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Diagnosis of Cutaneous Leishmaniasis and Species Discrimination of Parasites by PCR and Hybridization

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The aim of this study was to assess the efficacy of PCR methodology in establishing the diagnosis of cutaneous leishmaniasis in patients from areas of endemicity in Venezuela. Biopsies from 233 patients with cutaneous ulcers suggestive of leishmaniasis were analyzed by PCR, employing oligonucleotides directed against conserved regions of kinetoplast DNA (kDNA), and the PCR products were then hybridized to nonradioactively labeled, species-specific, cloned kDNA fragments. The ability of PCR to detect *Leishmania* cells was compared with those of the conventional methodologies: skin testing with killed promastigotes (Montenegro test), examination of Giemsa-stained biopsy smears, and in vitro culture of biopsy tissue. The PCR-hybridization technique detected the presence of *Leishmania* cells in 98% of patients clinically diagnosed as having leishmaniasis and also positive by the Montenegro skin test. In comparison, leishmania positivity was found in only 42% of cultures and 64% of biopsy smears. By hybridizing the PCR product to new kDNA probes specific for either *Leishmania mexicana* or *Leishmania braziliensis*, we found that both species are major causes of cutaneous leishmaniasis in Venezuela, and the species identification was confirmed by restriction enzyme analysis of kDNA from biopsy cultures. This work demonstrates that PCR coupled with hybridization is useful not only for the diagnosis of cutaneous leishmaniasis but also for the taxonomic discrimination essential for both epidemiology and therapy. This technique can be used to diagnose leishmaniasis in a country in which the disease is endemic and can perhaps be adapted for use in a rural clinic.

Leishmaniasis represents a major public health problem. It is estimated that there are 12 million cases worldwide, and approximately 350 million people are thought to be at risk (10). In Venezuela alone, a mean of 2,000 cases per year was reported during the period 1988 to 1990 (files, Epidemiology Section, Instituto de Biomedicina, Caracas, Venezuela). The etiologic agents, protozoal parasites of the genus *Leishmania*, are transmitted by infected sand flies. About 20 species of *Leishmania* are known to infect humans, causing a spectrum of symptoms ranging from simple self-healing skin ulcers to disseminated cutaneous lesions and life-threatening visceral disease.

We have previously shown (9) that different clinical forms of leishmaniasis can be produced by morphologically similar parasites, and a major problem over the past decade has been to develop the means to identify and differentiate the species of parasites responsible for the various clinical forms of the disease. Because mixed infections, containing other members of the order *Kinetoplastida* (19, 32) or more than one leishmanial species, also occur, precise taxonomic identification of parasites is essential for epidemiologic studies (1, 2, 5, 20).

The diagnosis of leishmaniasis (13) has traditionally been made by direct identification of leishmania amastigotes in tissue samples or smears, coupled with delayed-type hypersensitivity skin testing (25) by using killed, cultured leishmanial promastigotes. The skin test is simple and fairly sensitive but cannot distinguish between active, inactive, new, and past infections. Direct microscopic identification of leishmania amastigotes is rapid and easy to perform but does not distinguish between species, and the sensitivities of direct staining and smears are only 60 to 65% (12).

In Latin America, *Leishmania braziliensis* and *Leishmania mexicana* are the two species complexes that cause cutaneous and mucosal disease. The importance of discriminating between these two complexes for therapeutic decisions was confirmed in a recent report from Guatemala, which showed that the natural history and response to chemotherapy appear to be related to the species of organism found in an ulcer, *L. mexicana* or *L. braziliensis* (16, 28). Each of these species complexes comprises several subspecies (19), whose identification is critical for epidemiology of vectors and reservoirs but probably is not essential in choosing appropriate therapy.

It is clear that better methods are needed that will allow accurate identification of *Leishmania* spp. and also satisfy the following criteria: (i) direct and rapid identification of parasites in vectors, reservoirs, and tissue biopsies, especially biopsies of early or self-healing lesions; (ii) the ability to distinguish different pathogenic species; and (iii) ease of application to countries where the disease is endemic.

Over the past several years, significant progress in developing techniques for epidemiology and diagnosis has been made. One approach has been the development of monoclonal antibodies for the identification of *Leishmania* species and subspecies (24). Another technique has been the use of total mitochondrial DNA (kinetoplast DNA) (kDNA) or cloned fragments of this DNA as molecular hybridization probes that can discriminate among species and subspecies (3, 4, 6, 7, 18, 21, 22, 31, 38). While these techniques allow a high degree of specificity, many require a quantity of parasites obtainable only

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from cultures and are not suitable for direct use with clinical specimens.

The PCR, with its extraordinary capacity to amplify even a single copy of a DNA sequence into millions of copies, offers new possibilities for clinical and epidemiological studies of leishmaniasis (14, 15). Rodgers et al. (30) described PCR primers targeted to a sequence of minicircle kDNA that is broadly conserved among *Leishmania* spp. While these primers will amplify kDNA from all *Leishmania* species, Rodgers et al. (30), using biopsies from a very small series of Brazilian patients, found that by hybridizing the PCR products to species-specific kDNA probes, they could discriminate between *L. braziliensis* and *L. mexicana*. The work we report here uses the PCR primers developed by Rodgers et al. but employs new species-specific probes with a large series of ulcer biopsies obtained from patients in areas of Venezuela where leishmaniasis is endemic. We evaluated the ability of PCR-hybridization to detect parasites and discriminate between species and compared it with detection of parasites by microscopy and species identification by restriction enzyme analysis of kDNA isolated from biopsy cultures. Our findings suggest that the PCR-hybridization technique is highly sensitive, can accurately discriminate between species, and has an ease of application that should make it useful for diagnostic and epidemiologic studies of cutaneous leishmaniasis.

MATERIALS AND METHODS

Patient selection. Patients were obtained by a program of active case finding in the states with the highest incidence of leishmaniasis in Venezuela: Anzoategui, Amazonas, Merida, Miranda, Tachira, Trujillo, Lara, and Sucre. All patients included in this study were examined by a dermatologist (B.G. or A.R.) and judged to have an ulcer consistent with leishmaniasis, and all gave a positive skin test with killed promastigotes—*L. mexicana* cultured and prepared at Instituto de Biomedicina, Caracas, Venezuela (25).

Skin biopsies of 5 to 10 mm in diameter were taken under sterile conditions from the border of the ulcer and divided into several segments, one each for PCR, smear, histopathology, and culture. The smear was performed by touching the biopsy to a glass microscope slide and then staining it with Giemsa. Subsequently, 50 high-power fields (magnification, 100×) were searched for *Leishmania* organisms with a light microscope. The biopsy segment for PCR was placed directly in lysis buffer (10 mM Tris-HCl, 10 mM EDTA). Biopsies can be taken in the field and kept in lysis buffer at ambient temperature for up to 1 week and still yield results by microscopy and PCR equivalent to those obtained when the sample is processed immediately. Cultures of biopsies were performed as described elsewhere (36).

PCR. In the laboratory, proteinase K was added to the samples in lysis buffer to a final concentration of 200 μg/ml. The samples were then heated to 56°C for 30 min, after which the temperature was increased to 94°C for 30 min to inactivate the enzyme. After centrifugation at 800 × *g* for 1 min, the supernatant was transferred to a clean tube and the tissue pellet was discarded. The PCR mixture (25 μl of 10 mM Tris [pH 9]–5 mM KCl–2 mM $MgCl_2$–0.1% gelatin–2 mM deoxynucleoside triphosphates–2.5 U of *Taq* polymerase [Promega]–300 ng of each primer) was transferred to a 500-μl Eppendorf tube containing 3 μl of biopsy supernatant and then vortexed briefly and centrifuged for 1 min, after which 15 μl of mineral oil was layered on top. The amplification sequence was 1 min at 94°C to denature, 1 min at 50°C to anneal, and 1 min at 72°C for extension, performed in a Thermocycler (Cetus Corporation) for a total of 25 cycles. The oligonucleotides for PCR, 13A (5′-TTGACCCCCAACCACATTATA) and 13B (5′-GTGGGGGAGGGGCGTTCT), were developed, synthesized, and kindly supplied by M. Rogers of the Molecular Biology Laboratory of the Harvard School of Public Health (30). Additional quantities of the same oligonucleotides were synthesized at Albert Einstein College of Medicine.

A sample (8 μl) of the amplified reaction mixture was analyzed by electrophoresis in a 1% agarose gel in TAE buffer (0.05 M Tris, 0.02 M sodium acetate, 0.002 M EDTA [pH 8]). Two negative controls were included each time that the PCR was performed. One consisted of the reaction mixture alone, and the other was the reaction mixture with human lymphocyte DNA. Both were always negative.

Hybridization. Aliquots (4 μl) of the amplified PCR mixtures were denatured with an equal volume of 1 N NaOH and placed on ice for 5 min. They were then neutralized with 4 μl of 2 M ammonium acetate for 5 min and spotted on a nitrocellulose membrane (Sigma), which was then baked for 1 h at 80°C. The nitrocellulose membrane was prehybridized at 42°C overnight in prehybridization buffer (50% formamide, 5× Denhardt's solution, 5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% sodium dodecyl sulfate [SDS], and 100 μg of salmon sperm DNA per ml) (33). The probe was labelled with dioxigenin-UTP (Genius Kit; Boehringer Mannheim) and then added to the prehybridization solution and hybridized overnight at 42°C. Both the labelling of the probe and the detection of the label were carried out according to the manufacturer's instructions.

Isolation of kDNA species-specific probes. kDNA was isolated from reference strains of *L. mexicana* (PH8) and *L. braziliensis* (M2903), digested to completion with *Eco*RI, and ligated to pBR322 cut with *Eco*RI and treated with calf intestine alkaline phosphatase (Boehringer Mannheim). The ligation mixture was transformed into *Escherichia coli* JM105 and plated on Luria broth (LB)-ampicillin plates. The colonies were transferred to nitrocellulose discs, denatured, and renatured as described elsewhere (33) and then hybridized to total kDNA of the parent strain labelled with [^{32}P]dATP (Amersham) by using a nick translation kit (Amersham). Those colonies hybridizing to total parental kDNA were selected and grown in LB containing ampicillin (50 μg/ml) for miniprep plasmid DNA isolation. DNA from the minipreps was electrophoresed in an agarose gel, blotted onto nitrocellulose filters, and hybridized to ^{32}P-labelled total kDNA from both *L. mexicana* and *L. braziliensis*. One clone containing *L. mexicana* kDNA, pALM3, did not hybridize to *L. braziliensis* kDNA, and one clone containing *L. braziliensis* kDNA, pALB5, did not hybridize to *L. mexicana* kDNA.

kDNA isolation. Cultured promastigotes (approximately 10^9/ml) were collected by centrifugation at 1,000 × *g* for 10 min, resuspended, and washed three times in phosphate-buffered saline, pH 7.2. After the third wash, the parasite pellet was resuspended in 1 ml of lysis buffer (0.2 M NaCl, 0.05 M EDTA, and 10% SDS), left at room temperature for 15 min, and then passed through a 22-gauge needle. Following an incubation with proteinase K (10 mg/ml) at 42°C for 1 h, the kDNA was extracted with phenol-chloroform–isoamyl alcohol (25/24/1 [vol/vol/vol]) and centrifuged for 10 min. The aqueous phase was removed and centrifuged at 20,000 × *g* for 45 min. The supernatant was discarded, and the kDNA pellet was dissolved in 100 μl of TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA). The concentration of the kDNA was determined by

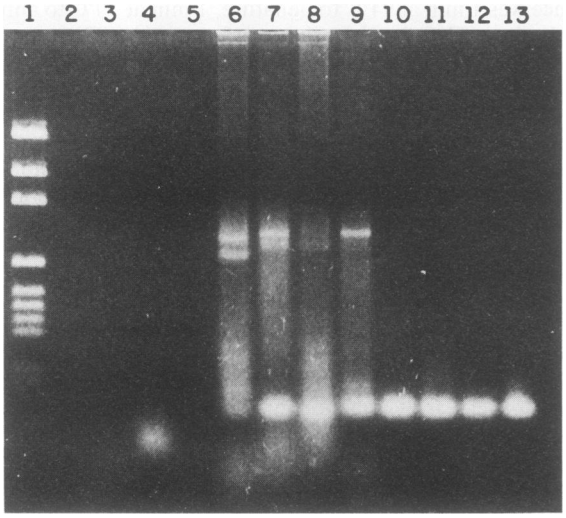

FIG. 1. Amplification of biopsies with primers 13A and 13B, showing the specificity of PCR. Lanes: 1, DNA molecular weight markers; 2, reaction mix; 3, reaction mixture with human genomic DNA; 4, reaction mixture with cultured *T. cruzi*; 5, reaction mixture with skin biopsy from a leprosy patient; 6 through 13, products of PCRs using samples from processed patient ulcer biopsies.

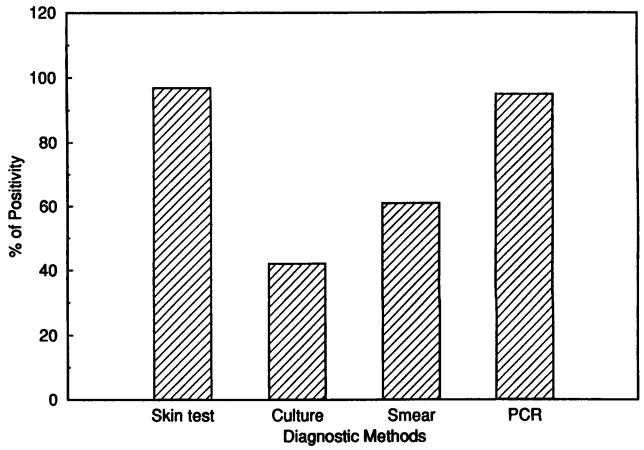

FIG. 2. Comparison of the abilities of various techniques to detect *Leishmania* parasites in ulcer biopsies from 233 patients. *Leishmania* parasites were detected in 63% of patients by smear, 43% by culture, and 97% by PCR. All patients had positive Montenegro skin test results.

measuring optical density at 260 nm (OD_{260}) (1 OD_{260} unit = 50 μg of double-stranded DNA).

Restriction enzyme analysis of kDNA. Approximately 1 μg of kDNA was digested with 2 U of *Msp*I (Bethesda Research Laboratories) at 37°C overnight. After the addition of a 1/10 volume of sample buffer (0.05% xylene cyanol, 0.05% bromophenol blue, 50% glycerol), the digested kDNA samples were electrophoresed on a gradient polyacrylamide gel (4.5 to 10% polyacrylamide) in TAE buffer at 8 mA overnight. The gels were then stained with 10% silver nitrate ($AgNo_3$) by the method described by Beidler et al. (8). kDNA isolated from positive biopsy cultures was cut with restriction enzyme *Msp*I, and the resulting digest patterns were compared with patterns obtained with *Msp*I digests of kDNA from international reference strains PH8 and M2903.

RESULTS

Detection of leishmania parasites. The first objective of this study was to assess the ability of PCR to detect leishmanial parasites in clinical biopsies and to compare PCR with other commonly used detection methods. Biopsies were taken from 233 patients presenting with ulcers consistent with leishmaniasis and also reacting positively to a Montenegro skin test. For the PCR amplification, we used primers 13A and 13B (30) (see Materials and Methods), which are targeted to a region of minicircle kDNA that is broadly conserved in leishmanial species and yield a PCR product 120 bp in length (Fig. 1). In the PCR, leishmanial parasites were detected in biopsies from 97% (226 of 233) of patients, yielding a PCR product 120 bp in length (Fig. 1). Control PCRs using 20 biopsies of healthy skin and five biopsies of lepromatous skin, as well as human lymphocytes and cultures of *Trypanosoma cruzi*, were all negative.

In comparison (Fig. 2), leishmanial organisms were seen in touch preparation smears from only 63% of these biopsies. Fragments of the biopsy tissue were also placed onto media: 43% grew leishmania colonies, 27% were negative, and 30% of the cultures were contaminated and discarded. Of the seven patients whose biopsies were negative by PCR, none was positive by smear or culture; two were diagnosed as having mycotic infections, and five were lost to follow-up, left untreated.

Taxonomy. The PCR primers used detected the presence of all *Leishmania* species and do not distinguish among them. However, we sought to clone fragments of kDNA that are species specific and could be hybridized to the PCR product to identify the species in the biopsy (30). Therefore, total kDNA from reference strains PH8 and M2903 was digested with restriction enzyme *Eco*RI, and the fragments were ligated into plasmid pBR322. Recombinant plasmids were then hybridized to total kDNA of both species in order to select clones that hybridized only with their parent species. One clone, pALB5,

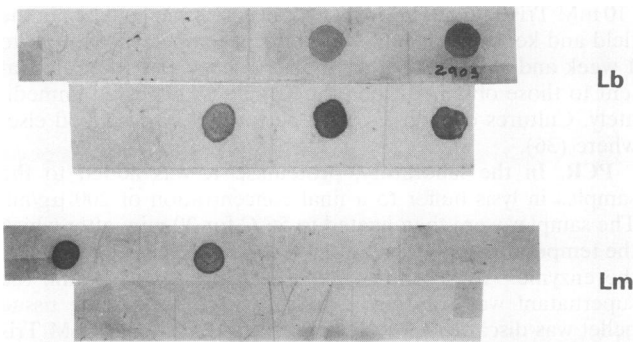

FIG. 3. *Leishmania* species determination by hybridization of PCR products to species-specific probes. Replica dot blots of completed PCRs, using patient biopsies from Miranda State, hybridized to *L. braziliensis* (Lb)-specific probe pALB5 and *L. mexicana* (Lm)-specific probe pALM3. Sample 2903 is a reference strain of *L. braziliensis*.

which hybridizes only to *L. braziliensis* and another, pALM3, which hybridizes only to *L. mexicana* were obtained. Neither of these clones hybridizes to kDNA from either *Leishmania donovani* or *Leishmania major* (data not shown).

In preliminary studies, it was found that the PCR products amplified with primers 13A and 13B hybridized strongly to one or the other of the two clones, but not to both. The PCR products amplified from patient biopsies were then spotted on duplicate nitrocellulose filters and hybridized to both clones. Biopsies were classified as containing *L. mexicana* or *L. braziliensis* according to the clone to which their PCR product hybridized (Fig. 3 and 4). Of the 226 total positive biopsies, 117 hybridized to the *L. mexicana*-specific clone pALM3 and 109 hybridized to *L. braziliensis*-specific clone pALB5. As can be seen from the distribution of these results by states of Venezuela (Table 1), *L. braziliensis* appears to be the predominant cause of leishmanial ulcers in Venezuela. It is of interest that while *L. mexicana* was found in all 90 biopsies from the state of Lara, *L. braziliensis* was predominant in biopsies from all other states.

Restriction enzyme analysis of kDNA. In order to confirm the taxonomic designation given above, we digested kDNA isolated from the reference strains PH8 and M2903 with several restriction enzymes, seeking one that would yield restriction patterns sufficiently different to allow easy discrimination of *L. mexicana* from *L. braziliensis*. *Msp*I gave readily distinguishable patterns (Fig. 5A), but *Hin*fI, *Hae*III, *Eco*RI, and *Hin*dII did not (17).

kDNA was then isolated from over 60 positive biopsy cultures and digested with restriction enzyme *Msp*I, and the resulting patterns were compared with *Msp*I digests of kDNA from the reference strains. In all cases, the species designation from the kDNA *Msp*I restriction pattern was in agreement with the identification obtained by hybridization of the PCR product with the species-specific clones (Fig. 5B). In addition, when kDNA from the positive cultures was hybridized by dot blot to total kDNA of both *L. mexicana* and *L. braziliensis* (38), the hybridization to the species found to be present by both kDNA restriction enzyme digest and the PCR-hybridization technique was invariably much stronger (data not shown).

TABLE 1. Distribution of cases of cutaneous leishmaniasis in eight states of high incidence in Venezuela, 1988 to 1990

State	No. of registered cases[a]	No. of patients positive by PCR	No. of patients with indicated *Leishmania* species identified by hybridization	
			L. braziliensis	*L. mexicana*
Anzoategui	164	25	19	6
Amazonas	3	3	3	0
Merida	189	5	5	0
Miranda	212	50	47	3
Tachira	185	22	12	10
Trujillo	224	19	16	3
Lara	418	90	0	90
Sucre	156	12	7	5
Total		226	109	117

[a] Average number of cases registered by the dermatology health service.

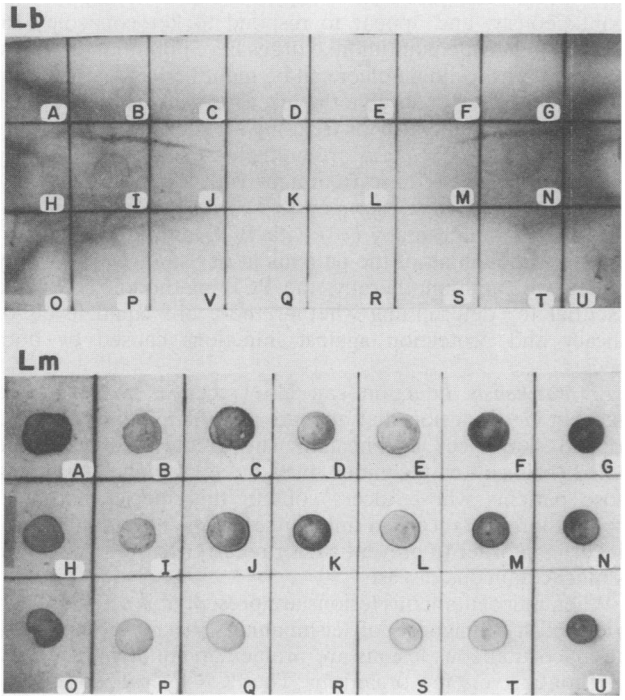

FIG. 4. Replica dot blots of completed PCRs from biopsies taken in Lara State were hybridized to *L. braziliensis*-specific probe pALB5 (Lb) and *L. mexicana*-specific probe pALM3 (Lm). Sample R is a negative control. Sample V is not shown hybridized to *L. mexicana*.

DISCUSSION

New World cutaneous leishmaniasis predominantly afflicts rural populations in developing countries, and new diagnostic techniques must be proven applicable in this setting. The traditional diagnostic methods are easily employed—direct microscopy can be performed at any clinic with a reasonable microscope, and cultures can be set up in a central laboratory—but their sensitivities are unacceptably low. When we attempted to detect parasites by these methods, we obtained results comparable to those described in other reports (11, 23, 27, 37): 64% of our biopsy smears were positive by direct microscopy, and *Leishmania* parasites were grown from only 42% of biopsy cultures. The low percentage of positives by both techniques is probably due to the paucity of parasites present in the lesions and the low sensitivity level of the tests. Culture, which might be expected to be more sensitive, actually gave fewer positive results, perhaps because the ulcers are often secondarily infected with bacteria or fungus, and many cultures are contaminated and discarded.

In contrast, the PCR-hybridization technique detected the presence of leishmania parasites in 97% of our ulcer biopsies. PCR is especially suited to situations where few organisms are found, and it has been shown (30) that the primers we used can detect the presence of as few as 10 leishmanial parasites. Another advantage of the PCR-hybridization technique is that it does not require rigorous sterility; in our study, the same biopsy fragment was routinely touched to a microscope slide for smears and then placed in buffer and kept for up to 10 days before being processed for PCR. Because the probes are specific for leishmanial targets, moderate contamination of the biopsies with other organisms should not affect PCR results.

Of the seven patients whose biopsies were negative, two

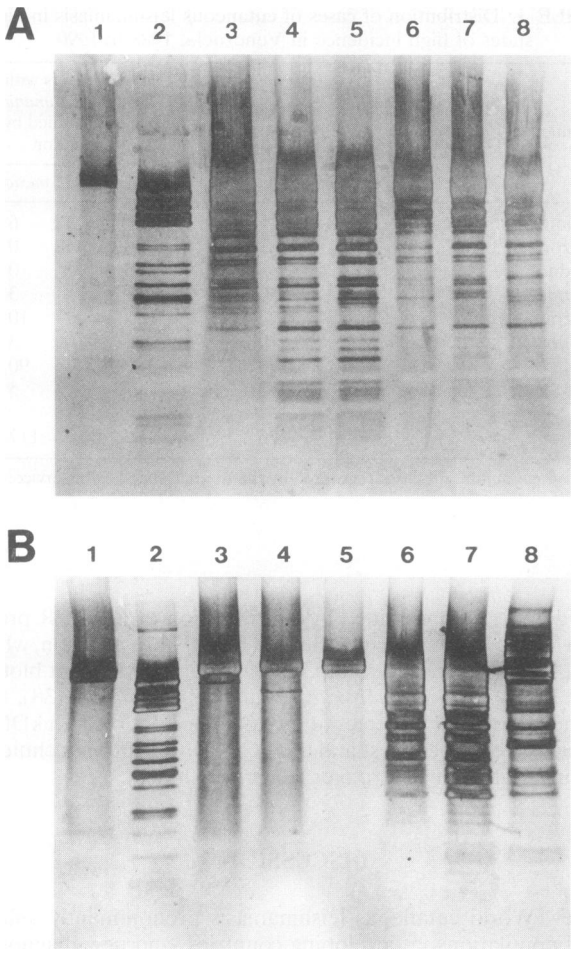

FIG. 5. Acrylamide gradient (4.5 to 10% acrylamide) gel electrophoresis of fragments generated by *Msp*I restriction digestion of kDNA. (A) Lanes: 1, kDNA of *L. braziliensis* reference strain 2903; 2, kDNA of *L. mexicana* reference strain PH8; 3 through 8, kDNA of isolates from Lara State, all showing the *L. mexicana* restriction pattern. (B) Lanes: 1 and 2, same as in panel A; 3 through 8, kDNA of isolates from Tachira and Trujillo states digested with *Msp*I.

were subsequently diagnosed as having mycotic ulcers and the other five failed to return to the clinic and were lost to follow-up, suggesting that perhaps their ulcers had other causes and healed spontaneously or were leishmanial but in the process of resolution. Their positive skin test results could be explained as evidence of previous exposure. All other patients received treatment, and all ulcers were resolved completely. Because of the low sensitivity level of the conventional tests for identification of *Leishmania* spp., there is no "gold standard" with which to evaluate the PCR-hybridization method and determine the number of false positives and negatives. Nevertheless, on the basis of clinical histories and the fact that PCR-hybridization detected parasites in every biopsy that was positive by culture or direct smear, we believe this technique to have higher degrees of sensitivity and specificity than does currently available methodology.

In addition to detecting the presence of parasites, it is also important to identify the *Leishmania* spp. as belonging to either the *L. mexicana* or the *L. braziliensis* species complex. The different species and subspecies of *Leishmania* can be distinguished by monoclonal antibodies (24), isoenzyme analysis, or restriction digests of kDNA (26, 29, 34, 35), but these techniques require a quantity of parasites obtainable only by in vitro culture.

In our study, we identified the species of the parasites by hybridizing the PCR product to species-specific clones of kDNA, thus eliminating the need for biopsy cultures. These probes were selected empirically as kDNA fragments of *L. mexicana* and *L. braziliensis* that will hybridize much more strongly to their own species. The accuracy of species identification by using these probes was confirmed by restriction enzyme digestion of total kDNA isolated from over 60 positive cultures. The minor differences in the restriction patterns of the Venezuelan isolates from those of the reference strains, or from each other (for example, in Fig. 5A compare lanes 3 through 8 with each other and with lane 2), likely reflect differences at the subspecies or strain level. Lane 2 in Fig. 5A contains *L. mexicana* subsp. *amazonensis*, but the clinical isolates in lanes 3 through 8 have not been characterized at the subspecies level and may belong to a different subspecies. Nevertheless, all have patterns clearly distinguishable from the pattern of *L. braziliensis* in Fig. 5A, lane 1. In addition, species assignment by this analysis was invariably in agreement with the species assignment by PCR-hybridization. In future work, we hope to isolate kDNA fragments that are specific for subspecies and can be hybridized to the PCR product to allow subspecies identification. It may also be possible to develop new PCR primers that will amplify only one subspecies.

While leishmania epidemiology and control are aided by subspecies discrimination, therapeutic decisions currently require only distinction of *L. braziliensis* from *L. mexicana*. The ulcers caused by *L. braziliensis* are generally more virulent and respond only to pentavalent antimonial drugs, while *L. mexicana* ulcers tend to be less severe, are more likely to heal spontaneously, and appear to respond to ketoconazole (16, 28). The pentavalent antimonial drugs have potential toxicities, so if the parasites in an ulcer can be identified as *L. mexicana*, it might be better to observe the ulcer for a few weeks to see if it will heal spontaneously or treat it with ketoconazole. Ulcers infected with *L. braziliensis* are unlikely to heal spontaneously and will probably require treatment with a pentavalent antimonial drug. A new therapeutic approach with promising results is immunotherapy (10) with BCG and killed promastigotes. Although all of the patients in our study were initially treated with immunotherapy, the PCR methodology will be essential for determining whether there are equal levels of efficacy and protection against infections caused by both species.

L. braziliensis infection can also recur as a destructive mucocutaneous lesion that appears months to years after the original skin ulcer has healed. The greatest value of the PCR-hybridization technique may be in rapidly identifying those patients whose ulcers contain this species and who should therefore receive a full course of a pentavalent antimonial drug in order to prevent a later recurrence of the infection as mucocutaneous disease.

When mucocutaneous lesions are present, it is often difficult to establish a diagnosis of leishmaniasis, because biopsies of the mucocutaneous lesions are problematic and characteristically contain very few organisms. The PCR-hybridization technique, with its high degree of sensitivity, may be especially useful in diagnosing this disease. A clinical study evaluating the use of this technique in diagnosing mucocutaneous leishmaniasis is currently in progress.

We believe that this work demonstrates not only that the PCR-hybridization technique can accurately and reliably detect *Leishmania* parasites and identify their species, but also that it can readily be employed in a country where the disease is endemic. The digoxigenin-labelled probes, which can be made in advance and reused, allow a high degree of sensitivity without the use of radioactivity. It is even possible that the entire protocol could be adapted for use in a rural clinic with three water baths replacing the thermocycler and the gel electrophoresis omitted. This technique, or modifications of it, should rapidly alter the epidemiology, identification of vectors and reservoirs, diagnosis, and treatment of leishmaniasis in Latin America.

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