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# SENSITIVE DETECTION OF TRYPANOSOMES IN TSETSE FLIES BY DNA AMPLIFICATION

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**Abstract**—MASIGA D. K., SMYTH A. J., HAYES P., BROMIDGE T. J. and GIBSON W.C. 1992. Sensitive detection of trypanosomes in tsetse flies by DNA amplification. *International Journal for Parasitology* **22**: 909–918. African trypanosome species were identified using the Polymerase Chain Reaction (PCR) by targeting repetitive DNA for amplification. Using oligonucleotide primers designed to anneal specifically to the satellite DNA monomer of each species/subgroup, we were able to accurately identify *Trypanosoma simiae*, three subgroups of *T.congolense*, *T.brucei* and *T.vivax*. The assay was sensitive and specific, detecting one trypanosome unequivocally and showing no reaction with non-target trypanosome DNA or a huge excess of host DNA. The assay was used to identify developmental stage trypanosomes in the tsetse fly. The use of radioisotopes was not necessary and mixed infections could be detected easily by incorporating more than one set of primers in a single reaction. The use of crude preparations of template made the process very rapid. The methodology should be suitable for large-scale epidemiological studies.

INDEX KEY WORDS: Polymerase Chain Reaction; *Trypanosoma congolense*; *Trypanosoma brucei*; *Trypanosoma vivax*; *Trypanosoma simiae*; satellite DNA.

## INTRODUCTION

THE accurate identification of different trypanosome species, subspecies and strains is a fundamental problem in studies of the epidemiology of trypanosomiasis in Africa. Indeed the risk to livestock and man can only be fully appreciated if pathogenic trypanosomes are identified unequivocally. Yet species with different pathogenicities are difficult to distinguish by morphology of bloodstream forms or developmental stages in the tsetse fly vector. Until recently identification of the latter relied on location in the fly, which is only accurate to subgenus level. This method also fails to detect mixed infections, e.g. of *Nannomonas* and *Duttonella*, or to identify immature infections where trypanosomes are found only in the midgut.

The development of species-specific DNA probes to identify trypanosomes (Gibson, Dukes & Gashumba, 1988; Kukla, Majiwa, Young, Moloo & Ole Moi-Yoi, 1987) went some way to improving the accuracy of identification. Based on repeated sequences, it offered both sensitivity and specificity. However, the limit of detection is about 100 trypanosomes by dot blot hybridization, so the technique is only useful for the identification of trypanosomes in the fly midgut where they are present in large numbers. Proboscis infections are seldom heavy enough for reliable dot blot identification, which is unfortunate, since these are mature infections representing actual challenge to livestock.

It is not desirable to resort to the outgrowth of parasites in culture or laboratory animals before characterization, because (a) some isolates do not grow or grow poorly (Hoare, 1972; Dukes, McNamara & Godfrey, 1991), and (b) this may incur selection, the results obtained not accurately reflecting the original population of parasites (Sturm, Degrave, Morel & Simpson, 1989).

The Polymerase Chain Reaction (PCR) is a technique used for DNA amplification *in vitro* (Mullis & Faloona, 1987; Saiki, Gelfand, Stoffel, Scharf, Higuchi, Horn, Mullis & Erlich, 1987; Saiki, Scharf, Faloona, Mullis, Horn, Erlich & Arnheim, 1985) and has already been applied to the identification of *T.congolense* and *T.brucei* in rodent blood (Moser, Cook, Ochs, Bailey, McKane & Donelson, 1989). We

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TABLE 1—PRIMER SEQUENCES AND PREDICTED AMPLIFICATION PRODUCT SIZES

| Trypanosome species or subgroup | | Primer sequence* | Amplification product (bp) |
|---|---|---|---|
| *T.simiae* | TSM1: | CCGGTCAAAAACGCATT | |
| | TSM2: | AGTCGCCCGGAGTCGAT | 437 |
| *T.congolense* | TCF1: | GGACACGCCAGAAGGTACTT | |
| forest | TCF2: | GTTCTCGCACCAAATCCAAC | 350† |
| *T.congolense* | TCS1: | CGAGAACGGGCACTTTGCGA | |
| savannah | TCS2: | GGACAAACAAATCCCGCACA | 316 |
| *T.congolense* | TCK1: | GTGCCCAAATTTGAAGTGAT | |
| Kenya Coast | TCK2: | ACTCAAAATCGTGCACCTCG | 294 |
| *T.brucei* | TBR1: | GAATATTAAACAATGCGCAG | |
| | TBR2: | CCATTTATTAGCTTTGTTGC | 164 |
| *T.vivax* | TVW1: | CTGAGTGCTCCATGTGCCAC | |
| West Africa | TVW2: | CCACCAGAACACCAACCTGA | 150† |

*Primer sequences are shown in the 5′–3′ orientation. TCS1, TCS2, TBR1 and TBR2 are five bases shorter than primers used by Moser *et al.* (1989).

†Sizes approximate since the complete sequences are unavailable (see text).

describe here methodology using PCR to identify small numbers of trypanosomes in tsetse by amplifying the satellite DNA.

## MATERIALS AND METHODS

*Tsetse and trypanosomes. Glossina morsitans morsitans*, strain FX9, maintained in the tsetse fly colony at the Tsetse Research laboratory (TRL), Bristol, was used routinely. The trypanosomes used are listed below and are maintained in the WHO cryobank at TRL: *T.congolense* savannah WG 81 (MCAP/KE/81/WG 81), 1/148 fly (MBOI/NG/60/1-148[FLY]), TSW 13 (MSUS/LR/75/TSW 13); *T.congolense* Kenya Coast WG 84 (MOVS/KE/81/WG 84), WG 5 (MCAP/KE/80/WG 5); *T.congolense* forest TSW 103 (MSUS/LR/77/TSW 103-A); *T.b.brucei* 427 (MOVS/UG/60/S 427), *T.b.rhodesiense* EATRO 2340 (MHOM/KE/77/EATRO 2340); *T.vivax* Y58 (MBOI/NG/72/Y58), Y486 (MBOI/NG/73/Y486), NIMR 17 (MOVS/NG/50/Desowitz); *T.Simiae* BAN 7 (GMOS/GM/88/BAN 7) and KEN 2 (GMOS/GM/88/KEN 2).

*Infection and dissection of tsetse.* Teneral flies were infected by feeding on horse blood containing procyclic or bloodstream form trypanosomes via a silicone membrane, except for *T.vivax* where flies were fed on anaesthetized infected mice. Mixed infections were established by first infecting flies with *T.congolense* via a membrane and then with *T.vivax* after starving for 2 days. The flies were thereafter maintained at 28°C by membrane-feeding on horse blood. Flies infected with *T.vivax* were dissected after 2 weeks and those infected with *T.brucei*, *T.congolense* or a mixture of *T.congolense* and *T.vivax* after 4 weeks. The proboscis or salivary glands were removed from each fly before removing the gut and care taken to clean dissecting instruments in between by immersing in detergent (3–5% sodium hypochlorite) for about 2 min followed by a brief rinse in water and in normal saline (0.9% NaCl). Dissected organs were examined by microscopy before preparation of samples for PCR.

*Preparation of crude DNA templates.* Individual tsetse organs were put in microcentrifuge tubes containing PCR template preparation buffer (10 mM-Tris–Cl, pH 8.3, 50 mM-KCl, 1.5 mM-$MgCl_2$ and 0.9% Nonindet P40), 30 $\mu$l for proboscides or salivary glands and 100 $\mu$l for midguts. These preparations were either used fresh or stored at −20°C. Proteinase K (2 $\mu$g) was added to each tube and the mixture incubated at 55°C for 1 h, followed by 10 min at 95°C to denature the proteinase K. Midgut preparations were further processed by standard phenol extraction and alcohol precipitation procedures (Maniatis, Fritsch & Sambrook, 1982). Trypanosomes grown in culture were prepared in the same way, washing once in PBS (pH 7.2), before resuspending in template preparation buffer at a concentration of $10^6$ trypanosomes per 170 $\mu$l buffer.

*Primers.* Oligonucleotide primers were synthesized using a DNA synthesizer (Gene Assembler Plus, Pharmacia-LKB Biotechnology). The DNA base sequences of the primers are shown in Table 1 and their locations on satellite repeat monomers and direction of extension in Fig. 1. Primers for *T.congolense* savannah and *T.brucei* were based on Moser *et al.* (1989) while the others were chosen using a primer design computer programme.

*PCR cycling.* Standard PCR amplifications were carried out in 50-$\mu$l reaction mixtures containing as final concentrations, 10 mM-Tris–Cl, pH 8.3, 50 mM-KCl, 1.5 mM-$MgCl_2$, 200 $\mu$M each of the four deoxynucleoside triphosphates (dNTPs), primers at 1 $\mu$M, DNA template and 2.5 units of Taq DNA polymerase (Amplitaq, Perkin-Elmer Cetus). DNA templates consisted of 1–200 ng purified DNA or plasmid clones of satellite DNA. For crude template preparations, 0.1 vol of template prepared from tsetse organs or 1 $\mu$l template from culture-grown or bloodstream trypanosomes was used. Primer concentrations were varied between 1 and 10 $\mu$M, but standard reactions carried out using primers at 1 $\mu$M (final) gave good results. The reaction mixtures were overlaid with about 40 $\mu$l paraffin oil to prevent evaporation and cycled in a programmable heating block as follows: samples were

*T.simiae* Ban 7

GATCGACTCC GGGCGACTGT TCTACCTCGA CGTCCGGCTG ACAGGGGGTA CCCCGTACAC
GGCCATTTTG GCCCCTGACC CCCCCCAGGC CGGTCGGCCG GACACCGGTC AAAAACGCAT
TTTGGCGCAA AAAAAAAATT TTTTACTGAT TGACGCGCGC TGCATATCCA GCCAGTAAAT
TCGATTTTCA GCCAATTTGG ACACCGCTAC GTGCCGGACC GACCGCTTGG CCACCACGGA
GTTGACGGGC CGGGGCCAAA AATCCAAAAT CGTAAATCGA CCCCACCAAT TCTGAGCGCA
TTGCTCATAG AGATGGTGAG TTCGCGCTTT AATCGAAATC CGTTCGAAAA ACGCTTGTGT
CTAGCGCCGT AACGGGCTGT ACCGCACCTT GGCCGAAATG GGCCAAATGG CCGTTTTCAG
CCTTAAGGCC GGTTTGCCCG TTACAGACTA TGCCGAGAGC TCTTCCAAAA CGGATTTAAA
ATGCGCAATG CCACGGTCCG CCGCATGGAG AAACGCTGTC T [521]

*T.congolense* savannah EATRO 325

AAATTTCGAG CGAGAACGGG CACTTTGCGA TTTTCCCAAA ATTCACCTTT TTGGGCCCAA
ATGGGCAAAA ACCGGTTTTT TTGAAAATGG TCAAAAATGT CAAAAACGCA AAAATTCGAA
AAACGCGTAT TTGGCACGTA TTTGTCGTTT TCGGGCCTAT TTGACGCGCA TAGTGATTTT
TCAAAATTTT GCAAAAAATT GTGTCAAAAA CTTTTTCTAA TTTTTGCAAA TTTTCAAAAA
AAAATTTGTA AAAAAATATT TTTTTTTGAC TTTTTGGGCG AAAATTTTTT CTGTTCCAAA
AATGGTTGTG CGGGATTTGT TTGTCCCTAA TTTTCATCGA AAACGCCGAA ATGCGTTTTA
AAAATGATC [369]

*T.vivax* Y486

CCTTCTTCAG GTTGGTGTTC TGGTGGCCTG TTGCCCCCCA CCCGCTCCCA GACCATATGG
TCCTGAGTGC TCCATGTGCC ACGTTGGCAC GCTCCACTGT CTAGCGTGAC GCGATGGCCC
GTGCACTGTC CCGCACCCCT TCCCCACTCC CTCTTGCACC TCTCGCTCCG GCCA [174]

*T.congolense* Kenya Coast WG 84

CTAGAAGAAC GCAAAAATAT TTTTAAAAGA TGCATTTTAA ACCCCCGAGG TGCACGATTT
TGAGTTTCCT GTGCGTTTTT GACCATCACA CAATTTTTGA CAAAAATGTC AATTTTTGTA
ATTTCGGGGC CCCCCAAAAG TGCCCAAATT TGAAGTGATT TTTCGTCAAA AAAATGAAAA
AACTAAAATG GCACAAAATG AACGTGGCAA ACTCACGGGT TGAGTAATTT GATGTATTGG
TGCATTTTTT TTATTTTTGT GTTTTTTGCA TATTTTTGTC AAAATCGAAT AATGCGTTTT
TTGGGCCCAA AAGTGCCAAA AACGCAAAAA CACTAAAAAA CACAAAAAAA TAAAATGCTC
GTTCTCAG [368]

*T.congolense* forest TSW 103

AAAAAATGAA TAGGCGGGTC AAAATGGCCT CTTTGAGACG TTTAAAATGC TTTTTGAAAT
TTGAAATTTG GTTGGATTTG GTGCGAGAAC GGTCATTTTC CGTTTTTGTT GGACACGCCA
GAAGGTACTT TGGGCGCCAA AATGGCCCAA AACGCCTTTT TTTGAAAATG GTCAAAAACG
TGAAAAACGC CAAAATTCGG AAAATGCGTG TTTTTACAAA TTGGTCGTTT TTGGGCGTTT
TTGACCCGGC ATAGTGGTTT TTTGAAATTT TTCAAAAAAA AAGGTCAAAA TTTTTTTCAA
ATTTTTGTGT AAAAAACAAC TTTTTTTTCG ACTTTTTGGG GAAAATTTTT TCG [353]

*T.brucei* EATRO 427

CGAATGAATA TTAAACAATG CGCAGTTAAC GCTATTATAC ACAATAACTT TTAATGTGTG
CAATATTAAT TACAAGTGTG CAACATTAAA TACAAGTGTG TAACATTAAT TTGCAAGTTT
GCAACGCTGT TCTTTAGTGT TTAATGTGTG CAACAAAGCT AATAAATGGT TCTTATA [177]

FIG. 1A. DNA sequences of repeats targeted for amplification in the Polymerase Chain Reaction. Each sequence shows one repeat unit except those of *T.congolense* forest and *T.vivax* which are incomplete (see text). Primers used for PCR are shown in bold type with arrowheads indicating direction of extension (see Table 1 for primer sequences). *Trypanosoma congolense* savannah sequence is from Gibson *et al.* (1987), *T.brucei* sequence from Sloof *et al.* (1983) and *T.vivax* sequence from Dickin & Gibson (1989). TCS1, TCS2, TBR1 and TBR2 are five bases shorter than primers used by Moser *et al.* (1989).

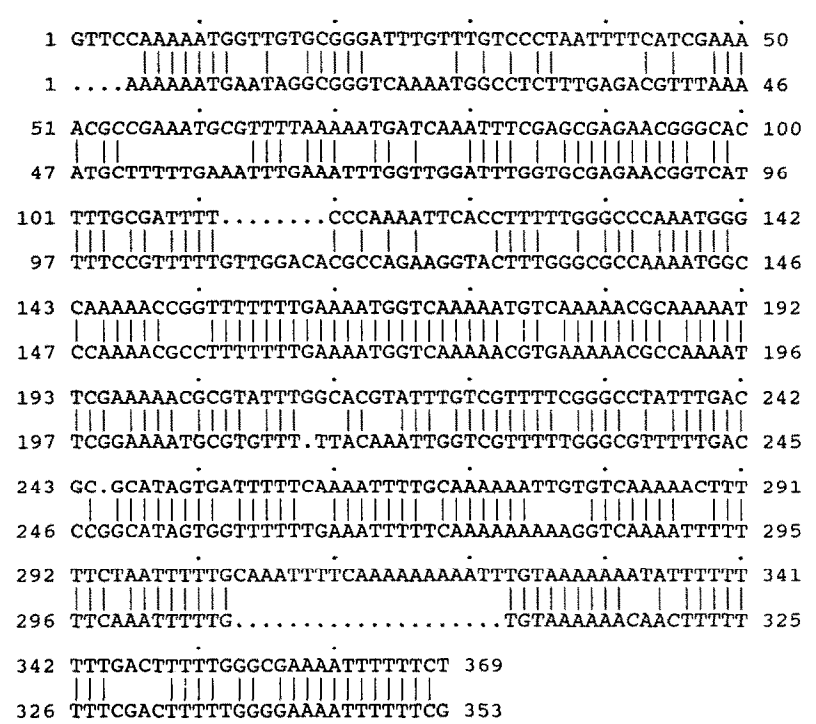



FIG. 1B. Sequence alignment of repeats from *T.congolense* savannah and *T.congolense* forest using GAP option in software (Devereux *et al.*, 1984). Dots denote spaces created to maximize homology. The *T.congolense* savannah sequence shown here starts from base position 293 in Fig. 1A.

incubated at 94°C for 3 min in an initial denaturation step and subjected to 30 cycles involving denaturation for 1 min at 94°C, annealing at 60°C for 2 min and extension at 74°C for 30 s. Fifteen microlitres of each sample was then electrophoresed through 1.5% or 2% agarose containing 0.5 $\mu$g $ml^{-1}$ ethidium bromide.

*Cloning and sequencing satellite DNA.* The major satellite DNA monomers from *T.congolense* Kenya Coast WG 84, *T.congolense* forest TSW 103 and *T. simiae* Ban 7 were cloned as described by Gibson *et al.* (1988) and sequenced by the chain-termination method (Sanger, Nicklen & Coulson, 1977) using Sequenase T7 DNA polymerase enzyme according to the manufacturer's instructions (United States Biochemicals). Percentage similarities of satellite DNA monomer sequences were calculated using the GAP option in the software package detailed by Devereux, Haeberli & Smithies (1984). The comparison is based on the largest number of nucleotides of one sequence that can be matched with those of a second sequence, allowing for interruptions in either sequence.

*Blotting and hybridization.* DNA was transferred onto nitrocellulose paper essentially by the method of Southern (1975). Blots were hybridized as described by Gibson *et al.* (1988) with the following species-specific probes labelled with $^{32}P$ (Feinberg & Vogelstein, 1983): 1. pNgSAT-sav, cloned satellite DNA monomer from *T.congolense* savannah; 2. pNgSAT-KC16, cloned satellite DNA monomer from *T.congolense* Kenya Coast; 3. pNgSAT-sim 160, cloned 160 bp CfoI fragment from the *T.simiae* satellite DNA monomer (Gibson *et al.*, 1988). Blots were washed stringently to 0.1 × SSC (0.15 M NaCl, 0.015 M Na citrate, pH 7), 0.1% SDS (sodium dodecyl sulphate) at 65°C.

## RESULTS

### *DNA sequences of repeats*

Figure 1 shows the DNA sequence of the satellite DNA monomers from three subgroups of *T.congolense*, *T.brucei*, *T.simiae* and *T.vivax*. Two of the sequences are not quite complete (*T.congolense* forest and *T.vivax*) because the satellite DNA monomers were cloned using S1 nuclease to remove terminal nucleotides (Gibson *et al.*, 1988; Dickin & Gibson, 1989). The three *T.congolense* monomers are of similar

TABLE 2—PERCENTAGE SIMILARITY (MAXIMUM HOMOLOGY) OF SATELLITE DNA SEQUENCES

| | TC-S | TC-F | TC-K | TSM | TV-W | TBR |
|---|---|---|---|---|---|---|
| TC-S | | 71 | 48 | 44 | 37 | 44 |
| TC-F | | | 47 | 41 | 41 | 42 |
| TC-K | | | | 37 | 38 | 41 |
| TSM | | | | | 44 | 36 |
| TV-W | | | | | | 34 |
| TBR | | | | | | |

TC-S, *T.congolense* savannah; TC-F, *T.congolense* forest; TC-K, *T.congolense* Kenya Coast; TSM, *T.simiae*; TV-W, West African *T.vivax*; TBR, *T.brucei*.

size (about 370 bp), are 65% AT with many A and T homopolymeric stretches. The other member of subgenus *Nannomonas*, *T. simiae*, has a much larger monomer, 521 bp in size (Ban 7 used here; KETRI 2431, Majiwa & Otieno, 1990). The sequence derived from the Gambian isolate Ban 7 is 55% GC and shares 84% homology with that of KETRI 2431, a stock from Kenya. The *T.brucei* and *T.vivax* monomers are both comparatively small at about 180 bp, but that of *T.brucei* is 71% AT (Sloof, Bos, Konings, Mehnke, Borst, Gutteridge & Leon, 1983) while that of *T.vivax* is 65% GC (Dickin & Gibson, 1989).

Table 2 shows the percentage similarity of satellite DNA monomer sequences. Similarity is on average about 40–45%, except for *T.congolense* savannah and *T.congolense* forest, although admittedly sequences of similar size are more realistically aligned. The *T.congolense* savannah and forest sequences, compared in Fig. 1B, are 71% similar and contain long blocks of homology. In contrast, comparing *T.congolense* Kenya Coast with either savannah or forest sequences produces an alignment that simply involves the lining up of homopolymeric stretches of bases, mostly A and T, with no runs greater than seven consecutive bases (not shown).

*Specificity and sensitivity of PCR*

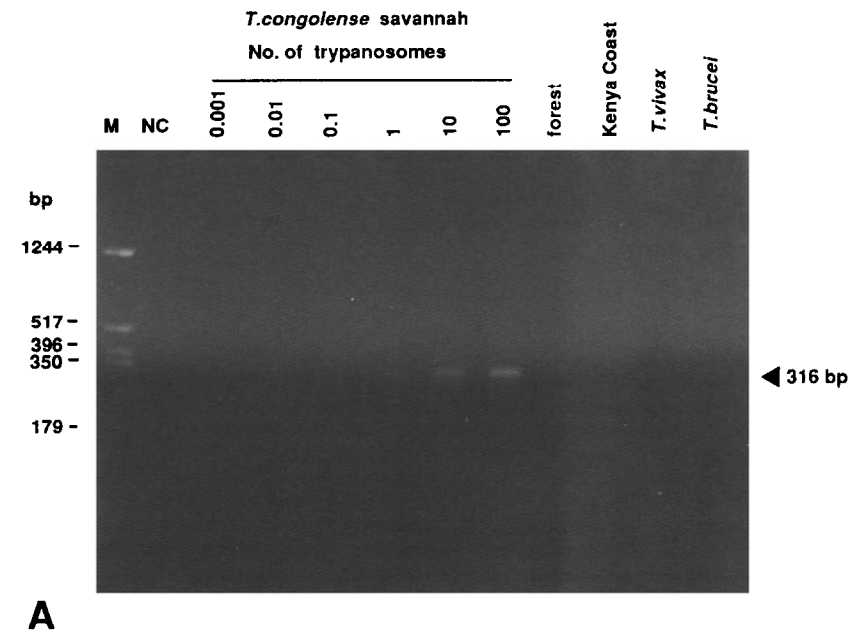
A set of primers was chosen for each repeat sequence (see Table 1 and Fig. 1). Primers were selected on the basis of: (a) length of predicted product to allow for the identification of different species in a single reaction; (b) near parity in melting temperature ($T_m$) and GC:AT ratio (c) where possible, the absence of more than four runs of any single base and, in particular, runs of G or C at the 3′-end; and (d) absence of stable secondary structures between primers. Additionally, primers for *T. simiae* were chosen in regions of homology between the sequences from East and West African stocks (KETRI 2431, Majiwa & Otieno, 1990; and Ban 7, respectively) to allow for the detection of *T.simiae* stocks of wide geographical occurrence.

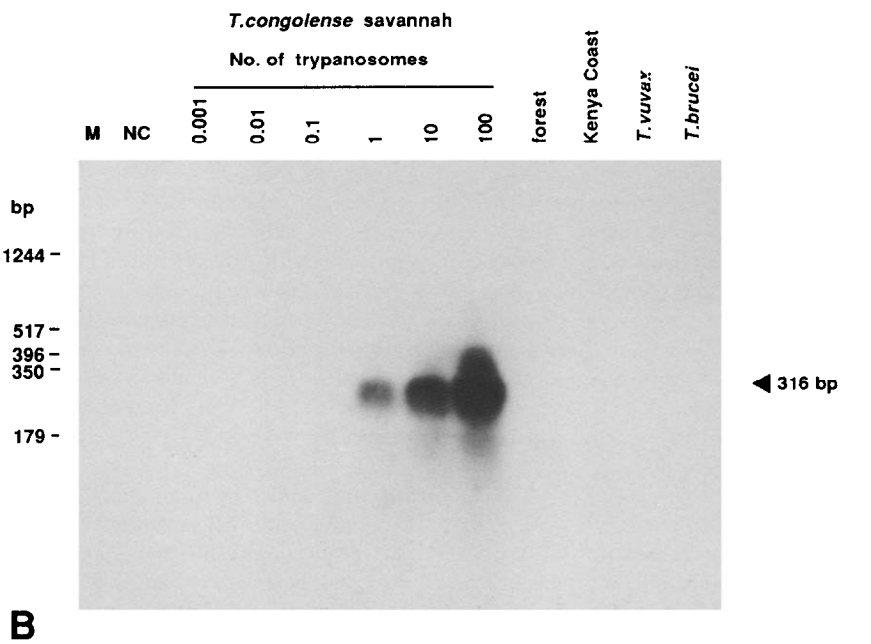
To test whether DNA amplification from the chosen primers was specific, each primer pair was tested against self DNA and DNAs from a range of trypanosome stocks. The sensitivity of each primer set was tested against a dilution series of purified DNA or of a lysate prepared from whole trypanosomes. Figure 2A shows results using specific primers for *T.congolense* savannah. The product from the amplification of one trypanosome of stock WG 81 is clearly visible on the gel, but no amplification is seen in lanes with (excess) template from other trypanosome species. Hybridization with the cloned satellite DNA monomer from *T.congolense* savannah did not enhance the detection level beyond one trypanosome (Fig. 2B). However, cutting the same template preparation with *Sau3AI* before PCR enabled detection down to 0.01 trypanosome by hybridization (Fig. 2C). Similarly, for other trypanosome species and subgroups, one trypanosome was detectable by simple gel electrophoresis of amplified DNA and the signal could not be enhanced by Southern blotting and hybridization (not shown).

*Identification of trypanosomes*

Figure 3 shows the results of the PCR identification of trypanosomes in the three tsetse organs tested: midguts, proboscides and salivary glands. Amplification products of expected size for *T.congolense* are clearly visible in the midguts and proboscides while *T.brucei* was easily identified in infected salivary glands. Oligomers were also amplified in some reactions, e.g. Figs. 3A, C and 4.

Amplification of crude preparations of template from proboscides and salivary glands was possible without further purification, while PCR from midguts prepared in the same way was inhibited (Fig. 3A, lane 1). This inhibition was easily overcome by phenol extraction and alcohol precipitation of crude midgut


T.congolense savannah
No. of trypanosomes
M
NC
0.001
0.01
0.1
1
10
100
forest
Kenya Coast
T.vivax
T.brucei
bp
1244
517
396
350
179
316 bp
A


T.congolense savannah
No. of trypanosomes
M
NC
0.001
0.01
0.1
1
10
100
forest
Kenya Coast
T.vuvax
T.brucei
bp
1244
517
396
350
179
316 bp
B

FIGS. 2A, B.

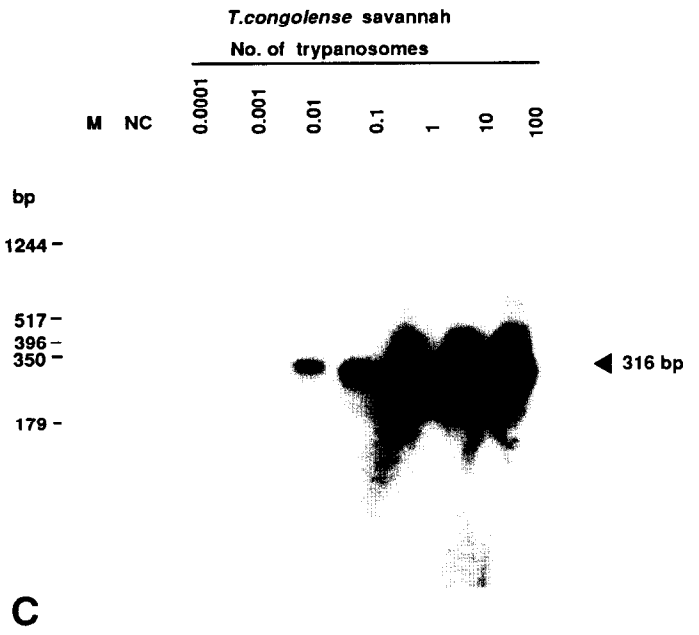



FIG. 2C.

FIG. 2. Sensitivity and specificity of PCR methodology. (A) Ethidium bromide stained 1.5% agarose gel showing WG 81 trypanosomes amplified using TCS1 and TCS2 primers specific for *T.congolense* savannah. The dilution series was made from a crude lysate of a known number of trypanosomes. 316 bp amplification product is clearly visible for one trypanosome. Templates from other trypanosome species (10 trypanosomes per reaction) show no amplification. M, marker, *Hinf*I digest of pGEM-3 (Promega-Biotech); NC, no template control. (B) Southern blot of gel shown in A hybridized with cloned repeat of *T.congolense* WG 81 and washed to 0.01 × SSC / 0.1% SDS at 65°C. The 316-bp fragment is the *T.congolense* savannah specific product of amplification. (C) Southern blot hybridized as for B carrying a dilution series of a *Sau3AI* digested crude preparation of *T.congolense* savannah WG 81 trypanosomes. Predigestion of the DNA in this way enhances sensitivity of the PCR test by at least two orders of magnitude.

preparations (Fig. 3A, lane 2). Similar methodology was used successfully to prepare templates for trypanosome identification from tsetse proboscides and midguts infected with *T.vivax* and *T.congolense* savannah, respectively, which had been air-dried and stored at room temperature for 5 months.

*Detection of mixed infections*

In order to assess the prevalence of mixed trypanosome infections in tsetse in the field, the methodology should reliably identify mixed infections. In the case of PCR, more than one primer set can be included in a single reaction (Chamberlain, Gibbs, Ranier, Nguyen & Caskey, 1988) giving the added advantage of reduced cost.

We assessed the ability of a combined PCR test to identify mixed infections of *T.congolense* and *T.vivax*, a combination likely to occur in the field and difficult to resolve by dissection or dot-blotting. Both trypanosome species infect the proboscis, and *T.congolense* additionally the midgut. Of 45 flies dissected and examined by microscopy 4 weeks after infection with both species, 10 (22%) had trypanosomes in both the midgut and proboscis, while 14 (31%) had only the proboscis infected.

The proboscides from all 24 infected flies were subjected to PCR analysis in the presence of the combined primer sets for *T.congolense* savannah and *T.vivax*. The 14 samples from flies with only an infected proboscis tested positive for *T.vivax* alone as expected. Of the 10 proboscides with an accompanying midgut infection, four had *T.congolense* only and six had both infections. Some of these results are shown in Fig. 4. The 316-bp and 150-bp amplification monomers are well resolved on the gel while the *T.vivax* dimer can also be seen as a larger 330-bp faint

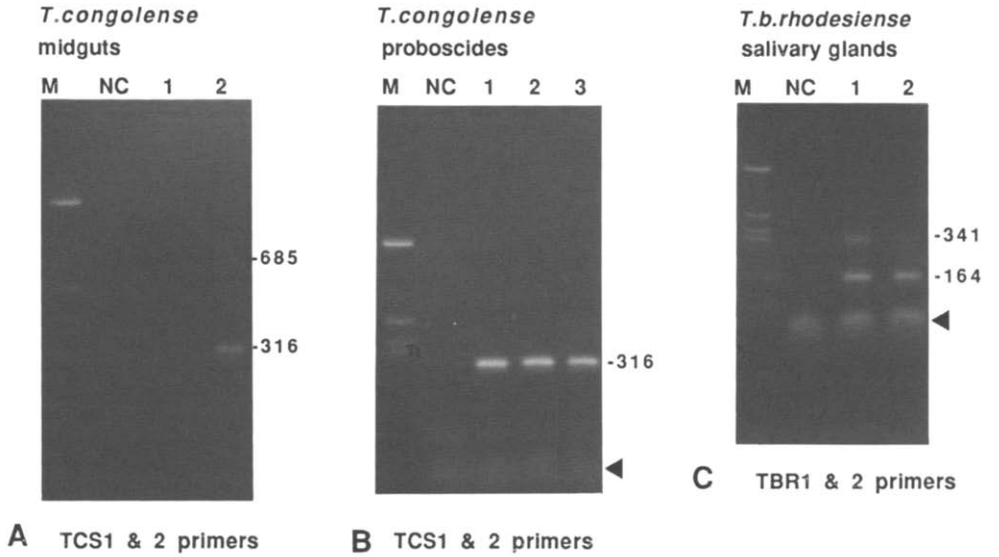



FIG. 3. PCR identification of trypanosomes in tsetse organs. A and B show amplification products from midguts and proboscides infected with *T.congolense* savannah WG 81 and TSW 13, respectively, C from salivary glands of flies infected with *T.b.rhodesiense* EATRO 2340. Products were electrophoresed through 1.5% agarose gels. Templates were as follows: A. 1. midgut crude preparation, 2. midgut crude preparation after phenol extraction and alcohol precipitation. B. 1–3. single proboscides, crude preparations. C. 1,2. salivary glands crude preparations. *Trypanosma congolense* lanes show 316-bp and *T.brucei* lanes 164-bp bands expected for monomer; bands representing amplification dimers can additionally be seen in some lanes. Arrowhead indicates free primer in B and C. Primer concentrations were 1 $\mu$M for A and B, 10 $\mu$M for C. NC, no template control; M, pGEM-3 × *Hinf*I marker (see Fig. 2 for band sizes).

band in some lanes. The two trypanosome species are clearly distinguishable, allowing the easy identification of mixed infections.

## DISCUSSION

We have shown that trypanosomes can be identified by amplification of satellite DNA sequences using the Polymerase Chain Reaction in a simple and rapid reaction. The technique is sensitive—one trypanosome can be identified unequivocally without resort to radioactivity. This is at least two orders of magnitude better than dot blot hybridization using $^{32}$P-labelled probes (Gibson *et al.*, 1988). The ability to identify trypanosomes in a sensitive test without resort to radioactivity makes the technique feasible in areas where radioactive labelling reagents are difficult to obtain or use, such as most areas of endemic trypanosomiasis in Africa.

The reaction worked well with simply prepared crude preparations of infected tsetse proboscides and salivary glands, while infected tsetse midguts required further purification prior to PCR. This is not a drawback, since we envisage that the technique will be most valuable for identifying trypanosomes in the proboscis. Here, mixed infections of *T.vivax* and *T.congolense* in tsetse proboscides were detected simply by incorporating more than one primer set in the reaction. This could be particularly useful for field studies, where identification of three or four trypanosome species might be required. Chamberlain *et al.* (1988) successfully used five primer pairs in a multiplex amplification. Here, not all combinations of amplified products are easily resolved by electrophoresis and so it may be necessary to positively identify PCR products by hybridization or subsequent use of a single primer set.

Hybridization with radiolabelled probes did not significantly enhance the level of detection, which was at best 0.1 trypanosome. This is surprising considering the abundance of satellite DNA repeats, which constitute the bulk of the mini-chromosomes of Salivarian trypanosomes (Gibson *et al.*, 1988). In our titrations we used crude preparations of whole trypanosomes, which presumably contained largely intact mini-chromosomes. The level of detection could be enhanced by subjecting the crude preparation to restriction enzyme digestion, thereby effectively increasing the primer–template molecular ratio. For practical purposes, however, a sensitivity of one trypanosome is sufficient, since only the presence of

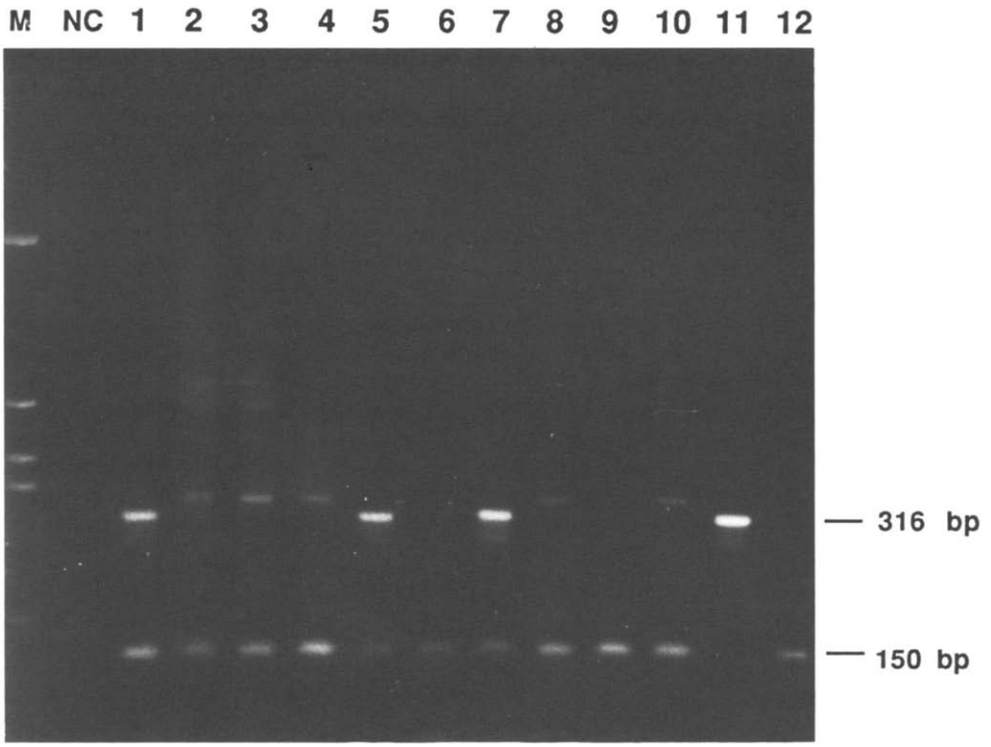



FIG. 4. Ethidium bromide-stained 2% agarose gel showing PCR amplification of template from proboscides of flies infected with *T.congolense* savannah TSW 13 and/or *T.vivax* Y58 using both primer sets. Flies 2, 3, 4, 6, 8, 9, 10 and 12 had proboscis infections only and these lanes show the 150-bp product resulting from specific amplification from *T.vivax* primers. Flies 1, 5, 7 and 11 had both midgut and proboscis infections. Lane 11 shows only the 316-bp band resulting from specific amplification from *T.congolense* primers, while lanes 1, 5 and 7 show both 316-bp and 150-bp products, i.e. these flies were infected with both *T.congolense* and *T.vivax*. The faint 330-bp band represents the *T.vivax* amplification dimer. Primer concentrations were 1 $\mu$M for *T.congolense* and 5 $\mu$M for *T.vivax*. M, pGEM-3 X *Hinf*I marker (see Fig. 2 for band sizes); NC, no template control.

whole organisms represents an infection in the tsetse fly.

In some reactions, amplification oligomers were formed in sufficient quantity to be detected by electrophoresis, despite the presence of a huge excess of primer molecules in relation to template binding sites. This implies that primer molecules did not bind to all sites on the template for some reason. Low frequency of collisions between primer and template is unlikely to be the cause, since oligomers were more prominent at higher template concentrations with the same primer:template ratio. The loss of some primer annealing targets due to point mutations would lead to oligomer formation and also reduced sensitivity of the PCR test. Scattered point mutations were found in two clones of the *T.simiae* repeat sequenced here, and also between three different versions of the *T.congolense* savannah monomer sequence (Gibson *et al.*, 1987; Moser *et al.*, 1989; Majiwa & Otieno, 1990). We also noticed considerable divergence between *T.simiae* sequences from East and West Africa. Although cloned repeats from East and West Africa cross-hybridize (Gibson *et al.*, 1988), there is only 84% homology between the two sequences, with few extensive blocks. Indeed initial PCR primers chosen from the East African sequence (Majiwa & Otieno, 1990) failed to work on West African material. The primer pair used here was chosen in regions of homology between the two sequences and should work on both East and West African *T.simiae*, although we were only able to try the latter. Similar geographical divergence may also be present in the *T.brucei* complex (Masiga & Gibson, unpublished) and *T.vivax* as observed by isoenzyme characterization (Fasogbon,

Knowles & Gardiner, 1990) which will obviously complicate choice of primers.

The comparison of DNA sequences of *T.congolense* forest and *T.congolense* savannah satellite repeats revealed a high level of similarity, echoing that found by isoenzyme electrophoresis, where a number of different isoenzyme bands are shared (Young and Godfrey, 1983). This contrasts with the distinctness of *T.congolense* Kenya Coast for which no isoenzyme bands are shared (Gashumba, Baker & Godfrey, 1988) and the sequence of the DNA repeat is at maximum only 48% similar to those of the savannah and forest sub-groups. *Trypanosoma congolense* Kenya Coast in fact appears to be as distantly related to the other sub-groups of *T.congolense* as *T.simiae*. Our results thus provide a further clue to the interrelationships of trypanosomes within sub-genus *Nannomonas*.

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