MAJOR ARTICLE

Clinical Utility of Polymerase Chain Reaction–Based Detection of *Leishmania* in the Diagnosis of American Cutaneous Leishmaniasis

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We evaluated the use of polymerase chain reaction (PCR) for diagnosis of American cutaneous leishmaniasis (ACL) in an area in Bahia, Brazil, where *Leishmania braziliensis* is endemic. *Leishmania* DNA was detected in 50 cases, yielding a positivity rate of 100%, which was higher than the rates for all of the other diagnostic methods studied—namely, the Montenegro skin test, anti-*Leishmania* serological testing, and microscopic examination of lesion biopsy specimens. These findings have led us to propose guidelines for the diagnosis of ACL that use PCR as the principal means of parasitological confirmation of cases.

Leishmania, a protozoan parasite, is the cause of leishmaniasis, a human disease with diverse clinical manifestations. An estimated 12 million people are currently infected with *Leishmania* species, whereas another 350 million people live at risk of infection [1]. American cutaneous leishmaniasis (ACL) is characterized by a cutaneous ulcer with elevated borders and a sharp crater, which, when caused by parasites of the *Leishmania braziliensis* complex, may spontaneously heal, disseminate, or metastasize to the nasopharyngeal tract. When metastasis occurs, severe disfigurement and secondary complications may also occur, with a fatal outcome [2]. Therefore, it is important to have an accurate diagnosis of infection to avoid the unnecessary use of drugs that, at this moment, still involve high costs and significant toxicity. Furthermore, it is most important to avoid the development of a long-lasting chronic disease, which is common in infections caused by New World *Leishmania* parasites.

Definitive diagnosis of ACL is made by demonstration of *Leishmania* parasites in tissue samples or by culture or by animal inoculation of material extracted from lesions. These methods are time-consuming and require experienced personnel, as well as special facilities. The main drawback of these methods, however, is the low sensitivity. To overcome such difficulties, new molecular approaches to the diagnosis of leishmaniasis have been developed [3]. Among these, the most widely used is PCR. In fact, a number of PCR assays have been developed for the detection of *Leishmania* species in clinical samples [4–9]. These assays target the amplification of rRNA, miniexon genes, kinetoplast DNA (kDNA), and repetitive nuclear DNA sequences. These methods have varying specificities: some identify the parasite to the level of species complex, whereas others identify all *Leishmania* species. This information is sufficient for diagnostic purposes; however, higher specificity can contribute directly to understanding the epidemiology of leishmaniasis.

Several research groups have examined the use of PCR as a diagnostic tool in areas where leishmaniasis is endemic; however, despite the many favorable results, diagnosis of leishmaniasis is still based on indirect tests,

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such as serological tests and the Montenegro skin test. The aim of the present study was to evaluate the contribution of PCR to the diagnosis of ACL and, on the basis of the results we obtained, to provide experimental evidence of the effectiveness of this detection tool.

PATIENTS, MATERIALS, AND METHODS

Study area and patients. Biopsy specimens were obtained from patients with ACL who attended the Pirajá da Silva Reference Center (PIEJ) for endemic diseases during the period of September 2000 through April 2001. The PIEJ is located in the town of Jequié, where *L. braziliensis* is endemic; Jequié is in southeastern Bahia, Brazil. The PIEJ is responsible for treating patients with ACL from ⩾20 small nearby municipalities.

Research was conducted in accordance with the Fundação Oswaldo Cruz (FIOCRUZ) guidelines for human experimentation and the Brazilian Ministry of Health regulations for research involving humans. Clinical research was approved by the Ethics Committee of the Centro de Pesquisas Gonçalo Muniz–FIOCRUZ (Salvador, Brazil), and informed consent was obtained from all individuals enrolled in the study. All patients underwent a complete physical examination, as well as clinical and laboratory evaluations. The Montenegro skin test, in which a delayed-type hypersensitivity reaction is provoked, was performed with a soluble *Leishmania* antigen that was prepared as described elsewhere [10], and *Leishmania* parasites were used to perform an indirect immunofluorescense assay (IFA) test, to detect the presence of anti-*Leishmania* antibodies, as described elsewhere [11].

The clinical characteristics of the 50 patients with ACL enrolled in this study are shown in table 1. All patients were treated with intravenous pentavalent antimony (Glucantime; Rhodia) at a dose of 20 mg Sb^{+5}/kg per day for 20 days. All patients were cured, as shown by the presence of healed lesions; cure was defined as the complete scarring of lesions, without induration and without recurrences up to 1 year after completion of treatment. The definition of a confirmed case of ACL was, therefore, based on the presence of typical lesions, a compatible epidemiological history, a positive Montenegro skin test or the presence of circulating antibodies (as detected by IFA), and clinical response to specific treatment.

Sample collection. After the administration of a local anesthetic, biopsy samples were obtained from the border of the lesion using a disposable 3.5-mm punch. Biopsy tissue specimens were fixed in 10% buffered formalin (pH, 7.0) and were processed using standard techniques for paraffin embedding. Tissue sections were stained with hematoxylin-eosin (HE), and immunohistochemical testing was performed using a polyclonal antibody against *Leishmania* species, as described elsewhere [12]. For negative controls, we used 10 biopsy specimens that were obtained from a collection of lesions caused by dermatological diseases not related to leishmaniasis, as well as biopsy specimens obtained from healthy patients who had undergone plastic surgery, all of whom were living in the same study area as the patients enrolled in our study.

Table 1. Clinical characteristics and laboratory findings for 50 patients with American cutaneous leishmaniasis enrolled in a study of diagnosis of *Leishmania* by PCR.

Characteristic or finding	Value
Male sex	34 (68)
Positive Montenegro skin test	36 (72)
Enlarged lymph nodes	21 (42)
Age, years	27.5 (3–87)
Duration of disease, days	47.5 (4–120)
Size of lesion,[a] cm	3 (1–12)
No. of lesions	1 (1–5)
Dose of intravenous pentavalent antimony, ampoules	80 (16–210)
Time to clinical cure, days	60 (20–360)

NOTE. Data are no. (%) of patients or median (range).

[a] Largest lesion from each patient.

Enlarged lymph nodes, draining the lesion site, were aspirated using a 1-mL syringe with a 25-gauge needle containing 500 μL of RPMI 1640 medium (Invitrogen). Aspirate was later transferred to sterile Vacutainer tubes (BD Biosciences) containing Nicole, Novy, and McNeal culture medium. Cultures were observed weekly for ⩾4 weeks before the results were considered to be negative.

PCR. Thirty-micrometer sections of the paraffin blocks containing patients' biopsy specimens were used for DNA extraction. DNA extraction and purification were performed using the Nucleon HT kit (Amersham Biosciences), in accordance with the manufacturer's instructions. Samples were eluted in 50 μL of TE (10 mmol/L Tris-HCl [pH, 8.0] and 0.1 mmol/L EDTA [pH, 8.0]). PCR was performed with the primers 5′-GGG(G/T)AGGGGCGTTCT(G/C)CGAA-3′ and 5′-(G/C)(G/C)(G/C)(A/T)CTAT(A/T)TTACACCAACCCC-3′, which target the amplification of the 120-bp conserved region of the *Leishmania* kDNA minicircle of all *Leishmania* species. A reaction mixture was prepared that contained 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH, 8.0), 0.2 mmol/L each deoxyribonucleotide (Invitrogen), 1 μmol/L each primer, 1.25 U of Taq polymerase (Invitrogen), and 2.5 μL of DNA sample in a final volume of 25 μL. The PCR conditions were as follows: denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 94°C for 45 s, with a final extension of 72°C for 10 min. The amplification reactions were analyzed by agarose gel electrophoresis, followed by ethidium bromide staining and

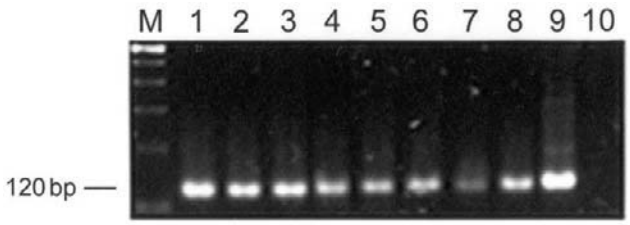

Figure 1. PCR amplification of *Leishmania* in biopsy samples obtained from patients with American cutaneous leishmaniasis (ACL). *M,* 100-bp DNA ladder DNA size marker; *lanes 1–8,* biopsy specimens obtained from patients with ACL; *lane 9,* positive control; *lane 10,* negative control.

visualization under UV light. DNA from the reference *L. braziliensis* strain MHOM/BR/75/2904 was used as a positive control.

RESULTS

Clinical characteristics of patients with ACL. Biopsy samples were obtained from 50 patients with ACL, all of whom were clinically cured after completion of the therapeutic regimen, as shown by complete healing of the lesion. Table 1 shows the clinical characteristics of this patient population. The Montenegro skin test was positive for 36 patients (72%) and negative for 16 patients (28%). In all patients with a negative Montenegro skin test result, the appearance of the lesion was recent (<55 days). Enlarged lymph nodes were observed in 21 (42%) of 50 patients.

Conventional methods of detection of* Leishmania *species. Table 2 shows the results obtained by conventional diagnostic methods for detection of *Leishmania.* Anti-*Leishmania* antibodies were detected in 21 (42%) of 50 serum samples, and parasites were isolated in 12 (57%) of 21 lymph node aspirates. Parasites were successfully isolated at a high rate mainly because the cultivation material came from enlarged lymph nodes, satellite to the lesion; therefore, the material was free of contaminating microorganisms that are commonly present in skin lesions. A high rate of lymph node enlargement has been observed in this area of endemicity, which confirms previous data on the presence of lymphadenopathy as an initial sign of ACL and as sometimes the only manifestation of *L. braziliensis* infection [13].

The 50 biopsy samples were submitted for histopathological examination, which consisted of both HE staining and immunohistochemical testing of biopsy sections. Direct visualization of parasites by HE staining was possible for only 8 (16%) of 50 samples. When immunohistochemical testing was used, parasites were detected in 33 (66%) of 50 samples.

PCR detection of* Leishmania *species. PCR was performed using primers that amplify a conserved region of *Leishmania* kinetoplast minicircles. In all 50 biopsy samples examined, the characteristic 120-bp amplification product was observed (figure 1). Positive and negative controls were included in all amplification reactions to ensure the amplification of the correct *Leishmania* product. No amplification of the 120-bp fragment was produced by PCR of biopsy samples obtained from patients with vascular ulcers or stasis ulcers, which are frequently mistaken for leishmaniasis by the patients, or by PCR of biopsy samples obtained from healthy individuals (who had either positive or negative skin test results) who live in the area of endemicity in Jequié (figure 2*A*); these results confirm that PCR detects the presence of active disease only. To ensure that this lack of amplification was not associated with the presence of inhibitors in the DNA sample, purified *Leishmania* DNA was added to each sample as an inhibition control (figure 2*B*), and the 120-bp fragment was seen in all of these samples. Therefore, PCR for the detection of *Leishmania* species in biopsy samples yielded a positivity rate of 100%, compared with the rates obtained by immunohistochemical testing (66%) and anti-*Leishmania* serological testing (42%) (table 2). The specificity of PCR for detection of *Leishmania* species was found to be 100%, because no amplification product was detected in samples obtained from patients with cutaneous ulcers that were not related to *Leishmania* infection or in biopsy specimens of healthy skin.

DISCUSSION

In the present study, we investigated the benefits of PCR as a tool for the detection of *Leishmania* parasites in biopsy specimens obtained from patients with ACL. In terms of clinical utility, PCR offers several advantages: it is highly sensitive and specific, and, most importantly, it is more rapid than the currently available methods—namely, serological tests, the Montenegro skin test, and microscopic examination of lesion biopsy specimens with HE staining and immunostaining [6, 9, 14–16]. Our results show that PCR results were positive for all biopsy samples obtained from patients with ACL, regardless of the results obtained with other diagnostic procedures, yielding a rate of positivity of 100%. This positivity rate was calculated on the basis of the fact that all patients in this study had clinical cure, defined as a total scarring of the initial lesions without

Table 2. Results of tests used for diagnosis of American cutaneous leishmaniasis.

Method	No. of positive results/ total no. of tests performed (%)
Serological testing	21/50 (42)
Culture	12/21 (57)
Hematoxylin-eosin staining	8/50 (16)
Immunohistochemical testing	33/50 (66)
PCR	50/50 (100)

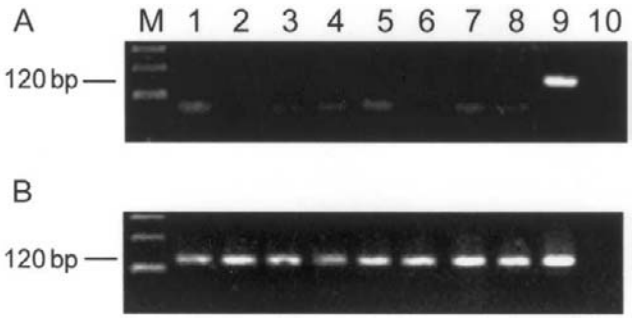

Figure 2. PCR amplification of *Leishmania* in biopsy samples obtained from patients with ulcers not related to *Leishmania* infection and from healthy individuals. *A, Lane M,* 100-bp DNA ladder DNA size marker; *lanes 1–3,* biopsy specimens obtained from patients who had vascular or stasis ulcers diagnosed; *lanes 4–8,* biopsy specimens obtained from healthy individuals; *lane 9,* positive control; *lane 10,* negative control. *B,* The same DNA resubmitted for PCR in the presence of 100 ng of DNA from the reference *Leishmania braziliensis* strain.

the appearance of other lesions up to 1 year after the end of treatment. Although clinical cure cannot be considered a "gold standard" for the diagnosis of ACL, it is a strong indicator that the patient was indeed infected with *Leishmania* parasites. Furthermore, all the patients studied presented with a compatible epidemiological history (i.e., typical ACL-associated ulcers), and all patients had either positive results of the Montenegro skin test or had detectable anti-*Leishmania* antibody.

The PCR was 100% specific: no amplification product was obtained using biopsy specimens from lesions with other causes or biopsy specimens obtained from healthy individuals. Parasitological confirmation had a rate of positivity (66%) comparable to that found elsewhere [7, 15], although, in this case, a laborious technique (immunohistochemical testing) had to be used, because the results of HE staining were positive in very few samples (16%), confirming the scarcity of parasites in *L. braziliensis* infections. Diagnosis of ACL based on in vitro culture of lesion material was positive in one-half of the samples tested (54.6%), which is in accordance with the findings of other reports [14, 17–19]. This variation in sensitivity has been linked to the degree to which bacterial contamination can be avoided and to the fact that some *Leishmania* strains are difficult to obtain by culture.

The Montenegro skin test is simple and sensitive, and, to date, it is the main indirect test used for confirmation of disease in areas of endemicity, especially in rural areas where resources for laboratory diagnosis are scarce or nonexistent [20]. In this study from the Jequié area of endemicity, we used a soluble *Leishmania* antigen [10], and we obtained a positive result for 72% of patients. Although the remaining patients presented with a cutaneous ulcer, all reported being recently infected (infection had occurred <2 months before the study). Serological tests are less informative in cases of ACL because of the characteristic low titers of specific antibodies. Accordingly, 42% of patients had positive results of IFA.

In conclusion, the present data support the view that PCR is the best approach to diagnosis of ACL, compared with parasitological confirmation by direct microscopy, detection of anti-*Leishmania* antibodies, and isolation of parasites in culture. However, major hurdles, such as cost and the need for laboratory facilities, must be overcome before this approach can be widely implemented in areas of endemicity. Therefore, PCR can be an alternative diagnostic procedure in cases of ACL in which the patient presents with a typical cutaneous ulcer and has both negative results of the Montenegro skin test, which can occur in recent infection, and negative results of anti-*Leishmania* serological tests.

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References

1. World Health Organization. The leishmaniases. World Health Organ Tech Rep Serv **1990**; 753:154.
2. Marsden PD. Mucosal leishmaniasis ("espundia" Escomel, 1911). Trans R Soc Trop Med Hyg **1986**; 80:859–76.
3. Degrave W, Fernandes O, Campbell D, Bozza M, Lopes U. Use of molecular probes and PCR for detection and typing of *Leishmania*—a mini-review. Mem Inst Oswaldo Cruz **1994**; 89:463–9.
4. Harris E, Kropp G, Belli A, Rodreiguez B, Agabain N. Single-step multiplex PCR assay for characterization of New World *Leishmania* complexes. J Clin Microbiol **1998**; 36:1989–95.
5. Uliana SR, Nelson K, Beverley SM, Camargo EP, Floeter-Winter LM. Discrimination amongst *Leishmania* by polymerase chain reaction and hybridization with small subunit ribosomal DNA derived oligonucleotides. J Eukaryot Microbiol **1994**; 41:324–30.
6. de Bruijn MH, Barker DC. Diagnosis of New World leishmaniasis: specific detection of species of the *Leishmania braziliensis* complex by amplification of kinetoplast DNA. Acta Trop **1992**; 52:45–58.
7. Belli A, Rodriguez B, Aviles H, Harris E. Simplified polymerase chain reaction detection of new world *Leishmania* in clinical specimens of cutaneous leishmaniasis. Am J Trop Med Hyg **1998**; 58:102–9.
8. Castilho TM, Shaw JJ, Floeter-Winter LM. New PCR assay using glucose-6-phosphate dehydrogenase for identification of leishmania species. J Clin Microbiol **2003**; 41:540–6.
9. Rodgers MR, Popper SJ, Wirth DF. Amplification of kinetoplast DNA as a tool in the detection and diagnosis of *Leishmania*. Exp Parasitol **1990**; 71:267–75.
10. Reed SG, Badaro R, Masur H, et al. Selection of a skin test antigen for American visceral leishmaniasis. Am J Trop Med Hyg **1986**; 35:79–85.
11. Cuba Cuba CA, Marsden PD, Barreto AC, et al. Parasitologic and immunologic diagnosis of American cutaneous leishmaniasis [in Spanish]. Bol Oficina Sanit Panam **1980**; 89:195–208.
12. Barral A, Pedral-Sampaio D, Grimaldi Junior G, et al. Leishmaniasis

in Bahia, Brazil: evidence that *Leishmania amazonensis* produces a wide spectrum of clinical disease. Am J Trop Med Hyg **1991**; 44:536–46.
13. Barral A, Guerreiro J, Bomfim G, et al. Lymphadenopathy as the first sign of human cutaneous infection by *Leishmania braziliensis*. Am J Trop Med Hyg **1995**; 53:256–9.
14. Pirmez C, da Silva Trajano V, Paes-Oliveira Neto M, et al. Use of PCR in diagnosis of human American tegumentary leishmaniasis in Rio de Janeiro, Brazil. J Clin Microbiol **1999**; 37:1819–23.
15. Rodrigues EH, Felinto de Brito ME, Mendonca MG, et al. Evaluation of PCR for diagnosis of American cutaneous leishmaniasis in an area of endemicity in northeastern Brazil. J Clin Microbiol **2002**; 40:3572–6.
16. Romero GA, Guerra MV, Paes MG, et al. Sensitivity of the polymerase chain reaction for the diagnosis of cutaneous leishmaniasis due to *Leishmania (Viannia) guyanensis*. Acta Trop **2001**; 79:225–9.
17. Aviles H, Belli A, Armijos R, et al. PCR detection and identification of *Leishmania* parasites in clinical specimens in Ecuador: a comparison with classical diagnostic methods. J Parasitol **1999**; 85:181–7.
18. Weigle KA, Labrada LA, Lozano C, et al. PCR-based diagnosis of acute and chronic cutaneous leishmaniasis caused by *Leishmania (Viannia)*. J Clin Microbiol **2002**; 40:601–6.
19. Isaza DM, Arboleda M, Restrepo M, et al. Validation of the polymerase chain reaction for the diagnosis of human cutaneous leishmaniasis in north-west Colombia. Trans R Soc Trop Med Hyg **2002**; 96(Suppl 1): S165–8.
20. Gontijo CM, da Silva ES, de Fuccio MB, et al. Epidemiological studies of an outbreak of cutaneous leishmaniasis in the Rio Jequitinhonha Valley, Minas Gerais, Brazil. Acta Trop **2002**; 81:143–50.