Evaluation of PCR for Diagnosis of American Cutaneous Leishmaniasis in an Area of Endemicity in Northeastern Brazil

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PCR-based approaches targeting kinetoplast DNA were evaluated for the diagnosis of American cutaneous leishmaniasis (ACL) in regions of endemicity in northeastern Brazil. A total of 119 cutaneous biopsy specimens from patients with ACL and nonleishmaniasis cutaneous lesions were studied. Two PCR-based systems were used; one was specific for the subgenus Viannia, and the other was specific for the genus Leishmania. The PCR specific for the subgenus Viannia had a sensitivity of 95.4%, whereas the genus-specific PCR detected the target DNA in 88.2% of the samples tested. The specificities of the assays, determined with samples from a group with nonleishmaniasis cutaneous lesions, was 100%. The results of the conventional tests indicate that the sensitivities of the PCR-based methods were significantly higher than those of smear examination, histological staining, and isolation by culture (P < 0.05). Antibodies specific for Leishmania braziliensis were detected by indirect immunofluorescence in 82.9% of the patients tested. Parasites were isolated from 40 of 86 patients (46.5%). Sixty-seven percent of dermal scrapings and 66.2% of stained tissue sections were positive by microscopy. Amplified products from the subgenus-specific PCR hybridized with the Leishmania panamensis minicircle, confirming infection consistent with L. braziliensis. The evidence available at present incriminates L. braziliensis as the only causative agent of ACL in the state of Pernambuco in Brazil.

American cutaneous leishmaniasis (ACL) is an anthropo-zoonotic disease caused by protozoans of the genus Leishmania, which infect the vertebrate host after a bite by infected phlebotomus insects of the genus Lutzomyia. Human infections may be apparent or display a clinical spectrum ranging from localized, sometimes self-healing cutaneous lesions to severe mutilating mucocutaneous lesions to diffuse cutaneous leishmaniasis. In Brazil, ACL is widely distributed from the south of the Amazon Basin to the southeast of Brazil (Leishmaniasis Section, Special Programme for Research and Training in Tropical Diseases, World Health Organization, 2001 [http://www.who.int/tdr/index.html], with most cases being caused by Leishmania (Viannia) braziliensis (17). However, Leishmania amazonensis is also important as a causative agent of ACL, having been reported in the northeast, southeast, and west-central parts of Brazil (3, 24, 29, 36). According to the National Health Foundation, the numbers of cases of ACL in 2000 were 27,502, 10,868, and 943 in Brazil, northeastern Brazil, and Pernambuco State, respectively (Fundação Nacional de Saúde, 2001 [http://www.funasa.gov.br]). In this context, cross-sectional epidemiological surveys developed in an area of endemicity in Pernambuco confirmed the high current rate of infection and an approximately 10-fold increase in rates of transmission during the last 10 years (5).

The diagnostic methods available at present are based on clinical and epidemiologic features, parasitologic detection (stained smears, culture, and histopathology), and immunologic methods. These traditional laboratory methods have several limitations; prominent among them is low sensitivity (1, 38). The need for more sensitive methods has prompted the development of DNA-based diagnostic techniques. The kinetoplast, an organelle unique to the kinetoplastids, contains approximately 10,000 small circular DNAs, known as kinetoplast DNA (kDNA) minicircles, which are between 600 and 800 bp in members of the genus Leishmania. The abundance and other characteristics of these molecules have made them the target for a number of PCR-based techniques. However, the wide range of sensitivities (63 to 97%) and specificities (60 to 100%) that have been reported (1, 11, 12, 14, 22, 27, 32) clearly justify additional tests in regions of endemicity. In the study described in the present paper we evaluated PCR-based approaches that target kDNA for the diagnosis of ACL in regions of endemicity in Pernambuco State, Brazil, and compared them to conventional diagnostic techniques. In addition, the use of specific PCR systems provided evidence that L. braziliensis is the only etiologic agent of ACL in Pernambuco.

MATERIALS AND METHODS

Study area and patients. A total of 119 cutaneous biopsy specimens were studied from two groups of subjects: (i) 88 patients with confirmed ACL living in the Amaraji Municipality and neighboring regions (Pernambuco State), where L. braziliensis is endemic, and (ii) 31 patients with nonleishmaniasis cutaneous lesions (control group), including tuberculosis, sporotrichiosis, epidermoid carcinoma, leprosy, tropical ulcer, zygomycosis, epidermal cyst, amyloidosis, and several types of nevi. The project was approved by the Ethical Committee of Centro de Pesquisas Aggeu Magalhães-FIOCRUZ, and all individuals enrolled...
membranes (Hybond N+; Amersham Pharmacia Biotech) with a VaucGeneXL vacuum blotting system (Amersham Pharmacia Biotech). Hybridization was carried out with probes labeled with alkaline phosphatase, which allows visualization by chemiluminescence, with the AlkosPhos direct kit (Amersham Pharmacia Biotech). Both the labeling of the probes and detection of the label were performed according to the instructions of the manufacturer. Probed membranes were exposed to radiographic films ( Biomax; Kodak).

**Calculation of test performance indices and statistical analysis.** The sensitivities and specificities of the diagnostic tests were calculated as described by Feinstein (15). The sensitivities of the PCR tests were assessed with samples from the patients with confirmed ACL, whereas the specificities were calculated on the basis of the results for patients without leishmaniasis living in leishmaniasis-free regions. These indices were compared by significance tests by using normal approximation, assigning statistical significance to differences with P values <0.05. EPI Info (version 6.04b) software and Statistica for Windows software (Stastsoft, Tulsa, Okla.) were used. The sample size used for calculation of these indices was not necessarily the same because some diagnostic tests could not be performed with samples from all individuals selected for the study.

**RESULTS**

**Evaluation of sensitivities and specificities of PCR-based methods.** Eighty-eight patients met the diagnostic criteria for ACL described above and were therefore considered to have confirmed cases of ACL. These cases were used as the “gold standard” in calculation of the sensitivity. The subgenus *Viannia*-specific PCR detected DNA in 84 of 88 of the patients with confirmed cases, resulting in a sensitivity of 95.4%, whereas the genus-specific PCR detected the target DNA in 60 of 68 patients (88.2%) (Table 1). The difference between sensitivities was not significant (P > 0.05). The specificities calculated on the basis of the results for the 31 patients with nonleishmaniasis cutaneous lesions were 100% for both PCR systems used. Figures 1 and 2 present representative results obtained by the two PCR approaches. Spurious bands larger than 120 bp could be seen for some samples (Fig. 2).

**Comparison of sensitivities of PCR-based techniques with sensitivities of classical diagnostic methods.** Because of the low sensitivities of the conventional tests for detection of *Leishmania*, there is no gold standard of general acceptance to be used for the evaluation of new diagnostic tests. Thus, the definition of a confirmed case of ACL was based upon a combination of elements (see Materials and Methods). Compared with confirmation of a case by use of the designated combination of elements (Table 1), the sensitivities of the PCR-based methods were significantly higher than those of smear examination, histological staining, and isolation by culture (P < 0.05). Parasites were isolated from 40 of 86 patients (46.5%).

Microscopy of dermal scrapings and examination of stained
tissue sections were positive for *Leishmania* for 66.7 and 66.2% of the patients, respectively. Antibodies specific for *L. braziliensis* were detected by IIF in 82.9% of the patients tested, yielding a sensitivity significantly lower than that observed for the subgenus *Viannia*-specific PCR ($P < 0.05$).

**Preliminary evaluation of PCR-hybridization with nonradioactive probes for diagnosis of leishmaniasis.** The primers specific for the genus *Leishmania* (34) enable the amplification of a 120-bp fragment for all *Leishmania* species, and the product can be typed as a member of the subgenus *Viannia* after hybridization with *L. panamensis* minicircles (30). For this preliminary experiment, samples from 11 patients with confirmed ACL and 4 patients with other cutaneous diseases were tested. Amplicons that hybridized with the *L. panamensis* minicircle were detected in all patients previously diagnosed with ACL by the subgenus *Viannia*-specific PCR, confirming infection with organisms consistent with *L. braziliensis* (Fig. 3). The analysis of the bands amplified by PCR suggested that some of them were spurious, being derived from host DNA. In fact, the hybridization corroborates this idea, showing that most of the nonspecific products were derived from DNA not related to kDNA sequences and possibly derived from human DNA. However, some products >120 bp hybridized with the probe, indicating that, although they are nonspecific, they are related to minicircle sequences.

**DISCUSSION**

Parasites of the subgenus *Viannia* produce chronic cutaneous and mucocutaneous lesions, but the organisms that cause the lesions are very difficult to detect and treat. Indeed, the importance of establishing control strategies makes diagnosis and typing of the cause of leishmaniasis critical (28). This is particularly relevant for two reasons: (i) increased human mobility and long-distance travel and (ii) the possibility of coexistence of AIDS and leishmaniasis (20). Increased human mobility and long-distance travel may contribute to the spread of leishmaniasis to areas where the disease was not endemic, whereas AIDS may increase the severity of the parasitic disease. In addition, accurate diagnosis is important because lesions caused by epidermoid carcinomas, *Paracoccidioides brasilensis*, tuberculosis, syphilis, and leprosy, which are all common in Brazil, have some resemblance to those found in patients with ACL (30, 32). Direct methods of identification of leishmania in tissue samples by microscopic smear examination or histological staining of tissue sections, which are simple and cheap, have limited sensitivities (11, 22, 30, 32, 37). Because of its low sensitivity, histopathology is more useful for the discrimination of ACL from other diseases causing similar macroscopic lesions. Isolation methods such as in vitro culture of parasites from punch biopsy specimens or lesion aspirates can be sensitive but are time-consuming and costly and have variable success rates (1, 37). Indirect tests for circulating antibodies against *Leishmania*, such as IIF, are sensitive but fail to distinguish between past and present disease, are unable to
detect early infections, and have significant cross-reactions to antibodies against other pathogens, including some which resemble *Leishmania* in terms of clinical presentations (19, 37). Nevertheless, serological diagnosis is expected to improve with the identification of new relevant antigens and the possibility of designing tests for the detection of antibodies against the purified molecules (8, 9). PCR is claimed to be very sensitive and specific, but there are conflicting results from different groups. The technique was found to be not very sensitive (63 to 80%) or specific (60%) with biopsy specimens from patients with cutaneous leishmaniasis (11, 23, 25). In other studies, PCR showed detection rates of 97% with samples from patients in the New World (31). A possible explanation for these discrepancies is a difference in the quality of the DNA samples used for PCR (the samples may contain different amounts of DNA polymerase inhibitors). Thus, to optimize the PCR results, we worked with high-quality DNA preparations obtained with a commercial DNA purification kit. Ten femtograms of total DNA (equivalent to approximately 1 fg of kDNA) was the detection limit of the subgenus *Viannia*-specific PCR. Thus, this assay could theoretically detect DNA corresponding to that from less than one parasite per reaction tube (16), in agreement with the sensitivities reported previously (11). Thus, use of this method for the diagnosis of ACL is appropriate, since it is known that parasites are scarce in New World cutaneous leishmaniasis caused by *Viannia* parasites. The detection limit for the genus-specific PCR was 1 pg of total DNA.

The sensitivity of PCR was significantly higher than that of the conventional direct parasite detection tests used (histopathological examination, microscopic smear examination, and culture). This is in line with the results of other studies showing that PCR is consistently more sensitive than conventional methods (1, 12, 30). The present study was based upon the analysis of 119 cutaneous biopsy specimens (with 88 samples coming from patients with confirmed cases of ACL). This sample is larger than the ones used in several previous studies (1, 12, 29).

Although it is possible that four confirmed cases of ACL were not identified by the PCR system proposed by de Bruijn and Barker (11) due to infection with non-*Viannia* species (Table 1), a more reasonable explanation is that the PCR has suboptimal sensitivity, since the genus-specific PCR was also not able to detect one of these four confirmed cases. The PCR systems used were negative for lesions with nonleishmanial etiologies, resulting in 100% specificities.

The PCR primers used in the PCR described by de Bruijn and Barker (11) were based on sequences present in World Health Organization reference strains (12). Although they also anneal to DNA from strains from Colombia, Venezuela, Ecuador, Peru, and Brazil (11), this had not been formally demonstrated in isolates from regions of endemicity in Pernambuco. This is particularly relevant, since several *L. braziliensis* variants from this region have recently been characterized (7, 10; M. E. F. Brito et al., unpublished data).

The potential confusion between the clinical presentations of ACL caused by *L. amazonensis* and *L. braziliensis* coupled with the overlapping distribution of *L. amazonensis* and *L. braziliensis* in some regions poses diagnostic problems. Although patients with ACL caused by species of the subgenera *Viannia* and *Leishmania* generally have similar clinical presentations, the clinical evolution and response to chemotherapy may differ for ACL caused by the two subgenera (24). Thus, approaches allowing the discrimination between the New World dermotropic leishmaniasis (subgenus *Viannia* and subgenus *Leishmania*) are relevant (12, 14, 18, 30, 32). In the state of Minas Gerais, Brazil, clinical epidemiological and molecular evidence supported a conclusion (29) that *L. (Viannia) braziliensis* and *L. (Leishmania) amazonensis* were the species present, although *L. (Viannia) braziliensis* was the predominant species. This species is also predominant in the states of São Paulo, Rio de Janeiro, Espírito Santo, and Bahia (24). Studies characterizing *Leishmania* species in Pernambuco are relatively scanty. The previous studies had shown that *Leishmania* isolated from patients with ACL and rodents in areas of endemicity in Pernambuco were of the species *L. braziliensis*, suggesting that this species is the only causative agent of ACL in this region (4, 7, 10; Brito et al., unpublished). The results described herein support this hypothesis, as DNA products specific for the subgenus *Viannia* were amplified from 95.4% of the samples from patients with confirmed cases of ACL. Genus-specific PCR followed by hybridization with specific minicircles confirmed the presence of *Leishmania* organisms of the *L. braziliensis* complex in 11 patients. Although we did not formally rule out the possibility of mixed infections in all patients, the parasite was isolated and characterized from 40 patients with ACL, confirming infection with *L. braziliensis* on the basis of analysis with monoclonal antibodies and isoenzyme analysis (Brito et al., unpublished). Thus, typing of *Leishmania* by PCR yielded results in agreement with those obtained by typing of cultured parasites. The evidence available at present incriminates *L. braziliensis* as the only causative agent of ACL in Pernambuco. In line with this, *Lutzomya whitmani*, the local vector in Pernambuco (6), has not been incriminated in the transmission of *L. amazonensis* (21, 35).

The genus-specific primers described by Schubach et al. (34) enable the amplification of a 120-bp fragment for all *Leishmania* species. The specificity of the assay can be guaranteed by molecular hybridization experiments performed with three distinct cloned minicircle molecules from *Leishmania* (*Viannia*) (*L. panamensis* minicircle), *Leishmania mexicana* complex (*L. amazonensis* minicircle), and *Leishmania chagasi* complex (*L. chagasi* minicircle) (13, 29, 30). The use of hybridization after PCR improves the sensitivity and specificity of the approach. However, most of these studies have used probes labeled with radioactive material (29, 30), which is not convenient for work in areas where leishmaniasis is endemic, as the disposal of radioactive waste in the environment is difficult and expensive. Thus, we adapted a PCR-hybridization assay (34) so that probes labeled with nonradioactive material could be used. This approach confirmed the presence of *Leishmania* (*Viannia*) in 100% of the samples from 11 patients with confirmed cases of ACL tested.

PCR is an extremely sensitive approach, having the potential to provide specific results in less than 1 day. PCR systems for detection of different species of *Leishmania* are likely to be valuable tools not only for differential diagnosis of ulcer-forming skin diseases but also for investigation of relationships between causative agents and the clinical manifestations and epidemiology of the disease. The possibility of PCR automation, the simplification of sample collection and processing, as


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REFERENCES


10. Eresh, S., S. M. McCullum, and D. C. Barker. 1994. Identification and