Short Report: Use of kDNA-based Polymerase Chain Reaction as a Sensitive and Differentially Diagnostic Method of American Tegumentary Leishmaniasis in Disease-Endemic Areas of Northern Argentina

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Abstract. We evaluated the polymerase chain reaction (PCR) for the diagnosis of endemic American Tegumentary Leishmaniasis in Salta, Argentina. Diverse *Leishmania* species, coexistence of mycotic and varicose ulcers, and high endemicity *T. cruzi*, represent diagnostic challenges in the region. We performed a simplified PCR using sensitive, generic primers on samples obtained by a non-invasive method. We tested different culture types and clinical specimens with other microorganisms that induce leishmaniasis-like lesions. The PCR had a sensitivity and specificity of 100%. Forty-five patients with presumptive leishmaniasis were compared to the PCR, smears, and the Montenegro skin test (MST). In the same population, the PCR had an increased sensitivity, detecting 25 of 45 cases compared with 16 of 45 for smears and had a higher sensitivity in detecting mucocutaneous lesions. Diagnosis by PCR was supported by clinical presentation, positive MST results, compatible epidemiology, and in some cases histopathologic results or isolation of parasites by culture. These findings indicate the convenience of incorporating this PCR into diagnostic strategies for detecting leishmaniasis in northern Argentina.

Leishmaniasis is a parasitic disease caused by hemoflagellates of the genus *Leishmania* and transmitted to humans by the bites of infected phlebotomine sandflies. Depending on the causative species, they can manifest as cutaneous, mucocutaneous, and visceral clinical forms.[1] In Latin America, cutaneous, mucocutaneous and mucosal forms of leishmaniasis are referred to as American tegumentary leishmaniasis (ATL).[2] This infection may be inapparent or may display a wide clinical spectrum, ranging from localized, sometimes self-healing cutaneous ulcers to severe mutilating mucosal lesions or diffuse cutaneous forms.[3] American tegumentary leishmaniasis is found from Mexico to northern Argentina.[4–7] In the province of Salta in northwestern Argentina, ATL is an endemic disease. In recent years, there has been a steady increase in the prevalence and/or recognition of the disease in this area, causing a negative socioeconomic impact.[2,7–9]

Diagnostic methods for ATL in this region are based on clinical evaluation, Montenegro skin test (MST), and demonstration of amastigotes in skin lesions by microscopic examination of cutaneous scrapings. Because the geographic distributions of leishmaniasis and Chagas disease overlap in many areas of northern Argentina, the MST may show cross-reactivity, which leads to misdiagnosis.[2,8] Similarly, histoplasmosis, paracoccidioidomycosis, dermal tuberculosis, and other diseases may be misdiagnosed as early lesions of cutaneous leishmaniasis.[1,10–12] Conventional smears display low sensitivity in detecting occult and subclinical infections.[1] Moreover, this method is time-consuming in lesions with few parasites. To overcome these difficulties, new molecular approaches in the diagnosis of leishmaniasis have been developed. Among these, the polymerase chain reaction (PCR) is the most widely used. The PCR has been evaluated as a diagnostic tool in areas where leishmaniasis is endemic.[13]

Our group in collaboration with Hospital del Milagro (Salta, Argentina) has investigated the use of a low-cost, simplified PCR procedure that may be used in central reference laboratories for confirmation of results of conventional methods and/or more sensitive detection.[14] Most of the patients attending this hospital were either residents or had spent some time in disease-endemic zones and had chronic lesions. Based on previous work, we developed a PCR procedure using generic primers.[14–16] Because most *Leishmania* species, such as *L. (Viannia) braziliensis*, its variants, and *L. (Leishmania) amazonensis*, in Salta cause mucosal disease, systemic treatment is proposed for all species described.[2,17,18] Primers 13A and 13B amplify a specific 120-basepair kinetoplast DNA sequence common to all *Leishmania* species.[16] This sequence is highly repetitive and provides a basis for sensitive PCR detection. The use of these generic primers provides a sensitive technique for obtaining a rapid diagnosis and preventing development of mucosal disease.

The aim of the present study was to validate the PCR as diagnostic method in a field setting in which high sensitivity and specificity are required and other pathogens that produce clinically similar lesions are present. This is the first study using the PCR as a routine diagnostic tool for leishmaniasis in clinical practice in Argentina. Rather than screen for positive PCR results in large populations of patients, we tested the diagnostic usefulness of this method with selected bacterial and fungal diseases in the area. To optimize the PCR and measure its detection sensitivity for *Leishmania* sp., we used serial, calibrated dilutions of laboratory samples of *Leishmania pifanoi* promastigotes diluted in 200 µL of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) as described.[19]

This study included 82 patients, 20 with a parasitologically confirmed diagnosis of leishmaniasis (positive controls), 17 with a confirmed diagnosis of other ulcerative dieseases (negative controls), and 45 with suspected leishmaniasis. All patients were recruited from areas of vectorial transmission of *L. (Viannia) braziliensis* within three phytogeographically regions described previously in the province of Salta, Argentina.[18] They attended the Instituto de Enfermedades Tropicales in Oran, Salta, or the Hospital del Milagro in Salta. All patients provided written informed consent. The study was

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reviewed and approved by the Ethical Committee of the Faculty of Health at the National University of Salta and the Ministry of Public Health.

Results in the 20 positive control patients had been confirmed by microscopy and clinical examination. Eighteen had cutaneous ulcers and 2 had mucocutaneous lesions. Among the 17 negative controls, 11 had lesions clinically compatible with ATL, but they were diagnosed as having other skin diseases such as myiasis (n = 2), leprosy (n = 2), varicose ulcer (n = 2), histoplasmosis (n = 2), paracoccidioidomycosis (n = 2), and traumatic ulcer (n = 1). The 45 patients with suspected leishmaniasis (Table 1) underwent clinical examinations and laboratory evaluations. They were also tested by PCR for *Leishmania* and subclassified into groups with alternative diagnoses by additional parasitologic, bacteriologic, and mycologic tests. We also tested culture isolates of *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, *Mycobacterium tuberculosis*, *Trichophyton* sp., and *Candida* sp. obtained from six patients. A search for inhibitory factors was performed in all negative controls by adding to each PCR sample a potential inhibitor and positive sample in a proportion 10 to 1.

For calculation of sensitivity and specificity, the unit of analysis was the patient, not the specimen or test. Simultaneous samples obtained from the same patient were often not all positive. In such patients, a positive test result was considered evidence of infection.

Dermal scrapings from the bottom and margin of lesions were obtained as described, stained, and examined by microscopy.[20,21] All samples, including cultures, were collected with a sterile wooden toothpick, placed in an Eppendorf (Hamburg, Germany) tube containing 200 μL of TE buffer, heated for 10 minutes in a boiling water bath, and frozen at −20°C until use. After centrifugation at 14,000 rpm for 1 minute, 10 μL of supernatant was used for the PCR.[14,22,23] Triplicate samples from each lesion were usually collected for smears and the PCR. The MST was performed by injecting 0.1 mL of leishmanin (40 μg of protein nitrogen/mL) intradermally into the forearm of a person as described.[2]

The PCR with primers 13A (5′-GTGGGGGAGGGGCGTTCT-3′) and 13B (5′-ATTTTACACCAACCCCCAGTT-3′) was performed according to the procedure of Belli et al;[14] all positive samples amplified a 120-basepair product.[14,16] Precautions were taken to avoid contamination of specimens and amplicons, as described.[24]

The detection limit of the PCR was 1 parasite/200 μL of TE (Figure 1). The 120-basepair amplification product was ob-

FIGURE 1. Sensitivity of the polymerase chain reaction. Lane b, blank; lane N, negative control (*Trypanosoma cruzi* DNA); lane P, positive control (*Leishmania* sp. DNA); lane 1, 10 parasites; lane 2, 5 parasites; lane 3, 1 parasite; lane 4, 0.5 parasites; lane, M, 100-basepair molecular mass marker.

served in all positive control patients, which indicates a PCR sensitivity of 100% (Figure 2). No product was observed from amplification of the negative control samples, which indicated a PCR specificity of 100% (Figure 3A and B). These results showed that PCR detects only *Leishmania* sp. and is useful for differential diagnosis. Lack of amplification was not associated with the presence of inhibitors (Figure 3C).

A diagnostic classification was made using microscopic and PCR results for the 45 patients with suspected leishmaniasis. All had ulcerations compatible with leishmaniasis and 41 (91.1%) of 45 had acquired their disease in the leishmaniasis-endemic zone (Table 1). Microscopic results were positive in 16 patients and negative in 29 patients The PCR results were positive in 25 patients and negative in 20 patients. All patients with positive microscopic results were also positive by PCR. Stained smears detected only 16 (64%) of 25 cases positive by PCR; this method detected an additional 9 of the 25 infected patients. The PCR protocol also showed higher sensitivity with mucocutaneous lesions, which confirmed results of previous studies in which PCR was the best method for diagnosis of mucosal disease.[25]

Assessment of the correlation between microscopic and PCR results and MST results indicated that most (22 of 32) patients positive by either by microscopy, PCR, or both methods were also positive by the MST; only 1 of 11 was negative by the MST. The 20 patients with negative PCR and microscopic results were sub-classified as follows. Ten had a positive result in the MST, 3 with bacteriologic infections, 1 with a mycologic infection, and 6 in which no specific pathogens were demonstrated. Ten had a negative result in the MST: 3 with bacteriologic infections, 3 with mycologic infections, and 4 in which no specific pathogens were demonstrated. On the asis of clinical and epidemiologic data, the 10 patients with a positive result in the MST, especially 6 with no other specific diagnosis, were most likely infected with *Leishmania* at low parasite loads.

TABLE 1
Clinical and epidemiologic characteristics of patients (n = 45)

Characteristics	% (n)	
Sex	Female	Male
	15.6% (7)	84.4% (38)
Type of lesion	Cutaneous	Mucocutaneous
	60% (27)	40% (18)
Time of lesion	Acute: ≤ 50 days	Chronic: > 50 days
	22.2% (10)	77.8% (35)
Residence	Salta City	Province/periphery
	28.8% (13)	71.2% (32)
Place where disease was acquired	Endemic zone	Non-endemic zone
	91.12% (41)	8.88% (4)

FIGURE 2. Polymerase chain reaction amplification of *Leishmania* DNA in patients who were microscopically positive. Lane b, blank; lane N, *Histoplasma capsulatum* in TE buffer; lanes 1–4, dermal scraping samples; lane P, *L. pifanoi* parasites in TE buffer; lane M, 100-basepair (bp) molecular mass marker.
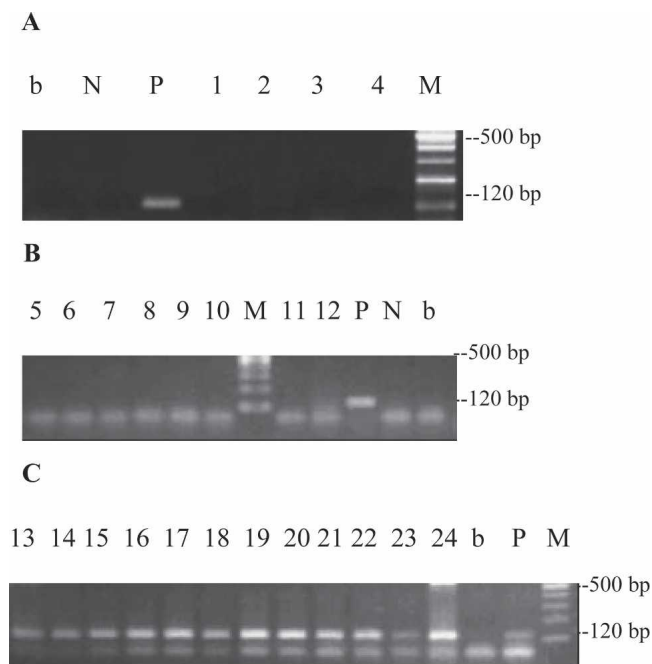
FIGURE 3. **A**, Polymerase chain reaction (PCR) amplification of *Leishmania* DNA in patients with lesions clinically compatible with leishmaniasis but with a laboratory-confirmed diagnosis of another disease. Lane b, blank; Lane N, DNA from a human blood sample, lane P, dermal scraping from a leismaniasis patient positive by microscopy; lane 1, leprosy; lane 2, traumatic ulcer; lane 3, myiasis; lane 4, varicose ulcer; lane M, 100-basepair (bp) molecular mass marker. **B**, PCR amplification for *Leishmania* DNA in mycologic and bacteriologic samples. Lane 5, histoplasmosis; lane 6, paracoccidioidomycosis; lane 7, paracoccidioidomycosis; lane 8, *Candida* sp.; lane 9, *Tricophyton* sp.; lane 10, *Histoplasma capsulatum*; lane M, 100-bp molecular mass marker; lane 11, *Paracoccidioides brasiliensis*; lane 12, *Mycobacterium tuberculosis*; lane P, dermal scraping from a leishmaniasis patient positive by microscopy; lane N, *Trypanosoma cruzi* DNA; lane b, blank. **C**, PCR inhibition test. Lane 13, leprosy; lane 14, traumatic ulcer; lane 15, myiasis; lane 16, varicose ulcer; lane 17, histoplasmosis; lane 18, paracoccidioidomycosis; lane 19, paracoccidioidomycosis; lane 20, *Candida* sp.; lane 21, *Tricophyton* sp.; lane 22, *H. capsulatum*; lane 23, *P. brasiliensis*; lane 24, *M. tuberculosis*; lane b, blank; lane P, dermal scraping from a leishmaniasis patient positive by microscopy; lane M, 100-bp molecular mass marker. Note that all samples did not contain any factors that interfered with amplification of *Leishmania* DNA.

Cure after treatment has been used as diagnostic evidence for leishmaniasis.[26] However, we cannot compare this diagnostic evidence with PCR results; treatment is sporadic because most of our patients do not live in Salta and must travel considerable distances for treatment and hospital resources are insufficient. Moreover, these patients are often resistant to treatment because of reactivation of their lesions.

In the 9 patients who had positive results only by PCR, the diagnosis of *Leishmania* infection was also supported by clinical presentation (9 of 9), positive MST result (8 of 9), or epidemiology compatible with ATL (8 of 9). Histopathologic results showed a correlation with a diagnosis by PCR in some patients, which confirmed the results of a study conducted in an adjacent area endemic for leishmaniasis.[27] Moreover, of the nine patients, four had the mucocutaneous form of the disease with a clinical presentation similar to mycosis, but results of mycologic diagnosis were negative in all nine patients. Of three 3 patients with cutaneous disease, one had a

positive *Leishmania* culture, and the remaining two had a diagnosis by histopathologic results.

Although the PCR is an expensive method, it would be useful if it were available in developing countries such as Argentina where the areas of leishmaniasis transmission overlap with areas of other endemic ulcerative diseases. Use of this method would increase the number of positive diagnoses in patients in which smears are negative, which would enable them to receive adequate treatment and avoid development of mucocutaneous complications.

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