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Use of PCR in Diagnosis of Human American Tegumentary Leishmaniasis in Rio de Janeiro, Brazil

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In Brazil, the most common etiological agent of American tegumentary leishmaniasis is *Leishmania* (*Viannia*) *braziliensis*. In general, diagnostic techniques envisage the visualization of the parasite, but that technique has a low sensitivity. The main purpose of the present work was to evaluate the PCR as a routine tool for the diagnosis of leishmaniasis. Biopsy specimens from cutaneous or mucosal lesions were taken from 230 individuals from areas where *Leishmania* is endemic: 216 patients who had a clinical picture suggestive of leishmaniasis and 14 individuals with cutaneous lesions due to other causes. Each specimen was processed for histopathologic examination, culture, touch preparation, and DNA isolation. Oligonucleotides that amplify the conserved region of the minicircle molecules of *Leishmania* were used in a hot-start PCR. While at least one conventional technique was positive for *Leishmania* for 62% (134 of 216) of the patients, PCR coupled to hybridization was positive for 94% (203 of 216) of the patients. The 14 patients whose clinical picture was not suggestive of leishmaniasis had negative results by all techniques. The impact of the PCR was striking in mucosal disease. While the disease in only 17% (4 of 24) of the patients could be diagnosed by conventional techniques, PCR was positive for 71% (17 of 24) of the patients. Hybridization showed that all cases of disease were caused by parasites belonging to the *Viannia* subgenus. Altogether, the results indicate that PCR is a valuable tool for the diagnosis of leishmaniasis on a routine basis and is likely to provide valuable epidemiological information about the disease in countries where it is endemic.

Diagnosis of infectious diseases is ideally performed through the direct demonstration of the respective etiologic agent. However, the small number of pathogens in clinical samples and/or the fastidious and cumbersome in vitro growth can hamper the use of parasitological diagnostic techniques. In order to overcome such difficulties, several molecular techniques have been developed in the last decade. DNA amplification through the PCR has several advantages compared to traditional techniques, such as the ability to detect infectious agents present at very low copy numbers and the ability to be performed with a broad range of clinical specimens (2, 12, 17, 21, 25).

Human American tegumentary leishmaniasis (ATL) is endemic in Brazil, with approximately 25,000 new cases per year. Cutaneous lesions characterize the disease in most of the patients, about 3 to 5% of whom develop metastatic mucosal lesions. The vast majority of lesions are caused by *Leishmania* (*Viannia*) *braziliensis* (8). Current parasitological diagnosis of New World leishmaniasis, which is caused by *Viannia* parasites, is performed by means of biopsy of the lesions and then processing of the specimens for histopathologic examination, touch preparations, and axenic cultures. These methods demand well-trained personnel, lack sensitivity, are time-consuming, and require culture facilities. In addition, the precise identification of *L.* (*Viannia*) *braziliensis* parasites, which are known for their paucity within the lesions, is also important due to epidemiological implications. The difficulty in establishing a correct diagnosis might be one of the reasons why the incidence of leishmaniasis in the New World has been underestimated (1).

Several research groups have pursued the amplification of different *Leishmania* DNA targets in order to establish PCR for the diagnosis of ATL as a feasible method in the routine clinical laboratory. The aim of this study was to evaluate the efficacy of PCR compared to those of traditional techniques for the diagnosis of tegumentary leishmaniasis in patients examined in Rio de Janeiro State, Brazil, where the incidence is estimated at approximately 300 new cases per year.

MATERIALS AND METHODS

Patients. A prospective study was conducted with patients selected from the Dermatology Outpatient Unit of the Evandro Chagas Hospital, FIOCRUZ, Rio de Janeiro, Brazil. A complete dermatological examination was performed, and all patients presenting with lesions suggestive of leishmaniasis were enrolled in the diagnostic protocol. Any active lesion or scars were photographed, and a description and the number of lesions were recorded. Independent of clinical complaints, an otorrhinolaryngologic examination (Hopkins optics, 0 to 90°; Storz, Mainz, Germany) was performed for all patients. Montenegro skin testing was performed by intradermal injection of 0.1 ml of leishmanin (40 μg/ml; kindly provided by W. Mayrink, Federal University of Minas Gerais, Belo Hirozonte, Brazil) on the volar surface of the forearm, and the reaction was measured after 48 h. The test was considered positive if indurations were more than 5 mm in diameter.

During the period from January 1995 to May 1997, a total of 230 patients who presented with cutaneous and/or mucosal lesions were examined. All patients were from areas where *Leishmania* is endemic. Patients were divided into three groups according to the clinical presentation: (i) localized cutaneous leishmaniasis (LCL) (Fig. 1A) when lesions were confined to skin sites (184 patients); (ii) mucosal leishmaniasis (ML) (Fig. 1B) when only the mucosal site was involved, with or without cutaneous scars (24 patients); and (iii) mucocutaneous leishmaniasis (MCL) when active cutaneous and mucosal lesions were present simultaneously (8 patients). A fourth group consisted of individuals who presented with

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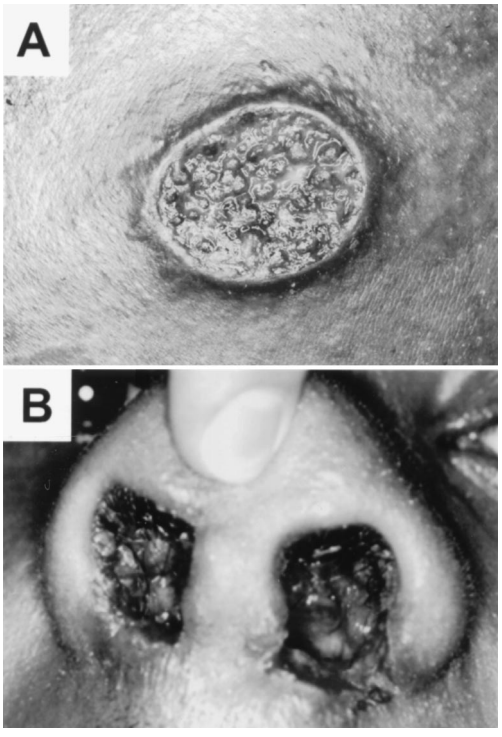

FIG. 1. Typical clinical picture of a cutaneous ulcer of LCL (A) and an ulcerated vegetative lesion of the nasal mucosa (B).

cutaneous lesions which were not clinically suggestive of leishmaniasis (14 patients). Samples from all patients were handled in the laboratory without previous knowledge of the clinical diagnosis. A positive Montenegro skin test was observed in 212 of 216 (98%) patients and in 9 of the 14 (64%) patients without leishmaniasis. The skin test was positive for all patients with mucosal lesions.

Biopsy specimens were taken at the border of the lesions after the administration of a local anesthetic (2% lidocaine [Xylocaine]). A scalpel was used for cutaneous lesions, and a laryngoscope forcep was used for mucosal lesions. All specimens were obtained for diagnosis with the approval of the ethical committee of FIOCRUZ, Ministry of Health, Rio de Janeiro, Brazil, and biopsies were performed according to the rules of the National Counsel of Health/Brazil (16a). Informed consent was obtained from all patients.

Conventional methods. Specimens were divided into three fragments. The first fragment was processed for both (i) histopathologic examination after the sample was embedded in paraffin and stained with hematoxylin-eosin (H&E) and (ii) touch preparation, in which the sample was stained with Leishman stain before fixation in buffered formalin. The second tissue fragment was used for cultivation in NNN medium, and the third one was processed for PCR analysis. The three conventional methods were performed for most cutaneous specimens; however, in the case of mucosal biopsy specimens, touch preparations were usually not performed.

PCR. A fragment of approximately 1 mm^3 was collected in an Eppendorf tube and frozen at −20°C. All specimens were handled in the laboratory without previous knowledge of the clinical diagnosis or the results of conventional methods. DNA isolation was performed with an anion-exchange chromatography spin column following the manufacturer's instructions (Pharmacia, Uppsalla, Sweden). The final DNA pellet was resuspended in 20 μl of 10 mM Tris-HCl (pH 8.0)–1 mM EDTA (pH 8.0) and was stored frozen at −20°C until use. A hot-start PCR was performed with oligonucleotides that anneal to the origin of replication of both strands of the minicircle molecule, which is one component of the mitochondrial DNA of the parasite. This amplifies the conserved region of the molecule. The reaction mixture contained 100 ng of 5′ and 3′ oligonucleotide primers, a 200 μM concentration of each deoxynucleoside triphosphate (Pharmacia), 2.5 U of *Taq* polymerase (Perkin-Elmer, Norwalk, Conn.) in the buffer recommended by the manufacturer (1.5 mM $MgCl_2$), and 2 μl of the DNA sample. PCR amplification was carried out in a DNA thermocycler (Perkin-Elmer) with 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s, with a final cycle of 10 min at 72°C. All recommended precautions were taken to avoid PCR artifacts. Each assay contained a negative control, in which no DNA was added to the reaction mixture, and a positive control, in which 10 fg of parasite DNA was included as a template in the PCR. Ten microliters of the amplified products was analyzed by agarose gel electrophoresis, ethidium bromide staining, and visualization under UV light. All products were either applied to a nylon membrane with a dot blot apparatus or capillary transferred and hybridized with probes from a cloned minicircle of *L.* (*Viannia*) *panamensis* IPAN V or *L.* (*Leishmania*) *amazonensis* IFLA/BR/67/PH8 (7).

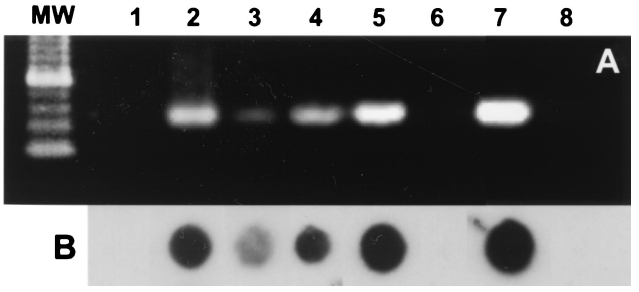

FIG. 2. Detection of *Leishmania* kinetoplast DNA by PCR of ATL lesions obtained by biopsy. (A) Agarose gel stained with ethidium bromide. Lane 1, stasis ulcer; lanes 2 to 6, lesions clinically suggestive of leishmaniasis (for lanes 2, 4, and 5, samples from patients with LCL; for lanes 3 and 6, samples from patients with ML); lane 7, DNA from *L. braziliensis* M2903; lane 8, negative control for reagent contamination (water, no DNA added); lane MW, 50-bp ladder. (B) Dot blots prepared with the same samples for which the results are shown in panel A and which were hybridized with an *L.* (*Viannia*) *panamensis* probe.

RESULTS

PCR was positive for 94% (203 of 216) of the patients with a clinical diagnosis suggestive of leishmaniasis, whereas parasites were detected by at least one conventional method in 62% (134 of 216) of the patients (Fig. 2 and 3). Specimens from all 14 patients with nonleishmanial cutaneous ulcers were negative by all methods, including PCR. When only those patients who had at least one positive result by any conventional method were considered to be true positives, the sensitivity of the PCR was 97% and the negative predictive value was 78%. Table 1 shows the results of each individual diagnostic test for specimens from patients with all three clinical forms of ATL.

LCL. A total of 184 patients presented exclusively with cutaneous lesions and were thus classified as having LCL. Parasites were demonstrated by at least one conventional method in 67% (124 of 184) of these patients, whereas PCR was positive for 97% (178 of 184) of the patients with LCL. Furthermore, 93% (56 of 60) of the cases with negative results by all

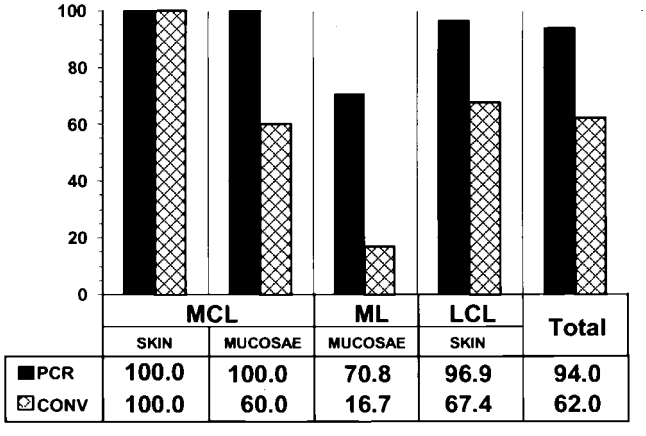

FIG. 3. Percentage of positive results for skin or mucosal biopsy specimens from patients with MCL, ML, or LCL by PCR or by at least one conventional (CONV) method.

TABLE 1. Results of conventional methods and PCR for diagnosis of LCL, ML, or MCL

Method	Skin biopsy specimen from patients with LCL (n = 184)		Mucosal biopsy specimen from patients with ML (n = 24)		Patients with MCL[a] (n = 8)				Total	
					Skin biopsy specimen		Mucosal biopsy specimen			
	No. positive/ no. tested	% Positive	No. positive/ no. tested	% Positive	No. positive/ no. tested	% Positive	No. positive/ no. tested	% Positive	No. positive/ no. tested	% Positive
H&E staining	65/175	37	2/23	9	5/7	71	1/3	33	73/208	35
Touch preparation	76/176	43	0/6	0	4/7	57	0/2	0	80/191	42
Culture[b]	86/144	60	3/13	23	0/1	0	2/4	50	91/162	56
PCR	178/184	97	17/24	71	7/7	100	6/6	100	208/221	94

[a] For five patients, biopsy specimens from both mucosal and skin lesions were obtained.
[b] The average rate of culture contamination was 22% (a total of 47 of 209 cultures: 34 of 178 for patients with LCL, 11 of 24 for patients with ML, and 2 of 7 for patients with MCL).

three conventional methods were positive by PCR. Six of the patients with LCL were negative by PCR. All but two also showed negative results by any of the conventional tests: one with a positive touch preparation and one by culture. Despite the negative results, the disease in the remaining four patients was still considered leishmaniasis because of the typical clinical and epidemiological histories and the positive skin test. In addition, the lesions on these four patients healed after specific therapy.

All positive specimens hybridized only with the *L. panamensis* probe and not with the *L. amazonensis* or *L. chagasi* probe, therefore indicating that the organism causing all cases of LCL belonged to the subgenus *Viannia* (Fig. 2).

The three conventional methods were performed with specimens from 162 of the 184 patients with LCL. Parasites could be detected by all methods in 19% (31 of 162) of the patients, by two methods in 24% (39 of 162) of the patients, and by only one method in 27% (43 of 162) of the patients. Although the results were not statistically significant, conventional methods tended to detect parasites in patients who had lesions of less than 3 months' duration and who presented with multiple lesions. Culture of the biopsy fragment gave the best rate of positivity (60%; 86 of 144), despite the relatively high level of bacterial contamination (19%; 34 of 178). Touch preparation and histopathologic examination had lower degrees of positivity (Table 1).

In 14 patients the diagnoses were other entities, as follows: pyoderma (n = 5), pyoderma gangrenosum (n = 1), ecthyma (n = 2), stasis ulcer (n = 2; one was due to falcemic anemia), human immunodeficiency virus infection-related folliculitis (n = 1), seborrheic dermatitis (n = 1), and Bazin's erythema induratum (n = 2). This fourth group of the study consisted of patients from areas of endemicity who presented with cutaneous ulcers which could be distinguished from leishmaniasis by either clinical means or a negative skin test. PCR and standard methods were negative for all these patients.

ML. Of the 24 mucosal biopsy specimens from patients without active cutaneous lesions, only 4 (17%) were positive by standard methods (2 by culture only, 1 by H&E staining only, and 1 by H&E staining and culture). Touch preparations were performed for only five patients, and all were negative. Cultures from mucosal biopsy specimens were often contaminated with bacteria (46%; 11 of 24), and only three patients (23%) were positive by this method. The PCR method showed a band of the expected size in agarose gels for 15 of 24 (62%) patients. However, after hybridization with the *L. panamensis* probe, a signal was detected for two additional patients, raising the rate of positivity to 71%.

MCL. Eight patients presented with concomitant active mucosal and cutaneous lesions. Two patients underwent a skin biopsy only, one underwent a mucosal biopsy only, and five underwent biopsies of the skin and mucosal lesions. In brief, a total of six mucosal biopsies and seven skin biopsies were performed. Conventional methods were positive for three of five mucosal biopsy specimens, whereas PCR was positive for all mucosal biopsy specimens. Skin lesions were all positive by at least one conventional method and by PCR (Table 1). Hybridization confirmed that the PCR products belonged to the subgenus *Viannia*.

DISCUSSION

Leishmaniasis is now considered a reemerging disease due to the increase in travel throughout the world, bringing the possibility of leishmanial infections to areas of nonendemicity (16). The diagnosis of tegumentary leishmaniasis can be based on the clinical presentation of patients in geographical regions where the infection typically occurs. However, epidermoid carcinomas or lesions caused by *Paracoccidioides brasiliensis*, tuberculosis, syphilis, and leprosy, which are all relatively common in Brazil, are other diseases or infections that should be considered in the differential diagnosis. Furthermore, the identification of the causative agent as *Viannia* is important due to the likelihood of metastatization of species belonging to this subgenus.

The standard methods more often used to diagnose leishmaniasis are the Montenegro skin test, touch preparations, histopathology, and culture. Skin testing is simple and has a high sensitivity and specificity, but it cannot distinguish active, inactive, or past infection (24). The paucity of parasites within the lesions is a hallmark of *L.* (*Viannia*) *braziliensis* species, which is responsible for the vast majority of human cases of leishmaniasis in Brazil (10). Therefore, methods that require direct visualization of the parasites such as touch preparations or histopathology usually have low sensitivities (5, 23). Cultivation of a biopsy fragment or aspirates from the lesion is probably the best method for a parasitological diagnosis since it allows species identification, but it has the disadvantage of being expensive and time-consuming. Serology has a low sensitivity and specificity since it cross-reacts with other pathogens (9).

The results presented here demonstrate that the leishmanial etiology was established in 62% of the patients by conventional methods. This relatively high rate of positivity is, however, a result of the use of a combination of three different methods, therefore increasing tremendously the cost of diagnosis. If only

one method is to be chosen, cultivation seems to be the best choice since it gave the highest rate of positivity (56%; 91 of 162). However, bacterial contamination is also high since lesions very often have secondary bacterial infections (4).

Methods that require visualization of the parasite had low rates of positivity. These methods, particularly H&E staining, require experienced histopathologists because of the scarcity of the parasites and their small size. On the other hand, histopathology allows one to identify other diseases that show clinical features similar to those of leishmaniasis (14, 20). Touch preparations in general give a fair degree of positivity and are less expensive methods, but they can be time-consuming (3).

The main limitations of these classic diagnostic methods are the requirement for a large sample of tissue and the need for specially trained personnel to perform all the three methods for each patient. In recent years, several other methods were attempted to improve diagnostic sensitivity. PCR is one of these methods, but the results from various investigators conflict. In similar studies, the technique was found not to be highly sensitive (60 to 80%) or specific (50%) with biopsy specimens from patients with cutaneous leishmaniasis (6, 13, 15). In other studies, PCR showed detection rates of 97% with samples from patients in the New World, even when the initial tissue sample contained few amastigotes (22). One of the factors that may influence the sensitivity is the DNA extraction protocol (11). In order to achieve reproducible results and avoid inhibitory factors in the PCR, column chromatography was used to extract the DNA from lesions. Our results showed 97% sensitivity for cutaneous lesions, although more specimens from patients with lesions not caused by *Leishmania* should be examined for a better evaluation of specificity. By considering a multiple-alignment analysis performed with several minicircle sequences from New World *Leishmania* species (7), oligonucleotides that were previously described for PCR amplification of the conserved region (21) of the mitochondrial DNA were adapted for use in a PCR. The new PCR primers enable the amplification of a 120-bp fragment for all New World *Leishmania* species. The specificity of the assay is guaranteed by molecular hybridization experiments performed with three distinct cloned minicircle molecules: *Leishmania* subgenus *Viannia* (*L. panamensis* minicircle) and *L. mexicana* complex (*L. amazonensis* minicircle).

Mucosal disease is particularly difficult to diagnose by conventional methods (5). Our results have shown that PCR is a useful tool for the diagnosis of this clinical form since conventional methods were positive for only 17% of the patients with ML, whereas PCR coupled with hybridization was positive for 71% of the patients. On the other hand, despite the high sensitivity of PCR, almost one-third of these patients remain negative. For the positive patients, the 120-bp band visualized in agarose gels tends to be of lower intensity than the band for patients with cutaneous disease (Fig. 2, lane 3), and for two samples positivity was detected only after hybridization, suggesting that parasites are present within the lesions at low numbers. It has been demonstrated that mucosal lesions present a mixture of type 1 and 2 cytokines (19). The type 1 response promotes the production of gamma interferon, a key cytokine for parasite killing, which is likely to be efficient in destroying the parasites. The nonhealing pattern of ML could thus be the result of the presence of interleukin-4, a type 2 cytokine, which is present in much higher amounts in mucosal lesions than in cutaneous lesions (18, 19).

In our region of endemicity in Rio de Janeiro State, Brazil, PCR has proven to be an excellent tool for improving the rate of correct diagnosis leishmaniasis and in the differential diagnosis of ulcerative cutaneous lesions of other etiologies in patients living in areas of endemicity. However, the use of PCR as a routine diagnostic method still requires a well-prepared laboratory and well-trained personnel, therefore hampering the feasibility of using the technique directly in the field. The present data from tests with specimens from a large number of patients validate the PCR as a diagnostic method in the New World, particularly for mucosal leishmaniasis.

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