR products were electrophoresed (30 min at 180–200 V) in 2% agarose gels (Biozym) previously stained by submersion in an ethidium bromide solution (1.5 mg/l Tris acetate EDTA buffer, pH 8.0). Signals were made visible by UV illumination and photographed with a Polaroid camera.

3. Results

The parasitological and PCR results are summarized in Table 1. The first trypanosome findings were made after 2 weeks of infection. Parasitaemia was generally low, never exceeding one trypanosome per 20 microscopic fields in the wet blood film preparation. The overall positivity rate of all the parasitological tests together was 74%. Per individual technique positivity rates were respectively 49% for the classical wet blood film, 72% for the SDS clarified WBF, 69% for the buffy coat wet film version and 69% for the buffy coat in situ examination.

After 1 week of infection, the PCR with ORPHON5J primers was positive in six of the seven goats, while trypanosomes were not detected at that time. Later on, the ORPHON PCR consistently gave positive results with all blood samples, thus yielding an overall positivity rate of 97%. The single amplification band obtained (Fig. 1) corresponds to the expected 246 bp sequence also revealed in pellets of various *Trypanozoon* stocks (Fig. 2). The TVW primers earlier evaluated for PCR in goats infected with *T. vivax* (de Almeida et al., 1997) yielded no amplification signals. Both with pre- and post-infection blood samples, PCR with the *T. congolense* GOL primers yielded some non specific amplification bands (not shown), which could easily be distinguished from the *T. congolense* specific 314-base band described by Moser et al. (1989).

PCR with ORPHON5J primers on pellets of *T. congolense* and *T. theileri* did not yield amplification bands with 50, 2500 or 50000 organisms (Fig. 2). All *Trypanozoon* pellets yielded a single amplification band in the theoretically expected 250 bp region (Fig. 2), even with as few as 50 organisms (not shown). As regards

Table 1
Summary of the results obtained with four different parasitological techniques and with ORPHON5J PCR

Week	WBF/BC/PCR						
	Goats						
	A	B	C	D	E	F	G
−1	0/−/− Infection	0/−/−	0/−/−	0/−/−	0/−/−	0/−/−	0/-/-
1	0/−/+	0/−/+	0/−/+	0/−/+	0/−/+	0/−/+	0/−/−
2	0/−/+	1/+/+	2/+/+	0/−/+	0/−/+	136/+/+	2/+/+
3	2/+/ND	4/+/ND	0/+/ND	1/+/ND	47/+/ND	4/+/ND	10/+/ND
4	24/+/+	61/+/+	45/+/+	46/+/+	*	10/+/+	7/+/+
5	25/+/+	52/+/+	9/+/+	16/+/+	*	123/+/+	16/+/+
6	13/+/+	195/+/+	145/+/+	36/+/+	*	35/+/+	3/+/+

WBF, wet blood film; *n*, total number of trypanosomes detected in both preparations examined (classical and SDS version); BC, buffy coat, global result of both test versions, i.e. wet film and in situ examination; +, trypanosomes detected; −, negative; PCR, polymerase chain reaction; +, positive result; −, negative; ND, not done; *, animal died.

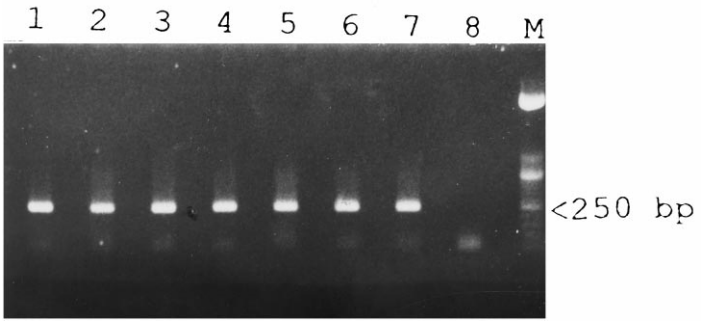

Fig. 1. Example of positive PCR results obtained with the ORPHON5J primers. Lanes 1–7, goats A–G 2 weeks post-infection; Lane 8, negative control (distilled water); M, ' marker XIII' (Boehringer, Mannheim).

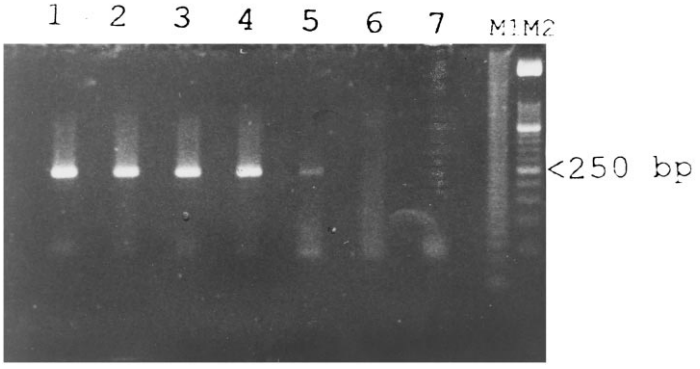

Fig. 2. PCR results obtained with trypanosome pellets (50000 organisms) in combination with ORPHON5J primers. (1) *T. b. brucei* AnTat 5.3 (2) *T.b. rhodesiense* AnTat 25.1 (3) *T.b. gambiense* AnTat 8.1 (4) *T. evansi* RoTat 1.2 (5) *T. vivax* ILRAD 700 (6) *T. congolense* LION 231 (7) *T. theileri*; M1, 'superladder-low' marker (GenSura, USA); M2, 'marker XIII' (Boehringer, Mannheim).

T. vivax, no amplification signals where obtained with 50 or 2500 organisms. With 50000 organisms, however, a faint band was obtained in the 250 bp region (Fig. 2).

4. Discussion

The diagnostic sensitivity of the PCR dried blood protocol in combination with ORPHON5J primers looks quite satisfactory. Only one of the 32 post-infection samples tested yielded a negative result (goat G, week 1). Trypanosomal DNA was detected in nine samples showing no parasites upon thorough examination of six buffy coats and two wet blood film preparations, as well as in seven samples showing only one to ten trypanosomes in the two wet blood films together (15 μl blood examined).

Sensitivity and specificity of PCR for diagnosis of African trypanosomiases remain to be evaluated on field materials. The development of subgenus specific assays raises a particular problem. Quite encouraging in this respect are the negative results now obtained in *T. brucei* infected goats both with the *T. vivax* TVW primers, which have been earlier evaluated during analogous experiments in goats (de Almeida et al., 1997), and with the *T. congolense* GOL primers. The putatively *Trypanozoon* specific ORPHON5J primers, on the other hand, cross-reacted with *T. vivax* organisms in the 250 bp region. However, according to our experience, the more conventional *T. brucei* primers described by Moser et al. (1989) also cross-react with this *T. vivax* stock and with all the four *T. congolense* stocks examined, whereas the ORPHON5J primers do not react with any of these *T. congolense* (results not shown).

The ORPHON5J primers are targeting a DNA sequence putatively present in all *Trypanozoon* organisms. All the 20 *Trypanozoon* stocks recently examined (5 *T. b. brucei*, 5 *T. b. rhodesiense*, 5 *T. b. gambiense* and 5 *T. evansi*) yielded identical PCR results (partially shown in Fig. 2). Thus, the assay presented here might serve for spotting infections with these organisms in animals or man.

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