

Detection and Identification of Human Pathogenic *Leishmania* and *Trypanosoma* Species by Hybridization of PCR-Amplified Mini-exon Repeats

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RAMOS, A., MASLOV, D. A., FERNANDES, O., CAMPBELL, D. A., AND SIMPSON, L. 1995. Detection and identification of human pathogenic *Leishmania* and *Trypanosoma* species by hybridization of PCR-amplified mini-exon repeats. *Experimental Parasitology* 82, 242-250. A single pair of PCR primers within a conserved region of the mini-exon repeat was used to amplify the repeats from 10 species of pathogenic *Leishmania* belonging to four major clinical groups and also from three species of *Trypanosoma*. Oligonucleotide hybridization probes for the detection and identification of the PCR-amplified repeats were constructed from alignments of mini-exon intron and intergenic sequences. The probes generated from mini-exon intergenic regions of the *L. (V.) braziliensis*, *L. (L.) donovani*, and *L. (L.) mexicana* species hybridized specifically to their cognate groups without discriminating between the species within the groups. The probes for *L. (L.) major* and *L. (L.) aethiopica* were species-specific, while the *L. (L.) tropica* probe also hybridized with the *L. (L.) aethiopica* mini-exon repeat. The mini-exon intron-derived probes for *T. cruzi*, *T. rangeli*, and *T. brucei* were species-specific. This method involving the detection of specific PCR-amplified products produced using a single primer set represents a novel sensitive and specific assay for multiple trypanosomatid species and groups. © 1996 Academic Press, Inc.

INDEX DESCRIPTORS AND ABBREVIATIONS: *Leishmania*; *Trypanosoma*; oligonucleotide probe; Polymerase chain reaction (PCR); mini-exon gene repeat.

INTRODUCTION

Pathogenic *Leishmania* and *Trypanosoma* species are the causal agents of several important human and animal diseases, such as dermal and visceral leishmaniasis, Chagas' disease, and African sleeping sickness. Rapid detection and identification of pathogenic trypanosomatids in patients, animal reservoirs, or insect vectors represent an important problem. PCR amplification of specific parasite DNA sequences has the advantage over serological assays of providing direct evidence for the presence of parasites in the host and also, in view of the inherent

sequence polymorphisms of the parasite genome, of allowing a classification of the species or possibly even the strain of the parasite.

Several methods utilizing PCR amplification of parasite nuclear DNA or kinetoplast minicircle DNA target sequences have been reported for *Trypanosoma cruzi* (Gonzalez *et al.* 1984; Sturm *et al.* 1989; Britto *et al.* 1995; Wincker *et al.* 1994; Avila *et al.* 1993; Laskay *et al.* 1991) and pathogenic *Leishmania* species (Wirth and Pratt 1982; Lopes *et al.* 1984; Lopes and Wirth 1986; Wirth *et al.* 1986; Rodgers *et al.* 1990; Howard *et al.* 1991; Uliana *et al.* 1994). A common feature of these methods is the use of specific primer sets and reactions for each particular parasite or species. The use of multiple PCR amplifications substantially increases the cost and difficulty of analysis. Multiplex PCR, a method in which a mixture of primer sets is used, with each specific for the DNA from a particular organism, should theo-

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retically overcome this problem and allow the detection of multiple etiological agents in the same patient, animal, or insect with a single assay. However, attempts in our laboratory to coamplify several sequences (*T. cruzi* minicircle DNA, hepatitis B DNA, cytomegalovirus DNA, HIV DNA) led to a substantial loss of sensitivity and specificity, apparently as a result of interactions of heterologous primers and templates (L. Simpson, D.A. Maslov, and A. Ramos, data not shown).

The mini-exon genes of the trypanosomatid protozoa represent a good target for a PCR-based multiple detection and identification assay, which can overcome the inherent problems of multiplex PCR. Mini-exon-derived RNA is involved in *trans*-splicing of mRNA, a process restricted to the kinetoplastid protozoa and some worms (De Lange *et al.* 1983, 1986; Agabian 1990; Murphy *et al.* 1986; Sutton and Boothroyd 1986; Campbell *et al.* 1984; Nilsen 1993). Mini-exon genes are absent from the vertebrate hosts and invertebrate vectors, but are present as multiple tandem repeats in the parasite genomes. Each repeat contains a highly conserved 39-nt exon sequence, a moderately variable 55- to 101-nt transcribed intron sequence, and a highly variable 250- to 1350-nt nontranscribed intergenic region. The length and nucleotide sequence of the mini-exon repeat was shown to vary between different groups of pathogenic *Leishmania* species (Hassan *et al.* 1993; Fernandes *et al.* 1994) and between the trypanosomes *T. cruzi* and *T. rangeli* (Murthy *et al.* 1992). In this paper we show that specific and sensitive detection of mini-exon repeats PCR-amplified from pathogenic *Leishmania* and *Trypanosoma* species using the conserved exon sequence can be accomplished using specific capture oligomers derived from the polymorphic intron and intergenic regions.

MATERIALS AND METHODS

Strains of parasites and growth conditions. The strains of *Leishmania* and *Trypanosoma* and their sources are as follows: *L. (L.) amazonensis* PH8 (Ralph Lainson, Instituto Evandro Chagas, Belem, Brazil), JOS (R. McMaster, University of British Columbia, Canada), M2269 (G. Grimaldi, FIOCRUZ, Brazil); *L. (L.) mexicana* M379 (D. McMahon-

Pratt, Yale University, U.S.A.), BEL21 (G. Grimaldi); *L. (V.) braziliensis* M2903 (D. McMahon-Pratt), LT300 (R. McMaster); *L. (L.) chagasi* BA3 (R. McMaster); *L. (V.) panamensis* WR120 (D. McMahon-Pratt); *L. (V.) guyanensis* M4147 (G. Grimaldi); *L. (L.) aethiops* L100 (G. Grimaldi); *L. (L.) major* LT252 (S. Beverley, Harvard University, U.S.A.), NIH-S (R. McMaster), A2 (R. McMaster); *L. (L.) tropica* OD (G. Grimaldi); *L. (L.) donovani* DD8 (G. Grimaldi), MRC-74 (D. McMahon-Pratt); *L. (L.) infantum* ITMAP263 (K. Stuart, Seattle Biomedical Research Institute, U.S.A.), IPTI (G. Grimaldi); *T. (T.) brucei* 427 (Campbell *et al.* 1984); *T. (S.) cruzi* Sylvio (Dvorak *et al.* 1980, 1982), MT01, MT03, MT05-26 (J. Coura, FIOCRUZ, Brazil); *T. (H.) rangeli* Ev26 (N. Saravia, CIDEIM, Colombia) (Holguin *et al.* 1987), MT02 and MT04 (J. Coura).

Leishmania promastigotes were grown at 26°C in HOMEM (Berens *et al.* 1976) medium supplemented with 10% heat-inactivated fetal bovine serum (Gemini Bio-Products, Calabasas, CA). *T. brucei* procyclics were grown in SDM-79 medium (Brun and Schoenenberger 1979). Epimastigotes of *T. cruzi* and *T. rangeli* were grown in LIT medium (Castellani *et al.* 1967) supplemented with 10 µg/ml hemin and 10% serum.

Isolation of DNA. Cells from 10-ml stationary phase cultures were pelleted at 2000g for 10 min and washed with an equal volume of SET (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 100 mM EDTA). The final pellet was suspended in 0.5 ml SET and lysed with 2% Sarcosyl and 0.5 mg/ml pronase at 65°C for 30 min. The lysate was phenol-chloroform extracted and the DNA was precipitated with an equal volume of isopropanol.

Oligonucleotides. The oligonucleotides shown in Fig. 1 were used in this study.

PCR amplification. Reaction mixtures contained 20 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, 25 mM KCl, 0.05% Tween-20, 0.1 mg/ml bovine serum albumin, 10% dimethylsulfoxide (for *Leishmania* species only), 250 µM of each dNTP, 20 µM of each amplification primer and 2.5 U of *Taq* DNA polymerase. Conditions included initial denaturation at 95°C for 5 min followed by 5 cycles at 95°C for 1 min, 45°C for 30 sec, 65°C for 1 min and 35 cycles at 95°C for 1 min, 50°C for 30 sec, 72°C for 1 min, and a final extension at 72°C for 10 min.

Blotting and hybridization. Aliquots (4–10%) of the amplified material were separated on 2% agarose gels and blotted onto a nylon filter (Micron Separations). Oligonucleotides were labeled by [γ -³²P]ATP and T4 polynucleotide kinase to specific activities of 10⁸–10⁹ cpm/µg. Hybridizations in Figs. 2 and 4 were performed at 37°C in 6× SSC, 1× Denhard's solution, 0.1% SDS, and 100 µg/ml of sonicated salmon sperm DNA in rotating bottles. Filters were washed briefly in 3× SSC (1× SSC contains 150 mM NaCl and 15 mM sodium citrate) at room temperature followed by washes in 3 M tetramethylammonium chloride (Fisher), 50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 0.5% SDS at 55°C. High-stringency washes were performed in 2× SSC, 0.1% SDS at 65°C. Hybridizations in Fig. 3 were

1. Overlapping PCR primers from highly conserved region of exon.

S-1629: GGAATCAATA (A/T) AGTACAGAACTG

S-1630: GGAAGCTTCTGTACT (A/T) TATTGGTA

2. *Viannia* group (*L. brasiliensis*, *L. guyanensis*, *L. panamensis*)

Intergenic region

Lb1 AGCGGCACCCCTCACAGCGACCTGGGCA

Lb2 .A.....

Lg .A.....

Lp .A.....

Ln .AT.....

S-1593: A (A/G) (C/T) GGCACCCCTCACA (G/A) CGACCTGGGCA

3. Viscerotropic group (*L. chagasi*, *L. donovani*, *L. infantum*)

Intergenic region

Ld1 CGGCCATGGTGGTGACGCGGGCCCGTGC

Ld2

Ld3

LiA.....

Lc

S-1698: CGGCCATGGTGGTGAC (G/A) CGCGGGCCCGTGC

4. New World dermatropic group (*L. amazonensis*, *L. mexicana*)

Intergenic region

Laz1 GGGCGCCGGCAGCCGTGACACGTGGCCCGG

Laz2

Lmx1A.....T.....

Lmx2T.....

S-1595: GGGCG (C/A) CGGC (A/G) GCCGTGAC (A/G) CGTGG (C/T) CCGG

5. Old World dermatropic group (*L. aethiopica*)

Intergenic region

S-1932: GCGTGC GCGGAGAACATCCA

6. Old World dermatropic group (*L. major*)

Intergenic region

S-1933: GCGTGC GCGGGAACGGCCA

7. Old World dermatropic group (*L. tropica*)

Intergenic region

S-1981: GCGTGC GCGGAGAATCCATCAA

8. *T. eruzi*

Intron region

S-2005: TATGTTGGTCAATTTCTTTGACCGG

9. *T. brucei*

Intron region

S-1607: GTAGCAGGCCAACACACGCATTGTGCT

10. *T. rangeli*

Intron region

S-1598: GATGCGTCCGAACGGTCGTGTCTGGTA

FIG. 1. Oligonucleotides used for amplification and hybridization of mini-exon repeats. The alignments used for the construction of primer sequences are shown. Dots indicate matches with the sequence of one repeat. Degenerate nucleotides in the primers are indicated by (X/X). Lb, *L. brasiliensis*; Lg, *L. guyanensis*; Lp, *L. panamensis*; Ld, *L. donovani*; Li, *L. infantum*; Lc, *L. chagasi*; Laz, *L. amazonensis*; Lmx, *L. mexicana*; Lae, *L. aethiopica*; Ltrop, *L. tropica*; Lmj, *L. major*.

performed at 37°C overnight in BLOTTO (Johnson *et al.* 1984). The filter was washed for 1 hr at 65°C in 0.5× SSC/0.1% SDS.

RESULTS

Amplification of Mini-exon Repeats

Previous work showed that a single pair of overlapping oligonucleotides from the conserved exon region of the mini-exon repeat (S-1629 and S-1630 in Fig. 1) could be used to amplify mini-exon repeats from a broad range of kinetoplastid protozoa (Fernandes *et al.*

1994; Murthy *et al.* 1992). We found in this work that the optimal amplification of mini-exon repeats from various pathogenic *Leishmania* species required the addition of 10% dimethylsulfoxide to the reaction mixture. This concentration of DMSO, however, adversely affected the mini-exon amplification from several *Trypanosoma* species. Therefore, two separate PCR reactions were performed to obtain the panel of PCR products shown in Fig. 2.

The size of the amplified mini-exon repeat was consistent for each group of species and, in

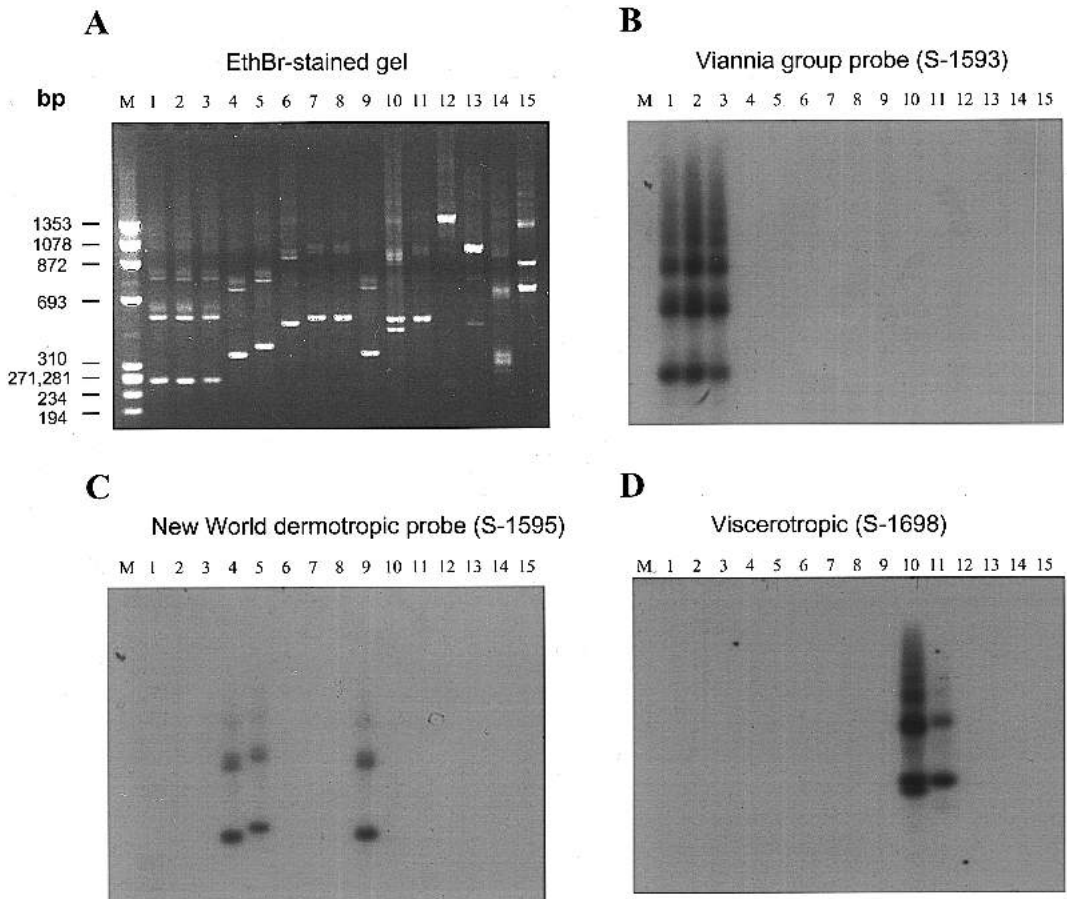


FIG. 2. Hybridization of PCR-amplified mini-exon repeats from a panel of trypanosomatids with group-specific and species-specific oligonucleotide probes. (A) Ethidium-stained. Lane M, ϕ X174/*Hae*III size standard; 1, *L. brasiliensis* M2903; 2, *L. panamensis* WR120; 3, *L. guyanensis* M4147; 4, *L. amazonensis* PH8; 5, *L. mexicana* M379; 6, *L. aethiopica* L100; 7, *L. major* LT252; 8, *L. tropica* OD; 9, unknown; 10, *L. donovani* MRC-74; 11, *L. infantum* ITMAP263; 12, *T. brucei* 427; 13, *T. rangeli* Ev26; 14, *T. cruzi* Sylvio; 15, *Trypanoplasma horreli* Pg-JH. (B) Hybridized with S-1593. (C) Hybridized with S-1595. (D) Hybridized with S-1698.

some cases, for each species (Fernandes *et al.* 1994). Three species representing the New World viscerotropic *Leishmania* (subgenus *Viviania*)—*L. brasiliensis*, *L. panamensis*, and *L. guyanensis*—showed a 260-nt monomeric repeat (expected size from Fernandes *et al.* (1994), 253–261 bp). Multimers are due to the genomic tandem arrangement of the repeats. Two representative species of the New World dermatotropic *Leishmania*—*L. mexicana* and *L. amazonensis*—generated products of 320 and 340 nt, respectively (expected size, 316–332 bp). In three Old World dermatotropic species (*L. aethiopica*, *L. major*, and *L. tropica*), the products were 440–460 nt in size (expected size, 436–460 bp), which is similar to the size of the products from Old World viscerotropic species (*L. donovani* and *L. infantum*) (expected size, 458, 411 bp). An unknown species of *Leishmania* yielded a product of 330 nt, which is close to that obtained for a species from the *L. mexicana* group. %All three trypanosome species generated PCR products of the expected size: 1350 nt for *T. brucei*, 860 nt for *T. rangeli*, and 600 nt for *T. cruzi* (expected sizes from Murthy *et al.* (1992) are 1350 for *T. brucei* (Campbell *et al.* 1984), 858 for *T. rangeli*, and 582–609 for *T. cruzi*).

Specificity of the Oligonucleotide Probes

A. *Leishmania* species. The probes for *Leishmania* were designed on the basis of the sequence alignments of Fernandes *et al.* (1994). Since the mini-exon gene introns of all species of *Leishmania* are highly conserved, probes for specific groups were derived from the less conserved intergenic regions. Several gels identical to the one shown in Fig. 2A were blotted and hybridized with labeled oligonucleotide probes. %The moderately degenerate oligonucleotide S-1593 and the less degenerate S-1698 were designed for the New World viscerotropic *Viviania* or the Old World viscerotropic *L. donovani* groups, respectively (Fig. 1). As shown in Figs. 2B and 2D, both probes hybridized only to their cognate groups.

The degenerate oligonucleotide S-1595 (Fig.

1) specifically hybridized as predicted to the New World dermatotropic *L. mexicana* PCR product (Fig. 2C). In addition, this probe hybridized to the product from an unknown *Leishmania* species and this identification was also consistent with the sizes of the amplified mini-exon repeats (Fernandes *et al.* 1994).

Intergenic regions from species belonging to the Old World cutaneous *Leishmania* group are less conserved (Fernandes *et al.* 1994), and hence no group-specific probe could be derived. Instead, we attempted to design species-specific probes for these parasites using variable intergenic sequences (Fig. 1). As shown in Fig. 3, under stringent hybridization conditions, the oligonucleotide S-1933 produced a specific signal with three different isolates of *L. major* (Fig. 3B), and the oligonucleotide S-1932 hybridized only with *L. aethiopica* (Fig. 3C). However, the oligonucleotide S-1981, which was designed as a probe for *L. tropica*, hybridized also to the *L. aethiopica* PCR product (Fig. 3D). A weak hybridization signal was also seen with one isolate of *L. donovani*, and this signal was even stronger under nonstringent wash conditions (data not shown). However, differential identification of *L. tropica* and *L. donovani* can be made with oligonucleotide S-1698 (Fig. 3E).

B. *Trypanosoma* species. Oligonucleotides S-2005, S-1598, and S-1607, which were derived from equivalent regions of the respective mini-exon intron sequences of *T. cruzi*, *T. rangeli*, and *T. brucei* (Fig. 1), hybridized only to PCR products from their cognate species (Figs. 4A, 4B, 4C).

We have found that isolates of *T. cruzi* fall within two major groups with regard to the sequences of the mini-exon repeats obtained by PCR amplification (D. Campbell and O. Fernandes, in preparation). Therefore, the specificity of the S-2005 probe was examined with several *T. cruzi* isolates. The slot-blot experiment shown in Fig. 4D shows that, under nonstringent hybridization conditions, this probe reacted with amplified mini-exon repeats derived from isolates from both major groups of *T. cruzi*, although to a variable extent. The most intensively hybridizing slots (1, 3, and 16) belonged

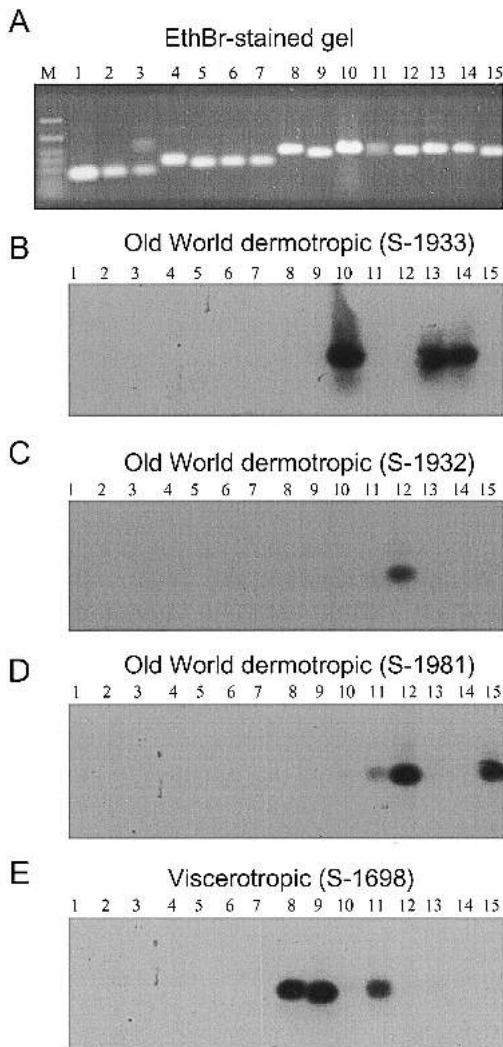


FIG. 3. Hybridization of PCR-amplified mini-exon repeats from a panel of *Leishmania* species with group-specific probes. (A) Ethidium-stained gel. Lane 1, *L. brasiliensis* LTB300; lane 2, *L. brasiliensis* M2903; lane 3, *L. guyanensis* M4147; lane 4, *L. mexicana* BEL21; lane 5, *L. mexicana* M379; lane 6, *L. amazonensis* M2269; lane 7, *L. amazonensis* JOS; lane 8, *L. chagasi* BA3; lane 9, *L. infantum* IPTI; lane 10, *L. major* NIH-S; lane 11, *L. donovani* DD8; lane 12, *L. aethiops* L100; lane 13, *L. major* LT252; lane 14, *L. major* A2; lane 15, *L. tropica* OD. (B) Hybridized with S-1933. (C) Hybridized with S-1932. (D) Hybridized with S-1981. (E) Hybridized with S-1698.

to Group 2 isolates. The *T. cruzi* probe did not hybridize with amplified mini-exon repeats derived from two isolates of *T. rangeli* (slots 2 and

4), nor was there any detectable hybridization of amplified *T. cruzi* mini-exon DNA with the *T. rangeli* probe (data not shown).

DISCUSSION

The mini-exon repeat was PCR-amplified using a single primer set from the conserved exon region from *Leishmania* species representing the four major clinically important groups and also from *T. brucei*, a variety of *T. cruzi* strains, and from the nonpathogenic *T. rangeli*. In *Leishmania* the level of mini-exon intergenic sequence variability is sufficient to identify major species or groups of closely related species, whereas mini-exon intron sequences are nearly identical. Most variability was seen in the group of Old World cutaneous *Leishmania*, for which we have generated individual species-specific probes. In other groups of pathogenic *Leishmania* (*Viannia*, *L. donovani*, *L. mexicana*), sequences of mini-exon intergenic regions are highly similar within the group, but differ between the groups, making it possible to generate group-specific probes. The existence of sequence variability within the intergenic regions provides the potential for the generation of hybridization probes which would discriminate between species, but this has not yet been tested.

In terms of nuclear ribosomal RNA sequences and other genetic markers, the trypanosomes have been shown to exhibit a higher level of genetic distances between species than do the *Leishmania* (Landweber and Gilbert 1994; Maslov *et al.* 1994; Fernandes *et al.* 1993). This is consistent with our finding that variability of the mini-exon intron sequences proved to be sufficient to discriminate *T. cruzi*, *T. rangeli*, and *T. brucei*. In regard to *T. cruzi*, the specific mini-exon intron-derived probe generated was shown to recognize amplified mini-exon DNA derived from both groups of cells which differ in the type of mini-exon repeat. The ability to distinguish *T. cruzi* and the nonpathogenic *T. rangeli* (Guhl *et al.* 1987) in vectors should prove useful in epidemiological studies. Furthermore, as in the case of *Leishmania*, it is likely that sequences from the intergenic re-

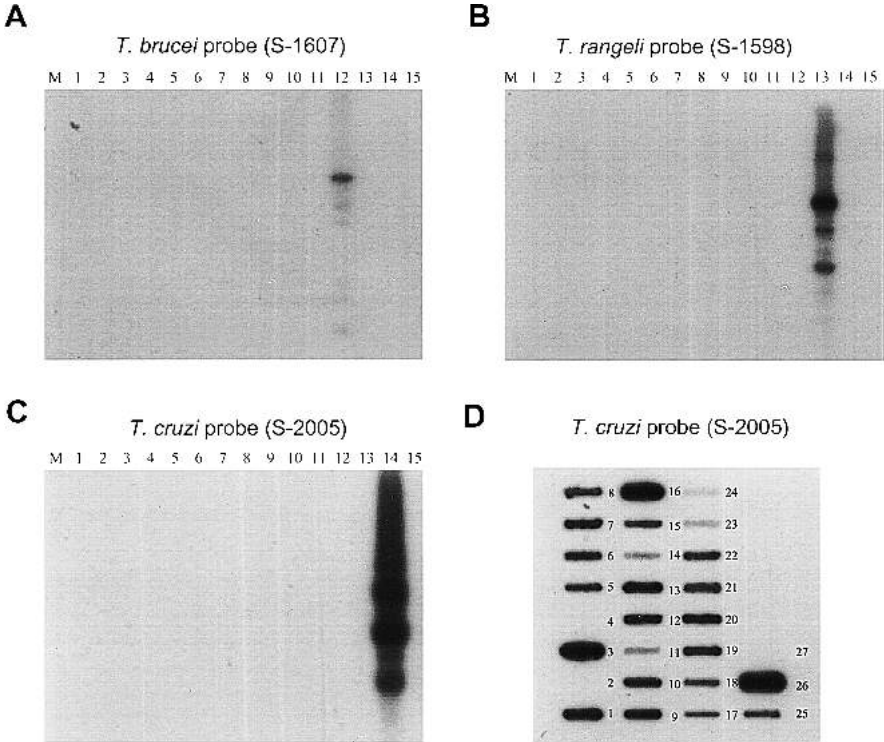


FIG. 4. Hybridization of PCR-amplified mini-exon repeats from a panel of trypanosomatids with species-specific oligonucleotide probes for *Trypanosoma* species. The gels in A–C are identical to that in Fig. 2. See legend to Fig. 2 for identification of lanes. (A) Hybridized with S-1607. (B) Hybridized with S-1598. (C) Hybridized with S-2005; an identical result was obtained using probe S-1597. (D) Slot-blot of amplified mini-exon DNA from strains of *T. cruzi* belonging to the two major groups with different mini-exon sequences and from two strains of *T. rangeli* hybridized with S-2005. Slots 2, 4, *T. rangeli*; slots 3, 16, 26, *T. cruzi* Group 2; slots 1, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 17, 19, 20, 21, 22, 23, 24, 25, *T. cruzi* Group 1; slot 27, negative hybridization control.

gions of *T. cruzi* could be used to identify individual isolates or specific groups, and this is being tested.

These results constitute an initial step toward the development of a PCR-based detection–identification system useful for clinical and epidemiological applications. The next step is to link covalently the capture oligonucleotides specific for each trypanosomatid group or species to microtiter wells and to employ nonradioactive methods for the detection of hybridized PCR-amplified mini-exon DNA (Murthy *et al.* 1992; Galindo *et al.* 1993). This work is in progress.

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