

PCR-Based Diagnosis of Acute and Chronic Cutaneous Leishmaniasis Caused by *Leishmania (Viannia)*

Kristen A. Weigle,¹ Luz Angela Labrada,^{2*} Caterin Lozano,² Cecilia Santrich,² and Douglas C. Barker³

*Department of Epidemiology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina*¹; *Corporacion CIDEIM, Cali, Colombia*²; and *MRC Outstation of NIMR, Molteno Laboratories, University of Cambridge, Cambridge, United Kingdom*³

Received 4 June 2001/Returned for modification 23 September 2001/Accepted 2 December 2001

We evaluated PCR methods for diagnosis of acute and chronic cutaneous leishmaniasis (CL) in an area of Colombia where *Leishmania (Viannia)* is endemic. The PCR method specifically amplified whole linearized minicircle kinetoplast DNA (kDNA) of the *Leishmania* subgenus *Viannia* from biopsy lysates. PCR products were detected in agarose gels. For 255 acute cases, this PCR method had greater sensitivity (75.7%) than each conventional method, i.e., microscopic examination of Giemsa-stained lesion scraping (46.7%), biopsy culture (55.3%), aspirate culture (46.3%), and the conventional methods combined (70.2%). Among 44 cases of chronic CL, amplification of biopsy DNA was more sensitive (45.5%) than the individual (4.5 to 27.7%) and combined (27.3%) conventional methods. The detection of kDNA in biopsies from chronic lesions was enhanced by a chemiluminescent dot blot hybridization, which produced a sensitivity of 65.8% when alone and 90.9% when in combination with DNA extraction of biopsy lysates ($P < 0.001$). Three biopsies from 84 skin lesions of other etiologies were falsely positive by PCR (specificity, 96.4%). PCR detected kDNA more frequently in biopsies (detection level, 83.9%) than in aspirates (74.7%) from 103 cases of acute CL. Among aspirates from 53 chronic cases of CL, the alternative methods, DNA extraction and hybridization, increased sensitivity from 41.5 to 56.6% ($P > 0.05$). This enhanced PCR method in chronic biopsies was so much more sensitive than conventional methods that it should be considered the preferred diagnostic method for chronic CL. These findings support the appropriate incorporation of PCR into diagnostic strategies for cutaneous leishmaniasis.

The leishmaniasis are a group of illnesses of the skin, oral and respiratory mucosae and the reticuloendothelium caused by protozoa of the genus *Leishmania*. Of these, the cutaneous form is the most widespread, afflicting primarily rural and periurban populations exposed to the infected sand fly vector. Cutaneous leishmaniasis (CL) is most frequently diagnosed by clinical evaluation, either alone or in combination with the leishmanin skin test. Clinical evaluation usually suffers from lack of standardization (13, 38) and is hampered by the fact that even fairly typical acute lesions can be confused with other dermatological problems, such as sporotrichosis (9). The leishmanin skin test is highly sensitive but lacks specificity when employed in areas of endemicity because it cannot distinguish acute lesions from past infection. A definitive, laboratory diagnosis of mucosal or cutaneous leishmaniasis traditionally requires either the visualization of amastigotes or the isolation of replicative *Leishmania* from lesions (24). The most widely employed laboratory methods for CL are microscopic examination of lesion scrapings, biopsy impression smears, and histopathology. The most sensitive conventional diagnostic methods, culture of lesion biopsies and aspirates, are available only in reference laboratories. Even these less available, more sensitive methods are positive in only 70 to 75% of acute cases when optimally performed (21, 38).

Due to these difficulties several new approaches to the diagnosis of CL were developed, including DNA probes and PCR (15, 29, 31). Of these, the PCR method is the most

promising (43). The minicircle of kinetoplast DNA (kDNA) and ribosomal DNA are ideal targets for amplification because they are present in multiple copies and have both conserved and variable regions (7, 18, 31, 36). Primers that amplify either segments of kDNA or the entire minicircle have great potential as diagnostic tools because they detect minute quantities of DNA and as little as 1/10 of a cultured *Leishmania* organism (7, 29). When applied to patient samples the PCR continues to show promise as a practical and sensitive diagnostic tool for CL (2, 8, 16, 20, 26, 30).

Despite these encouraging findings, essential questions remain to be solved before PCR can be incorporated routinely into diagnostic protocols for CL. Working with a large group of well-characterized patients, we addressed the following questions. (i) To what extent does PCR improve upon three currently available, conventional diagnostic methods for CL? (ii) When the PCR is positive but conventional methods are negative, do these patients truly have leishmaniasis or are the PCR results falsely positive? (iii) Does the type of specimen (biopsy or aspirate) affect the PCR results? (iv) How does PCR perform in detecting *Leishmania* spp. in chronic lesions? (v) How did alternative methods of sample preparation and product detection affect clinical performance of the PCR in chronic CL? We emphasized the application of PCR to chronic lesions because these patients provided the greatest diagnostic challenge. Chronic lesions are easily misdiagnosed by clinical criteria because they are often atypical, giving rise to terms such as carcinoma-like, lupoid, sporotrichoid, and verrucoid leishmaniasis (9, 23, 42). Moreover, due to the low density of *Leishmania* in chronic disease, conventional laboratory methods are much less sensitive in detecting the protozoans in lesions of more than 6 months' duration (38). Because we were

* Corresponding author. Mailing address: Corporacion CIDEIM, Avenida 1N #3-03, Centenario, Apartado Aéreo 5390, Cali, Colombia. Phone: 57 2 5581931. Fax: 57 2 6672989. E-mail: luzlabrada@hotmail.com.

interested in PCR methods that could be most readily used in settings of high endemicity, we utilized amplicon detection methods that did not require radioactive reagents and used relatively simple sample preparation methods.

MATERIALS AND METHODS

Study design. The sensitivities of PCRs and three conventional methods for diagnosis of CL were compared against a reference standard used to identify cases of CL among those evaluated, because none of the conventional methods is sufficiently sensitive to serve as a reference standard (28; W. C. Miller, Reply to letter, *Clin. Infect. Dis.* 27:1186–1193, 1998). The reference standard for cases of leishmaniasis consisted of combined laboratory criteria (conventional laboratory methods or supplemental laboratory methods) and previously evaluated clinical criteria (40). A case of CL was required to meet either the laboratory or the clinical criteria. For calculation of sensitivity the unit of analysis was the patient, not the specimen or test. The numbers of cases of CL identified by the reference standard provided the denominators of the sensitivity calculation. A case of CL was considered to be test positive for the tests being compared, PCR and the conventional methods, only if the specimen obtained during the patient's first pretreatment evaluation was positive. The numbers of these test-positive cases of CL provided the numerators of the sensitivity calculations.

Study population. All patients who presented to the CIDEIM clinics in Cali or Tumaco, Colombia, for diagnosis of their skin lesions between 30 August 1990 and 20 April 1994 were evaluated by clinical scores and by the conventional diagnostic methods prior to receiving antileishmanial treatment. Altogether 299 patients met the reference standard for CL and the inclusion criterion that their first specimens were evaluated by all three conventional diagnostic methods and PCR. Of these, 255 presented with acute lesions while 44 presented with chronic lesions.

Patients who did not meet the criteria of the reference standard could not be considered noncases due to the small possibility that they had undiagnosed leishmaniasis. Therefore, we identified a group of 84 noncases among those who presented either to the CIDEIM clinic in Cali for suspected leishmaniasis or to the Dermatology Clinic of the Hospital Universitario del Valle for evaluation of skin lesions due to other causes. The criteria for noncases were negative conventional exams for leishmaniasis, never having resided in an area where leishmaniasis is endemic, a negative reaction to the leishmanin skin test (39), and a laboratory-confirmed diagnosis of another etiology of their skin lesions. These other causes included sporotrichosis, leprosy, seborrheic keratosis, vascular ulcer, and basocellular carcinoma.

Specimen collection. After explaining the study and obtaining the patient's consent, a standardized history was recorded, a physical exam was performed, and the following samples were obtained as previously described (9, 24): four lesion scrapings, eight lesion aspirates, and two punch biopsies. Dermal scrapings were air dried, fixed in methanol, Giemsa stained, and examined for amastigotes under oil immersion. Four randomly chosen aspirates were cultured in Senekjie's medium; the other four were pooled and frozen at -70°C for later evaluation by PCR. One randomly chosen biopsy was fixed in formalin and examined as needed to confirm other etiologies. The other biopsy was divided longitudinally and randomized, and one half was processed for *Leishmania* culture, while the other half was stored at -70°C . Specimens collected for PCR in Tumaco were stored at 4°C and transported by air and frozen within 24 h.

Diagnostic methods. The three conventional methods, Giemsa-stained lesion scrapings, culture of lesion aspirates, and macerated biopsies in Senekjie's media (9, 24, 33), and PCRs were performed on clinical specimens collected during each patient's initial diagnostic evaluation. Bacterial contamination of *Leishmania* cultures was minimized by treating patients with obviously purulent or impetiginous lesions with systemic antibiotics and local debridement before obtaining specimens, by cleansing lesions with 70% alcohol, by using sterile procedures for biopsies and aspirates, and by adding penicillin G and streptomycin to the phosphate-buffered saline solution utilized in the aspiration and the biopsy maceration.

In order to apply the reference standard and thereby identify additional cases of CL, supplemental laboratory procedures were performed and clinical prediction rule criteria established in a prior study (40) were utilized when all conventional methods were negative for the initial clinical sample. The additional specimens were obtained and evaluated by the three assays described above and by inoculation of macerated biopsies into hamsters. Supplemental laboratory results were not used to determine if a case of CL was test positive. The clinical criteria for the reference standard were met if either of two previously evaluated scores was above their cut points: the Clinical_Historical_MN (Montenegro

skin test) score (≥ 15) or the Clinical_Historical score (≥ 12). The variables used to calculate these scores were collected on a standardized form using uniform definitions.

Preparation of specimens for PCR testing. One-fourth of each 4-mm-diameter punch biopsy was vortexed in 25 μl of lysis buffer (10 mM Tris-HCl [pH 8.0], 10 mM EDTA, and 50 μg of proteinase K [Boehringer-Mannheim]) for 10 s, digested at 65°C for 2 h, and then boiled for 15 min; the debris was sedimented at $12,000 \times g$ for 10 min (37). The supernatants were diluted 1:10 in Tris-EDTA (TE) buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) and stored at -20°C . Fifty microliters of lesion aspirates was precipitated at $12,000 \times g$ for 10 min. The pellet was resuspended in 30 μl of the lysis buffer containing 80 μg of proteinase K, and then it was lysed, diluted, and stored as described for biopsies. As an alternative method for sample preparation, we extracted DNA from selected biopsy and aspirate lysates using a rapid phenol-chloroform-isoamyl alcohol (25:24:1) extraction and precipitation in ethanol with 250 mM sodium acetate and 1 μg of glycogen per μl (32). The remaining pellet was dried in a vacuum desiccator and dissolved in 10 μl of TE buffer, and 1 μl (1 to 10 pg) of DNA was added to PCR vials.

Positive controls of kDNA were prepared from promastigotes of *Leishmania (Viannia) braziliensis* World Health Organization reference strain no. MHOM/BR/75/M2904, grown in Schneider's *Drosophila* medium at 28°C for 5 days, and extracted as previously described (24). To examine the specificity of these primers, kDNA was similarly extracted from *Leishmania* reference strains and Colombian *Leishmania* isolates.

PCR conditions. After the PCR conditions were optimized, as described in Results, the following routine methods were used for processing lysates and DNA extracts and compared to alternative methods. B1 and B2 primers used in this study specifically amplify the entire kDNA of *Leishmania (Viannia)* to a 750-bp band product (7). A reaction mixture was prepared in a 50- μl final volume containing 50 mM KCl, 1.5 mM MgCl_2 , 10 mM Tris-HCl (pH 8.3), 0.01% gelatin, a 0.2 mM concentration of each deoxyribonucleotide, 100 pmol of each primer, 2.5 U of Ampli-Taq DNA polymerase (Perkin-Elmer Cetus or Boehringer Mannheim), and either 3 μl of diluted biopsy lysate or 10 μl of diluted aspirate lysate. PCR amplification was carried out in a DNA thermal cycler (MJ Research) with a hot-start step to denature DNA for 5 min followed by 35 cycles of 95°C for 30 s and 60°C for 1 min and then a final prolonged cycle of 95°C for 1 min, 60°C for 3 min, and 72°C for 3 min. In the routine assays, 10 μl of the amplification products was analyzed by agarose gel electrophoresis, ethidium bromide staining, and visualization under UV light and recorded on Polaroid film (8).

Precautions taken to avoid contamination during specimen collection included use of disposable gloves, vials, biopsy punch, needles, syringes, and scalpels and use of DNA-free solutions. The risk of amplicon contamination was minimized by designating four separate areas as already described (11). Lots of PCR mixture were prepared, tested for contamination by PCR, and stored at -20°C in aliquots, to avoid repeated handling.

The detection limit of the PCR was monitored with low-copy-number positive controls, 1.0 and 0.1 fg of kDNA, in each assay. If a PCR product appeared less dense than the product of the 0.1-fg positive control, it was considered to be weakly positive and the assay was repeated. Only those products that remained weakly positive or positive on repeat testing were considered positive. A negative control consisting of the reaction mixture was tested for every 15 clinical specimens to monitor contamination. In each assay up to 30 clinical specimens (which always included negative specimen controls [3] from noncases) were evaluated without knowledge of patient number, group, or the results of other diagnostic methods, by assigning random numbers to each specimen.

Dot blot hybridization and chemiluminescent detection. The probe was prepared from kDNA, extracted from amplification product of *L. (V.) braziliensis* promastigotes, labeled with biotin using random primers according to the manufacturer's instructions (BIO-PRIME labeling system; GIBCO-BRL, Grand Island, N.Y.). We denatured 25 μl of PCR products with an equal volume of 0.5 M NaOH–1.5 M NaCl for 10 min at room temperature, neutralized them with 50 μl of 2 M ammonium acetate (pH 8.0) on ice for 10 min, and then spotted them onto a nylon membrane (GIBCO-BRL) presoaked in water. The DNA was fixed to the membranes by UV cross-linking. Prehybridization and hybridization conditions were performed under conditions recommended by the manufacturer (Photogene Nucleic Acid Detection System; GIBCO-BRL), and results were visualized on X-ray film.

Data analysis. Results of clinical evaluation, diagnostic tests, and PCR were analyzed using Statistical Package for the Social Sciences software. The sensitivity (test positives/cases of CL identified by reference standard) of PCR of specimens taken during the patients' first evaluations was compared to that of each conventional method. The sensitivity of PCR was also compared to the

TABLE 1. Comparison of PCR of skin biopsies with conventional diagnostic methods for cutaneous leishmaniasis, by lesion duration

Lesion duration (mo)	Reference standard criteria	% Sensitivity (no. of cases testing positive ^a /no. of cases evaluated ^b)				
		PCR	Lesion scraping	Biopsy culture	Aspirate culture	Conventional methods combined
<6	Laboratory	83.9 (177/211)	56.4 ^c (119/211)	66.8 ^c (141/211)	55.9 ^c (118/211)	84.8 (179/211)
<6	Clinical score	36.4 (16/44)	0.0 ^c (0/44)	0.0 ^c (0/44)	0.0 ^c (0/44)	0.0 ^c (0/44)
<6	Combined	75.7 (193/255)	46.7 ^c (119/255)	55.3 ^c (141/255)	46.3 ^c (118/255)	70.2 ^d (179/255)
≥6	Laboratory	60.9 (14/23)	8.7 ^c (2/23)	43.5 (10/23)	34.8 ^d (8/23)	52.2 (12/23)
≥6	Clinical score	28.6 (6/21)	0.0 ^c (0/21)	0.0 ^c (0/21)	0.0 ^c (0/21)	0.0 ^c (0/21)
≥6	Combined	45.5 ^d (20/44)	4.5 ^c (2/44)	22.7 ^c (10/44)	18.2 ^c (8/44)	27.3 ^d (12/44)

^a No. of patients whose lesions were test positive by one or more of the three conventional methods.

^b No. of patients who were evaluated initially by all three conventional methods (lesion scraping, biopsy culture, and aspirate).

^c $P \leq 0.01$, McNemar chi-square test, comparison with PCR.

^d $P \leq 0.05$, McNemar chi-square test, comparison with PCR.

sensitivity of the combined conventional methods. The combined conventional methods were considered test positive if any of the three conventional methods was test positive. The group of noncases served to identify false-positive PCR results and estimate the specificity of the routine PCR (test negative/noncases). Statistical methods included estimation of sensitivity and specificity as described above and comparing the sensitivity of PCR with that of other methods using the McNemar chi-square test, because the pairs of tests were performed on specimens from the same patient (10). One-sided hypothesis tests were performed to test the hypothesis that the routine PCR was more sensitive than the individual and combined conventional methods.

RESULTS

Biological specificity. These primers were previously demonstrated to amplify four species (*panamensis*, *peruviana*, *braziliensis*, and *guyanensis*) of *Leishmania* subgenus *Viannia* but not *Leishmania* subgenus *Leishmania* *amazonensis*, *L. (L.) mexicana*, *L. (L.) tropica*, *L. (L.) major*, *L. (L.) chagasi*, *L. (L.) donovani*, or *L. (L.) infantum* (7). We confirmed these specificities and also demonstrated that these primers did not amplify human DNA or the DNA from pathogens in the differential diagnosis of CL: *Sporothrix schenckii*, *Trypanosoma cruzi*, *Escherichia coli*, *Staphylococcus aureus*, *Mycobacterium tuberculosis*. To assess whether these primers amplified all *Leishmania (Viannia)* organisms from this region of Colombia, they were evaluated with lysed promastigotes of 10 strains of *L. (V.) guyanensis*, 45 strains of *L. (V.) panamensis*, and 11 strains of *L. (V.) braziliensis*, which represent the range of enzyme variants isolated from Colombian patients. Only 2 of these 66 strains were not amplified even after repeat testing; both were *L. (V.) panamensis*.

Optimization of PCR. Amplification of 126 lysates of lesion biopsies indicated that detection limits lower than the 1 to 10 fg reported earlier (7) would be needed. This was achieved by beginning the PCR with a hot start to ensure complete denaturation prior to the first prolonged annealing cycle (25), increasing the number of cycles from 30 to 35 and prolonging the last cycle to 3 min each of annealing and extension. These enhanced conditions consistently amplified 0.1 fg of kDNA. Forty-one biopsies from cases of CL sensitivity increased from 44 to 78% ($P = 0.0015$). These modified PCR conditions were employed in subsequent routine amplifications.

PCR of skin biopsies. The sensitivity of the routine PCR method for lesion biopsies was compared with that of each of the three conventional methods and of the methods combined (Table 1). Because the conventional methods are known to be

less sensitive in chronic lesions, i.e., of 6 or more months' duration (38), the comparisons are presented separately for patients with acute and chronic lesions. For acute CL, the PCR was much more sensitive than any single conventional method. The sensitivity of the PCR (75.7%) was slightly greater than that of the conventional methods combined (70.2%) in acute CL. However, for chronic CL the sensitivity of PCR (45.5%) far exceeded the sensitivities of both the single and combined conventional methods, which ranged from 27.3 to 4.5%. Inclusion of the clinical score as a criterion for the reference standard allowed the assessment of the gain in sensitivity afforded by PCR in 65 cases of CL that were negative by both conventional and supplemental laboratory methods. Bacterial contamination was observed in the aspirate and/or biopsy cultures of only 10 of the 108 patients with negative results by the combined conventional methods.

Among the biopsies from the 84 patients with diagnoses other than leishmaniasis, three specimens were considered to be false positives: two positives and one weakly positive that remained weakly positive on repeat testing, resulting in an overall specificity of 96.4% (81/84) for this PCR. In order to understand the origin of these false positives, both stored lysates and newly prepared lysates were retested. Two of the three stored lysates were weakly positive when retested, but none of the three fresh lysates were positive, indicating that contamination had occurred during lysate preparation or use, but not during sample collection.

Alternative PCR methods for biopsies. In order to achieve a greater sensitivity of the PCR for chronic lesions, we employed DNA extraction and chemiluminescent dot blot hybridization

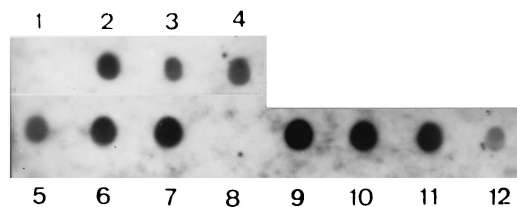


FIG. 1. Analysis of PCR products by chemiluminescent dot blot hybridization. Dot 1, PCR control; dots 2 and 3, PCR products of 1.0 fg and 0.1 fg, respectively, of *Leishmania (Viannia)* kDNA; dots 4 to 12, PCR products from biopsy lysates of chronic lesions of patients 1847, 2996, 1791, 1813, 1837, 1839, 4055, 2932, and 4091, respectively. Dots 1 and 8 were read as negative; the remainder were read as positive.

TABLE 2. Alternative methods for sample preparation and detection of PCR product from chronic lesions

Specimen	Sample preparation	Product detection	No. of cases testing positive by PCR/no. of cases evaluated ^a (%)
Biopsy	Diluted lysate ^b	Agarose gel ^b	15/33 (45.5)
	Diluted lysate	Dot blot	21/33 (63.6)
	DNA extract	Agarose gel	13/33 (39.4)
	DNA extract	Dot blot	30/33(90.9 ^c)
Aspirate	Diluted lysate ^b	Agarose gel ^b	22/53 (41.5)
	Diluted lysate	Dot blot	27/53 (50.9)
	DNA extract	Agarose gel	28/53 (52.8)
	DNA extract	Dot blot	30/53 (56.6)

^a No. of cases evaluated initially by all three conventional methods.

^b Routine methods.

^c In chi-square test for multiple proportions, $P < 0.01$ compared with all other combinations of biopsy sample preparation and product detection methods.

(Fig. 1). When the PCR products were evaluated by this hybridization dot blot, the PCR detection limit was consistently 0.01 fg of kDNA. We compared two options for detection of the PCR products: the dot blot hybridization and the routine agarose gel in combination with two sample preparation methods, DNA extraction and the routine method, a diluted lysate. Detection of the products by dot blot hybridization improved the sensitivity of the PCR, regardless of how the sample was prepared (Table 2), whereas DNA extraction improved sensitivity only when the product was detected by dot blot.

PCR of lesion aspirates. Because biopsy specimens are invasive and sometimes unaccepted (14, 17), we also evaluated aspirate specimens as PCR targets. After trials of several alternatives for sample preparation, we selected the method of diluting the aspirate lysates 1:10 in TE buffer and adding 10 μ l of the diluted lysate to the PCR vial because undiluted aspirate lysates were very inhibitory, presumably due to their high hemoglobin content. This routine method amplified the target kDNA in 80 of 103 (77.7%) aspirates from a random sample of cases of CL but in none of the aspirates for nine noncases. Among aspirates from patients with acute CL this routine PCR was more sensitive than the lesion scraping and aspirate culture methods (Table 3). PCR detected kDNA more frequently in biopsies (83.9%) than in aspirates (74.7%) from these 103 cases of acute CL ($P < 0.05$). For cases of chronic CL, PCR of aspirate samples did not improve upon the low sensitivity of conventional methods.

Alternative methods for lesion aspirates. We examined the effect of DNA extraction in combination with two methods for amplicon detection (either dot blot hybridization or the routine agarose gel) on the PCR results of aspirates from 53

chronic lesions (Table 2). The net effect of these three alternatives was an increased sensitivity, ranging from 50 to 56%, relative to the 40% sensitivity of the routine PCR of aspirates from chronic CL cases.

DISCUSSION

We modified an existing PCR method and determined the extent to which PCR could improve upon the sensitivity of conventional diagnostic methods for CL. Because diagnosis is more challenging for chronic CL, we examined the performances of PCR methods separately for acute and chronic cases. We emphasized detection of kDNA in biopsy specimens, because these are more frequently obtained in areas where leishmaniasis is endemic. For acute CL, the routine PCR method applied to biopsies was more sensitive than each of the three individual conventional methods and the combination of the three conventional methods. PCR of biopsies from chronic lesions was twice as sensitive as conventional methods. DNA extraction and product detection by a dot blot hybridization greatly enhanced the ability of this PCR to detect kDNA in chronic lesions. This enhanced PCR method was so much more sensitive than conventional methods that it should be considered the preferred diagnostic method for chronic CL.

The advantages of PCR for the detection of *Leishmania* is most striking in clinical specimens with scarce amastigotes, for which conventional methods are very insensitive. PCR detected *Leishmania* (*Viannia*) in scars of healed treated cases of CL (34). Pirmez et al. found PCR to have its greatest impact in the diagnosis of mucosal lesions, where conventional methods are very insensitive due to the paucity of amastigotes and frequency of bacterial contamination (26). Diagnosis of chronic CL of the Old World, lupoid leishmaniasis, caused by *L. major* and *L. tropica*, is also hampered by the scarcity of amastigotes. A PCR method using paraffin-embedded biopsies was reported to detect kDNA in 53% of 20 culture-positive cases and 48% of clinically suspect cases of lupoid leishmaniasis (19). The PCR method was more sensitive than histopathology. Half of the false-negative PCRs were attributed to poor stability of DNA in paraffin-embedded biopsies.

The two types of specimens useful for the diagnosis of CL, aspirates and biopsies, have not been previously compared as targets for PCR. For acute CL, PCR of aspirates was more sensitive than two conventional assays; however, PCR detected kDNA more frequently in biopsies than in aspirates. Therefore, aspirates should be used as amplification targets only for patients with acute CL for whom biopsy is unacceptable or not feasible (4). For acute CL, dermal scrapings from the bottom of the ulcer may yield viable specimens for PCR, although

TABLE 3. Comparison of PCR of lesion aspirates with conventional diagnostic methods for leishmaniasis, by lesion duration

Lesion duration (mo)	Reference standard criteria	% Sensitivity (no. of cases testing positive ^a /no. of cases evaluated ^b)				
		PCR	Lesion scraping	Biopsy culture	Aspirate culture	Conventional methods combined
<6	Laboratory	77.7 (80/103)	46.6 ^c (48/103)	68.9 (71/103)	58.3 ^c (60/103)	87.4 (90/103)
≥6	Laboratory	38.1 (8/21)	4.8 ^c (1/21)	42.8 (9/21)	33.3 (7/21)	52.4 (11/21)

^a No. of patients whose lesions were positive by one or more of the three conventional methods.

^b No. of patients who were evaluated initially by all three conventional methods.

^c $P \leq 0.01$, McNemar chi-square test, comparison with PCR.

results of this method have not been compared with those obtained with other types of specimens (27).

The lack of an ideal "gold standard" for CL poses problems for estimating the sensitivity and specificity of PCR methods (27, 43; Miller, reply to letter). Some studies have used all suspect cases as the gold standard (16, 26, 30), which may be of low sensitivity and low specificity, depending on the diversity of clinical cases (12). Others have used conventional methods, which lack sensitivity (6, 27). Because PCR methods can have extremely low detection limits, they can detect kDNA in specimens that are negative by conventional methods. If insensitive conventional methods are used to define cases, then the potential gain in sensitivity afforded by PCR cannot be determined. The question "When the PCR is positive but the conventional methods are negative, does the patient have leishmaniasis or is this a false-positive PCR?" is challenging. Discrepant analysis, which uses a third assay to decide these discrepant cases, is frequently employed but is inherently biased (6; Miller, reply to letter). We reduced these problems by recognizing that the conventional methods were inadequate reference standards. Instead, we defined a reference standard that followed guidelines for an expanded gold standard (Miller, reply to letter) and exploited the strength of multiple tests: conventional assays, supplemental laboratory assays, and a sensitive clinical score. As recommended, this reference standard was applied to all patients, not only to discrepant cases. To assess specificity we identified a group of noncases that had lesions caused by other etiologies.

The relative gain in sensitivity achieved by PCR varies according to the conventional methods to which this procedure is compared (4). When compared to microscopic methods requiring abundant parasites, the PCR uniformly produces a marked gain in sensitivity (16, 30). When compared to *Leishmania* culture, the relative gain in sensitivity achieved by PCR depends on the degree to which culture contamination was avoided. In four studies in which cultures were frequently contaminated, PCR appeared much more sensitive than cultures (16, 26, 30, 34). Our measures to decrease bacterial contamination of cultures may have increased the sensitivity of culture methods and minimized relative gain in sensitivity achieved by PCR to a degree that cannot be generalized to many settings.

Regular monitoring of specificity of PCRs for leishmaniasis by processing blinded, negative specimen controls in parallel with all steps utilized for clinical specimens and in each assay warrants serious consideration (11, 25). To date, other evaluations of PCR for CL have included reaction controls, but not specimen controls (2, 4, 6, 8, 12, 16, 26, 27, 30, 34). A blinded evaluation of samples distributed through a World Health Organization-sponsored *Leishmania* PCR network indicated that false positives were unexpectedly high within several of the participating molecular biology laboratories in Latin America (L. Labrada, personal communication). In another study in which 30 laboratories were sent blinded samples for evaluation by PCR for *Mycobacterium tuberculosis*, the false-positive rate ranged from 0 to 80% (22). It appears that the negative-reaction controls used by these laboratories were not sufficient to detect what occurred during sample preparation. These multi-institutional studies demonstrate that a system of exter-

nal quality control is required before PCR methods can be routinely incorporated into diagnostic strategies (22).

The advantages of PCR for the diagnosis of leishmaniasis should be considered in the context of the existing diagnostic services for CL and the clinical and epidemiological patterns (5, 41). If most patients reside at some distance from reference laboratories, the logistical problem of storing and shipping frozen biopsies could be avoided by performing PCR on specimens stored in lysis buffer at ambient temperatures, in ethanol, in paraffin-embedded biopsies, or on stained smears (1, 14, 19, 35). However, these more logistically feasible methods of collecting and storing *Leishmania* PCR targets require further evaluation before they can be used. Where an active case detection program regularly identifies early cases, a microscopic evaluation may suffice, provided that species identification is not required for case management (27). PCR should be used selectively, for chronic cases or when other assays are negative. In areas in which several *Leishmania* species that require different management are endemic, a PCR that both detects and identifies kDNA should be utilized routinely (12, 26, 28).

ACKNOWLEDGMENTS

This study was supported by the National Institutes of Health grant P50-AI30603, the U.S. Public Health Service, and COLCIENCIAS project 22-90-400-392. D. C. Barker was supported by the Medical Research Council (United Kingdom).

We are grateful to the members of the Clinical Units of CIDEIM for provision of specimens and clinical data and for performing the conventional diagnostic methods under the direction of G. Palma and C. Rojas, including M. Escobar, L. Rosas, and J. C. Sepulveda. We thank W. Lopez, W. Cortes, A. L. Arias, D. Mejia, and M. C. Acosta for their technical assistance in laboratory procedures. We are indebted to L. Valderrama, D. Lorena, and E. Muñoz of CIDEIM's Biometry Unit for managing data files and calculating the clinical scores. We thank the Dermatology Clinical Unit of the Hospital Universidad del Valle for provision of selected biopsy specimens, M. Lopez and J. Arevalo (Universidad Peruviana Cayetano Heredia, Lima, Peru) and N. Rodríguez (Instituto de Biomedicina, Caracas, Venezuela) for their technical advice, and Helen Santrich and Anne Bean for their secretarial assistance. Finally, we are most grateful to N. Saravia for her critical suggestions and encouragement.

REFERENCES

1. Alger, J., M. C. Acosta, C. Lozano, C. Velasquez, and L. A. Labrada. 1996. Stained smears as a source of DNA. Mem. Inst. Oswaldo Cruz 91:589-591.
2. Andresen, K., A. Gaafar, A. M. El-Hassan, A. Ismail, M. Dafalla, T. G. Theander, and A. Kharazmi. 1996. Evaluation of the polymerase chain reaction in the diagnosis of cutaneous leishmaniasis due to *Leishmania major*: a comparison with direct microscopy of smears and sections from lesions. Trans. R. Soc. Trop. Med. Hyg. 90:133-135.
3. Arévalo, J., R. Inga, and M. López. 1993. PCR detection of *Leishmania braziliensis*, p. 456-461. In D. H. Persing, T. F. Smith, F. C. Tenover, and T. J. White (ed.), Diagnostic molecular microbiology: principles and applications. American Society for Microbiology, Washington, D.C.
4. Aviles, H., A. Belli, R. Armijos, F. Monroy, and E. Harris. 1999. PCR detection and identification of *Leishmania* parasites in clinical specimens in Ecuador: a comparison with classical diagnostic methods. J. Parasitol. 85: 181-187.
5. Barker, R. H., Jr. 1994. Use of PCR in the field. Parasitol. Today 10:117-119.
6. Belli, A., B. Rodriguez, H. Aviles, and E. Harris. 1998. Simplified polymerase chain reaction detection of new world *Leishmania* in clinical specimens of cutaneous leishmaniasis. Am. J. Trop. Med. Hyg. 58:102-109.
7. de Bruijn, M. H. L., and D. C. Barker. 1992. Diagnosis of new world leishmaniasis: specific detection of species of the *Leishmania braziliensis* complex by amplification of kinetoplast DNA. Acta Trop. 52:45-58.
8. de Bruijn, M. H. L., L. A. Labrada, A. J. Smyth, C. Santrich, and D. C. Barker. 1993. A comparative study of diagnosis by the polymerase chain reaction and by current clinical methods using biopsies from Colombian patients with suspected leishmaniasis. Trop. Med. Parasitol. 44:201-207.

9. Escobar, M. A., F. Martinez, D. S. Smith, and G. I. Palma. 1992. American cutaneous and mucocutaneous leishmaniasis (tegumentary): a diagnostic challenge. *Trop. Doc.* **22**(Suppl. 1):69-78.
10. Fleiss, J. L. 1981. Statistical methods for rates and proportions. John Wiley & Sons, New York, N.Y.
11. Fredricks, D. N., and D. A. Relman. 1999. Application of polymerase chain reaction to the diagnosis of infectious diseases. *Clin. Infect. Dis.* **29**:475-488.
12. Harris, E., G. Kropp, A. Belli, B. Rodriguez, and N. Agabian. 1998. Single-step multiplex PCR assay for characterization of New World *Leishmania* complexes. *J. Clin. Microbiol.* **36**:1989-1995.
13. Kubba, R., Y. Al-Gindan, A. M. El-Hassan, and A. H. S. Omer. 1987. Clinical diagnosis of cutaneous leishmaniasis (oriental sore). *J. Am. Acad. Dermatol.* **16**:1183-1189.
14. Laskay, T., T. L. Mikó, Y. Negesse, W. Solback, M. Rölinghoff, and D. Frommel. 1995. Detection of cutaneous *Leishmania* infection in paraffin-embedded skin biopsies using the polymerase chain reaction. *Trans. R. Soc. Trop. Med. Hyg.* **9**:273-275.
15. Lopez, M., Y. Montoya, M. Arana, F. Cruzalegui, J. Braga, A. Llanos-Cuentas, G. Romero, and J. Arevalo. 1988. The use of nonradioactive DNA probes for the characterization of *Leishmania* isolates from Peru. *Am. J. Trop. Med. Hyg.* **38**:308-314.
16. Lopez, M., R. Inga, M. Cangalaya, J. Echevarria, A. Llanos-Cuentas, C. Orrego, and J. Arevalo. 1993. Diagnosis of *Leishmania* using the polymerase chain reaction: a simplified procedure for field work. *Am. J. Trop. Med. Hyg.* **49**:348-356.
17. Matsumoto, T., Y. Hashiguchi, E. A. Gomez, M. H. Calvopiña, S. Nonaka, H. Saya, and T. Mimori. 1999. Comparison of PCR results using scrape/exudate, syringe-sucked fluid and biopsy samples for diagnosis of cutaneous leishmaniasis in Ecuador. *Trans. R. Soc. Trop. Med. Hyg.* **93**:606-607.
18. Meredith, S. E. O., E. E. Zijlstra, G. J. Schoone, C. C. M. Kroon, G. J. J. M. van Eys, K. U. Schaeffer, A. M. El-Hassan, and P. G. Lawyer. 1993. Development and application of the polymerase chain reaction for the detection and identification of *Leishmania* parasites in clinical material. *Arch. Inst. Pasteur Tunis* **70**:419-431.
19. Mimori, T., J. Sasaki, M. Nakata, E. A. Gomez, H. Uezato, S. Nonaka, Y. Hashiguchi, M. Furuya, and H. Saya. 1998. Rapid identification of *Leishmania* species from formalin-fixed biopsy samples by polymorphism-specific polymerase chain reaction. *Gene* **210**:179-186.
20. Momeni, A. Z., S. Yotsumoto, D. R. Mehregan, A. H. Mehregan, D. A. Mehregan, M. Aminjavaheri, H. Fujiwara, and J. Tada. 1996. Chronic lupoid leishmaniasis. Evaluation by polymerase chain reaction. *Arch. Dermatol.* **132**:198-202.
21. Navin, T. R., F. E. Arana, A. M. de Merida, B. A. Arana, A. L. Castillo, and D. N. Silvers. 1990. Cutaneous leishmaniasis in Guatemala: comparison of diagnostic methods. *Am. J. Trop. Med. Hyg.* **42**:36-42.
22. Noordhoek, G. T., J. D. A. van Embden, and A. H. J. Kolk. 1996. Reliability of nucleic acid amplification for detection of *Mycobacterium tuberculosis*: an international collaborative quality control study among 30 laboratories. *J. Clin. Microbiol.* **34**:2522-2525.
23. Oliveira-Neto, M. P., M. Mattos, C. S. Souza, O. Fernandes, and C. Pirmez. 1998. Leishmaniasis recidiva cutis in New World cutaneous leishmaniasis. *Int. J. Dermatol.* **37**:846-849.
24. Palma, G., and Y. Gutierrez. 1991. Laboratory diagnosis of *Leishmania*. *Clin. Lab. Med.* **11**:909-922.
25. Persing, D. H., and G. D. Cimino. 1993. Amplification product inactivation methods, p. 114-115. *In* D. H. Persing, T. F. Smith, F. C. Tenover, and T. J. White (ed.), *Diagnostic molecular microbiology: principles and applications*. American Society for Microbiology, Washington, D.C.
26. Pirmez, C., V. da Silva Trajano, M. Paes-Oliveira Neto, A. M. Da-Cruz, S. C. Goncalves-da-Costa, M. Catanho, W. Degraive, and O. Fernandes. 1999. Use of PCR in diagnosis of human American tegumentary leishmaniasis in Rio de Janeiro in Brazil. *J. Clin. Microbiol.* **37**:1819-1823.
27. Ramirez, J. B., S. Agudelo, C. Muskus, J. F. Alzate, C. Berberich, D. Barker, and I. D. Velez. 2000. Diagnosis of cutaneous leishmaniasis in Columbia: the sampling site within lesions influences the sensitivity of parasitologic diagnosis. *J. Clin. Microbiol.* **38**:3768-3773.
28. Ransohoff, D. F., and A. R. Feinstein. 1978. Problems of spectrum and bias in evaluating the efficacy of diagnostic tests. *N. Engl. J. Med.* **299**:926-930.
29. Rodgers, M. R., S. J. Popper, and D. F. Wirth. 1990. Amplification of kinetoplast DNA as a tool in the detection and diagnosis of *Leishmania*. *Exp. Parasitol.* **71**:267-275.
30. Rodríguez, N., B. Guzman, A. Rodas, H. Takiff, B. R. Bloom, and J. Convit. 1994. Diagnosis of cutaneous leishmaniasis and species discrimination of parasites by PCR and hybridization. *J. Clin. Microbiol.* **32**:2246-2252.
31. Rogers, W. O., and D. F. Wirth. 1987. Kinetoplast DNA minicircles: regions of extensive sequence divergence. *Proc. Natl. Acad. Sci. USA* **84**:565-569.
32. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
33. Schubach, A., F. Haddad, N. M. P. Oliveira, W. Degraive, C. Pirmez, G. Grimaldi, and O. Fernandes. 1998. Detection of *Leishmania* DNA by polymerase chain reaction in scars of treated human patients. *J. Infect. Dis.* **178**:62-66.
34. Senekjic, H. 1943. Biochemical reactions, culture characteristics and growth requirements of *Trypanosoma cruzi*. *Am. J. Trop. Med. Hyg.* **23**:523-531.
35. Uezato, H., K. Hagiwara, A. Hosokawa, M. Maruno, S. Nonaka, M. Oshiro, Y. Nakashima, M. Furuya, and Y. Hashiguchi. 1998. Comparative studies of the detection rates of *Leishmania* parasites from formalin, ethanol-fixed, frozen human skin specimens by polymerase chain reaction and Southern blotting. *J. Dermatol.* **25**:623-631.
36. Uliana, S. R. B., K. Nelson, S. M. Beverley, E. P. Camargo, and L. M. Floeter-Winter. 1994. Discrimination amongst *Leishmania* by polymerase chain reaction and hybridization with small subunit ribosomal DNA derived oligonucleotides. *J. Eukaryot. Microbiol.* **41**:324-330.
37. Warhurst, D. C., F. M. Awad el Kariem, and M. A. Miles. 1991. Simplified preparation of malarial blood samples for polymerase chain reaction. *Lancet* **337**:303-304.
38. Weigle, K. A., M. de Dávalos, P. Heredia, R. Molineros, N. G. Saravia, and A. D'Alessandro. 1987. Diagnosis of cutaneous and mucocutaneous leishmaniasis in Colombia: a comparison of seven methods. *Am. J. Trop. Med. Hyg.* **36**:489-496.
39. Weigle, K. A., L. Valderrama, A. L. Arias, C. Santrich, and N. G. Saravia. 1991. Leishmanin skin test standardization and evaluation of safety, dose, storage of reaction and sensitization. *Am. J. Trop. Med. Hyg.* **44**:260-271.
40. Weigle, K. A., M. Escobar, A. L. Arias, F. Martinez, and C. Rojas. 1993. A clinical prediction rule for American cutaneous leishmaniasis in Colombia. *Int. J. Epidemiol.* **21**:548-558.
41. Weigle, K. A., C. Santrich, F. Martinez, L. Valderrama, and N. G. Saravia. 1993. Epidemiology of cutaneous leishmaniasis in Colombia: a longitudinal study of the natural history, prevalence, and incidence of infection and clinical manifestations. *J. Infect. Dis.* **168**:699-708.
42. Weigle, K. A., and N. G. Saravia. 1996. Natural history, clinical evolution, and the host-parasite interaction in new world cutaneous leishmaniasis. *Clin. Dermatol.* **14**:433-450.
43. Wilson, S. M. 1995. DNA-based methods in the detection of *Leishmania* parasites: field applications and practicalities. *Ann. Trop. Med. Parasitol.* **89**:95-100.