Improving *Leishmania* Species Identification in Different Types of Samples from Cutaneous Lesions

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The discrimination of *Leishmania* species from patient samples has epidemiological and clinical relevance. In this study, different gene target PCR-restriction fragment length polymorphism (RFLP) protocols were evaluated for their robustness as *Leishmania* species discriminators in 61 patients with cutaneous leishmaniasis. We modified the hsp70-PCR-RFLP protocol and found it to be the most reliable protocol for species identification.

Human infections by *Leishmania* spp. produce a pleomorphic syndrome in which symptomatology depends on the parasite species and the immunological stage of the host. The symptoms range from completely asymptomatic to cutaneous, mucocutaneous, and visceral (1). Several authors have reported differences in the treatment outcomes linked to the parasite species (2–5). Furthermore, mucocutaneous leishmaniasis (MCL) is a belated complication associated with specific parasite species (3, 6) most commonly occurring in infections caused by the *Leishmania* (*Viannia*) subgenus. American cutaneous leishmaniasis (ACL) cases are usually the result of infections produced by this subgenus, and species identification is useful for treatment and prognosis. Molecular techniques may become a routine way to confirm suspected cases of ACL (7–10); the present study describes the best PCR-restriction fragment length polymorphism (RFLP) gene target for determining the species of *Leishmania* present in clinical samples from ACL lesions in a set of Colombian patients.

The study was approved by the boards of ethical conduct of the Hospital Militar Central-Bogota-Colombia (HOMIC) and Centro Dermatologico Federico Lleras Acosta Bogotá-Colombia (CDFLL) in accordance with national (resolution 008430 of the Colombian Health Ministry) and international (Declaration of Helsinki and amendments, World Medical Association, South Korea, 2008) guidelines. DNA was extracted from skin biopsy specimens from the internal border of the lesions from 42 adult patients with a clinical diagnosis of ACL. The diagnosis was confirmed microscopically in 35 patients and by PCR detection of the parasite in 7 patients. All patients voluntarily participated in the study and signed an informed consent.

The CDFLL biobank provided 19 Giemsa-stained slide smears from cutaneous lesions. In 17 of them, the presence of *Leishmania* sp. amastigotes was microscopically confirmed, and in 2 smears, the detection of the parasite was established by PCR. DNA was recovered from the Giemsa-stained smears.

All PCRs performed included DNA from 2 negative-control patients from CDFLL (with confirmed diagnoses of sporotrichosis and echyyma gangrenosum) and from 3 healthy volunteers. The entire group of patients had been infected within the Colombian borders.

We selected genes and sequences previously reported to be useful markers for species identification by PCR-RFLP of *Leishmania* species for further evaluation. We analyzed zinc-metallo-protease (*gp63*) (11), spliced leader (SL) (12), cysteine protease B1 (*cpb*) (13), and heat shock protein 70 (*hsp70*) (14, 15) for their ability to discriminate the *L.* (*Viannia*) subgenus in clinical samples. Using the PCR-RFLP protocols previously reported for each gene, we extracted DNA from the reference strains of *Leishmania* species commonly associated with ACL in Colombia (*Leishmania* (*Viannia*) panamensis, *L.* (*Viannia*) braziliensis, *L.* (*Viannia*) guyanensis, *Leishmania* (*Leishmania*) amazonensis, and *L.* (*Leishmania*) mexicana) (5, 16, 17). Other *Leishmania* species circulating in South America were not considered in the present study.

*gp63* PCR amplification of DNA from the reference strains produced an expected fragment of 870 bp, in agreement with previous reports (11) (data not shown). However, when the fragments were digested with SalI and ApaI, only the *L.* (*Leishmania*) amazonsensis amplicon digestion behaved as described previously (11).

Amplification of the SL sequence was performed with organisms of the *Leishmania* (*Viannia*) and *Leishmania* (*Leishmania*) subgenera, as described previously (12). Using genomic DNA from *L.* (*Leishmania*) amazonsensis and *L.* (*Leishmania*) mexicana reference strains, products of 300 and 320 bp were identified, respectively. For species belonging to the *Leishmania* (*Viannia*) subgenus, an expected 226-bp fragment was obtained. Further digestion of those fragments using HaeIII allowed for the identification of *L.* (*Viannia*) braziliensis but could not discriminate between *L.* (*Viannia*) guyanensis and *L.* (*Viannia*) panamensis. We successfully amplified the genomic DNA of cultured promastigotes; however, when we amplified up to 200 ng of genomic DNA from various clinical samples, the results were negative.

Amplification of the cysteine protease B (*cpb*) gene from the genomic DNA derived from parasite cultures, according to the
protocol reported elsewhere (13), was successful. When patient samples were used, the amplification of human DNA was also obtained. A fragment of about 1 kb was present in all human DNA negative controls and in clinical samples (data not shown). We isolated and cultured parasites from patient 8 and amplified the cpb gene. This amplification yielded a unique 1.3-kb band of the expected size. However, when DNA was extracted directly from the patient biopsysample, we detected a light specific band and a strong band of about 1 kb.

Using DNA from reference strains and 10 clinical samples randomly selected from this patient’s cohort, the hsp70 gene was PCR amplified and digested with HaeIII and BcI, as previously reported (14, 15). This protocol was applied to the entire set of clinical samples, and the hsp70 gene was chosen for further evaluation.

In 39 out of 61 samples with clinically suspected ACL, no obvious amplification products of the hsp70 gene fragment were observed. A nested-PCR protocol was designed to improve the yield of hsp70 DNA. After cleaning the PCR product, amplification yielded the original hsp70 fragment, as previously described (14, 15), and this product was used as the DNA template for a second round of amplification. After determining the ideal amount of DNA (ranging from 1.9 ng to 19.2 ng), nested-PCR was performed, using the described conditions for the first round of amplification (15). The following internal primers were used for the second round of amplification: Fw (5’-ACTTCAACGACTCGCA GGCACA-3’) and Rv (5’-ATCGGTTGCGATGTCCTCCA-3’). The amplification products were digested with HaeI11 and Bc1I (14, 15) (Fig. 1).

The approach described in this report allowed for the identification of Leishmania species in clinical samples (Table 1). The predominance of L. (V.) braziliensis associated with ACL in our patients contrasts with previous reports that describe L. (V.) panamensis as being the main species producing ACL in Colombia (5, 16, 17). This might be a result of a bias of the present study in the selection of patients, given that most of them come from eastern Colombia, where L. (V.) braziliensis is predominant.

The identification of Leishmania species of the subgenus Leishmania (Viannia) in clinical samples has been challenging with the current PCR-based protocols (18). Even with rigorous approaches, such as stepwise PCR, samples with a weak-positive target gene signal after diagnostic PCR have not enabled identification at the species level (19). The nested-PCR for hsp70 implemented in the present study successfully identifies Leishmania species in clinical samples, even from low concentrations of parasite DNA, as was the case for the direct smears. This is a significant contribution for species differentiation in cases with little parasite DNA in the specimen, such as samples obtained by noninvasive diagnostic sampling.

**TABLE 1 Distribution of Leishmania species identified by hsp70-RFLP and hsp70-nested-PCR-RFLP**

<table>
<thead>
<tr>
<th>Leishmania species identified</th>
<th>No. of patients</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. braziliensis</td>
<td>46</td>
<td>75.4</td>
</tr>
<tr>
<td>L. panamensis</td>
<td>9</td>
<td>14.8</td>
</tr>
<tr>
<td>L. guyanensis</td>
<td>2</td>
<td>3.3</td>
</tr>
<tr>
<td>Undefined pattern</td>
<td>3</td>
<td>4.9</td>
</tr>
<tr>
<td>No amplification of hsp70</td>
<td>1</td>
<td>1.6</td>
</tr>
</tbody>
</table>

*From a total of 61 samples, the method improved in the present study allowed species identification in 93.5% of the samples. The undefined patterns in 4.9% might correspond to mixed infections or rare gene polymorphisms. A DNA sample from a patient whose direct skin smear was positive for microscopy did not amplify using either the hsp70 or the hsp70-nested-PCR.

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REFERENCES


