Molecular Detection of Leishmania infantum in Atypical Cutaneous Lesions from Paraguayan Patients

Oscar Daniel Salvioni¹, José Pereira¹, Marco González Sander¹, and Celeste Vega Gómez*²

¹Center for the Development of Scientific Research, CEDIC, Paraguay
²Center for Dermatological Specialties - National Leprosy Control Program, Paraguay

Abstract

Leishmaniasis is one of the most important vector-borne human diseases. Different parasite species are associated with the various forms of the disease. In Paraguay, visceral leishmaniasis is caused by L. infantum, whereas tegumentary leishmaniasis is due mainly to L. braziliensis. Recently, two cases of patients with atypical cutaneous lesions have been described in which the causative agent was determined to be L. infantum. Based on these findings, we aimed to determine the leishmania species of all cutaneous and mucosal biopsy lesions submitted to our research center during 2016 using real-time PCR. We found that 20% of all tegumentary leishmaniasis were due to L. infantum and most of these patients presented with atypical cutaneous lesions and no visceral involvement.

ABBREVIATIONS

ACL: Atypical Cutaneous Leishmaniasis; CL: Cutaneous Leishmaniasis; TL: Tegumentary Leishmaniasis; VIH: Human Immunodeficiency Virus; PCR: Polymerase Chain Reaction; WHO: World Health Organization

INTRODUCTION

The WHO considers leishmaniasis one of the seven most important tropical diseases. It has been estimated worldwide that 350 million people are at increased risk of contracting leishmaniasis, with a total incidence of 2 million cases per year. This infection is caused by an obligate protozoan parasite of the genus Leishmania, transmitted through the bite of dipterous insects belonging to the family Phlebotominae [1].

In Latin America, leishmaniasis is mainly caused by two subgenera, L. (Viannia) and L. (Donovani). The subgenus L. (Viannia) includes L. (V.) braziliensis, L. (V.) panamensis, L. (V.) guyanensis which are the causative agents of tegumentary leishmaniasis (TL). Species within the L. (Donovani) subgenus include L. (L.) infantum (synonym of L. (L.) chagasi) which is the causative agent of visceral leishmaniasis, L. (L.) mexicana and L. (L.) amazonensis, which are responsible for the cutaneous form of the disease [2].

In Paraguay, the phlebotomines and fly Lutzomyia longipalpis is the main vector of leishmaniasis, being L. infantum the causative agent of visceral leishmaniasis, L. braziliensis, and in some cases L. guyanensis, these of the majority of cases of mucocutaneous leishmaniasis [3].

A recent report has shown for the first time in Paraguay, two cases of atypical cutaneous leishmaniasis (ACL), where the causative agent of the lesions was determined to be L. infantum. One of the reported cases was an HIV-immunocompromised adult patient [4]. The other reported case was an immunocompetent adult patient [5].

Based on these reported cases in Paraguay, we aimed to investigate this disease using real-time PCR to avoid false negatives, and to determine the species of all biopsy samples submitted to our laboratory that were positive for the genus Leishmania spp.

MATERIALS AND METHODS

Samples

Cutaneous and mucosal lesion biopsies were obtained from a total of 54 patients with suspected leishmaniasis, submitted from all over the country to the Centro de Especialidades Dermatológicas (San Lorenzo, Paraguay) from January to December 2016. For each patient, the following data were collected: age, gender, pet ownership, and occupation.

The biopsies were obtained using a 4mm biopsy punch and sent in 70% ethanol to the Centro para el Desarrollo de la Investigación Científica (CEDIC), where they were stored at -20°C until DNA was extracted.

DNA extraction

Genomic DNA from cutaneous and mucosal lesions was extracted and purified with Genejet Genomic DNA Purification Kit (#K0722 Thermo Scientific) according to the manufacturer’s instructions. The purity of the DNA samples was assessed with a
Real-Time PCR reactions and sequencing

For the detection of Leishmania spp DNA by real-time PCR, the following primers that amplify the ribosomal RNA internal transcribed spacer 1 (ITS-1) were used: LSGTTS1F: 5’CATTCTCGGATTTACAC 3’, and LSGTTS1R: 5’ GTTTATGGACCCGTTA 3’, described by Almeida et al. [6,7]. For amplification, Maxima Sybr Green qPCR (2X) (#K0251 Thermo Scientific®) was used.

For the determination of the L. (viannia) complex, the following pairs of primers that amplify a region of the flagellar actin gene were used: LVIAN36ACTF:

5’ CAA TGC GAC AATTGATGATGTCGGA 3’ and LVIAN122ACTR:5’ CGCTCCGGCAGATKTC 3’. Determination of L. (donovani) complex was performed using the following protein partial mRNA: 5’ CGCGTGCGCTGTGCTG 3’ and 5’ CCCACAACGGCGGGAACCT 3’. Both pairs of primers were previously described by Colombo et al. [9-10]. For amplification, Maxima Probe qPCR (2X) Mix (#K0261 Thermo Scientific®) was used.

Reactions were performed using a Rotor Gene 6000 (Qiagen) thermocycler in a final reaction volume of 20µL. In each of the amplification reactions positive and negative controls were added.

To confirm the presence of L. infantum, a PCR reaction using pairs of primers that amplify the ribosomal RNA internal transcription spacer 2 (ITS-2) was performed, according to the conditions previously described by Almeida et al. [6]. The purified product was submitted to Macrogen (Korea) for sequencing and the results were analyzed by sequence alignment using the BioEdit Sequence Alignment Editor software.

Statistical analysis

The chi-square test ($\chi^2$) was used to assess whether patients, men and women working in high-risk occupations (farmers, carpenters, etc.), presented statistical significance ($p < 0.05$) with respect to patients (men and women) working in risk-free areas.

RESULTS AND DISCUSSION

54 samples from patients of both sexes with suspected leishmaniasis were analyzed, and 15 of them (28%) presented positivity for the genus Leishmania spp, of whom 13 (87%) are men and 2 (13%) are women, with an average age of 42.53% of the positive cases reported being pet owners.

Of the 54 patients, 15 (28%) are men working in high-risk occupations (farmers) and 18 (33%) are men with occupations that pose no apparent risk; 5 (9%) are women working in high-risk occupations (farmers), and 16 (30%) are women with occupations that pose no apparent risk. After analyzing the variables between gender and type of occupation, a chi-square value of 2.56 was obtained which led us to reject the hypothesis that the gender variable and risky occupation are independent, and that leishmaniasis has been historically regarded in America as a rural and sylvatic disease.

Of the 15 positive cases for the genus Leishmania spp, 12 (80%) corresponded to the L. viannia (L. braziliensis) complex and 3 (20%) to the L. donovani (L. infantum) complex. The great majority of the positive patients were male (Table 1).

The three samples positive for the L. Donovani complex were submitted for sequencing. Once the obtained sequences were aligned, it was confirmed that all of them had a 99% similarity to L. infantum(GenBanK accession number KT438781.1.)

As for the geographical distribution, positive patients for the L. Viannia(L. braziliensis) complex live or work in rural areas of the country with agricultural activities, coinciding with a previous report by Velez ID et al. [7] and with a 2015 report by the Servicio Nacional de Erradicación delpaludismo (SENPA, Paraguay) [11]. Whereas of the three cases positive for L. infantum, one patient resides in a rural area and the other two reside in urban areas of the Central Department. In 2015, SENPA reported that the majority of the visceral leishmaniasis cases (61.3%) occur in the Central Department [12].

In patients that tested positive for L. braziliensis, single or multiple skin and mucosal lesions were observed (Figure 1), in agreement with a description by Chena L et al. [13]. However, in those positive that tested positive for L. infantum, crusted ulcers, papules and skin nodules were observed (Figure 2 and 3), coinciding with a description by Rosal Rabes T et al. [14].

One of the patients that tested positive for L. infantum showed a personal history that might indicate an immunosuppression caused by the presence of HIV. However, the other two patients were immunocompetent adult individuals in which no visceral or systemic involvement was observed. This behavior was observed in regions of Central America and Venezuela where the high prevalence of visceral leishmaniasis caused by L. infantum produced cutaneous lesions such as papules, plaques or nodules, in immunocompromised patients without observable systemic involvement [1-17], in agreement with the results presented in this study.

The prevalence of ACL in areas of Honduras is 27.6% in children younger than 5 years old. This age distribution might be linked to the maturity of the immune system, since the immune status of the patients, their genetic identity, and the characteristics of the parasite, determine whether L. infantum causes visceral or cutaneous lesions [18].

CONCLUSION

These results show that Leishmania infantum must be considered as a potential causative agent of cutaneous leishmaniasis in areas where visceral leishmaniasis is endemic, and that real-time PCR is a sensitive and effective tool that should be used to determine leishmania species in atypical lesions.

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**Table 1: Leishmania detected by real-time PCR in skin and mucosal tissues.**

<table>
<thead>
<tr>
<th>Leishmania spp</th>
<th>L. viannia complex</th>
<th>L. donovani complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR negative</td>
<td>PCR positive</td>
<td>PCR positive</td>
</tr>
<tr>
<td>Men</td>
<td>26</td>
<td>13</td>
</tr>
<tr>
<td>Women</td>
<td>13</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>39</td>
<td>15</td>
</tr>
</tbody>
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Email: mcvegagomez@gmail.com

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Central


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Figure 1 Classical ulcer of cutaneous leishmaniasis caused by L. braziliensis.

Figure 2 Crusted ulcer of outer nose caused by L. infantum.

Figure 3 Purple papules on leg due to L. infantum.

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REFERENCES


