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Diagnostic evaluation of PCR in goats experimentally infected with *Trypanosoma vivax*

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Abstract

Six goats were experimentally infected with a stock of *Trypanosoma vivax*. Parasitaemia was weekly monitored by buffy coat and wet blood film examination during a period of 15 weeks and another 3 weeks following drug-treatment. Dried blood samples were tested by the polymerase chain reaction (PCR), using an extraction method with Chelex® 100 (BioRad). PCR proved consistently more sensitive than the parasitological techniques. © 1997 Elsevier Science B.V.

Keywords: Trypanosoma vivax; Goat; Diagnosis; PCR; Chelex® 100

1. Introduction

Trypanosoma vivax is one of the most important trypanosome species causing disease amongst cattle and other ungulates (Jordan, 1986). An efficient control strategy requires good diagnostic tools. Commonly used parasitological techniques, like wet blood film, thick blood smear and buffy coat examination are often unable to detect low parasitaemia, which is commonly present in chronic infections. Trypanosomal DNA amplification by PCR is a potential candidate system for

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developing highly sensitive and specific tests (Lessard et al., 1995). This possibility was studied in an experimental infection model of goats with *T. vivax*. The use of Chelex® 100, a resin with ion binding properties (Walsh et al., 1991), has enabled the development of a simplified DNA extraction method for dry blood spots on filter paper.

2. Materials and methods

2.1. Experimental infection

Six healthy female Saanen goats, about 1 year old, were kept in a flyproof stable near Antwerp. A first animal was inoculated both i.m. and i.v with 1 ml cryostabilate of *T. vivax* stock EATRO 1185 containing antilog 6.6 viable trypanosomes (matching method, Herbert and Lumsden, 1976). On day 10, when the parasitaemia was approximately antilog 8.1 per ml, 0.5 ml of blood was i.m. inoculated into each of the five other animals. After 15 weeks of infection all the animals were treated by a single i.m. injection of 7 mg/kg diminazene aceturate (Berenil®, Hoechst).

During a total observation period of 18 weeks, the evolution of parasitaemia was weekly monitored by a wet blood film (matching method, Herbert and Lumsden, 1976) and a microhaematocrit buffy coat examination (Murray et al., 1977). At each sampling about 30 μl of heparinized blood was spot onto filter paper Whatman no. 4 for PCR assays. Filter papers were air-dried, packed in plastic bags with silica gel and stored at -20°C for up to 2 months.

2.2. DNA extraction (modified from Walsh et al., 1991)

From each blood spot, two circular 6 mm confettis were cut out with a chromium-plated puncher which was intermittently sterilized above a Bunsen flame for about 2 s. Both confettis were eluted during 30 min with 1 ml distilled water, in an eppendorf tube with screw cap. After about 15 min, the tubes were inverted a few times to promote elution. Following centrifugation in a fixed angle rotor (10 min at $11\,000 \times g$) 850 μl supernatant was carefully removed by aspiration. To the undisturbed pellet and confettis, 200 μl of a Chelex® 100 (BioRad) 1% suspension in distilled water was added. This mixture was stirred up by rubbing the tube over the meshes of a tube rack, incubated at 56°C during 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 $11\,000 \times g$) 20 μl of the supernatant was used in the PCR.

2.3. DNA amplification and detection

The TVW-A and -B primers used have been previously described by Masiga et al. (1996). They recognized a genomic repetitive satellite DNA sequence of *T. vivax*. Amplification was carried out in 500 μl eppendorf tubes. Each tube received 30 μl

of a PCR cocktail consisting of: 0.5 μl of a 10 pmol/μl solution of each primer, 25 μl 2 × Goldstar PCR-mix (EuroGenTec) enriched with 400 μM of each of the four dNTPs (Boehringer) and 3.0 mM $MgCl_2$, 0.5 μl 1 U/μl Goldstar DNA polymerase (EuroGenTec) and 3.5 μl distilled water. The cocktails were overlaid with about 50 μl mineral oil (Sigma) and 20 μl of blood extract was pipetted through the oil layer. These final mixtures were subjected to 40 PCR cycles, each of them consisting of three steps: 30 s denaturation at 94°C, 1 min annealing at 60°C and 30 s polymerization at 72°C. The very last polymerization step lasted for 3 min.

Each PCR product (10 μl) was submitted to electrophoresis during 30 min at 200 V in a 2% agarose gel (Biozym agarose), previously stained by a 30 min submersion in a 1.5 mg/l ethidium bromide bath, using Tris acetate EDTA buffer (pH 8.0) throughout.

3. Results

Results are summarized in Table 1. All tests were intermittently positive for a varying period of time before drug treatment but never thereafter. PCR yielded twice as much positive results as the combined parasitological techniques. On one

Table 1
Weekly parasitological and PCR results for six goats experimentally infected with *T. vivax* and drug treated after 15 weeks

Weeks after infection	BC/WBF/PCR					
	Goat 1	Goat 2	Goat 3	Goat 4	Goat 5	Goat 6
1	2/3/+	3/3/+	2/2/+	2/2/+	1/1/+	2/2/+
2	−/−/+		−/−/+	1/-/+	−/−/+	3/2/+
3	−/−/+	−/−/+	1/1/+	2/1/+		3/3/+
4	−/−/+		2/1/+	1/−/+	1/1/+	3/2/−
5		−/−/+	−/−/+	−/−/+		−/−/+
6			3/2/+	1/1/+		
7			−/−/+		2/-/+	−/−/+
8	−/−/+					−/−/+
9				−/−/+		−/−/+
10					−/−/+	−/−/+
11					−/−/+	
12			2/1/+	−/−/+		
13						−/−/+
14				−/−/+		
15						
16						
17						
18						

+, positive; −, negative; blank, all tests negative; BC, buffy coat; WBF, wet blood film. Figures indicate the number of trypanosomes per microscopic field: 1, ≤1; 2, 1–10; 3, ≥10.

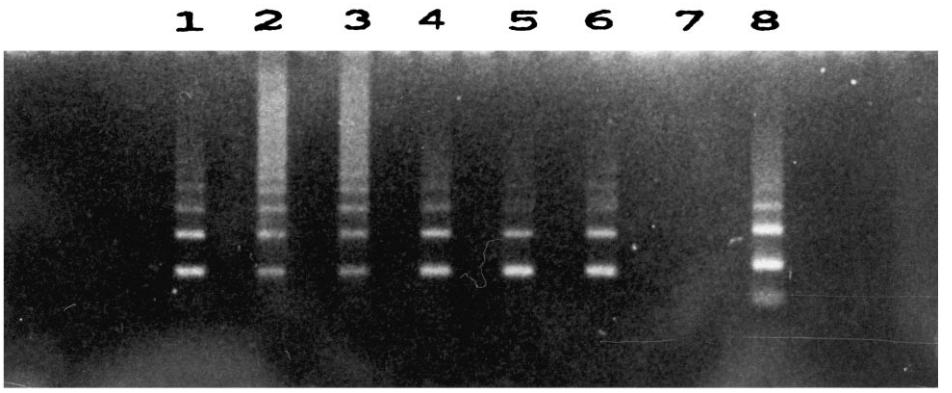

Fig. 1. PCR results obtained one week after infection. Lanes 1–6, goats 1–6. Ladder patterns are due to amplification of varying numbers of 175 bp repeat units. Lane 7, negative control—water; Lane 8, marker—a ladder of 180 bp multiples.

occasion however (goat 6, week 4), PCR was negative and parasitological tests positive. A paired sample t-test ($P = 0.0004$) gives a $25 \pm 7\%$ increased sensitivity for PCR in comparison with the combined parasitological techniques.

Figs. 1 and 2 show the PCR patterns obtained with blood samples after 1 and 8 weeks of infection, respectively. Due to the repetitive character of the target satellite sequences, a ladder pattern was frequently obtained, corresponding to the varying number of repeat units present in the amplification products.

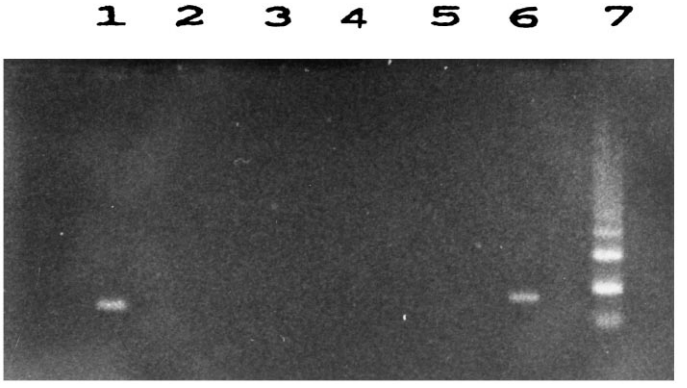

Fig. 2. PCR results obtained 8 weeks after infection. Lanes 1–6, goats 1–6. The single band seen in lanes 1 and 6 is the amplification product of one 175 repeat unit. Lane 7, marker—a ladder of 180 bp multiples.

4. Discussion

The present experiments suggest that it is possible to develop a relatively simple PCR protocol for detection of *T. vivax* DNA in dried blood samples. As compared with wet blood, dried blood spots can more easily be collected under field conditions and dispatched to the laboratory. Moreover, a better stability of the DNA is to be expected. The extraction procedure using Chelex® 100 seems to overcome the well known problems associated with the inhibitory effects of some blood components, such as haemoglobin and the anticoagulans heparin (Beutler et al., 1990).

Although superior to the trypanosome detection techniques, the diagnostic sensitivity of the present PCR version was not yet entirely satisfactory. This could be related to the small volume of blood processed, i.e. about 10 μl only. Towards further improvement of the test system it will also be useful to examine the relative contribution of whole trypanosomes, cell debris and DNA fragments thereof in the test sample.

As regards specificity, preliminary results obtained in Belgian goats are encouraging. Apart from the here described negativation of the assay after drug treatment, completely negative results have been obtained with the present *T. vivax* PCR version in seven other goats before and during eight consecutive weeks after experimental infection with *T. b. brucei*.

As a matter of fact, sensitivity and specificity of PCR essentially depend on the type of DNA sequences targeted. In the near future Masake et al. (1997) will present a PCR protocol for wet blood using a set of primers that recognizes a variety of *T. vivax* stocks from Africa and South America.

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