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Veterinary Parasitology 117 (2003) 185–193

veterinary parasitology

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PCR-RFLP using Ssu-rDNA amplification: applicability for the diagnosis of mixed infections with different trypanosome species in cattle

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Received 15 May 2003; received in revised form 25 July 2003; accepted 5 August 2003

Abstract

The use of a single restriction fragment length polymorphism (RFLP)-PCR assay which is able to characterise all important bovine trypanosome species was evaluated for the detection of mixed infections with *Trypanosoma brucei brucei*, *Trypanosoma theileri*, *Trypanosoma congolense* and *Trypanosoma vivax*. Results showed that mixed infections are detectable at a minimum ratio of 2%/98% of standardised DNA solutions with a concentration of 10 ng ml^{-1}. All mixed infections gave clear profiles that could be easily differentiated except with *T. theileri* and *T. congolense* where the *T. theileri* band was concealed by the *T. congolense* profile.
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Keywords: Cattle-protozoa; *Trypanosoma* spp.; RFLP-PCR; Species characterisation; rDNA; Diagnosis

1. Introduction

Diagnostic tools to gather accurate data regarding the epidemiology of trypanosomosis are crucial for the development of good treatment and control strategies to protect livestock against trypanosomosis. PCR assays for trypanosome detection have been developed (Kukla et al., 1987; Moser et al., 1989; Desquesnes and Tresse, 1996; de Almeida et al., 1997) using species-specific DNA hybridisation probes (Majiwa et al., 1985; Majiwa and Webster, 1987). These methods require a panel of probes for each sample to be tested. A 'pan-*Trypanosoma*' test based on the ITS 1 region of the ribosomal genes has been recently

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doi:10.1016/j.vetpar.2003.08.004

described by Desquesnes et al. (2001) replacing the various PCRs with a single assay. The authors concluded that this test has a great potential as diagnosis tool but lacked sensitivity, especially for *T. vivax*.

The association of PCR with restriction fragment length polymorphism (RFLP) (Geysen et al., 2003) provides a potential sensitive and specific tool for field surveys. The 18S ribosomal subunit is an ideal target sequence as it is a mosaic of highly conserved and species-specific sequences, present as a multi-copy locus. Although PCR and RFLP techniques need a well-equipped laboratory, the collection of field samples on filter paper is very simple (de Almeida et al., 1997, 1998), and the sample transportation and conservation is easy. However, the results obtained by Geysen et al. (2003) were from single infections. The objective of this study was to evaluate the PCR-RFLP (Geysen et al., 2003) for its capacity to detect mixed infections. Mixed infections were prepared in vitro, i.e. by mixing known quantities of DNA of two different species of trypanosome to avoid bias in the ratios of the two species. Some preliminary results of in vivo validation are also presented.

2. Materials and methods

2.1. DNA reference samples

Trypanosoma congolense and *Trypanosoma brucei brucei* pellets were obtained via rat inoculation and blood filtration through a mini anion exchange column (Lanham and Godfrey, 1970). *Trypanosoma theileri* was isolated from cattle in Burkina Faso. A KIVI (kit for in vitro isolation) (Aerts and Truc, 1992) was inoculated with 10 ml of blood and stored at 20 °C. After 10 days, the medium was checked microscopically for the presence of *T. theileri* and centrifuged for 10 min at $2500 \times g$. The supernatant was discarded and the pellet briefly vortexed to re-suspend the cells. *Trypanosoma vivax* pellets were obtained via cattle inoculation and blood filtration through a mini anion exchange column (Lanham and Godfrey, 1970).

All trypanosome pellets were subjected to DNA extraction using the QIAamp kit (Qiagen, US). The DNA concentration of the reference samples was estimated by optical density measurements at 260 and 280 nm as described by Warburg and Christian (1942) (spectrophotometer SPECTRONIC UNICAM GENESYS®) and standardised to 10 ng μl^{-1} by adequate dilution in milli-Q water. The origin and codes of the trypanosome isolates used to develop the PCR assay are described in Table 1.

Table 1
Description of the trypanosome isolates used in the PCR amplifications

Species	Code	Origin	Obtained
T.b. brucei	AnTat 1.8	Uganda	ITM[a]
T. congolense (savannah type)	IL 1180	Tanzania	ITM
T. theileri	ITGD2	Burkina Faso	ITM
T. vivax	ILRAD 700	Nigeria	ITM

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2.2. Field samples

Cattle were sampled from herds in the Abomey-Calavi area in Benin (Azando, 2002). Blood was collected into a micro-capillary tube from the ear vein and centrifuged at 12,000 × *g* for 5 min. The buffy coat has been transferred on a Whatman® 4 filter paper, air dried for 24 h in the shade, placed in plastic bag with silica gel till DNA extraction performed as described by Geysen et al. (2003).

2.3. DNA amplification

Standard PCR amplifications were carried out in 25 μl volume reaction containing 20 μl of a solution of 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). DNA template consisted of a 5 μl volume containing a mix of two DNA reference samples (10 ng μl^{-1}) in ratio varying according to Table 2. 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). 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. For the semi-nested runs, 1 μl of amplification product from the first run was added through the oil layer to 24 μl of PCR mix at 82 °C (hot start principle), containing the same ingredients and concentrations except for the primer pair. The amplification programme was identical

Table 2
DNA ratio used for in vitro mixed infections

DNA ratio (species 1 (%)/species 2 (%))	Volume of sample[a] (species 1) (μl)	Dilution	Volume of sample[a] (species 2) (μl)	Dilution
0.0002/∼99.9998	1	10^{-5}	4.9	1
0.002/∼99.998	1	10^{-4}	4.9	1
0.02/∼99.98	1	10^{-3}	4.9	1
0.2/99.8	1	10^{-2}	4.9	1
2/98	1	10^{-1}	4.9	1
6/94	3	10^{-1}	4.7	1
15.7/84.3	6.25	10^{-1}	3.75	1
25/75	1.25	1	3.75	1
50/50	2.5	1	2.5	1
75/25	3.75	1	1.25	1
84.3/15.7	3.75	1	6.25	10^{-1}
94/6	4.7	1	3	10^{-1}
98/2	4.9	1	1	10^{-1}
99.8/0.2	4.9	1	1	10^{-2}
∼99.98/0.02	4.9	1	1	10^{-3}
∼99.998/0.002	4.9	1	1	10^{-4}
∼99.9998/0.0002	4.9	1	1	10^{-5}
−control	0		0	
+control	5	1	0	
+control	0		5	1

[a] Concentration of the reference samples: 10 ng μl^{-1}.

except for 25 cycles. A negative control consisting of adding ultrapure water instead of template DNA to the PCR mixture was included in all PCR amplifications.

The samples were examined for the presence of DNA amplicons by loading 5 μl of each reaction mixture with 2 μl of 5× loading buffer onto 2% agarose gels. A 100 bp DNA ladder (MBI Fermentas, Lithuania) was included on every gel. The samples were run for 20 min at 100 V, stained in ethidium bromide for 20 min, washed under running tap water and photographed under UV illumination. RFLP was used for further typing of the fragments.

2.4. Primers used

The first amplification was done on the 18S gene using the forward primer 18STnF2 (CAACGATG-ACACCCATGAATTGGGGA) and 18STnR3 (TGCGCGACCAATAATTG-CAATAC) as reverse primer as described by Geysen et al. (2003). A semi-nested second amplification was done using the forward primer 18STnF2 of the first amplification with the reverse primer 18STnR2 (GTGTCTTGTTCTCACTGACATTGTAGTG).

2.5. Restriction fragment length polymorphism

Nested products were digested with *Msp*1 and *Eco*571 enzymes in buffer Y+/Tango with S-adenosylmethionine according to the manufacturer's specifications (MBI Fermentas, USA) using 10 U μg^{-1} DNA (0.6 U μl^{-1} PCR product) on 6 μl of amplified DNA in 15 μl total volume. The reaction was left overnight in a water bath at 37 °C. A volume of 4 μl of restricted sample was then mixed with 2 μl of loading buffer and transferred onto a 10% polyacrylamide gel together with a 100 bp DNA ladder (MBI Fermentas, Lithuania) for fragment size determination. DNA fragments were thereafter separated by horizontal electrophoresis in 0.5× TBE buffer at 100 V for 2.5 h. The gel was stained using a commercial silver staining kit (Silver staining kit DNA plusone, Pharmacia Biotech, Uppsala, Sweden) and mounted for storage.

3. Results

Six samples consisting of the following in vitro mixed infections were prepared and analysed by RFLP-PCR:

- *T. congolense–T. vivax* (Figs. 1 and 2).
- *T. brucei–T. congolense* (Fig. 3).
- *T. brucei–T. vivax* (Fig. 4).
- *T. brucei–T. theileri* (Fig. 5).
- *T. theileri–T. congolense* (Figs. 6 and 7).
- *T. theileri–T. vivax* (Figs. 8 and 9).

For all mixed infections, the lowest ratio which could be detected under the conditions described above was a ratio of 2%/98% for any two DNA mixtures with a concentration of 10 ng ml^{-1}. Profiles of simple and mixed infections of the field samples are shown in Fig. 10.

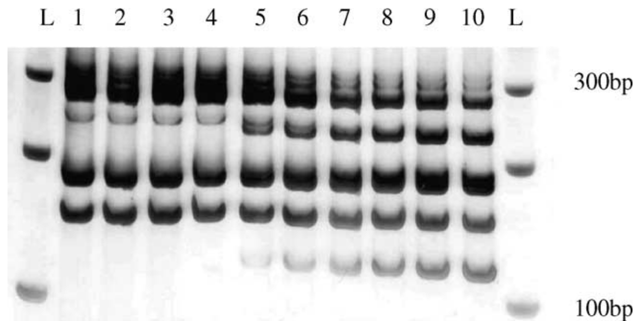

Fig. 1. In vitro mixed infections of *T. congolense* and *T. vivax* mixed in the following ratios: 99.9998% *T. congolense* in lane 1; 99.998% in lane 2; 99.98% in lane 3; 99.8% in lane 4; 98% in lane 5; 94% in lane 6; 85.7% in lane 7; 75% in lane 8; 50% in lane 9; 25% in lane 10. Lanes L contain a 100 bp DNA ladder.

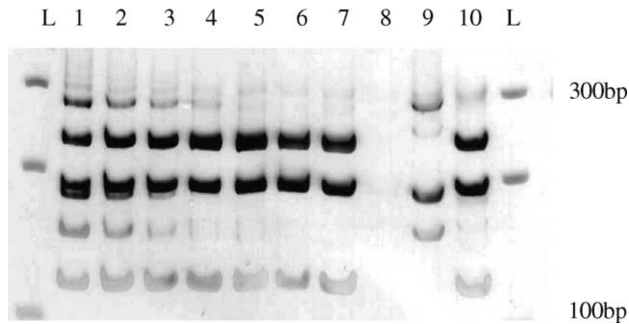

Fig. 2. In vitro mixed infections of *T. congolense* and *T. vivax* mixed in the following ratios: 85.7% *T. vivax* in lane 1; 94% in lane 2; 98% in lane 3; 99.8% in lane 4; 99.98% in lane 5; 99.998% in lane 6; 99.998% in lane 7; 75% in lane 8; positive control of *T. congolense* in lane 9; positive control of *T. vivax* in lane 10. Lanes L contain a 100 bp DNA ladder.

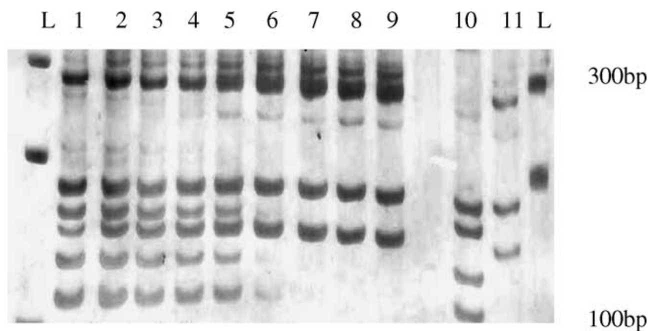

Fig. 3. In vitro mixed infections of *T. congolense* and *T. brucei* mixed in the following ratios: 50% *T. congolense* in lane 1; 75% in lane 2; 85.7% in lane 3; 94% in lane 4; 98% in lane 5; 99.8% in lane 6; 99.98% in lane 7; 99.998% in lane 8; 99.9998% in lane 9; positive control of *T. brucei* in lane 10; positive control of *T. congolense* in lane 11. Lanes L contain a 100 bp DNA ladder.

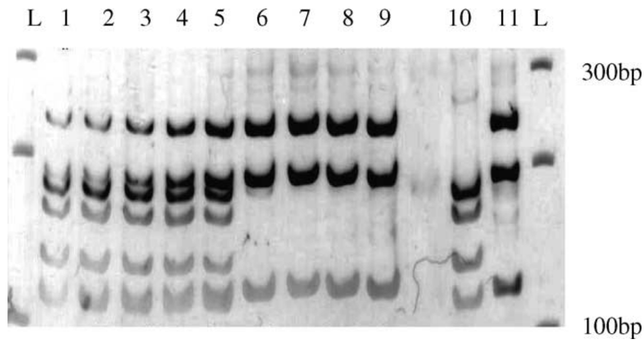

Fig. 4. In vitro mixed infections of *T. vivax* and *T. brucei* mixed in the following ratios: 50% *T. vivax* in lane 1; 75% in lane 2; 85.7% in lane 3; 94% in lane 4; 98% in lane 5; 99.8% in lane 6; 99.98% in lane 7; 99.998% in lane 8; 99.9998% in lane 9; positive control of *T. brucei* in lane 10; positive control of *T. vivax* in lane 11. Lanes L contain a 100 bp DNA ladder.

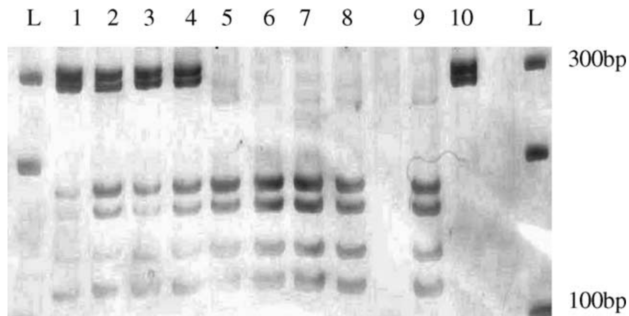

Fig. 5. In vitro mixed infections of *T. theileri* and *T. brucei* mixed in the following ratios: 75% *T. brucei* in lane 1; 85.7% in lane 2; 94% in lane 3; 98% in lane 4; 99.8% in lane 5; 99.98% in lane 6; 99.998% in lane 7; 99.9998% in lane 8; positive control of *T. brucei* in lane 9; positive control of *T. theileri* in lane 10. Lanes L contain a 100 bp DNA ladder.

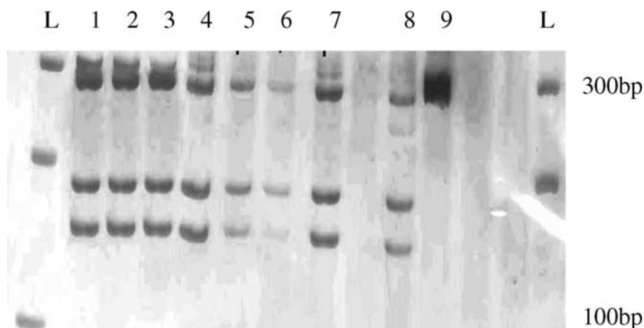

Fig. 6. In vitro mixed infections of *T. theileri* and *T. congolense* mixed in the following ratios: 85.7% *T. congolense* in lane 1; 94% in lane 2; 98% in lane 3; 99.8% in lane 4; 99.98% in lane 5; 99.998% in lane 6; 99.9998% in lane 7; positive control of *T. congolense* in lane 8; positive control of *T. theileri* in lane 9. Lanes L contain a 100 bp DNA ladder.

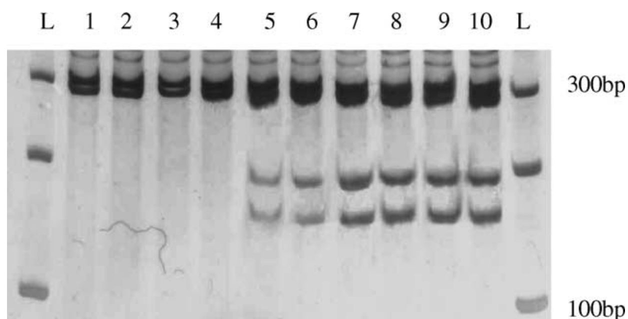

Fig. 7. In vitro mixed infections of *T. theileri* and *T. congolense* mixed in the following ratios: 99.9998% *T. theileri* in lane 1; 99.998% in lane 2; 99.98% in lane 3; 99.8% in lane 4; 98% in lane 5; 94% in lane 6; 85.3% in lane 7; 75% in lane 8; 50% in lane 9; 25% in lane 10. Lanes L contain a 100 bp DNA ladder.

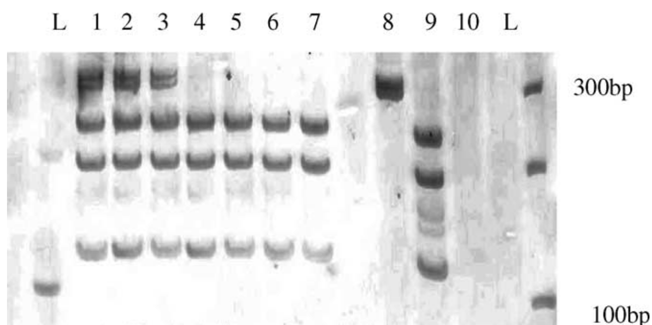

Fig. 8. In vitro mixed infections of *T. theileri* and *T. vivax* mixed in the following ratios: 85.7% *T. vivax* in lane 1; 94% in lane 2; 98% in lane 3; 99.8% in lane 4; 99.98% in lane 5; 99.998% in lane 6; 99.9998% in lane 7; positive control of *T. theileri* in lane 8; positive control of *T. vivax* in lane 9. Lanes L contain a 100 bp DNA ladder.

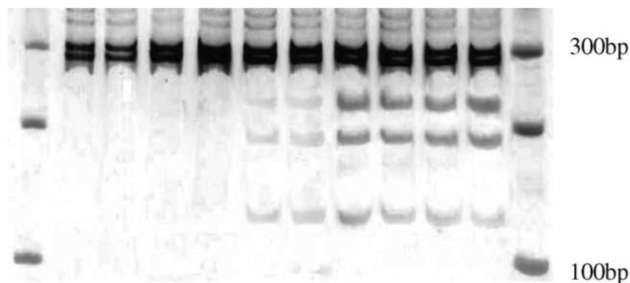

Fig. 9. In vitro mixed infections of *T. theileri* and *T. vivax* mixed in the following ratios: 99.9998% *T. theileri* in lane 1; 99.998% in lane 2; 99.98% in lane 3; 99.8% in lane 4; 98% in lane 5; 94% in lane 6; 85.3% in lane 7; 75% in lane 8; 50% in lane 9; 25% in lane 10. Lanes L contain a 100 bp DNA ladder.

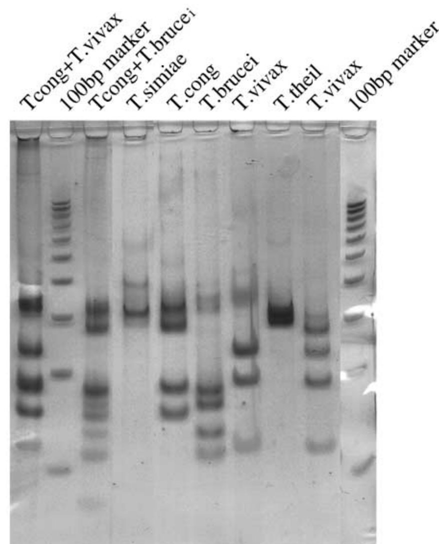

Fig. 10. Single and mixed infections in field samples from cattle sampled in Benin (Abomey-Calavi area).

4. Discussion

The single RFLP-PCR assay to identify all important bovine trypanosome species as described by Geysen et al. (2003), is a potential powerful tool for the analysis of large scale epidemiological surveys samples. The results of this study confirm that the test is equally useful for the detection of in vitro mixed infections and that the preliminary results for in vivo mixed infections are very promising. The restriction profiles of the different trypanosome species are easily recognised, as the profile for each individual species is not overlapping with the profile of the other species except for *T. congolense* and *T. theileri*. In this case, it is difficult to differentiate a single infection with *T. congolense* from a mixed infection *T. congolense* and *T. theileri*. The single restriction band of *T. theileri* (300 bp) is matching with the heaviest restriction band of *T. congolense* (Figs. 6 and 7). This is only a minor inconvenient as *T. theileri* is not pathogenic for cattle and is not affecting PCV or productivity, which would require therapeutic intervention.

For the mixed infections including *T.b. brucei*, this species was intentionally not tested at a higher ratio than 50% because if considering its normal tissular localisation, *T.b. brucei* is not expected to be predominant in normal conditions.

The perfect reproducibility of the detection limit in the different PCR mixtures is to be related to the standardisation of the DNA samples and seems to indicate that there is no difference in affinity among the primers for the different species.

5. Conclusions

The profiles of different mixed trypanosome infections in vitro and in vivo are easily recognisable and confirm that the single PCR-RFLP technique is a useful tool for the diagnosis of natural mixed infections in the field except in case of a *T. theileri* and *T. congolense* mixture. The detection limit of this test on blood on filter papers in the case of single infections is one trypanosome in 40 μl. The assay is highly specific as no cross reaction was observed with *Theileria parva* or *Babesia bigemina*, two other hemoparasites frequently observed in cattle (Geysen et al., 2003). The sensitivity of mixed infections should be further determined by inoculation in cattle or in a mice model where applicable with mixed parasite populations to complete this qualitative study with quantitative results.

Acknowledgements

This research was financed by the Belgian General Directorate for International Co-operation (DGIC). Special thanks to the technical staff of the Veterinary Department of ITM, Antwerp, in particular Rita Beudeker and Jacob De Witte. We also thank Ph. Büscher (ITM, Antwerp) for providing us the various trypanosome DNAs.

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