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Molecular Identification of *Leishmania* spp. DNA from Archived Giemsa-Stained Slides of Patients from Salta, Argentina

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Abstract. Cutaneous leishmaniasis is endemic in Salta province, which belongs to the northwest of Argentina. *Leishmania* spp. DNA from Giemsa-stained slides of up to 12 years in storage of patients from Salta was characterized through polymerase chain reaction (PCR) restriction fragment length polymorphism. One hundred smears positive for microscopy, classified in a semiquantitative scale for amastigote density, were analyzed. Also, Leishmanin skin test (LST) results were included. DNA extraction was carried out applying lysis buffer with proteinase K, and then DNA was amplified with ribosomal internal transcribed spacer 1 primers. PCR products were digested with HaeIII enzyme. All PCR-positive smears (74/100) belonged to *Viannia* subgenus. A statistically significant, directly proportional relationship between semiquantitative microscopy and PCR results was detected. All patients had LST-positive results (induration ≥ 5 mm), and the smears of those with smaller induration (LST < 19 mm) gave a higher proportion of positive PCR results. This study determined that smear age did not affect PCR positivity, which allows retrospective analyzes and suggests smears might be useful for molecular complementary diagnosis. Because *Leishmania* (*Viannia*) *braziliensis* is the main circulating species in the study area, determining *Viannia* subgenus in all analyzed samples confirms previous findings. PCR positivity showed statistically significant differences according to semiquantitative microscopy, highlighting the importance of parasite burden in the diagnostic sensitivity of the method. Considering that smears of patients with smaller LST induration were more positive in PCR, a negative smear from patients with positive LST response, but < 19 mm, could actually represent a false-negative result.

INTRODUCTION

Leishmaniasis is a group of parasitic diseases caused by kinetoplastid flagellates from the genus *Leishmania*, which is divided into three subgenera, *Leishmania*, *Viannia*, and *Mundinia*.[1,2] Leishmaniasis presents a variety of clinical manifestations, which include visceral leishmaniasis (VL), mucocutaneous leishmaniasis (MCL), diffuse cutaneous leishmaniasis (DCL), and cutaneous leishmaniasis (CL).[3] In the Americas, CL, MCL, and DCL together receive the denomination of American Tegumentary Leishmaniasis (ATL), which has a wide geographical distribution from southern United States to northern Argentina.[1]

American Tegumentary Leishmaniasis is endemic in the north of Argentina; in fact, the highest number of reported cases (53.1%) comes from Oran and San Martin departments in the north of Salta Province despite representing 0.7% of the country's population, being the primary focus of CL and MCL.[4] In Argentina, *Leishmania* (*Viannia*) *braziliensis* is the causative agent of the large majority of cases of ATL, but *Leishmania* (*Viannia*) *guyanensis*, *Leishmania* (*Leishmania*) *amazonensis*, and *Leishmania* (*Viannia*) *panamensis* have been rarely identified; a single case of VL due to *Leishmania* (*Leishmania*) *infantum* has also been reported from this area.[5–9]

Regarding traditional clinical diagnosis of leishmaniasis, microscopy is the most frequently used method and is based on the search of amastigotes in scrapings taken from the edges of ulcers. Although this technique is simple, expertise and experience are required to identify *Leishmania* spp. amastigotes in a smear because very few parasites may be present.[10,11] On the other hand, microscopy is not able to determine the infecting species because all *Leishmania* parasites are morphologically similar.[12] Generally, *Leishmania* spp. are determined according to their geographical distribution and clinical manifestations. However, geographical criterion becomes inadequate when several *Leishmania* spp. coexist in a particular geographic region.[13] The identification of parasitic species might be important and necessary for epidemiological reasons, treatment, and future control measures.[14]

Despite the disadvantages, Giemsa-stained slides can be easily stored without the need of special preservation conditions, such as low temperatures.[15] Hence, these samples have been used in molecular studies. Motazedian et al., 2002 confirmed CL-suspected cases through PCR, detecting *Leishmania* spp. DNA from smears; similarly, Brustoloni et al., 2007 worked with Giemsa-stained bone marrow slides to diagnose molecularly VL and to determine PCR sensitivity and specificity. Furthermore, identification of *Leishmania* spp. by PCR-restriction fragment length polymorphism (RFLP) was achieved from DNA extracted from smears.[12,16,17]

In this context, the aim of this work was to characterize *Leishmania* spp. DNA from archived Giemsa-stained samples of up to 12 years in storage of patients from Salta, Argentina, through PCR-RFLP. Furthermore, comparisons between molecular results and clinical characteristics of the patients were performed.

MATERIALS AND METHODS

Samples. One hundred Giemsa-stained slides of patients were analyzed in 2014. The patients were diagnosed at

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Instituto de Investigaciones de Enfermedades Tropicales, Oran, Salta. All the samples had been considered positive for CL by microscopy and had been originally taken in the years 2002, 2008–2012 (20 Giemsa-stained slides for each year).

DNA extraction. For cell lysis, each Giemsa-stained slide was covered with 300 µL lysis buffer (50 mM NaCl, 50 mM Tris HCl, 10 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4, 1% v/v Triton X-100 and 100 µg of proteinase k). After a short time, the smear was removed completely and transferred to a 1.5 mL reaction tube. Tubes were incubated at 56°C for 3 hours.[12] Then, DNA was extracted from the lysates by phenol/chloroform extraction. Finally, the pellets were redissolved in 30 µL tris-EDTA (TE) buffer (10 mM Tris and 1 mM EDTA, pH 8) and were kept at 4°C until tested.

Polymerase chain reaction. A PCR assay was carried out to amplify the ribosomal internal transcribed spacer 1 (ITS1) region, which separates the genes coding for the ssu rRNA and L5.8S rRNA using the primers *Leishmania* internal transcribed spacer and L5.8S.[18] Amplification reaction was performed in volume of 50 µL. Five microliters of extracted DNA was added to a PCR mixture, containing 1.5 mM $MgCl_2$, 200 µM deoxynucleotide triphosphates, 20 pmol of each primers and 2 U Taq polymerase (Promega, Madison, WI). Conditions for cycling were 94°C for 4 minutes, followed by 35 cycles of 95°C for 40 seconds, 53°C for 30 seconds, 72°C for 1 minute, and finally 72°C for 5 minutes.[12] *Leishmania* (*V.*) *braziliensis* DNA was used as positive control in every reaction and sterile Mili Q water (Genbiotech, Buenos Aires, Argentina) as negative control. After the amplification, PCR products were analyzed on 2% agarose gel by electrophoresis and visualized under ultraviolet light after staining in ethidium bromide.

Restriction fragment length polymorphism analysis of amplified ITS1. PCR products (15 µL) were digested with HaeIII restriction enzyme according to conditions recommended by the supplier (Thermo Fisher Scientific, Waltham, MA). The restriction fragments were subjected to electrophoresis in 2% agarose and visualized under ultraviolet light after staining in ethidium bromide. DNA from local reference strains was used for PCR and RFLP analysis to compare their restriction patterns with those obtained from patients' samples. The reference strains used were as follows: *L*. (*L*.) *infantum* MCAN/AR/10/NNP4, *L*. (*L*.) *amazonensis* MHOM/BR/73/M2269, *L*. (*V*.) *panamensis* MHOM/PA/71/LS94, *L*. (*V*.) *guyanensis* MHOM/AR/99/JDM1, and *L*. (*V*.) *braziliensis* MHOM/AR/03/OLO1 (Figure 1).[5–9]

Clinical information. Clinical data included semiquantitative microscopic diagnosis from smears, Leishmanin skin test (LST), diameter of the lesion, and age of the lesion. Semiquantitative microscopic diagnosis was undertaken by a technician who classified each smear according to an increasing amastigotes density in P1+: 1–10 parasites/1,000 fields, P2+: 1–10 parasites/100 fields, or P3+: ≥ 10 parasites/10 fields. Negative smear: no amastigotes/1,000 fields. Leishmanin skin test was applied by injecting intradermally 0.1 mL of Leishmanin (40 µg of protein/mL) into the forearm for evaluating the induration size after 72 hours. Leishmanin was locally prepared with a soluble extract of promastigotes of *L.* (*V.*) *braziliensis*, obtained in culture from a patient of our region (strain MHOM/AR/03/OLO1) as described elsewhere.[19] Indurations were considered positive if diameter was ≥ 5 mm. For comparison with other variables, the values of the LST induration diameter were categorized into two classes with 19 mm (< 19 and ≥ 19 mm) as the class mark.

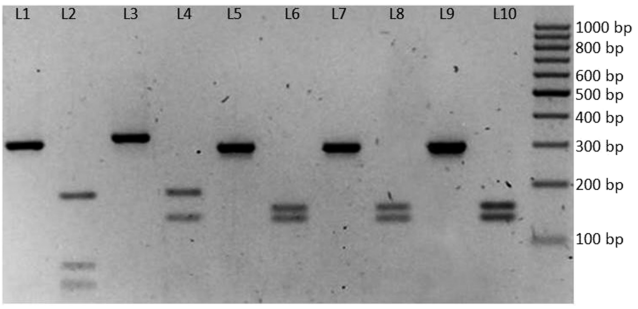

FIGURE 1. Digestion of amplified internal transcribed spacer 1 regions of *Leishmania* spp. with the restriction enzyme HaeIII. Lanes 1 and 2, undigested and digested *Leishmania* (*Leishmania*) *infantum* MCAN/AR/10/NNP4; lanes 3 and 4, undigested and digested *Leishmania* (*Leishmania*) *amazonensis* MHOM/BR/73/M2269; lanes 5 and 6, undigested and digested *Leishmania* (*Viannia*) *panamensis* MHOM/PA/71/LS94; lanes 7 and 8, undigested and digested *Leishmania* (*Viannia*) *guyanensis* MHOM/AR/99/JDM1; lanes 9 and 10, undigested and digested *Leishmania* (*Viannia*) *braziliensis* MHOM/AR/03/OLO1; lane 11, a 100-bp ladder was used as molecular size marker.

For determining the diameter of the lesion, which was scraped for microscopic diagnosis, the longest diameter between the edges of the ulcer was measured in centimeters. The diameter of the lesion was classified into three categories (< 10 mm, 10–25 mm, and > 25 mm). Finally, the age of lesion was recorded according to what patients expressed during the anamnesis, and was measured in days. It was classified into three classes (< 25 days, 25–30 days, and > 30 days).

Statistical analysis. Chi-squared test was used to compare the proportions of ITS1-PCR–positive results among the clinical variables analyzed. Chi-squared test was carried out by GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA). Spearman rank correlation was used to assess a possible association between two variables by R Studio version 0.99.903 (RStudio, Inc., Boston, MA). Significance was defined at P values ≤ 0.05.

Ethical considerations. All patients voluntarily accepted at the time of having their samples taken for diagnostic purposes, to participate in further research studies. The entire database was de-identified. The project was evaluated and approved by the Bioethics Committee of the Universidad Nacional de Salta as part of the research plan on entry to Consejo Nacional de Investigaciones Científicas y Técnicas Research Track of the coauthor A. J. K.

RESULTS

Patients were mainly from Oran and San Martin departments. The localities with higher amount of cases belonged to Oran department; San Ramon de la Nueva Oran city, was the most affected with 58% of the cases, and another 21% of cases were from the city of Hipolito Yrigoyen. The male: female ratio was 5:1. All cases had information on age, with a median interquartile range (IQR) of 37 years old (IQR: 26–50).

The analysis included 100 patients with Giemsa-stained slides taken from cutaneous lesions compatible with CL confirmed by positive microscopic examination. From them, 40 smears were P1+, 28 smears were P2+, and 32 smears were P3+ in the semiquantitative microscopic diagnostic scale. Seventy-four (74%) smears were positive for ITS1-PCR. No parasite DNA was detected by PCR in the negative

controls. Although the oldest samples were 12-year-old slides and newer samples were 2 years old, no statistically significant differences in the PCR results were appreciated ($P > 0.05$). Regarding species identification by ITS1-RFLP, 100% of samples belonged to *Viannia* subgenus (two DNA fragments of 157 and 141 bp) (Figure 2). When comparing the ITS1-PCR results with those of semiquantitative microscopy, it was seen a directly proportional relationship between parasite density detected by microscopy and PCR results. Through the chi-squared test, significant differences between P1+ and P3+ ($P < 0.001$), and P1+ and P2+ ($P = 0.006$) smears were observed. However, no significant differences between P2+ and P3+ categories were seen ($P > 0.05$) (Figure 3).

Considering the 75 cases with LST results available, all of them showed an induration ≥ 5 mm, hence being positive for LST. In the group with smaller induration (LST < 19 mm), smears showed a higher proportion (28/34) of positive PCR results; by contrast, the smears of the group with larger LST induration (≥ 19 mm) had a lower proportion (25/41) of positive PCR results ($P < 0.05$). Although lesion diameter and lesion age were also analyzed, statistically significant differences were not appreciated in the proportion of positive PCR results among these variables ($P > 0.05$). In addition, correlations between clinical variables of patients were performed. No significant correlation was found between semiquantitative microscopy and LST, diameter of the lesion and LST, diameter of the lesion and semiquantitative microscopy, and time of the lesion and semiquantitative microscopy.

DISCUSSION

The results and findings of this study contribute evidence on the possibility and value of applying molecular tests to archived samples, despite preservation at room temperature, for a better characterization of cases of leishmaniasis. DNA extraction from old Giemsa-stained slides was adequate enough to characterize *Leishmania* spp. DNA. Species identification becomes essential and relevant because treatment response is partially influenced by differential susceptibility of different *Leishmania* species to available drugs.[14,20,21] Currently, multilocus enzyme electrophoresis is the gold standard for *Leishmania* spp. assignation; but, this technique is demanding, laborious, and requires isolation and mass cultivation of the parasites.[7] Hence, PCR-RFLP has been used for *Leishmania* spp. identification, representing a simpler, faster, and cheaper alternative.[12,22]

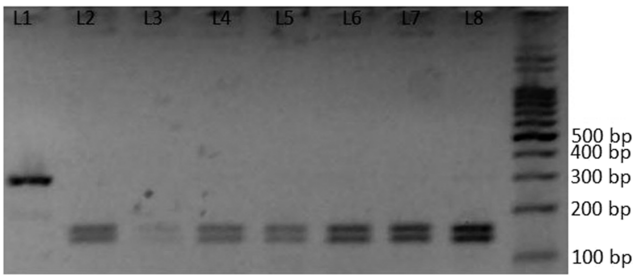

FIGURE 2. Characterization of *Leishmania* spp. parasites in clinical material by internal transcribed spacer 1–PCR and restriction enzyme analysis. Lane 1, *Leishmania* (*Viannia*) *braziliensis* undigested control; lanes 2–8, restriction pattern of parasites from patients' Giemsa-stained slides; lane 9, a 100-bp ladder was used as molecular size marker.

In Argentina, most of ATL causing parasites belong to *Viannia* subgenus. In fact, *L.* (*V.*) *braziliensis*, *L.* (*V.*) *panamensis*, and *L.* (*V.*) *guyanensis* have been recorded in Argentina.[7–9] Because the representatives of *L. braziliensis* complex have identical RFLP patterns even using a great variety of restriction enzymes, in this report parasites were characterized up to subgenus level (Figure 1). All 100% positive PCR products belonged to *Viannia* subgenus (Figure 2). Our results agree with those of Schönian et al., 2003 in the fact that species determination could not be achieved for *Viannia* ITS1 amplicons by HaeIII digestion despite the use of gels with higher agarose concentration (3% and 3.5%).

Although *L.* (*L.*) *amazonensis* has been determined as infectious species in Oran Department,[6,23] our results are consistent with several previous reports where most typed etiological agents belonged to *Viannia* subgenus in the same department.[7–9,23,24] Based on the clinical characteristics of the patients evaluated in our Center and on previous molecular identifications of parasites,[7–9,23,24] the prevalent disease form in the study area is compatible with *L.*(*V.*) *braziliensis* infection. Localized cutaneous form is frequently observed there, and it can be simple or multiple depending on the number of lesions[25–27]; also, mucocutaneous disease is found in the region.[7,8,19,24] Entomological surveys carried out there, have determined that the predominant species is *Nyssomyia neivai*,[28,29] which has been linked to outbreaks and human cases in our country.[29–31] In addition, this sand fly species is a proved vector of *L.* (*V.*) *braziliensis*.[32,33] However, further epidemiological explorations are needed for a better and deeper characterization of the study area.

When analyzing ITS1-PCR results within a set of clinical variables, PCR positivity showed statistically significant differences according to semiquantitative microscopic diagnosis, highlighting the importance of parasite burden in the diagnostic sensitivity of the method. The P3+ smears were more positive than P1+ smears for PCR, which is biologically logical because smears with higher parasite burden are more likely to be positive for PCR.[16]

Considering 19 mm as a class mark for LST, smears of patients with smaller induration diameters were more frequently positive in PCR ($P < 0.05$). It is widely known that LST is a delayed hypersensitivity reaction, which measures the host cellular response. Leishmanin skin test induration size has been related to different clinical forms, being nonreactive in diffuse forms where the immune response of the host is affected.[34] Induration size has been observed to be larger among cases with mucosal involvement in some reports but not in other larger case series.[19] Hence, for those patients (LST < 19), a milder cellular response might be responsible for a higher parasite burden, which would explain their higher PCR positivity and an increased amastigote density on microscopy, being all P2+ and P3+ smears positive for PCR (Figure 4). In agreement with this, treatment response has been reported to be higher among patients with larger LST induration,[35] which might be indicating an effective cellular response.

Our hypothesis is that a negative smear from patients with LST responses that are positive although < 19 mm of induration, could actually represent false-negative results. In the group of those patients (LST < 19 mm) there were more P1+

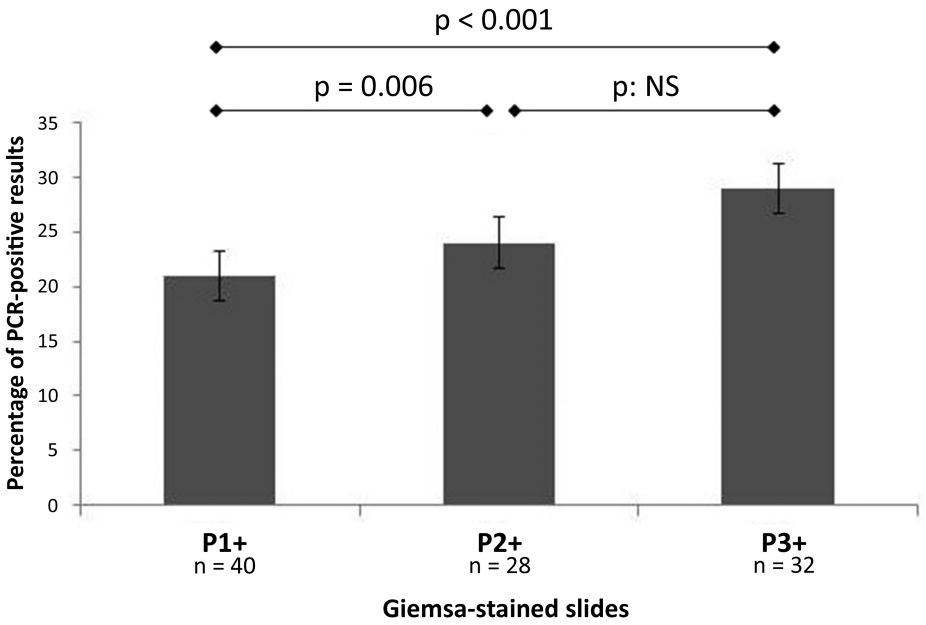

FIGURE 3. PCR-positive results according to semiquantitative microscopic diagnosis.

smears (the lowest amastigote abundance on microscopy in the semiquantitative scale) (Figure 4), which implies that a misdiagnosis might be caused by low parasite burden. As in our study PCR assays have been carried out from smears' DNA, the molecular results were highly dependent on the samples collected and prepared for microscopy; and because P1+ smears had the lowest proportion of positive PCR (Figure 3), the importance of considering LST as a complementary diagnostic tool is highlighted. In a previous study, our group has shown that the ratio of positive LST to positive smear was ≥ 1 and maintained across age groups,[19] supporting its diagnostic ability and suggesting that within an adequate epidemiologic frame, cases with positive LST but negative smear, can be related to the limited sensitivity of direct diagnostic methods rather than to LST sensitization due to exposure to parasite but with no clinical symptoms.

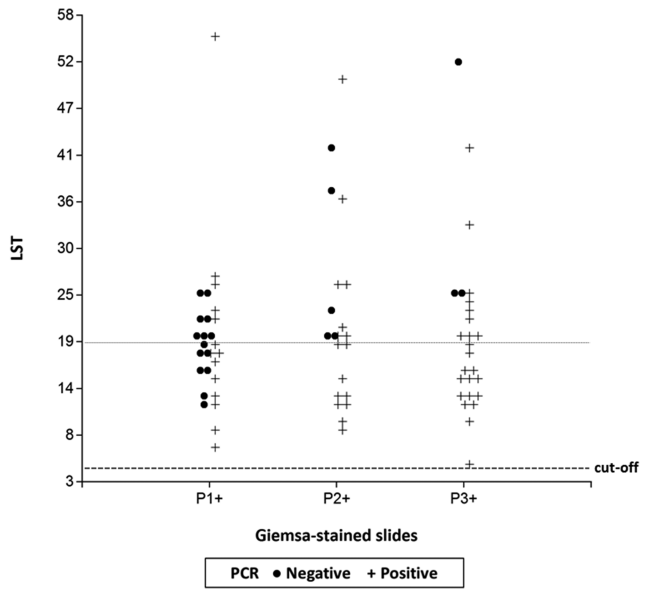

FIGURE 4. Distribution of PCR results in relationship to semiquantitative microscopy and Leishmanin skin test induration.

Internal transcribed spacer 1-PCR positivity has not shown significant statistically correlation with lesion size or lesion age. The fact that PCR positivity is not considerably influenced by age of the lesion might represent an important advantage of this method because chronic lesions are characterized by their low parasite burden which tends to render lower sensitivity of microscopic diagnosis.[36,37] Because Giemsa-stained slides are stored at room temperature, DNA degradation could explain the 26 negatives samples for ITS1-PCR.[15,17] However, according to smears age, no statistically significant differences were found in PCR positivity ($P > 0.05$), which allows epidemiologic retrospective studies such as this or even to work with 36-year-old Giemsa-stained lesion imprint slides.[38]

When correlations were analyzed, no statistical association was found for LST and semiquantitative microscopy, nor LST and diameter of the lesion. The results do not agree with recent research in the same study area in a larger group of patients.[19] No significant correlation was found for microscopic diagnosis and diameter of the lesion. The results agree with a previous work, where parasite burden was quantified by qPCR.[37] Also, the authors found that recent cases are associated with high parasite burden, whereas our results found no correlation between those variables probably because semiquantitative microscopy is not as accurate as qPCR.

To our knowledge, this is the largest study analyzing, retrospectively, Giemsa-stained slides from patients of an endemic area where *L*. (*V*.) *braziliensis* is the main circulating species. In summary, the findings of this study establish that the combination of molecular techniques such as PCR and RFLP can be carried out on archived Giemsa-stained slides. PCR-RFLP from smears is cheaper and less laborious than traditional typing methods and might be applied in endemic areas, which are characterized for working with a high volume of samples in resource-limited laboratories. Because using

the smears with diagnostic purposes was not the aim of this study, a limitation of this study was that sensitivity and specificity values were not determined for the molecular technique. However, according to previous reports, applying molecular-based techniques to smears might further represent a support for diagnosis in suspicious cases in view that PCR sensitivity is higher than microscopy and is not affected by improper staining,[39,40] or by the sampling site within lesions.[10] Other publications have also identified, false-negative smears through PCR.[11,15] In any case, the molecular analysis of smears maintained at room temperature even in tropical areas allows retrospective studies, as ours, for epidemiologic research purposes.

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