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Polymerase chain reaction (PCR) is highly sensitive for diagnosis of mucosal leishmaniasis

Jene Greyce S. Oliveira [a], Fernanda O. Novais [b], Camila I. de Oliveira [b], Antonio C. da Cruz Junior [a], Léon Fábio Campos [a], Any V. da Rocha [a], Viviane Boaventura [b], Almério Noronha [b], Jackson M.L. Costa [b], Aldina Barral [b,c,*]

[a] *Fundação Hospital do Acre, Rodovia BR364, km 2, Distrito Industrial, Rio Branco, AC 69914-220, Brazil*

[b] *Centro de Pesquisas Gonçalo Muniz, Fundação Oswaldo Cruz, Rua Waldemar Falcão, Av. Waldemar Falcão, 121 Brotas, CEP 40295-001, Salvador, BA, Brazil*

[c] *Faculdade de Medicina da Bahia, Universidade Federal da Bahia, Praça XV de Novembro, s/n Largo do Terreiro de Jeusus, Salvador, BA 40025-010, Brazil*

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Abstract

We evaluated the use of polymerase chain reaction (PCR) for diagnosis of mucosal leishmaniasis (ML) in an endemic area in Acre, Brazil, where *Leishmania braziliensis* is present. *Leishmania* DNA was detected 34 of 35 cases, yielding a positivity rate of 97.1%, which was higher than the positivity rates for all of the other diagnostic methods studied, namely Montenegro skin test (MST), anti-*Leishmania* serological testing and microscopic examination of lesion biopsy specimens. These findings have led us to propose guidelines for the diagnosis of ML that use PCR as the principal method of parasitological confirmation of cases.
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Keywords: Polymerase chain reaction; *Leishmania braziliensis*; Mucosal leishmaniasis

1. Introduction

Leishmania is a protozoan parasite and the causative agent of leishmaniasis, a human disease with diverse clinical manifestations. An estimated 12 million people are currently infected with *Leishmania* species and 350 million people currently live at risk of infection (http://www.who.int/tdr/dw/leish2004.htm). 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 to the skin or metastasize to the nasopharyngeal tract. This last manifestation, known as

* Corresponding author. Tel.: +55 71 356 8782x215; fax: +55 71 356 8782x261.
E-mail address: abarral@cpqgm.fiocruz.br (A. Barral).

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mucosal leishmaniasis (ML), occurs in 2–4% of patients with ACL. In this case, the lesions are chronic and spontaneous healing is rarely observed (Marsden, 1994). The number of ML cases has increased in the last few years in Northern Brazil, particularly in the state of Acre, where it accounts for 21% of the ACL cases (FUNASA, 2000).

Definitive diagnosis of leishmaniasis is made by the demonstration of *Leishmania* parasites in patients' tissue sample or by culture or, still, by inoculation of animals with material extracted from lesions. These methods are time consuming, laborious, and generally, require experienced personnel and special facilities in order to be performed. However, due to the paucity of parasites in lesions, these methods usually present low sensitivities. To overcome these obstacles, several methods based on the amplification of *Leishmania* DNA from patients' tissues have been developed (Belli et al., 1998; Aviles et al., 1999; Rodrigues et al., 2002; Weigle et al., 2002). These studies have collectively shown that polymerase chain reaction (PCR) sensitivity for *Leishmania* detection in biopsy samples ranges from 75.7 to 100% whereas specificity ranges from 96.4 to 100%. In terms of ML, reports in the literature have shown that the sensitivity of detection by PCR varied from 47.4 to 83.3% (Uezato et al., 1998; Piñero et al., 1999; Onuma et al., 2001). The detection of parasite DNA is sufficient for diagnosis purposes, however, higher specificity, such as identification of parasite species can contribute directly to the understanding of the epidemiology of leishmaniasis.

Several research groups have examined the use of the PCR as a diagnostic tool in areas where leishmaniasis is endemic, however, to this date diagnosis is still largely based on immunological tests such as serology and/or Montenegro skin test (MST). The aim of the present study was to evaluate the contribution of the PCR to the diagnosis of ML in an endemic area in Brazil.

2. Subjects, materials and methods

2.1. Study area and patients

Biopsy specimens were obtained from patients with ML who attended the otorhinolaryngology clinic of the Hospital Foundation of the State of Acre (FUNDAHCRE) and the Barral y Barral Health Care Centre during the period from 1999 to 2002. These health posts are located in Rio Branco, Acre, Northern Brazil, where *L. braziliensis* is endemic. Research was conducted in accordance with the Research Ethics Committee from the State Foundation Hospital of Acre guidelines for Human Experimentation and the Brazilian Ministry of Health regulations for research involving humans. 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. Patients were submitted to an anterior rhinoscopy and oral examination and, in some cases, patients were also submitted to endoscopic exploration with an optical fibroscope (0° or 70° telescope), in order to probe for extension of the lesion to the pharynx or larynx. Mucosal lesions were characterized by diffuse infiltration with a granular surface or ulceration. The clinical characteristics of the 35 patients with ML, enrolled in this study are shown in Tables 1 and 2. All patients were treated with intravenous pentavalent antimony (Glucantime®) at a dose of 20 mg Sb^{5+} (kg day)$^{-1}$ for 30 days. All patients were cured, as show by the presence of healed mucosal lesion, disappearance of inflammatory signs and the presence of a scar, without recurrences up to 1 year after treatment. The definition of a confirmed case of ML was, in this case, based on the presence of a compatible epidemiological history, the presence of circulating antibodies (as detected by immunofluorescense assay, IFA) or a positive Montenegro skin test and clinical response to treatment.

Table 1
Clinical epidemiological characteristics of 35 patients with mucosal leishmaniasis enrolled in a study of diagnosis of *Leishmania* by PCR

Characteristic or finding	Value
Male sex	28 (80%)
Age, years (median)	39 (6–80)
Occurrence of previous infection	33 (94.3%)
Presence of cutaneous scar	33 (94.3%)
Duration of disease, days (median)	35 (1–60)
Dose of intravenous pentavalent antimony, ampoules (median)	90 (20–180)
Positive Montenegro skin test	33 (94.3%)

Table 2
Clinical presentation of mucosal lesions in mucosal leishmaniasis patients enrolled in a study of diagnosis of *Leishmania* by PCR

Characteristic or finding	Value[a]
Site	
Nose	28 (80)
Nose and oropharynx	7 (20)
Type[b]	
Infiltrative/vegetating-ulcer	15 (42.9)
Mutilate/destroyed-ulcer	7 (20)
Dry atrophic	6 (17.1)
Associated types	4 (11.4)
Dry ulcer	2 (5.7)
Polypus	1 (2.9)

[a] Data are no. of patients (%).
[b] Classification proposed by Ribeiro and Lopes-Filho (1994).

2.2. MST and serological testing

The Montenegro skin test, in which a delayed-type hypersensitivity reaction is provoked, was performed with a soluble *Leishmania* antigen and it was prepared as described elsewhere (Reed et al., 1986). *Leishmania* parasites were used to perform an indirect immunofluorescense assay test to detect the presence of anti-*Leishmania* antibodies, as described elsewhere (Cuba Cuba et al., 1980).

2.3. Sample collection

After the administration of a local anesthetic, biopsy samples were obtained from the lesions using forceps or Takahachi tweezers. Biopsy tissue speciments 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 analysis was performed using a polyclonal antibody against *Leishmania*, as described elsewhere (Barral et al., 1991). For negative controls, biopsy specimens were obtained from a collection of lesions originated from mucosal diseases not related to leishmaniasis.

2.4. Polymerase chain reaction

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 (GE Healthcare), 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 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 containing 50 mmol/l KCl, 10 mmol Tris–HCl (pH 8.0), 0.2 mmol/l each deoxyribonucleotide (Invitrogen), 1 μmol/l each primer, 1.25 units 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 visualization under UV light. DNA from the reference *L. braziliensis* strain MHOM/BR/75/2904 was used as a positive control.

3. Results

3.1. Clinical characteristics of patients with ML

Biopsy samples were obtained from 35 patients with ML, all of whom were clinically cured after completion of the treatment regimen. Tables 1 and 2 show the clinical characteristics of this patient population. The MST was positive for 33 (94.3%) of 35 patients and negative for 2 (5.7%) of 35 patients. The lesion involved nose and oropharynx in 7 (20%) of 35 patients and 22 (63%) of 35 patients developed destructive or vegetating ulcers.

3.2. Conventional methods for detection of *Leishmania*

Table 3 shows the results obtained by conventional diagnostic methods of *Leishmania*. The 35-biopsy samples were submitted to histopathological examination, which consisted of both HE staining and immunohistochemical testing of biopsy sections. Direct visual-

Table 3
Results of tests used for diagnosis of mucosal leishmaniasis

Method[a]	No. of positive results/total no. of tests performed (%)
HE	20/35 (57)
IHQ	24/35 (68.6)
PCR	34/35 (97)

[a] HE: hematoxylin–eosin staining, IHQ: immunohistochemistry.

ization of parasites by HE staining was possible in 20 (57%) of 35 samples whereas immuohistochemistry allowed for the detection of parasites in 24 (68.6%) of 35 samples. Anti-*Leishmania* serological testing was performed in 12 samples and the sensitivity was 83.3% (data not shown).

3.3. PCR detection of Leishmania

PCR was performed using primers that amplify a conserved region of *Leishmania* kinetoplast minicircles. In 34 (97.1%) of 35 biopsy samples examined, the characteristic 120-bp amplicon was detected (Fig. 1). Positive and negative controls were included in all reactions to ensure correct amplification of the *Leishmania* product. The specificity of the PCR for the detection of *Leishmania* species was found to be 100% since no amplification product was detected in samples from patients with ulcers not related to leishmaniasis or biopsy samples from normal skin (data not shown). The sensitivity of the PCR was significantly higher (0.0001) when compared to MST.

4. Discussion

In the present study, we have investigated the benefits of the PCR as a diagnostic tool for ML, in patients' biopsy samples. In terms of clinical utility, the PCR offers several advantages such as high sensitivity and specificity. Most importantly, however, PCR is more rapid than currently available immunological (serology and Montenegro skin test) and parasitological methods (microscopic examination of lesion biopsy by HE or immunostaining) (de Bruijn and Barker, 1992; Rodgers et al., 1990; Pirmez et al., 1999; Rodrigues et al., 2002; Romero et al., 2001; Isaza et al., 2002). PCR has also been used for diagnosis of mucosal leishmaniasis in blood sample (Piñero et al., 1999).

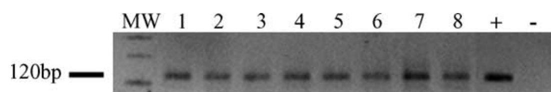

Fig. 1. PCR amplification of *Leishmania* in biopsy samples obtained from patients with mucosal leishmaniasis (ML). MW, 100-bp DNA ladder DNA size marker, lanes (1–8) biopsy specimens obtained from patients with ML; (+) positive control, (−) negative control.

Our results show that the PCR was positive in 34 of 35 biopsy samples obtained from patients with ML, regardless of the results obtained with other diagnostic procedures; therefore, positivity, in this case, was 97.1%. This positivity rate was calculated based on the fact that all patients in this study showed clinical cure, as shown by total scarring of the initial lesion. As previously reported, although clinical cure cannot be considered as "gold standard" for the diagnosis of ML or even cutaneous leishmaniasis, it is a strong indicator that the patient was infected with *Leishmania* parasites (de Oliveira et al., 2003). Moreover, patients enrolled in this study presented a compatible epidemiological history as evidenced by the presence of either lesion in mucosal areas, previous infection by *Leishmania*, positive serologic test and/or positive Montenegro skin test. The PCR was 100% specific since no amplification product was obtained in biopsies from dermatological lesions not caused by *Leishmania* or biopsies obtained from normal skin (data not shown). Parasitological confirmation by histopathology was possible in 57% of samples and this rate of positivity was increased to 68.6% when immunohistochemistry was used. This is compatible with previously published results (Belli et al., 1998; Rodrigues et al., 2002).

The Montenegro skin test is simple and sensitive and it is the main test used for confirmation of infection by *Leishmania*, especially in rural areas where resources for laboratory diagnosis are scarce or nonexistent (Aviles et al., 1999). In this study, we used a soluble *Leishmania* antigen (Reed et al., 1986) and we obtained a positive result in 94.3% of patients, similar to previously published reports (Passos et al., 2002). However, this test cannot discriminate between past and present infection, since we have observed that patients with cutaneous scar, indicative of *Leishmania* infection in the past, have positive skin reaction to *Leishmania* antigen.

In conclusion, the present data support the view that the PCR is the best alternative for diagnosis of ML, when compared to conventional parasitological and immunological methods. To our knowledge, this is the first report on the diagnosis of ML by PCR in an endemic area in Brazil. Despite the still elevated costs associated with molecular diagnosis, the continuous simplification of collection procedures and sample processing associated with the high sensitivity and specificity indicate that the PCR will indeed be the method of choice for the diagnosis of leishmaniasis, particularly ML, where a fatal outcome may occur.

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