Short Communication

Molecular diagnosis of cutaneous leishmaniasis in an endemic area of Acre State in the Amazonian Region of Brazil

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Abstract

Introduction: This study proposes to identify the Leishmania species found in the skin lesions of cutaneous leishmaniasis (CL) patients from Brasiléia municipality (Acre).

Methods: Skin biopsy imprints or biopsy fragments were assayed via kDNA-PCR/RFLP and FRET-real-time PCR.

Results: Of individuals with suspected CL, 18 were positive for Leishmania DNA. Leishmania (Viannia) braziliensis (61.1%) and Leishmania (Viannia) guyanensis (5.5%) were identified in the positive samples.

Conclusions: These results are congruent with the previous reports in Acre and Bolivia, revealing L. braziliensis as the most prevalent species. L. guyanensis identification also corroborates with the epidemiology of the disease in the Amazon Basin.

Keywords: Cutaneous leishmaniasis. Molecular diagnosis. Acre State.
Center from September 2013 to February 2015. All patients provided written informed consent for collection of samples and subsequent analysis.

The City of Brasília is located in the Northern State of Acre, Brazil [11°00′S 68°44′W] (Figure 1) and had a population of 23,378 inhabitants in 2015. The region exhibits an equatorial tropical climate with annual temperatures between 22°C and 33°C and annual precipitation around 1,900mm.

All patients reside in Brasília, Acre and attended to the Fernando Azevedo Correia Center of Health. They presented with skin lesions with characteristics of CL and were submitted to Montenegro skin test. Between September 2013 and February 2015, patients with suspected or confirmed CL were invited to participate in the study after providing signed, written informed consent. Participants provided sociodemographic information, including sex, age and location (rural or urban area) during the medical appointment. In addition, skin biopsy imprints on filter paper (FTA® cards) were collected from the lesions, and in some cases, biopsy fragments were obtained from the border of lesions after administering a local anesthetic [2% lidocaine (xylocaine)]. These samples were stored in liquid nitrogen until processing. All recommended measures (e.g. the use of individual disposable blades for filter paper cutting and careful individual packaging of material) were taken to prevent cross contamination.

Deoxyribonucleic acid (DNA) was extracted from FTA® cards (skin biopsy imprints) and biopsy fragments as described by Marques et al.9 The kinetoplast DNA (kDNA)-polymerase chain reaction (PCR) was performed as previously described10. Following amplification, kDNA products were digested with HaeIII (Invitrogen®, USA) for 3h at 37°C, in accordance with the manufacturer’s recommendations, and the resulting digestion fragments were analyzed on a 12% silver-stained polyacrylamide gel. A fluorescent resonance energy transfer (FRET) real-time

PCR based on *Leishmania* species-specific DNA polymorphisms in the genes encoding mannose phosphate isomerase (MPI) and 6-phosphogluconate dehydrogenase (6PGD) was carried out to support the identification of the *Leishmania* species in the kDNA-positive samples\(^1\).

**Twenty-two patients who provided clinical and sociodemographic information and presented positive results in the Montenegro skin test were included (Table 1).** Of those, 18 lived in rural areas (transversely situated along the Federal BR317 Trans Pacific highway, named Ramal or Ramais, based on the distance from the center of Brasiléia); three patients were from urban areas (center of Brasiléia), and one represented an imported case from Cobija, Pando District, in Bolivia (Figure 1 and Table 1). Most patients from rural areas (\(n=12\)) resided in isolated reserves located far away within the Ramais, in dense forest sites. The remaining patients from rural areas lived in kilometer 4 - *R*amal do Polo (\(n=5\)) and kilometer 5 - Ramal do Jarinal (\(n=1\)), less isolated than the reserves, but still close to the dense forest (Figure 1). The houses were simple dwellings without basic sanitation, with many peridomestic animals, providing a favorable environment for the attraction and multiplication of insect vectors.

The patients positive for the Montenegro skin test presented with lesions that were clinically characterized as simple or multiple ulcer-crusted lesions with 59% of them presenting irregular borders and were mostly observed on the lower limbs (Table 1). Detection of *Leishmania* genus parasites via kDNA-PCR resulted in 18 positive samples out of 22 patients with clinically suspected CL. The 4 negative samples (three biopsy fragments and one imprint) were obtained from individuals living in kilometer 4 - *R*amal do Polo (rural area; 3 males and 1 female) (Table 1). This negative result may probably be due to the lapse of time between lesion formation and the collection of clinical material, considering that parasite detection becomes more difficult as the number of parasites in the lesion decreases with the progression of the chronic granulomatous process\(^12\). Following HaeIII digestion of the kDNA amplified products from 18 positive samples, the restriction profile corresponding to *L. (V.) braziliensis* was identified in 11/18 samples (61.1%), namely the presence of two fragments of 40 and 80bp in 12% silver-stained acrylamide gel (data not shown).

FRET-based real-time PCR was used to confirm the identification of the species of *Leishmania* in the positive kDNA samples. This assay is based on the identification of

**TABLE 1:** Characteristics of the study group and diagnostic results of skin biopsy imprints and biopsy fragments from cutaneous lesions of patients with positive Montenegro test.

<table>
<thead>
<tr>
<th>Patients</th>
<th>sex</th>
<th>Variable</th>
<th>age</th>
<th>area</th>
<th>imported case</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>male</td>
<td>female</td>
<td>0-7</td>
<td>8-14</td>
<td>15-21</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>12</td>
<td>2</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>Distribution</td>
<td>Unique</td>
<td></td>
<td>7</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Multiple (2-7)</td>
<td></td>
<td>3</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Borders</td>
<td>Regular</td>
<td></td>
<td>5</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Irregular</td>
<td></td>
<td>5</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>Diameter</td>
<td>cm</td>
<td>1-7</td>
<td>0.5-4</td>
<td>1-4</td>
<td>1-3</td>
</tr>
<tr>
<td>Mean duration*</td>
<td>Days</td>
<td>15-120</td>
<td>30-120</td>
<td>15-120</td>
<td>30-120</td>
</tr>
<tr>
<td>Characteristics of the lesions</td>
<td>Upper limbs</td>
<td>X</td>
<td>-</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Localization</td>
<td>Lower limbs</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>Thorax</td>
<td>X</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Face</td>
<td>X</td>
<td>X</td>
<td>-</td>
<td>X</td>
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<tr>
<td>Positive for kDNA**</td>
<td>7</td>
<td>11</td>
<td>1</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Leishmania species</td>
<td><em>L.</em> <em>bra</em></td>
<td>3</td>
<td>8</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td><em>L.</em> <em>guy</em></td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>NI</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

\(L. *bra*\): *Leishmania braziliensis*; \(L. *guy*\): *Leishmania guyanensis*; NI: not identified; cm: centimeters; kDNA: kinetoplast deoxyribonucleic acid.

*Based on the Montenegro skin test. **Positive samples for the presence of *Leishmania* kDNA.
mutations in the 6PGD and MPI genes, yielding distinct melting peaks that are used to differentiate between five *Leishmania* (*Viannia*) species. The method detects down to 60 fg of parasite DNA, which is equivalent to less than five parasites per reaction. **Figure 2** shows an example of the results obtained by the coupled analysis of 6PGD and MPI loci. Three samples (II, VI and VIII) identified as *L. (V.) braziliensis* or *Leishmania (Viannia) peruviana* by the analysis of 6PGD gene (**Figure 2A**) were confirmed as *L. (V.) braziliensis* using the MPI locus (**Figure 2B**). The results of 6PGD analysis revealed infection by *L. (V.) guyanensis* (Floch 1954) in one sample (IX) (**Figure 2A**), whereas the same sample was identified

![Melting Peaks](image)

**FIGURE 2**: Example of melting curve analysis of FRET real-time PCR focusing on the 6PGD and MPI genes. Dotted lines represent cutaneous lesion samples from patients with suspected cutaneous leishmaniasis. Solid lines correspond to a set of New World *Leishmania* reference strains used as standard samples. *Leishmania* species were identified based on melting curves exhibiting the same dissociation temperature. **A. Melting curves corresponding to 6PGD-based amplification reactions**. Samples from three patients (II, VI and VIII) were identified as *L. braziliensis* or *L. (V.) peruviana*, and one sample (IX) revealed the same melting temperature attributed to *L. (V.) guyanensis* reference strain. **B. Melting curves corresponding to MPI-based amplification reactions**. Samples from patients (II, VI and VIII) were positioned at the same melting temperature corresponding to the *L. (V.) braziliensis* reference strain. Sample IX was identified as *L. (V.) panamensis* or *L. (V.) guyanensis*. *L. pan*: *Leishmania panamensis*; *L. guy*: *Leishmania guyanensis*; *L. lain*: *Leishmania lainsoni*; *L. bra*: *Leishmania braziliensis*; *L. per*: *Leishmania peruviana*; *L. ama*: *Leishmania amazonensis*; *L. mex*: *Leishmania mexicana*; *L. (V.)*: *Leishmania (Viannia)* FRET PCR; polymerase chain reaction; 6PGD: 6-phosphogluconate dehydrogenase; MPI: mannose phosphate isomerase.
as *Leishmania* (*Viannia*) panamensis or *L. (V.) guyanensis* through the dissociation profile of the MPI amplification products (Figure 2B). Using FRET real-time PCR, we were able to confirm *L. (V.) braziliensis* infection in 11 out of 18 samples, corroborating the results of HaeIII digestion of kDNA-amplified products (PCR-RFLP - Restriction Fragment Length Polymorphism). It also allowed the detection of *L. (V.) guyanensis* in one sample that did not exhibit a restriction profile congruent with *L. (V.) braziliensis* in the PCR-RFLP.

Infection with *L. (V.) braziliensis* in 61.1% of the patients suggests that this species is the main etiological agent responsible for CL cases in the municipality of Brasiélia, which agrees with previous findings in Rio Branco and other areas in Bolivia and Peru, bordering the State of Acre. This result was expected, considering that *L. (V.) braziliensis* is recognized as the predominant species associated with the evolution to the mucosal form of CL in Latin America. Herein, it is important to note that an imported case of *L. (V.) braziliensis* was identified in a Brazilian individual who became infected in Cobija, Pando district, a border City of Bolivia. In spite of the proximity between Cobija and Brasiélia, it is worth mentioning that the construction of the Pacific Highway BR317 promoted an increase in migration across the Brazil/Peru/Bolivia borders. Together with the growth of tourism, this may have had a role in the dissemination of CL in the region.

We were able to identify *L. (V.) guyanensis* as the parasite responsible for the infection in a female 13 years old patient who resided in the Guanabara extractive reserve, kilometer 67. All *Leishmania* species implicated in the different clinical forms of CL in Brazil have been registered in the Amazon Basin. *L. (V.) guyanensis* has been well characterized in Acre, Amapá, Roraima and Pará, and its main vector is *Nyssomyia umbratilis*, a sandfly recently observed at low density in Acre State. In Peru, *L. (V.) guyanensis* is the third most common species associated with CL cases, following *L. (V.) braziliensis* and *L. (V.) peruviana*.

In 6 samples positive for the presence of *Leishmania* kDNA, identification of the parasite species by both methods was unsuccessful, raising the possibility that other *L. (Viannia)* species could be circulating in Brasiélia, as observed in other regions of the Brazilian Amazon. The HaeIII/kDNA restriction profile was inconclusive, and the samples were not amplified following the 6PGD/MP1 real-time PCR analysis. A possible explanation could be the differences in copy number when comparing *Leishmania* kDNA minicircles and the 6PGD/MP1 loci. The first are represented as thousands of copies per parasite mitochondrial genome, whereas the MPI and 6PGD are present as single copy genes, which could explain the higher sensitivity of kDNA-PCR assay over the approach used in FRET real-time PCR. Further studies are necessary to improve the molecular identification of other *Leishmania* spp. implicated in the epidemiology of CL in the State of Acre.

In conclusion, in this study, most of the individuals with clinically suspected CL live in rural areas (81.8%), suggesting that these local cases are associated with activities in a forest environment, despite the finding of three individuals infected with *L. (Viannia)* living in urban areas in the center of Brasiélia.

The positivity for *Leishmania* kDNA was higher in women (91.7%) than men, and the ages of infected individuals varied from 3 to 30 years old (Table 1).

The study performed in the municipality of Brasiélia revealed the predominance of *L. (V.) braziliensis* in cutaneous lesions of individuals with clinically suspected leishmaniasis, and that cutaneous disease is probably associated with rural lifestyle, particularly with the activities in rubber extraction reserve sites. From an epidemiological aspect, there are a few studies carried-out in Acre, and this study is the first conducted in Brasiélia district despite the small number of individuals studied. Further investigation is needed, taking into account the vectors, reservoirs, the incidence of cutaneous disease, and its respective etiological agents, as well as surveillance activities, and the control of leishmaniasis in the State of Acre.

**Ethical considerations**

The study was approved by the Ethical Committee of the Oswaldo Cruz [Instituto Oswaldo Cruz (IOC)], Oswaldo Cruz Foundation [Fundação Oswaldo Cruz (Fiocruz)], Rio de Janeiro (RJ), Brazil; protocol CAAE2765312.7.0000.5248.

**Acknowledgements**

We would like to thank the Fernando Azevedo Correia Center of Health and in particular, Mrs. Solange Kurvski, who assisted patients with suspected leishmaniasis and those patients agreed to be included in the study. We are also grateful to Hugo Valdivia, Maxy B. De Los Santos, Erika Perez Velez, Andres G. Lescano and the United States Naval Medical Research Unit No. 6, Lima, Peru Departments of Parasitology for support with FRET real time PCR. Also, we would like to thank David M. Forrest for the English revision.

**Conflict of interest**

The authors declare that there is no conflict of interest.

**Financial support**

This work was supported by CNPq (grant# 473859/2013-0) and Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ CNE E_05/2015). C. Britto and R.P. Brazil are research fellows of Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). D. Pita-Pereira is a FAPERJ postdoctoral fellow. FRET real time PCR was supported by the U.S. DoD Armed Forces Health Surveillance Center, Global Emerging Infections Surveillance and Response System (AFHSC/GEIS) project P0372_14_N6.

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