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New molecular marker for *Trypanosoma (Duttonella) vivax* identification

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Abstract

Trypanosoma vivax is a widespread hemoparasite in tropical areas and is pathogenic to ruminant domestic livestock as well as wild ruminants. The accurate identification of parasites in both hosts and vectors is crucial for epidemiological studies and disease control programs. We describe here the development of molecular markers specific for *T. vivax* identification. These markers were used to identify mouthpart infections in field-collected tsetse flies from Cameroon. The markers target the genomic sequence of a species-specific antigen from the bloodstream stages. No cross amplification with other trypanosome species was observed, which makes the markers a reliable tool to detect *T. vivax* infections, both in hosts and vectors. The PCR-amplified sequence contains a $(CA)_n$ microsatellite repeat for which 11 different alleles were identified. This microsatellite, which showed high polymorphism, provides a suitable marker for population genetic studies. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: *Trypanosoma vivax*; PCR identification; Microsatellite; Polymorphism

1. Introduction

Trypanosoma (Duttonella) vivax Ziemann, 1905 is a protozoan parasite widespread in sub-Saharan Africa, and in central and south America. In Africa, the parasite is predominantly transmitted cyclically by tsetse flies, but other bloodfeeding insects such as horse-flies and stable-flies are capable of mechanical transmission (Hoare, 1972). In America, only mechanical transmission by tabanids or *Stomoxys* occurs.

This salivarian trypanosome is economically important because the disease it causes in ruminant livestock significantly affects growth and survival (Betancourt and Wells, 1979; FAO, 1994). The American isolates induce a disease generally less severe than those from Africa (Gardiner, 1989). Individual isolates of *T. vivax* show different levels of pathogenicity, infectivity for laboratory rodents and adaptation for in vitro cultivation (Fairbairn, 1953; Murray, 1982). De-

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spite these distinct characteristics, few phenotypic and karyotypic differences have been revealed by isoenzyme analysis or DNA fingerprinting (Fasogbon et al., 1990; Dirie et al., 1993a,b).

Diagnosis of *T. vivax* infections has historically been based on parasitological techniques (Woo, 1970; Murray et al., 1977). However, these methods lack adequate sensitivity and specificity as parasitaemia levels in chronically infected animals are often very low and species identification in vectors relies on parasite localization to different insect organs (Lloyd and Johnson, 1924). Although antibody detection ELISA has been developed (Luckins, 1977) this technique cannot differentiate current from past infections. Recently, trypanosome identification in hosts and vectors has been based on a PCR technique (Masiga et al., 1992, 1996; McNamara et al., 1995; Solano et al., 1995; Masake et al., 1997; Morlais et al., 1998a), and several primer sets originating from the same probe have been described for *T. vivax* characterization (Dickin and Gibson, 1989; Masiga et al., 1992, 1996). In Tanzania, those markers have been suitable for trypanosome identification in proboscis from field collected tsetse flies (Lehane et al., 2000). However, PCR assays, using the same markers, often fail with mouthparts identified microscopically as positive (Solano et al., 1995, 1997; Masiga et al., 1996; Reifenberg, 1996; Le Francois et al., 1998; Morlais et al., 1998b) and it has been shown that the DNA sequences amplified by these primers are not present in some *T. vivax* isolates (Masake et al., 1997).

In this study, we describe a new PCR assay for *T. vivax* specific identification, and we investigate its sensitivity on field collected tsetse flies with mouthpart infections. The amplification product is a fragment of the gene encoding a *T. vivax*-specific antigen (Masake et al., 1994). The antigen is recognized by the monoclonal antibody Tv27 employed in an Ag-ELISA (Nantulya et al., 1992). The sequence is present in *T. vivax* isolates from different origins (Masake et al., 1997) and the amplification product is specific for *T. vivax* identification. The nucleotide sequence contains a microsatellite motif that reflects considerable polymorphism within *T. vivax* populations.

2. Materials and methods

Tsetse flies were collected in Cameroon in previously described study areas (Morlais et al., 1998a). Fly organs (proboscis, midguts and salivary glands) found infected by microscopical examination were individually preserved in 70% ethanol in order to identify the trypanosome species responsible for the infection. As *T. vivax* epimastigote development takes place within tsetse fly mouthparts, only flies with mouthpart infections were retained for this study.

In the laboratory, samples were air-dried and homogenized in 1% lauryl-sarcosine/1% Triton X-100 buffer containing proteinase K (200 μg/ml), followed by standard phenol extraction and ethanol precipitation (Maniatis et al., 1982). Pellets were then resuspended in 60 μl of sterile water. Primers used to amplify target DNA were: TBR 1, 2 for *T. brucei* s.l. (Masiga et al., 1992), TCF 1, 2 for forest type *T. congolense* (Masiga et al., 1992), TCN 1, 2 for savannah type *T. congolense* (Moser et al., 1989), TCK 1, 2 for kilifi type *T. congolense* (Masiga et al., 1992), IL 1, 2 for *T. simiae* (Majiwa et al., 1994), DDG 1, 2 for *T. godfreyi* (Masiga et al., 1996) and TVW 1, 2 for *T. vivax* (Masiga et al., 1992).

Additional oligonucleotide primers for *T. vivax* identification were designed based on the nucleotide sequence of a gene encoding an antigen recognized by the monoclonal antibody (Tv27) employed in the *T. vivax* Ag-ELISA (Nantulya et al., 1992). Primer sequences are detailed in Table 2. The primers TVMF, TVMR allow the amplification of a 399 bp product. The cDNA sequence also contains an $(CA)_{11}$ microsatellite repeat motif for which a 3′ primer (TVMRb) flanking the repeat motif was designed.

Standard PCR was performed in a 25 μl reaction mixture containing 10 mM Tris–HCl, pH 9, 50 mM KCl, 3 mM $MgCl_2$, 200 μM of each of the four deoxynucleoside triphosphate (dNTPs), 15 pmoles of oligonucleotide primers and 0.25 U of *Taq* (*Thermus aquaticus*) DNA polymerase (Appligene-Oncor, USA). About 5 μl of the individual DNA samples were used as template in each reaction. Positive (1.0 ng of reference DNA) and negative (without DNA) controls were in-

cluded in each set of experiments. The reaction mixtures were placed in a programmable heating block (DNA thermal cycler, Perkin Elmer, USA). Tubes were incubated at 94 °C for 3 min in an initial denaturation step, followed by 30 cycles of 1 min at 94 °C, 1 min at 60 °C and 1 min at 72 °C and a final extension for 15 min at 72 °C. A 15 μl aliquot of each sample was analyzed on 2% agarose gels, stained with ethidium bromide and photographed under UV illumination.

With the microsatellite primers (TWM F/Rb) the PCR reactions were processed as described above except that the amplification cycles were increased to 35. Five μl of PCR products were first analyzed on 3% agarose gels in order to check the reaction. Then 2 μl of the amplified samples were resolved on 10% nondenaturing polyacrylamide gels, stained with ethidium bromide and photographed under UV illumination.

3. Results and discussion

Specificity of the TVM primers for *T. vivax* was tested individually against 2 ng aliquots of DNA from *T. brucei*, forest type *T. congolense*, savannah type *T. congolense*, Kilifi type *T. congolense*, *T. simiae* or *T. godfreyi*. None of these nontarget DNAs produced any amplification product. The TVM primers, therefore, appear specific to *T. vivax* species. Since the sequence encoding the Tv27 antigen has been reported in several *T. vivax* isolates from diverse areas of Africa and South America (Masake et al., 1997), the TVM primers are suitable for *T. vivax* diagnosis in hosts and vectors.

A total of 888 non-teneral field collected flies were dissected and examined microscopically for the presence of trypanosomes (see Morlais et al., 1998a). Among them, 110 (12.4%) were infected in one or more of the examined organs (proboscis, midguts and salivary glands). Of the infected flies, 50.9% (56/110) had mouthpart infections. In the present study, 36 flies with mouthpart infection were evaluated for trypanosome identification. PCR results are shown in Table 1.

The TVM primer set identified *T. vivax* infections with 31 of the 36 mouthpart infections (86%), while the TVW primers mis-classified 16 (44%) of these infections as negative. This result demonstrates that the DNA sequence targeted by the TVW primer set is not conserved in all *T. vivax* isolates and could explain the frequently reported failure to identify mouthpart infections (Solano et al., 1995, 1997; Masiga et al., 1996; Reifenberg, 1996; Le Francois et al., 1998; Morlais et al., 1998b). A recent study (Lehane et al., 2000) reports successful identification of mouthpart infections (87%) in Tanzania using the same TVW primers and we could assume that in this area the circulating *T. vivax* isolates carry the targeted DNA. Conversely, all samples identified as *T. vivax* positive with the TVW primer set were also positive with the new diagnostic primers (TVM). By targeting a species-specific antigen, the TVM primer set showed better sensitivity and seems to provide a reliable marker for *T. vivax* identification. In addition forest type *T. congolense* and *T. simiae* were identified in, respectively, three and one fly samples, reflecting a mixed-infection rate of 12.9% (4/31).

Of interest, five samples did not yield an amplification product with any of the primer sets tested. We can envision three possibilities to explain these results: (1) that trypanosome DNA was degraded; (2) because of low parasitaemia, DNA concentrations were insufficient to produce a visible PCR product on EtBr-stained agarose gels; or (3) different strains of parasites whose DNA is not amplified by the primers we used exist in our studied areas. In fact, for each trypanosome species or subspecies, primer nucleotide sequences have been determined from a distinct isolate and genetic differences between isolates exist, according to geographical location particularly (Fasogbon et al., 1990; Dirie et al., 1993a,b).

Microsatellite markers have been shown to be very useful molecular tools to measure genetic variation and gene flow in natural populations (Bruford and Wayne, 1993; Jarne and Lagoda, 1996). However, microsatellite markers have been developed for a limited number of arthropod disease vectors including *Anopheles* spp. (Lanzaro et al., 1995; Rongnoparut et al., 1996; Zheng et al., 1996; Kamau et al., 1998), *Glossina palpalis gambiensis* (Solano et al., 1997) and *Simulium*

damnosum (Dumas et al., 1998). Since the gene targeted by the TWM primers also contains a $(CA)_{11}$ microsatellite sequence, we investigated the utility of the repeat as a polymorphic marker.

To facilitate fragment analysis on nondenaturing polyacrylamide gels, we designed a second 3′-end specific primer (TVMRb) that allowed the amplification of a smaller DNA fragment. As shown in Fig. 1, a total of 11 distinct alleles are evident among the 31 *T. vivax* samples tested. Allele sizes ranged from 203 to 233 bp, reflecting a variation of 15 dinucleotide motifs. Multiple amplification products were observed in some samples and are likely due to either heterozygosity among individual parasites or to multiple infections. Indeed, no cross reaction between try-

Table 1
Results of parasitological examination and PCR identification in tsetse flies

Fly number	Dissection results		PCR results							
	Hypoph	Labrum	TBR	TCF	TCN	TCK	IL	DDG	TVW	TVM
3293	+	+	–	–	–	–	+	–	+	+
3389	+	+	–	–	–	–	–	–	–	+
5345	+	–	–	–	–	–	–	–	–	–
5353	+	–	–	–	–	–	–	–	–	+
7261	+	+	–	–	–	–	–	–	–	+
7417	+	+	–	–	–	–	–	–	–	–
7425	+	+	–	–	–	–	–	–	–	+
8789	+	–	–	–	–	–	–	–	–	+
8793	+	–	–	–	–	–	–	–	–	+
8821	+	–	–	–	–	–	–	–	–	–
8825	+	–	–	–	–	–	–	–	–	+
8933	+	–	–	–	–	–	–	–	–	+
8937	+	–	–	–	–	–	–	–	–	+
8973	+	–	–	–	–	–	–	–	–	+
9001	+	–	–	–	–	–	–	–	–	–
9021	+	–	–	–	–	–	–	–	–	+
9041	+	–	–	–	–	–	–	–	–	+
9045	+	–	–	–	–	–	–	–	–	+
9049	+	–	–	–	–	–	–	–	–	+
9065	+	–	–	–	–	–	–	–	–	–
9073	+	–	–	–	–	–	–	–	–	+
9081	+	–	–	–	–	–	–	–	–	+
9757	+	–	–	–	–	–	–	–	+	+
9869	–	+	–	–	–	–	–	–	+	+
9885	+	+	–	–	–	–	–	–	+	+
11 597	+	+	–	–	–	–	–	–	+	+
11 861	+	+	–	–	–	–	–	–	+	+
11 921	+	+	–	–	–	–	–	–	+	+
12 657	+	+	–	–	–	–	–	–	+	+
12 665	+	+	–	+	–	–	–	–	+	+
13 221	+	+	–	–	–	–	–	–	+	+
13 461	+	+	–	–	–	–	–	–	+	+
13 505	+	+	–	–	–	–	–	–	+	+
14 537	+	+	–	+	–	–	–	–	+	+
14 645	+	+	–	+	–	–	–	–	+	+
14 869	–	+	–	–	–	–	–	–	+	+

hypoph, hypopharynx; primer sets for: *T. brucei*, TBR; Forest type *T. congolense*, TCF; Savannah type *T. congolense*, TCN; Kilifi type *T. congolense*, TCK; *T. simiae*, IL; *T. godfreyi*, DDG; *T. vivax*, TVW and TVM.

Table 2
Oligonucleotide sequences for *T. vivax* identification

Code	Primer sequence	Reference
TVM F	5′-TCGCTACCACAGTCGC AATCGTCGTCTCAAGG-3′	In this study
TVM R	5′-CAGCTCGGCGAAGGCC ACTTGGCTGGGGTG-3′	Masake et al. (1994)
TVM Rb	5′-CCCGGCAGGTTGGCCG CCATC-3′	In this study
TVW 1	5′-CTGAGTGCTCCATGTG CCAC-3′	Masiga et al. (1992)
TVW 2	5′-CCACCAGAACACCAAC CTGA-3′	

panosome species was ever seen and the fly DNA can not have contaminated the reaction as no amplification was ever observed with the TVM primers on proboscis that were *T. vivax* negative, even on those that were also positive with another trypanosome marker, reflecting the DNA extraction efficiency.

Whereas isoenzyme patterns have shown limited variation between East and West african *T. vivax* isolates (Fasogbon et al., 1990), the microsatellite locus is highly polymorphic and should be useful for population studies.

In summary, we have identified nucleotide primers that are diagnostic for *T. vivax*-specific identification on field collected tsetse flies. Our results indicate that the PCR assay is specific and reliable for *T. vivax* characterization in trypanosomiasis endemic areas. Additionally, the target gene sequence contains a microsatellite that reflects considerable polymorphism among isolates, and, therefore, represents a useful tool for population genetic studies.

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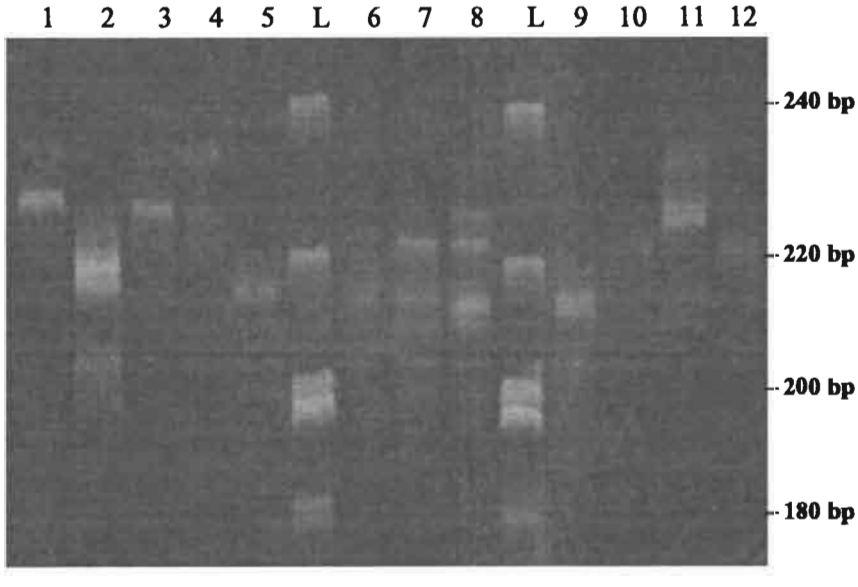

Fig. 1. Nondenaturing polyacrylamide gel showing microsatellite polymorphism. Lanes:1–12, PCR products corresponding to 12 infected flies; L, 20-bp ladder.

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