

SIMPLIFIED POLYMERASE CHAIN REACTION DETECTION OF NEW WORLD *LEISHMANIA* IN CLINICAL SPECIMENS OF CUTANEOUS LEISHMANIASIS

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Abstract. Polymerase chain reaction (PCR)-based detection of New World *Leishmania* from different types of clinical specimens has been further streamlined for field use by simplifying sample preparation and modifying published protocols. A multiplex PCR reaction was developed that allows simultaneous detection of the *Leishmania* genus and identification of the *L. braziliensis* complex. For typing isolates in culture, we found that simply boiling diluted cultured strains was sufficient preparation for the PCR. We have demonstrated that *Leishmania* parasites can be reliably detected from boiled dermal scrapings, instead of the more invasive skin biopsies often used as PCR specimens. The PCR of dermal scrapings yielded a sensitivity of 100% and a specificity of 100%, as compared with microscopic examination. In a study population, PCR was more sensitive than classic diagnostic techniques. The PCR detection of *Leishmania* in biopsies and peripheral blood mononuclear cells (PBMCs) was investigated. Diluting crude extracts of skin biopsies was sufficient to eliminate sample inhibition yet maintain required sensitivity. *Leishmania braziliensis* was also detected by PCR in PBMCs of individuals with active cutaneous leishmaniasis. The simplifications described here significantly improve the feasibility of using the PCR in endemic countries as the primary method for detection and preliminary characterization of *Leishmania* in clinical specimens of cutaneous leishmaniasis.

Leishmaniasis is an important public health problem in tropical and subtropical countries worldwide, involving 400,000 new cases annually, with 12 million people infected and 350 million at risk.¹ In the Americas, human leishmaniasis occurs from Mexico to Argentina and is caused by 13 distinct species grouped into three complexes. New World *Leishmania* produce several forms of disease, depending on the species of the parasite, the immune status of the host, and possibly factors deriving from its sandfly vector.² Symptoms range from self-curing cutaneous lesions (*L. mexicana* complex) to persistent and disfiguring cutaneous and mucocutaneous manifestations (*L. braziliensis* complex) to the potentially fatal visceral disease (*L. donovani* complex).

Parasitologic confirmation of diagnosis is critical because of the high cost and significant toxicity of current treatment regimens for leishmaniasis. It is also important to identify the species of *Leishmania* for both clinical and epidemiologic reasons. *Leishmania* species of different complexes found in the same geographic region can cause cutaneous lesions of similar appearance, yet the different complexes require distinct therapeutic approaches.³ The epidemiologic information furnished by the identification of parasites in a given region is critical for the design of appropriate control measures.

The classic diagnostic techniques for cutaneous leishmaniasis have a number of limitations. Microscopic examination of skin scrapings, though rapid and low-cost, has limited sensitivity, particularly in chronic lesions. In vitro culture techniques, while more sensitive, are susceptible to microbiologic contamination and are hampered by the particular growth requirements of different strains; in addition, since certain strains grow better than others in vitro, the dominant strains can be inadvertently selected when culturing mixed infections.⁴ The Montenegro skin test detects specific cutaneous delayed-type hypersensitivity but cannot distinguish between current and past infection. Serologic diagnostic techniques present drawbacks that include the cross-reactivity of leishmanial antigens with antibodies induced by other

kinetoplastids such as *Trypanosoma cruzi*,^{5,6} as well as poor sensitivity due to the low antibody titer characteristic of cutaneous leishmaniasis.²

Species identification has been conventionally achieved using isoenzyme electrophoresis (zymodeme analysis)⁷ or monoclonal antibodies (serodeme analysis).⁸ Zymodeme analysis is a lengthy and expensive process that requires large-scale cultivation of parasites. Monoclonal antibodies are useful for identification of species in cultured strains but are not as amenable to direct analysis of clinical specimens.

Molecular techniques, such as the polymerase chain reaction (PCR), offer an alternative approach to the demonstration of parasites in clinical samples.⁹ Due to their molecular specificity, detection and genetic characterization of *Leishmania* can be accomplished simultaneously. The PCR is a sensitive and rapid technique, which can be adapted for use under conditions of limited resources by applying a low-cost approach.^{10–12} A number of PCR protocols have been reported for the detection of *Leishmania* that cause cutaneous leishmaniasis, using either purified DNA^{13–18} or clinical specimens.^{19–22}

At the *Leishmania* Reference Laboratory of the Nicaraguan Ministry of Health, we have implemented the PCR for routine use because of the limitations we experienced with traditional diagnostic and characterization techniques in our field work.¹⁰ Several reported PCR protocols were useful;^{13,19} however, we found it necessary to further simplify sample preparation methods and amplification protocols. Here we describe the adaptations developed to streamline routine use of the PCR for detection and characterization of *Leishmania* in endemic countries.

MATERIALS AND METHODS

Specimen collection and preparation. Skin biopsies from lesions of suspected leishmaniasis were taken from patients in the northwestern region of Pichincha Province, Ecuador and tested during a PCR workshop in Quito.¹⁰ Skin

scrapings and blood samples were obtained from subjects at the Health Center in Los Chiles, Rio San Juan, Nicaragua and from Rancho Grande, Nicaragua. Ten milliliters of blood for isolation of peripheral blood mononuclear cells (PBMCs) were collected in Vacutainer® (Becton Dickinson, Franklin Lakes, NJ) tubes and transferred to tubes containing 10 ml of RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) with 2% fetal bovine serum (Sigma Chemical Co.) and 2,500 IU of heparin (Biochimie, Vienna, Austria). Patients are routinely tested in clinics conducted by the Ministry of Health in Nicaragua and the National Leishmania Reference Laboratory in Ecuador; unused portions of these specimens were used in the validation studies reported here.

Two to three millimeter skin biopsies were taken with a sterile scalpel from the border of the lesions and placed in 200 μ l of transport buffer consisting of 10 mM Tris-HCl (pH 8.0) and 10 mM EDTA. The samples were then heated at 65°C for 3 hr and frozen until use. Prior to PCR analysis, each biopsy was macerated and treated with proteinase K (200 μ g/ml) at 55°C for 1 hr; the proteinase K was then inactivated by incubation at 94°C for 30 min. Two skin scrapings were taken from the border of presumptive leishmanial lesions with both a sterile scalpel and a sterile wooden toothpick, stained with Giemsa, and examined by microscopy. For PCR, duplicate samples were collected from each lesion with a sterile toothpick, placed in 100 μ l of 5% Chelex 100 (Bio-Rad Laboratories, Richmond, CA),²³ and heated for 10 min in a boiling water bath. After centrifugation at 13,600 \times g for 1 min, 5 μ l of the supernatant were used for PCR amplification. Lesion aspirates were collected in 600 μ l of transport buffer (see above), heated at 65°C for 3 hr, and treated with 200 μ g/ml of proteinase K for 1 hr at 55°C. The PBMCs from 10 ml of the blood suspension described above were isolated using Histopaque 1077 (Sigma Chemical Co.) and washed three times in phosphate-buffered saline. Pelleted cells were resuspended in lysis buffer (50 mM Tris HCl [pH 8.0], 50 mM EDTA, 50 mM NaCl, 1% sodium dodecyl sulfate) and treated with 200 μ g/ml of proteinase K for 1 hr at 56°C. After heat inactivation of the proteinase K, the samples were extracted once with phenol/chloroform and again with chloroform, followed by ethanol precipitation.

In vitro cultivation. *Trypanosoma cruzi* (TCN), *Leishmania panamensis* (MHOM/NI/87/ZE09), and *L. braziliensis* (MHOM/NI/88/XD28 and MHOM/NI/91/ZF11) were isolated from clinical cases in Nicaragua. *Leishmania mexicana* (Honduras MHOM/HN/92/HN368), *L. amazonensis* (Panama, MHOM/PA/91/CIDEP006), and *L. chagasi* (Honduras, MHOM/HN/89/HN125) were isolated from clinical cases in the designated countries and form part of a Central America-wide strain bank.²⁴ *Leishmania guyanensis* (MHOM/BR/75/M4147) was obtained from the London School of Tropical Medicine and Hygiene. Two drops of *Leishmania* parasites grown in modified RPMI 1640 medium²⁵ or Senekjie's medium²⁶ were diluted in 200 μ l of sterile distilled water and heated at 100°C for 10 min. After centrifugation at 13,600 \times g for 1 min, 5 μ l of the supernatant were used for PCR amplification.

Polymerase chain reaction amplification. Primers MP3H (5'-GAACGGGGTTTCTGTATGC-3') and MP1L (5'-TACTCCCCGACATGCCTCTG-3')¹⁹ were used to am-

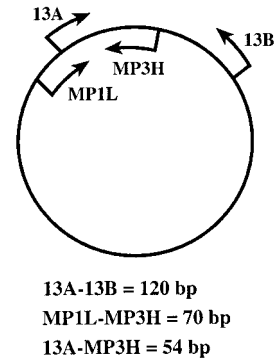


FIGURE 1. Schematic diagram of a *Leishmania* minicircle, indicating the position and direction of the polymerase chain reaction primers and the length of the amplification products. bp = basepairs.

plify a 70-basepair (bp) fragment of minicircle kinetoplast DNA specific to members of the *L. braziliensis* complex. The reaction mixture contained 10 mM Tris-HCl [pH 9.0], 50 mM KCl, 0.1% Triton X-100, 1 mM DTT, 2 mM MgCl₂, 0.2 mM of each of the four nucleotides (Promega Corp., Madison, WI), 1 μ M of each primer, and 0.625 units of *Taq* DNA polymerase (*Taq* DNA polymerase; Promega Corp. and AmpliTaq®; Perkin Elmer, Foster City, CA). Five microliters of sample were used in a final reaction volume of 25 μ l overlaid with 50 μ l of mineral oil (Sigma Chemical Co.). The amplification consisted of 35 cycles for 1 min each at 94°C, 54°C, and 72°C, preceded by an initial denaturation for 3 min at 94°C and followed by a final extension for 10 min at 72°C. In another PCR, a 120-bp sequence was amplified using primers 13A (5'-GTGGGGGAGGGGCGTTCT-3') and 13B (5'-ATTTTACACCAACCCCCAGTT-3'),¹³ which hybridize to a region of the minicircle conserved in all *Leishmania* species. The reaction conditions for this PCR were as in the *L. braziliensis*-specific PCR above, except that the annealing temperature was 52°C instead of 54°C. Lastly, a multiplex reaction was developed with the reaction mixture as described above, using primers 13A, 13B, and MP3H to generate a 54-bp fragment from members of the *L. braziliensis* complex and a 120-bp fragment from all other *Leishmania* complexes (Figure 1). The amplification consisted of 35 cycles for 1 min each at 94°C, 52°C, and 72°C, preceded by an initial denaturation for 3 min at 94°C and followed by a final extension for 10 min at 72°C. The PCR products were analyzed by electrophoresis on 1.6% or 1.8% agarose gels, using Amplisize DNA Size Standards (Bio-Rad Laboratories).

RESULTS

Simple preparation of cultured parasites. A simple alternative to extraction of DNA from cultured parasites was used for characterization of isolates. Cultured parasites were prepared for PCR amplification by diluting and boiling the cultured strains. Two PCR protocols were applied to type the isolates. One amplification, adapted from Rodgers and others,¹³ yields a 120-bp product from the minicircle region of all *Leishmania* species (primers 13A and 13B). The other protocol, a modification of Lopez and others,¹⁹ results in a

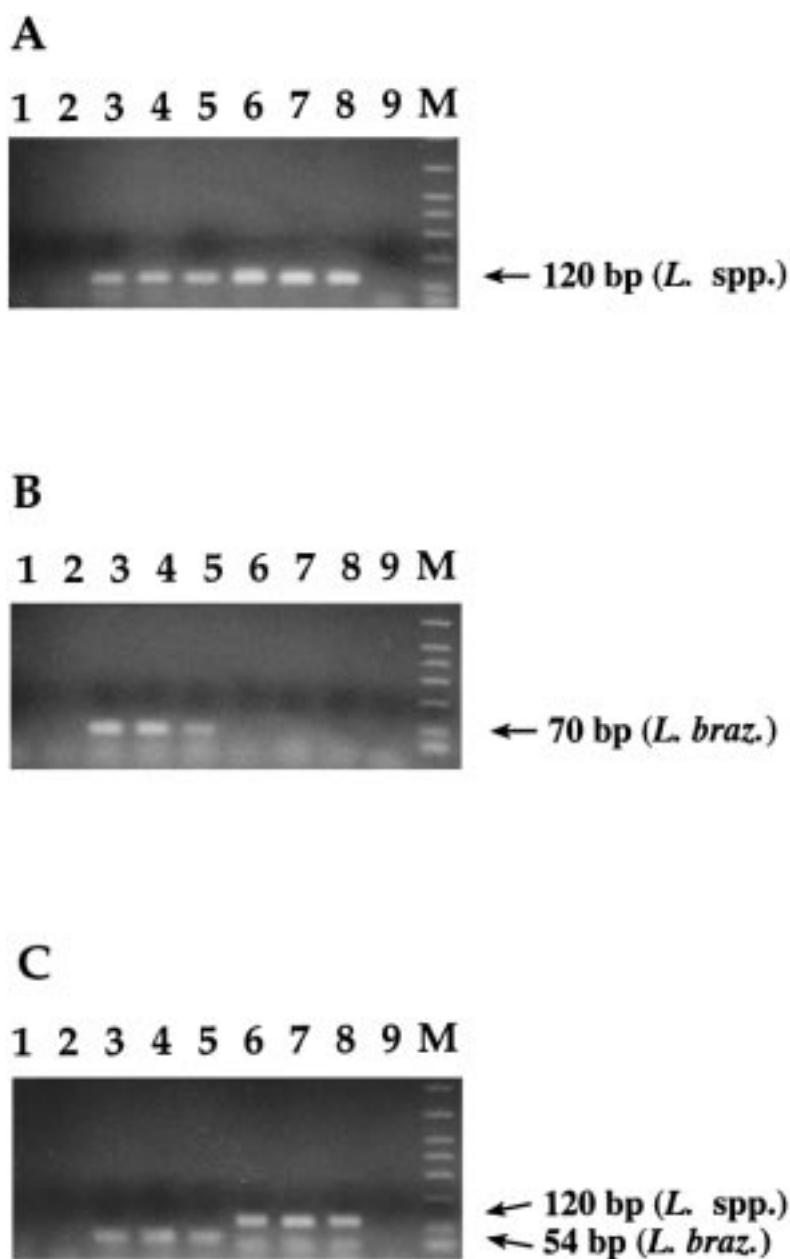


FIGURE 2. Rapid characterization of cultured *Leishmania* strains by the polymerase chain reaction (PCR). Crude extracts of cultured strains were amplified as follows: **A**, amplification with primers that generate a 120-basepair (bp) product from all *Leishmania* species; **B**, amplification of a 70-bp fragment of the minicircle with primers specific to members of the *L. braziliensis* (*L. braz.*) complex; **C**, multiplex PCR combining the two PCR protocols above to generate a 54-bp product from members of the *L. braziliensis* complex and a 120-bp product from other *Leishmania* complexes. Lane 1, negative control (RPMI 1640 culture medium); lane 2, *Trypanosoma cruzi*; lane 3, *L. panamensis* (Nicaragua 87ZE09); lane 4, *L. braziliensis* (Nicaragua 88XD28); lane 5, *L. guyanensis* (Brazil M4147); lane 6, *L. mexicana* (Honduras HN368); lane 7, *L. amazonensis* (Panama CIDEP006); lane 8, *L. chagasi* (Honduras HN125); lane 9, negative amplification control (water); lane M, Amplisize DNA size standards (50, 100, 200, 300, 400, 500, 700, 1,000, 1,500, and 2,000 bp).

70-bp fragment only from members the *L. braziliensis* complex (primers MP3H and MP1L). Characterized Central American strains in culture were prepared as above and amplified with both sets of primers. Products of the expected 120 bp were obtained from all *Leishmania* species with primers 13A and 13B, while similar preparations of cultured *T. cruzi* as well as negative controls resulted in no amplification (Figure 2A). Amplification with primers MP3H and MP1L resulted in the expected 70-bp product only in members of the *L. braziliensis* complex, while other *Leishmania*

species, *T. cruzi*, and negative controls yielded no product (Figure 2B).

Multiplex reaction for preliminary characterization. To minimize the number of PCRs necessary to characterize *Leishmania* strains, the two PCR protocols described above were combined in a single multiplex reaction. A single 5' primer (13A) was used with the two 3' primers (13B and MP3H) in a modified protocol (Figure 1), and the same cultured strains as above were analyzed. In members of the *L. braziliensis* complex, a single 54-bp fragment was prefer-

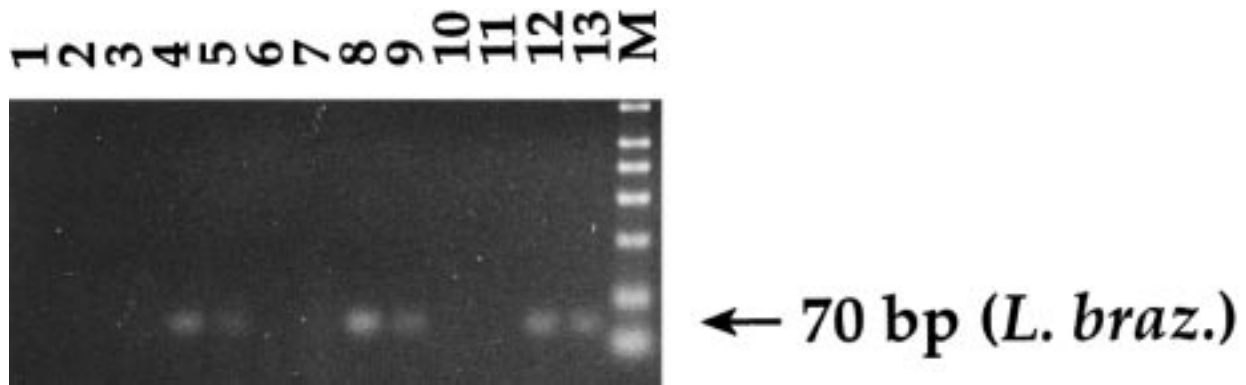


FIGURE 3. Detection of the *Leishmania braziliensis* (*L. braz.*) complex in biopsy samples by the polymerase chain reaction (PCR). Dilutions of crude extracts of biopsy samples were tested in a PCR protocol that amplifies the *L. braziliensis* complex. Lane 1, negative control for reagent contamination (water); lane 2, negative control for sample contamination (water); lanes 3–5, biopsy from patient 01 (neck lesion) at dilutions of 1:1, 1:5, and 1:10, respectively; lane 6, aspirate from patient 01 (neck lesion); lanes 7–9, biopsy from patient 01 (leg lesion) at dilutions of 1:1, 1:5, and 1:10, respectively; lane 10, aspirate from patient 01 (leg lesion); lanes 11–13, biopsy from patient 02 at dilutions of 1:1, 1:5, and 1:10, respectively; lane M, Amplisize DNA size standards (see Figure 2). The expected product size was 70 basepairs (bp).

entially amplified, a product of the complex-specific 3' primer MP3H and the genus-specific 5' primer 13A (Figure 2C). The other *Leishmania* species resulted in a 120-bp product using the two conserved genus-specific primers (13A and 13B). Again, negative controls were as expected. The primer-dimer artifacts observed in Figure 2C, lanes 6–9 are distinguishable from the positive controls (members of the *L. braziliensis* complex, lanes 3–5), which yielded the expected 54-bp *L. braziliensis*-specific product.

Detection of *Leishmania* in dermal biopsies by PCR.

Crude extraction of biopsies instead of DNA purification presents a quicker approach to specimen preparation, but may not remove inhibitors of amplification. A simple solution to this problem is the serial dilution of crude biopsy extracts, such that the potential inhibitors are no longer inhibitory, while sufficient sample DNA remains to generate a PCR product. In Figure 3, three biopsy specimens collected in Ecuador were macerated, treated with proteinase K, and subsequently diluted five- and 10-fold for PCR amplification with *L. bra-*

ziliensis-specific primers. In all three cases, the undiluted sample yielded no product, while dilutions of 1:5 and 1:10 resulted in the predicted 70-bp product characteristic of the *L. braziliensis* complex. Lesion aspirates yielded no product, most likely due to the excessive volume of sample collection solution. Parasite cultures were done on each patient, and the results coincided with those obtained by the PCR.

Standardization of PCR detection of *Leishmania* directly from dermal scrapings. Dermal scraping was examined as an alternative specimen for PCR since it requires a less invasive procedure. Skin scrapings from the border of presumptive leishmanial lesions were boiled and then amplified directly. Figure 4 shows the results of a representative amplification with the multiplex protocol, in which the undiluted specimen and a 1:5 dilution were tested. Amplification of scrapings that were found to be positive by microscopy resulted in the predicted 54-bp *L. braziliensis*-specific PCR product (lanes 5, 6, 8, and 9). Dermal scrapings negative by microscopy also yielded negative results by PCR (lanes 2 and 3). It appears that there are less inhibitory

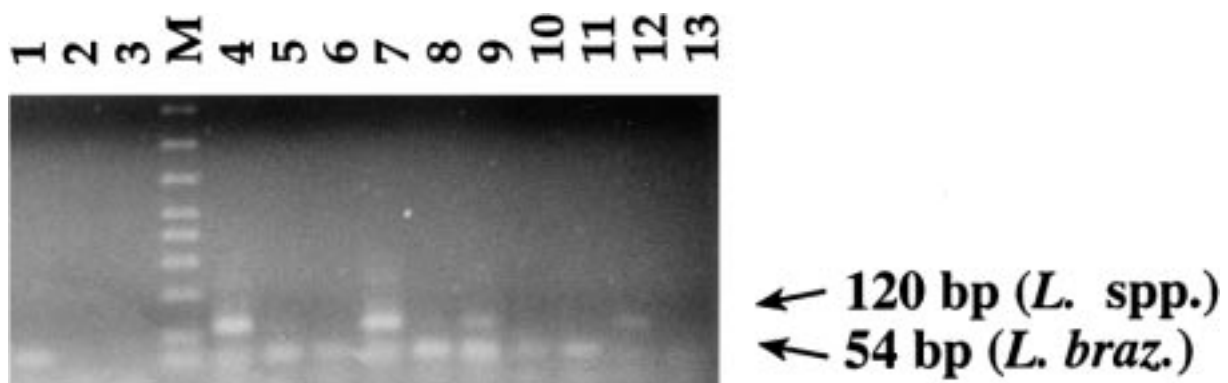


FIGURE 4. Polymerase chain reaction (PCR) amplification of *Leishmania* in dermal scrapings. Crude preparations of dermal scrapings were analyzed using the multiplex PCR protocol. Lane 1, undiluted skin scraping from patient 05 (negative) plus purified *L. braziliensis* (*L. braz.*) DNA (inhibition control); lanes 2 and 3, skin scraping from patient 05 (negative) undiluted and diluted 1:5, respectively; lane M, Amplisize DNA size standards (see Figure 2); lane 4, patient 07 inhibition control; lanes 5 and 6, skin scraping from patient 07 undiluted and diluted 1:5, respectively; lane 7, patient 10 inhibition control; lanes 8 and 9, skin scraping from patient 10 undiluted and diluted 1:5, respectively; lane 10, 5% Chelex 100 plus purified *L. braziliensis* DNA (inhibition control); lane 11, cultured *L. braziliensis* parasites; lane 12, cultured *L. chagasi* parasites; lane 13, negative amplification control (water). The expected product size was 54 basepairs (bp) for *L. braziliensis* and 120 bp for *Leishmania* spp.

TABLE 1

Comparison of the results of the polymerase chain reaction (PCR) and microscopy in the detection of *Leishmania* in dermal scrapings

PCR*	Microscopy†	
	Positive	Negative
Positive	16	4‡
Negative	0	4

* Duplicate aliquots from each sample were amplified on different days; results from duplicate aliquots were concurrent.

† Microscopy was performed on dermal scrapings taken from the border of presumptive leishmanial lesions with a sterile wooden toothpick.

‡ For cases that were positive by PCR (duplicate aliquots from one sample) and negative by microscopy, a second sample from the same lesion was amplified, again yielding positive results.

substances in dermal scrapings than in biopsy specimens, since undiluted preparations of the positive dermal scrapings as well as diluted samples resulted in amplification product. Purified leishmanial DNA was added to each sample as an inhibition control (lanes 1, 4, and 7). When the template is in excess, two products can be generated from *L. braziliensis*; namely, the 54-bp *L. braziliensis*-specific product and the 120-bp fragment representative of all *Leishmania* spp. (lanes 4, 7, and 9).

To assess the usefulness of the PCR of dermal scrapings as a reliable diagnostic test, several evaluations were performed. First, the sensitivity of the PCR test was determined by conducting PCR amplification of skin scrapings from 12 lesions shown by microscopy to be cutaneous leishmaniasis. Product of the expected size was observed in 12 of the 12 samples, for an overall sensitivity of 100%. Next, the specificity of the PCR protocol was evaluated by testing six lesions classified as negative by microscopy. These patients were diagnosed with other diseases, including leprosy, candidiasis, staphylococcal infection, and varicose ulcer. No product resulted from amplifications with these samples; thus, the specificity of the PCR test was 100%.

Lastly, PCR of dermal scrapings was compared with conventional parasitologic diagnostic techniques in a group of 24 patients from southern Nicaragua with presumptive cutaneous leishmaniasis (Table 1). During the course of the study, we observed that microscopy was more sensitive when dermal scrapings were taken with a wooden toothpick

(14 of 24 = 58% positive) than with a sterile lancet (11 of 24 = 46% positive). Leishmanial amastigotes were observed by microscopy in 16 of the 24 dermal smears examined. Two samples were taken from each lesion with a sterile toothpick for PCR analysis. Duplicate aliquots from one sample were amplified on different days. The PCR of dermal scrapings was positive in 16 of the 16 confirmed leishmanial lesions and negative in four of four negative lesions examined in this field study. The PCR yielded positive results in four lesions classified as negative by conventional procedures. In these cases, the second sample from the same lesion was subjected to PCR analysis, and again positive results were obtained in all four cases.

Attempts were made to culture parasites from the patients' lesions; however, cultured parasites were obtained from only one of the 24 samples, and therefore the culture results could not be compared with PCR and microscopy. This is consistent with previous observations that *Leishmania* parasites from southern Nicaragua (where this study was conducted) are notoriously difficult to isolate in culture, whereas it is easier to isolate *Leishmania* in culture from cases in northern Nicaragua (Belli A, unpublished data).

Detection of *Leishmania* parasites in PBMCs. In Figure 5, PBMCs from patients negative (lanes 1 and 2) and positive (lanes 3–8) for *L. braziliensis* by microscopic examination and PCR of lesion scrapings were collected, extracted, and subjected to amplification with *L. braziliensis*-specific primers. Dilutions of the samples (1:5) were examined (lanes 2, 4, 6, and 8) in addition to undiluted specimens (lanes 1, 3, 5, and 7). The characteristic 70-bp *L. braziliensis*-specific product was observed in the positive but not negative specimens. Controls were as expected.

DISCUSSION

The PCR offers certain advantages over classic techniques for diagnosis and characterization of infectious pathogens.⁹ When appropriately applied, the PCR can be more specific, sensitive, versatile, and rapid than conventional methods; in addition, genetic information can be obtained in the process.

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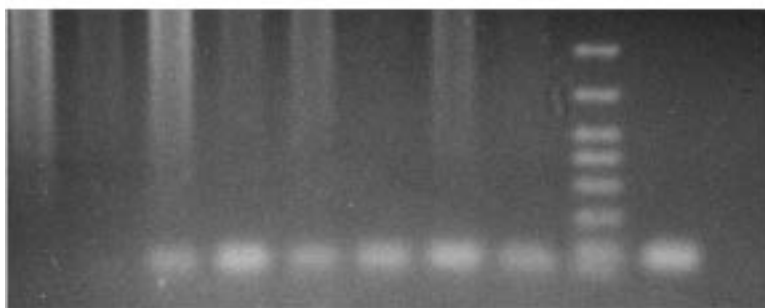


FIGURE 5. Polymerase chain reaction (PCR) amplification of *Leishmania braziliensis* (*L. braz.*) in peripheral blood mononuclear cells (PBMCs). Amplification of DNA extracted from human PBMCs was conducted with *L. braziliensis*-specific primers. Lanes 1 and 2, PBMCs from patient 13 (negative) undiluted and diluted 1:5, respectively; lanes 3 and 4, PBMCs from patient 03 undiluted and diluted 1:5, respectively; lanes 5 and 6, PBMCs from patient 04 undiluted and diluted 1:5, respectively; lanes 7 and 8, PBMCs from patient 08 undiluted and diluted 1:5, respectively; lane M, Amplisize DNA size standards (see Figure 2); lane 9, cultured *L. braziliensis* strain 91ZF11 (Nicaragua) prepared as in Figure 1; lane 10, negative amplification control (water). The expected product size was 70 basepairs (bp) for *L. braziliensis*.

Furthermore, simplification of procedures and in-house preparation of reagents can transform the PCR into a low-cost technology appropriate for use in situations of limited resources.^{10, 12, 27} The risk of sample cross-contamination, the principle disadvantage of the PCR, can be minimized by closely following a number of precautions. We use separate rooms and different sets of pipettors for 1) preparation of the PCR mixture, 2) sample preparation and DNA extraction, and 3) amplification and analysis of PCR products. Different gloves are used in each room and cleaned periodically throughout the work day with bleach, which cleaves DNA. Sodium hypochlorite is also used to cleanse work surfaces at the beginning and end of each day and to periodically treat pipettor shafts. All reagents are kept in small aliquots to avoid potential contamination of large volumes. Duplicate samples are amplified on different days to minimize the risk of artifactual results. Each amplification includes a number of negative controls, inhibition controls, and positive controls. Through strict adherence to these simple rules, we have avoided major contamination in years of routine diagnostic PCR work. Positive controls are always included in each amplification to ensure that the PCR is functioning correctly and to serve as a size control during gel electrophoresis to eliminate possible confusion with primer-dimer artifacts.

The PCR is particularly useful in the case of leishmaniasis, due to the requirement for parasitologic confirmation and to the limitations of classic methodologies. Demonstration of parasite DNA is an alternative to microscopic detection of the organism in clinical specimens or culture. Culture of lesion samples is complicated by the particular growth requirements of certain strains; for example, we found it difficult to culture parasites from an endemic region in southeastern Nicaragua in the same medium that was used successfully for the isolation of strains from the northern part of this country. Culture medium that is amenable to all strains, such as Schneider's *Drosophila* medium, is too expensive and therefore not feasible for routine use in our laboratory. In addition, strain preferences for particular growth conditions can result in inadvertent selection of specific isolates. Furthermore, long-term maintenance of the aseptic conditions required for culture can be difficult in tropical countries. Even under optimal conditions, the sensitivity of aspirate culture is reported as 58–83% and varies depending on a number of factors, including the species of *Leishmania*.^{28, 29}

Identification by PCR of the parasite at the molecular level is also an important advantage with leishmaniasis because different species can cause similar cutaneous lesions yet require distinct therapeutic regimens.³ In addition to its use at the clinical level, this information is epidemiologically relevant for identifying foci of active transmission and designing control strategies. Species identification using isoenzyme electrophoresis, the current gold standard, is an elaborate process that includes mass cultivation of the parasite, protein extraction and electrophoresis, and characterization of a number of enzymes.⁷ This technique is not feasible in many laboratories in endemic countries. Alternative methods for strain characterization include serodeme analysis with monoclonal antibodies;³⁰ however, this technique also requires cultured parasites and is less useful for direct analysis of clinical samples.

The PCR can be performed directly on clinical specimens and can be used for the simultaneous detection and typing of the parasite. By combining two existing amplification protocols,^{13, 19} we were able to simultaneously detect all *Leishmania* species and further identify those members of the *L. braziliensis* complex (Figure 2C). The PCR alone can be used for identification of *Leishmania* complexes, but does not yet replace zymodeme and serodeme analyses for characterization of *Leishmania* at the species level.

The PCR is also amenable to the analysis of different sample types. *Leishmania* isolates can be typed directly from culture without the need for DNA purification. Biopsy material can also be used for the PCR, following a simple extraction procedure.^{19, 20} While crude lysates can be prepared rapidly, potential inhibitors of the enzymatic amplification may not be eliminated. We found that a simple dilution procedure combined with the crude extraction protocol was sufficient to circumvent PCR inhibition while maintaining the required sensitivity (Figure 3). We have observed cases in which the PCR of biopsy material from presumptive cutaneous leishmaniasis yielded a positive result, while microscopy of specimens from the same lesion was not sensitive enough to detect the *Leishmania* parasites. Biopsies are the most commonly reported PCR specimen from cutaneous lesions^{19–22, 31, 32} and are the sample of choice for monitoring parasites in the lesion as an indicator of treatment efficacy. However, biopsies are traumatic for the patient and are associated with a high risk of bacterial infection. In addition, biopsy sampling requires experienced personnel and fairly expensive disposable punches or reusable scalpels, which require repeated sterilization.

Dermal scrapings are less invasive than biopsy samples, require less expertise, and can be taken with inexpensive, sterile, disposable lancets or toothpicks. In this study, we have shown that the PCR of dermal scrapings is more sensitive than direct microscopic examination. In an initial investigation, 12 of 12 smear-positive lesions were found positive by PCR while six of six nonleishmanial lesions were PCR-negative. When evaluated in a study population in an endemic area, PCR detection of leishmanial parasites in dermal scrapings was 100% sensitive and 100% specific when compared with microscopic detection ($n = 24$). The PCR resulted in a higher sensitivity than microscopy, detecting an additional 25% of the positive cases. This finding is consistent with previous reports that have found the PCR to be more sensitive than conventional methods for parasitologic detection in clinical specimens.^{19, 31–33} We used a multiplex protocol that simultaneously detects the *Leishmania* genus and types members of the *L. braziliensis* complex, thus ensuring that no leishmanial parasites were overlooked (e.g., *L. mexicana*) while permitting the identification of *L. braziliensis* (Figure 4).

Recently, it has been shown that *Leishmania* can be detected in PBMCs by the PCR. Most reports involve cases of visceral disease caused by members of the *L. donovani* complex^{21, 22, 34–37} (Videa E and Belli A, Nicaraguan Ministry of Health, unpublished data). However, one group has reported detection of *L. braziliensis* in blood samples by the PCR plus DNA probe hybridization.^{38–40} We have detected *L. braziliensis*-specific products by PCR analysis of PBMCs in a number of cases of active cutaneous leishmaniasis (Figure

5). *Leishmania braziliensis* has also been detected by the PCR in peripheral blood 10–30 years after treatment and healing of lesions³⁸ (Belli A, Rodriguez B, and Gomez A, Nicaraguan Ministry of Health, unpublished data). Reactivation of latent infection has been shown to be responsible for a large proportion of recurrent lesions in endemic areas.⁴¹ Furthermore, animal studies have demonstrated that *Leishmania* persist after treatment and that disease can be reactivated by immunosuppression.^{42, 43} Taken together, these findings imply that complete eradication of the parasite is difficult to achieve and suggest a number of applications for detection of *L. braziliensis* in PBMCs by PCR, including 1) monitoring blood supplies for the presence of *Leishmania* parasites, 2) investigating the presence of *L. braziliensis* in peripheral blood as a predictive factor for relapse, and 3) assessing therapeutic regimens for efficiency of parasite clearance.

There are numerous other uses for PCR detection of *Leishmania*. Direct identification of parasites in their sandfly vectors⁴⁴ furnishes crucial information regarding vector incrimination, which can help design behavioral control strategies. The PCR analysis of animal reservoirs can also serve an important epidemiologic role in disease control. We have implemented the PCR for routine diagnosis and preliminary strain characterization in the Leishmania Reference Laboratory of the Nicaraguan Ministry of Health. We are also using a *Leishmania* PCR in conjunction with a number of other techniques as part of a Central America-wide epidemiologic survey of leishmaniasis.²⁴ We have recently developed a multiplex PCR protocol for simultaneous characterization of all three *Leishmania* complexes in a single reaction (Harris E, Kropp G, Rodriguez B, Belli A, Agabian N, unpublished data). Thus, the PCR is a promising technique that can be appropriately adapted to provide important and timely information about leishmaniasis for clinical and epidemiologic purposes in endemic countries.

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