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Laboratory Monitoring of Individuals with Subclinical Cutaneous Leishmaniasis Infections in Southern Brazil

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Abstract

Background: Cutaneous leishmaniasis (CL) has a high incidence worldwide. Most individuals with CL develop the cutaneous form, but subclinical infections may occur.

Objective: The present study used different laboratory methods [polymerase chain reaction (PCR), indirect immunofluorescence (IIF) and enzyme reaction immunoassay (ELISA)] to investigate the incidence, and monitor individuals with subclinical infections.

Patients and methods: The six individuals with subclinical infections included residents of a CL endemic area. Collections of biological material were carried out for nearly three years.

Results: Two of these individuals were positive for *Leishmania*, one of them by PCR and the other by PCR, IIF and ELISA after previous negative results.

Conclusion: The presence of a subclinical infection suggests the development of an appropriate immune response that can control replication of the parasite and maintain the integrity of the tissue. Individuals with subclinical infections must be correctly diagnosed and monitored using different methods, because of the possibility of the development of the mucosal form, and may serve as reservoirs for the insect vector.

Keywords: Cutaneous Leishmaniasis; subclinical infections; indirect immunofluorescence; enzyme immunoassay reaction; polymerase chain reaction

Introduction

Leishmaniasis is a commonest parasitic disease in the world and a recent review shows that over 98 countries and territories are endemic for leishmaniasis [1]. Cutaneous leishmaniasis (CL) is a serious public-health problem because of its high incidence [1], and the chance of lesions and disabling and destructive forms, with great impact on the psychosocial situation of individuals [2]. CL has been reported worldwide, and is endemic in several regions [3]. The disease affects both sexes and all age groups. There is a higher incidence in males 60% of cases) [3].

CL normally occurs in the cutaneous form, and in Brazil is caused mainly by *Leishmania (Viannia) braziliensis*. Lesions caused by this parasite can result in the mucosal form, destroying cartilage and causing disfiguring lesions, and the disease is potentially fatal if untreated [3].

Laboratory diagnosis of CL is accomplished through a group of parasitological and immunological tests, with the combination of three techniques, as recommended by the Brazilian Ministry of Health regulations [3]: parasite direct search in the lesion (PD), Montenegro's skin test (MST) and the indirect immunofluorescence test (IIF).

The IIF, a sensitive serological method, can be used for laboratory monitoring of patients after treatment [3]. However, there is the possibility of cross reactions, especially with *Trypanosoma cruzi* [2]. This test can give variable results for CL, due to reduced antigenicity of the parasite or to low levels of circulating antibodies. Antibody titers are high in patients with multiple lesions [2]. In addition, the reactions tend to be negative in the beginning of the infection (1-3 months). After treatment and clinical cure, the titers may fall or disappear in a few months [2].

Yoneyama et al. [4] demonstrated that ELISA (enzyme immunoassay reaction) presents good sensitivity and it may become an important tool for diagnosis and epidemiological studies of CL endemic areas. The ELISA is more sensitive and faster, and tests using crude, purified, or recombinant antigens of *Leishmania* have shown sensitivity rates ranging from 85-100% [4,5].

Molecular techniques based on DNA detection, such as Polymerase Chain Reaction (PCR), have provided an alternative approach to the demonstration of the presence of parasites in clinical samples [6]. The PCR, because of its high sensitivity, has been widely used to detect and identify *Leishmania* species in different clinical samples [7-9].

Most individuals with CL develop the cutaneous form [10]. Subclinical or asymptomatic infections can occur, where populations exposed to the parasite acquire the infection without developing the disease [11-13]. These individuals should be monitored in order to evaluate the prognosis of the Leishmania infection and the relationship to their susceptibility or resistance to infection, as well as to determine the true incidence and epidemiology of CL. Costa et al. [14] describe that in asymptomatic infections, even if a small proportion of such asymptomatic persons are infective to sandflies, they represent a formidable reservoir of infection in endemic areas, and these patients should be monitored. Given the presence of residents in an endemic area of CL who showed anti-*Leishmania* antibodies, by means of the ELISA, but showed no lesion characteristic of the disease, this study investigate the incidence, and monitored such individuals with subclinical infections, using different laboratory methods.

Materials and Methods

Patient selection

The study group included six individuals who showed a positive ELISA for CL in 2006, but who had no lesions and were therefore considered to have subclinical infections. These individuals were residents of the Inocente Vilanova Junior Housing Complex in Maringá city (23º25'S and 51°57'W), State of Paraná, southern Brazil. This is within an endemic area of CL, where an outbreak occurred in 2003/2004 [15]. The individuals answered a questionnaire collecting clinical and epidemiological characteristics, and signed a free and informed consent form. This study complied with Resolution No. 196/1996 of the National Health Council of the Brazilian Ministry of Health (Brazil), and was approved by the Standing Committee on Ethics in Research involving Humans, Universidade Estadual de Maringá, opinion No. 251/2008.

Laboratory diagnosis

Samples were collected from June 2008 through May 2011. The examinations and tests were carried out by trained and certified professionals, according to the Brazilian Ministry of Health [3]. Part of the collected blood was used to obtain serum for IIF and ELISA tests for detection of IgG anti-Leishmania, and the other part of each blood sample was used for PCR.

-Indirect immunofluorescence. Promastigotes of L. (V.) braziliensis and conjugated anti-IgG human - fluorescein (Biolab-Mérieux, Brazil) were used as the antigen. Serum samples were diluted from 1/20; those with titers ≥ 40 were considered positive. To evaluate the cross-reactivity with Chagas disease, the IIF was carried out, using as the antigen epimastigotes of T. cruzi and conjugated anti-IgG human - FITC (Biolab-Mérieux, Brazil) [16].

-Enzyme immunoassay. Polystyrene plates of 96 wells (Nunc-Immuno Plate Maxisorp Surface, Denmark) were coated with 50 µL of antigen solution of L. (V.) braziliensis per well, diluted in carbonate-bicarbonate buffer, pH 9.6, and incubated overnight at 4°C. The plates were washed with PBS (saline solution buffered with 10 mM sodium phosphate, 0.15 M NaCl, pH 7.2), and blocked with 200 µL of 1% bovine albumin (Gibco, Invitrogen Corporation, NY, USA) in PBS [1% bovine serum albumin (BSA)] and washed again with PBS. The serum samples (100 µL) diluted 1/150 in 1% BSA were added to each well. The plate was incubated for 2 h at 25°C and washed. Conjugated anti-human IgG-peroxidase (Sigma-Aldrich) (1/1000) in BSA was added (100 µL per well). After 2 h of incubation at 25°C, the plate was washed with PBS. The reaction was revealed with 100 µL of OPD (o-phenylenediamine-2HCl, Sigma, St. Louis, USA) 1 mg/mL in 0.05 mol/L phosphate-citrate, pH 5.0 containing 0.03% H_2O_2 for 15 min at 25°C. The samples were read at 492 nm, and the cutoff point was 0.68 [4]. The cutoff point was set as the mean plus 2 standard deviations of values of the healthy controls to obtain maximum sensitivity and specificity.

-Molecular Diagnostics. To part of each blood sample (3 mL), 3 mL of ACD solution (25 mM citric acid, 50 mM sodium citrate, 81 mM D-glucose) was added and the buffy coat was obtained after centrifugation for 10 min at 1200 g. DNA extraction was analyzed by guanidine isothiocyanate-phenol [17]. DNA was hydrated with 50 µL of TE buffer (10 mM TRIS, 1 mM EDTA, pH 8.0) and stored at -18°C before the PCR was performed. The reaction mixture (final volume 25 µL) [9] was composed of 1 µM of each primer (Invitrogen Life Technologies, São Paulo, Brazil) for amplification of *Leishmania* DNA: MP3H (5'-GAA TTC GGT TGT CGG ATG C-3') and MP1L (5'-ACA TAC GCC TCC CTC TGC TG-3'), which amplify a 70-bp kDNA fragment of the subgenus *Leishmania (Viannia)*, 1.5 mM $MgCl_2$, 1 x enzyme buffer, 0.2 mM dNTP (Invitrogen, Carlsbad, CA, USA), 1 U Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA), and 2 µL of DNA obtained from blood [17]. DNA amplification was carried out in a PC Thermocycler (Biometra, Germany) at 95°C for 5 min, followed by 35 cycles: 1.5 min at 95°C, 1.5 min at 55°C, and 2 min at 72°C; finally for 10 min at 72°C. The product was kept at 4°C until analysis. Ten microliters of amplified products was submitted to electrophoresis in 3% agarose gel (Invitrogen, Paisley, Scotland, UK), stained with 0,1 µg/mL ethidium bromide, at 10-15 V/cm. A positive control [1 pg of *L. (V.) braziliensis* DNA] and a negative control (water) were added. The presence of bands was observed in a transilluminator (Macro VueTM UV-20, Hoefer).

As internal amplification control, the samples with negative PCR results for *Leishmania* were submitted to PCR for detection of possible inhibitors [18]. Specific primers for the human β-globin were used, which amplify a fragment of 268-bp (GH20:5'-GAA GAG CCA AGG ACA GGT AC-3', and PC04:5'-CAA CTT CAT CCA CGT TCA CC-3') [19]. The reaction mixture (final volume 25 µL) was composed of 1 mM of each primer (Invitrogen Life Technologies, São Paulo, Brazil), 3 mM $MgCl_2$, 1 x enzyme buffer, 0.2 mM dNTP (Invitrogen, Carlsbad, CA, USA), 1 U of Taq DNA polymerase (Invitrogen Life Technologies, São Paulo, Brazil) and 2 µL of DNA. The PCR was carried out in a PC Thermocycler (Biometra, Germany) by 40 cycles: 1 min at 95ºC, 1 min at 5 ºC, and 2 min at 72ºC; finally for 10 min at 72ºC. The product was kept at 4ºC until analysis. Ten microliters of amplified products was submitted to electrophoresis in 2% agarose gel (Invitrogen, Paisley, Scotland, UK), stained with ethidium bromide. The bands were observed in a transilluminator (Macro VueTM UV-20, Hoefer).

Results and Discussion

The high incidence rate and the outbreak of CL occurred in Maringá, State of Paraná, and the emergence of subclinical cases in this locality, the possibility of these individuals develop mucosal form of CL and these serve as reservoirs of the disease, the present study, therefore was necessaryto monitorthese individuals [14,19]

Most cases of CL in Brazil are caused by *L. (V.) braziliensis*, which unless properly diagnosed and treated, has the potential to develop a

mucosal form [2]. In Brazil as a whole, mucosal leishmaniasis occurs in 3-5% of cases of infection with *L. (V.) braziliensis* [3]. However, in northern Paraná, patients showed an 11.1% incidence of the mucosal form [20].

Subclinical or asymptomatic infections have been reported in residents of CL endemic areas. Novoa et al. [21] reported an approximately 10% incidence of subclinical infections in residents of endemic areas of *L. (V.) braziliensis*. Fagundes et al. [13] found 60 individuals with subclinical infections who showed a positive Montenegro skin test, as well as one individual with a positive PCR. Trujillo et al. [22] reported that about 30% of the population infected with *L. (V.) panamensis* did not develop clinical forms. Davies et al. [12] reported a subclinical infection rate of about 10% with *L. (V.) peruviana*. Carvalho et al. [23] found the agent of visceral leishmaniasis in healthy individuals who were residents of endemic areas of *L. (L.) chagasi*. The existence of a subclinical infection suggests the development of an appropriate immune response that can control the replication of the parasites and maintain tissue integrity (asymptomatic individuals produced more IL-10 than cured patients), hence the possibility of serologic study [24]. Although CL is endemic in several regions of Brazil, subclinical infections have been little studied, because the infection is difficult to investigate in individuals who do not exhibit clinical signs.

Following an outbreak of CL in the study area, subclinical infections were detected in individuals living in households with people who already had CL, or in their neighbors. These individuals, residents of a CL endemic area, reside in a single street that borders a forest reserve, and some of them enter the reserve, where they may be exposed to insect vectors (sandflies) and animal reservoirs of *Leishmania* [25]. We initially selected 11 individuals, but one, who had shown a positive result in ELISA for *Leishmania* and a titer of 80 in Chagas-IIF, died; two moved to another area or municipality; and two opted not to continue in the study. Therefore, six individuals were followed (one male and five females). These individuals did not show clinical signs or symptoms of CL and have never received specific treatments for this disease. The six individuals were tested for the possibility of cross-reactivity for Chagas disease, all with a negative result.

Although diagnostic and monitoring methods for patients with suspected CL have been studied, little is known about the results and changes in the results of laboratory diagnostic methods for individuals with subclinical infections. The knowledge of diagnostic methods to better detect subclinical cases aids in better targeting of treatment and monitoring of patients especially in endemic areas as referred to in this work.

Two females, with neighbors who have had CL, were positive. One of them (aged 87 years) had one positive PCR in a single sample, after previous negative results (Table 1). For the other individual (aged 66 years), the PCR was positive in one sample and the ELISA and IIF were positive in another sample, after previous negative results (Table 1)

In a new clinical examination 12 months after the last sample collection, neither individual showed clinical signs or symptoms of CL. In both cases, although not confirmatory serology, being in a sample positive and negative in the other, the simple presence of DNA in the samples of the patients may to leave to alert the possibility of developing the disease later.

Individuals	Date of collection	Laboratory results		
		ELISA	IIF	PCR
1	20-Jun-08	neg	neg	np
	3-Mar-09	neg	neg	neg
	7-Aug-09	neg	neg	neg
	23-Jul-10	neg	neg	neg
	5-May-11	np	np	np
2	20-Jun-08	neg	neg	np
	3-Mar-09	neg	neg	neg
	7-Aug-09	neg	neg	neg
	23-Jul-10	neg	neg	pos
	5-May-11	neg	neg	neg
3	20-Jun-08	neg	1/20	np
	3-Mar-09	neg	1/20	neg
	7-Aug-09	neg	neg	neg
	23-Jul-10	neg	1/20	neg
	5-May-11	neg	neg	neg
4	20-Jun-08	neg	neg	neg
	3-Mar-09	neg	neg	neg
	7-Aug-09	neg	neg	neg
	23-Jul-10	np	np	np
	5-May-11	np	np	np
5	20-Jun-08	neg	neg	neg
	3-Mar-09	neg	neg	neg
	7-Aug-09	neg	neg	neg
	23-Jul-10	neg	neg	pos
	5-May-11	(0,800)	1/40	neg
6	20-Jun-08	neg	neg	neg
	3-Mar-09	neg	neg	neg
	7-Aug-09	np	np	np
	23-Jul-10	np	np	np
	5-May-11	np	np	np

Table 1: Laboratory results, on different sampling dates, for samples from individuals with subclinical infections, resident in the state of Paraná, southern Brazil. Pos=positive; neg=negative; np=not performed.

Serological methods have been described for diagnosis of CL. IIF and ELISA show good positivity, but in patients with recent lesions (one to six months of evolution), is frequent serologic negativity by IIF [18]. In positive cases, the mean titers are significantly higher in those

with multiple lesions, reflecting the greater antigenicity induced higher number of parasites [2]. This may explain the low positive rate in individuals with no lesions. There are reports that individuals with subclinical infections by *L. (L.) chagasi* have low or immeasurable IgG antibodies to *Leishmania*, demonstrating the low sensitivity of serological tests for subclinical infections [14]. Arraes showed that among the 130 samples analyzed, one presented titer 40 and three presented titer 20 in the IIF, while 11 samples were positive in the ELISA, demonstrating that serologic tests can be used for the diagnosis of subclinical cases of CL.

PD, although it is rapid and easy to perform, has limited application in individuals without lesions, since without a lesion present; it is not possible to obtain material to perform a PD or PCR [2]. Here, the PCR was positive in blood from two individuals. All samples were positive by PCR for internal control of the reaction. PCR has been evaluated in endemic areas and in many clinical samples. This method offers advantages over the conventional tests, and is reliable because the finding of the DNA is synonymous with the presence of the pathogen [18,26]. PCR with MP3H/MP1L primers has good sensitivity for detecting *Leishmania (Viannia)*, and can detect 2 fg DNA, making this technique appropriate for diagnosis of CL [8]. Several investigators have suggested that tests based on PCR or skin tests are better than the serological tests for the detection of subclinical infections in monitoring studies, although it does not distinguish recent or past infection [14,18,27]. PCR can be a useful tool for surveillance in order to identify areas where the parasites circulate and individuals who are at risk of acquiring the disease [12].

In subclinical CL infections, which are distinguished by the absence of a skin and/or mucosal lesion combined with positivity in PCR, serological and/or skin tests, it is difficult to predict the potential for disease progression, since the determinants of its evolution are not yet clearly defined [28]. Auto-resolution or host-parasite equilibrium may occur, which can result in disease progression if there is a decline in local or systemic immunity, which makes it important to monitor these individuals [29]. Thus, future CL lesions can be detected early, to prevent the secondary lesions that may appear months or years after contact with the parasite.

The competence of individuals with subclinical infections to infect sand fly vectors is uncertain [14,30]. However, if even a small proportion of these individuals are infective to sandflies, they represent a reservoir of infection in endemic areas, especially if people with subclinical infections are present in high numbers [14]. Thus, it is debatable whether these individuals should be treated or whether they should simply be followed clinically.

The indication of subclinical infections with *Leishmania (Viannia)* was confirmed by PCR and serology. Individuals with subclinical infections must be correctly diagnosed and monitored with the use of different methodologies, since they may develop the mucosal form and even these may serve as reservoirs for the insect vector. It is advisable to follow these individuals for a period of years, because the symptoms of mucosal Leishmaniasis can arise years after the contact with the parasite. These results can serve as an indicator of the risk of transmission and maintenance of infection in urban areas, indicate the actual incidence of CL, and contribute to the assessment of resistance or susceptibility to disease. Further studies should be encouraged, in order to evaluate the diagnostic methods to be used for monitoring individuals with subclinical infections, to better understand the infection and the results of the laboratory diagnosis and to indicate risk factors for transmission of infection and assess the prognosis and the development of the mucosal form of the disease.

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