Original Research Paper

Serological and Molecular Investigation of Cutaneous Leishmaniasis in Healthy Individuals from an American Cutaneous Leishmaniasis-Endemic Region

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Abstract: American Cutaneous Leishmaniasis (ACL) is a major, clinically relevant zoonosis in Brazil. The disease spectrum includes single, localized, cutaneous ulcers, diffuse cutaneous leishmaniasis and mucosal disease. A subclinical form of the disease has also been described in individuals living in ACL-endemic regions. The goal of this study was to employ immunological and molecular diagnostic methods to evaluate the presence of subclinical ACL in healthy individuals from an ACL-endemic region in northwestern Paraná. Antibodies IgG were detected by enzyme-Linked Immunosorbent Assay (ELISA) and the positive samples were analyzed by indirect Immunofluorescence (IIF) for *Leishmania braziliensis* and *Trypanosoma cruzi*. Polymerase Chain Reaction (PCR), employing the *MP3H/MP1L* primers, amplified a fragment of *Leishmania (Viannia)* k-DNA. Of the 159 individuals analyzed, 31 presented ELISA-positive serology and 5 and 8 of these were IIF-positive for *Leishmania* and *T. cruzi*, respectively. All 159 individuals were PCR-negative. Most ELISA-positive individuals were males and the cities São Jorge do Ivaí and Doutor Camargo showed the highest prevalence of positive individuals. Our results reveal the presence of subclinical *Leishmania* infections in inhabitants of this region. Further investigation of this population may contribute to understanding the immune responses to ACL.

Keywords: Leishmaniasis, Subclinical Cases, ELISA, Polymerase Chain Reaction, Indirect Immunofluorescence

Introduction

American Cutaneous Leishmaniasis (ACL) is clinically one of the most important zoonoses in Brazil, with 635,399 recorded cases between 1990 and 2013 (Brasil, 2009; Brasil, 2013). A majority of the ACL cases recorded in southern Brazil originated in the state of Paraná. Data obtained from the Health Surveillance Secretariat revealed that 13,899 disease cases were recorded in southern Brazil between 1990 and 2013, 13,188 of which were from Paraná (Brasil, 2013). Several northwestern cities in the state of Paraná have shown a high incidence of ACL; therefore, it is an endemic region for this disease (Curti *et al.*, 2009; Monteiro *et al.*, 2009). *Leishmania braziliensis* is the main etiological agent of ACL in the state of Paraná (Lonardoni *et al.*, 2006; Szargiki *et al.*, 2009). The disease spectrum includes single, localized, cutaneous ulcers, diffuse cutaneous leishmaniasis and mucosal disease. A subclinical or asymptomatic form of this disease has also been observed in individuals living in the ACL-endemic regions (Sampaio *et al.*, 2009; Brito *et al.*, 2008; Arraes *et al.*, 2008).

ACL is routinely detected in the laboratory by the direct detection of the parasite and immunological methods, such as the Montenegro Skin Test (MST) and Indirect Immunofluorescence (IIF) (Brasil, 2009). Enzyme-Linked Immunosorbent Assay (ELISA)-based methods of diagnosis are known to be highly sensitive (Yoneyama *et al.*, 2007) and have been employed in the

diagnosis of both clinical and subclinical cases of ACL (Arraes *et al.*, 2008; Szargiki *et al.*, 2009). Previous reports have also confirmed the applicability of Polymerase Chain Reaction (PCR) in the diagnosis of ACL, although it is not routinely used for this purpose (Yoneyama *et al.*, 2007).

The aim of this study was to employ immunological and molecular diagnostic methods to evaluate the presence of subclinical ACL in healthy individuals native to an ACL-endemic region in northwestern Paraná.

Material and Methods

Study Design

A retrospective study was conducted using the epidemiological records of patients between the years 2005 and 2011, assisted by the Laboratório de Ensino e Pesquisa da Universidade Estadual de Maringá, Paraná (LEPAC/UEM), a Reference Center of the Ministry of Health for laboratory-based diagnosis of ACL. Individuals with ACL were identified from these records and grouped according to the municipality of their residence. Relatives and neighbors living within a 1 km radius of these patients, or individuals who frequently visited ACL-endemic areas (woods and rivers), aged 18 years or above, were invited to take part in this study. Blood samples were collected from these subjects (n = 159) for ELISA-IgG and PCR analyses. ELISA-positive samples were subsequently analyzed for *T. cruzi* and *Leishmania braziliensis* antibodies by IIF.

Sample Preparation and Storage

A 10 mL aliquot of the blood sample was collected and split in two 5 mL tubes, one containing Ethylenediaminetetraaceticacid (EDTA) and one without an anti-coagulating agent. Serum and buffy coat fractions were obtained by centrifuging the blood samples at 3,000 rpm for 15 min and stored at -30°C until further use.

ELISA

Promastigote forms of *L. braziliensis* (MHOM/BR/1987/M11272) were cultured at 25°C in 199 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) inactivated bovine fetal serum, penicillin G (100 UI mL^{-1}), streptomycin (100 µg/mL) and 1% (v/v) human urine. Parasites were washed in phosphate buffered saline (PBS; pH 7.2), centrifuged at 4°C and 1,700 × *g* for 10 min, lyophilized and stored at 4°C. The antigens were prepared according to the protocol described by Yoneyama *et al.* (2007). Briefly, the plates were sensitized with a dilution of the extract in carbonate-bicarbonate buffer (0.1 M, pH 9.6); the serum samples were diluted to a rate of 1:150 and

added to this plate. The detection reaction employed an anti-human IgG-peroxidase conjugate (Sigma-Aldrich, St. Louis, MO, USA) and O-Phenylenediamine Dihydrochloride (OPD; Sigma-Aldrich). The reaction was stopped with 3 M H$_2$SO$_4$ and the absorbance measured at 492 nm using a microplate reader (Reader ASYS Microplates v.1.4; Biochrom, Cambridge, Austria). All samples were analyzed in duplicate. Samples with an average absorbance greater than 0.68 were considered to be positive (Yoneyama *et al.*, 2007). The assay reactivity was confirmed by against the positive and negative controls provided in each plate.

DNA Extraction

The obtained buffy coat samples were washed with phosphate buffered saline (10 mM sodium PBS, 0.15 M NaCl, pH 7.2) and centrifuged at 3,500×*g* for 15 min. DNA was extracted by the guanidine-phenol method (Venazzi *et al.*, 2007), resuspended in 50 µL TE buffer (10 mM Tris, 1 mM EDTA; pH 8.0) and stored at 4°C until further use. One positive control (10^4 L. *(V.) braziliensis* promastigotes in normal human blood) and one negative control (normal human blood) was included for each group of samples extracted.

Polymerase Chain Reaction

The primers *MP3H* (5′-GAA CGG GGT TTC TGT ATG C-3′) and *MP1L* (5′-TAC TCC CCG ACA TGC CTC TG-3′) (Lopez *et al.*, 1993) were used to amplify a 70 bp fragment of the minicircle kinetoplast (kDNA) of sub-gender *Leishmania (Viannia)*. The samples were amplified and subsequently subjected to agarose gel electrophoresis according to the protocol provided by Nietzke-Abreu *et al.* (2013). The reaction mixture (25 µL) contained 1 µM of each primer (Invitrogen), 1.5 mM MgCl$_2$, 1X enzyme buffer, 0.2 mM dNTP (Invitrogen), 1 U Taq DNA polymerase (Invitrogen) and 2 µL of the sample DNA. Amplification was performed in a thermocycler (Biometra, Gottingen, Germany). The PCR conditions for primers *MP3H/MP1L* were set as follows: an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation (95°C; 1.5 min), annealing (55°C; 1.5 min) and extension (72°C; 2 min) and a final extension at 72°C for 10 min. The reaction products were stored at 4°C until further analyses. Ten microliters of the amplification products were analyzed by electrophoresis, on a 3% agarose gel stained with 0.1 µg mL^{-1} ethidium bromide, at a voltage of 10-15 V/cm. A positive control (1 pg *L. braziliensis* DNA) and a negative control (water) was added to each group of samples. DNA bands were visualized using a UV transilluminator (Macro Vue UV-20; Hoefer Inc., Holliston, MA, USA).

Indirect Immunofluorescence

Promastigote forms of *L. braziliensis* (MHOM/BR/1987/M11272) and an anti-human IgG-Fluorescein Isothiocyanate (FITC) conjugate (BioMérieux, Craponne, France) were employed for the detection of anti-*Leishmania* antibodies. Serum samples were serially diluted (starting from a 1/20 dilution) and titers ≥ 40 were considered to be positive (Silveira *et al.*, 1999). An Imunocruzi antigen (Biolab, Rio de Janeiro, Brasil) and an anti-human IgG-FITC conjugate (BioMérieux) was used in the detection of anti-*Trypanosoma cruzi* antibodies. Serum samples were serially diluted from a starting dilution of 1/20 and titers ≥ 40 were considered to be positive (Silveira *et al.*, 1999).

Statistical Analysis

The obtained data was introduced to a Microsoft Excel® 2010 worksheet. The Stata 9.1® (Stata Corporation, College Station, TX, USA) program was employed for data analyses. The data was analyzed by the Chi-squared test and Student's *t*-test for independent samples, with a significance level of 5%.

Ethical Considerations

All study participants were provided with detailed information regarding the details of the study; the patients who agreed to participate in this study were asked to sign an informed consent form. The study was approved by the Permanent Committee on Ethics in Human Research of the Maringá State University, according to the report n. 153/2009.

Results

One hundred and fifty nine patients, residing in six municipalities in northwestern Paraná, Brazil, were included in this study. Of these, 104 were male and 55 were female subjects. The patients were aged between 18 and 88 years, with an average age of 45.8±16.3 and a median age of 46 years.

Of the 159 samples analyzed, 19.5% (31) were determined to be positive for ACL by ELISA-IgG, with an absorbance ≥ 0.68 (Yoneyama *et al.*, 2007). Twenty (64.5%) and 11 (35.4%) of the ELISA-positive samples were obtained from males and females, respectively. There was no statistically significant correlation between the gender and ELISA positivity (p = 0.973). The age of ELISA-positive individuals ranged between 18 and 88 years, with an average age of 46.1±15.9 and a median age of 44 years; therefore, the age and ELISA positivity were not significantly correlated (p = 0.607).

The cities São Jorge do Ivaí (35.48%; 11) and Doutor Camargo (22.59%; 7) showed the highest number of subclinical cases (Table 1). No statistically significant differences were observed between the municipalities in terms of the distribution of ELISA-positive individuals (p = 0.447).

Of the 31 ELISA-positive samples identified, 5 were discovered to be IIF-positive for *Leishmania*, while 8 were IIF-positive for *T. cruzi*. All 159 individuals were PCR-negative for *Leishmania (Viannia)* (Fig. 1).
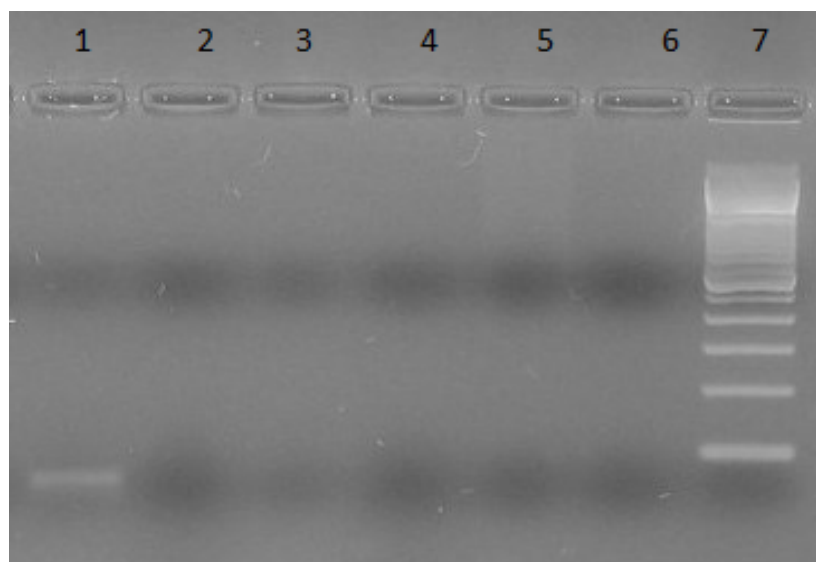

Fig. 1. Representative agarose gel showing PCR products of 70-bp (region of the minicircle kinetoplast kDNA of sub-gender Leishmania (Viannia), following amplification with MP3H and MP1L primers). Lane 1, positive control [DNA of L. (V.) *braziliensis* promastigote forms]; lane 2, negative control (water); lanes 3, 4, 5 and 6, DNA samples from study subjects; lane 7, MW, 100-bp molecular weight marker

Table 1. Distribution of 31 ACL-positive individuals, as determined by ELISA, according to the municipality of residence in the northwestern region of Paraná

Municipality	Number of individuals analyzed	Number of ACL-positive individuals (%)
Cianorte	22	2 (6,45)
Doutor camargo	29	7 (22,59)
Jussara	17	5 (16,12)
Maringá	4	2 (6,45)
São Jorge do Ivaí	63	11 (35,48)
Terra Boa	24	4 (12,91)
Total	159	31 (100)

The most common clinical manifestations of ACL are single, cutaneous ulcers, which correspond to localized lesions; these may evolve to healing and mucosal disease, which mainly affects the nasal and oral cavities and whose treatment may be difficult (Brasil, 2009; Curti *et al.*, 2009). Despite this, individuals living in endemic regions with no patent ACL-related lesions or scars may test positive for MST and serologically, suggesting a recent or subclinical infection (Nunes *et al.*, 2006; Arraes *et al.*, 2008; Sampaio *et al.*, 2009). This study examined antibodies of the IgG class. Thus, it is believed that positive cases are subclinical.

A majority of the individuals evaluated in this study were males (104/159); therefore, higher disease positivity was observed in males than females; for this reason, no statistically significant difference was observed between genders, in terms of ELISA-positivity. However, previous studies have reported greater frequency of the clinical manifestations of this disease in males than females (Monteiro *et al.*, 2009; Curti *et al.*, 2009; Murback *et al.*, 2011).

Our results regarding the age of the patients are not in agreement with those seen in a previous study aimed at identifying the subclinical cases of ACL in the Federal District, which reported an average incidence age of 21.16±14.01 and a median age of 19 years. This difference arises from the inclusion of children in the previous study, contrary to this study (Sampaio *et al.*, 2009).

The municipalities with the highest number of identified subclinical cases of were São Jorge do Ivaí and Doutor Camargo. However, no statistically significant differences were observed between the municipalities in terms of the distribution of detected subclinical cases. Previous studies have shown that ACL is endemic to the municipalities included in this study (Curti *et al.*, 2009). Between 1986 and 2006, 412 cases of ACL were reported in Maringá, 121 in São Jorge do Ivaí and 126 in Doutor Camargo, the three municipalities with the highest prevalence of ACL in northwestern Paraná. The high ACL endemicity may explain the presence of subclinical forms of the disease in these municipalities. Previous studies have also reported the presence of subclinical cases in municipalities with high ACL endemicity (Arraes *et al.*, 2008; Sampaio *et al.*, 2009).

A previous study identified 11 (8.5%) ELISA-positive samples from among 130 healthy individuals living in an endemic region in Maringá city, Paraná (Arraes *et al.*, 2008), which is in agreement with the results presented in this study (19.5%). Some studies identified higher percentages of subclinical cases in the metropolitan area of Recife and the Federal District (67% and 71.8%), respectively (Brito *et al.*, 2008; Sampaio *et al.*, 2009), which the authors attributed to peri-domicile transmission. This is in contrast to the ACL transmission characteristics observed in northwestern Paraná, where the occurrence of ACL has been linked to proximity to woods and rivers, as well as to rural and peri-urban sections of endemic areas (Curti *et al.*, 2009).

Several authors have described the detection of *Leishmania (Viannia)* DNA in peripheral blood as an appropriate tool for the diagnosis of ACL (Ferreira *et al.*, 2006; Venazzi *et al.*, 2007; Martins *et al.*, 2010). However, parasite DNA was not detected in the blood of individuals analyzed in this study. An analysis of patients with a clinical history of ACL conducted by Ferreira *et al.* (2006) did not reveal the presence of parasite DNA; however, the results of the ELISA conducted by Ferreira *et al.* (2006) were in agreement with those obtained in this study. Conversely, Camera *et al.* (2006) successfully detected parasite DNA in samples obtained from individuals (with no prior history of ACL) living in endemic regions.

In this study, DNA was extracted from the buffy coat extracted from the patient blood samples, which may have increased the difficulty of parasite DNA detection. Parasite DNA is frequently detected in whole blood and mononuclear cell samples, rather than in buffy coat samples (Camera *et al.*, 2006). In addition, the detection of parasites in blood samples is believed to be related to the amount of circulating parasites. Venazzi *et al.* (2012) identified parasite DNA solely in those patients in whom a direct examination of the lesion samples revealed a high number of amastigotes. The samples analyzed in this study originated from subclinical patients, who are likely to have low parasitemias, or may even show parasite clearance, rendering the detection of parasite DNA impossible.

The association of different serological tests is needed to increase the positivity of LTA in the laboratory diagnostics (Yoneyama *et al.*, 2007; Silveira *et al.*, 1999). Thus, IIF-*Leishmania* was performed to compare the positivity of the ELISA-positive samples. The results of IIF were not in agreement with those obtained by ELISA, which may be a consequence of the higher sensitivity of the latter (Szargiki *et al.*, 2009). Some studies have shown that IIF-negative samples could test positive for ACL in ELISA (Ferreira *et al.*, 2006), including the subclinical cases (Arraes *et al.*, 2008). Thus, the use of different antigens in sensitive techniques, such as ELISA, can provide more specific diagnosis.

In the routine, the IIF reaction is the most used for the diagnosis of ACL, but there are possibilities of cross-reactions, especially with Chagas disease, so, it was performed the IFI-*T. cruzi*. The number of individuals determined to be IIF-positive for *T. cruzi* was higher than those that were IIF-positive for *Leishmania*. However, all *T. cruzi* IIF-positive samples presented high absorbance values in the ELISA-*Leishmania* assay.

A major limitation of this study was the time span between ELISA and IIF, with the latter having been performed after the former. Although the samples were storage in ideal storage conditions (-30°C), antibody titers are known to decrease with time, which may lead to a higher number of negative results (Souza *et al.*, 2012).

Conclusion

This study demonstrates the occurrence of subclinical infections in the inhabitants of northwestern Paraná. Based on the complexity of the immunological events that take place throughout the clinical development of ACL, further immune-genetic studies of subclinical cases may help elucidate why some individuals present clinical manifestations of the disease, while others remain resistant to ACL.

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Author's Contributions

RSRC: Sample collection, laboratory tests, manuscript preparation; TRN: Laboratory tests, manuscript preparation; LSB: laboratory tests; EPL: Laboratory tests; CMS: Laboratory tests; CCC: laboratory tests; SDB: manuscript preparation and revision; TGV: Laboratory tests, manuscript preparation and revision.

All authors have read and approved the submitted version of the manuscript.

Competing Interest

The authors have no conflicts of interest to declare.

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