Comparison of Serological and Parasitological Methods for Cutaneous Leishmaniasis Diagnosis in the State of Paraná, Brazil

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We evaluated the effectiveness of serological and parasitological methods for cutaneous leishmaniasis (CL) diagnosis in patients from the central region of Paraná state, southern Brazil. Five groups were compared: clinical diagnosis, parasitological diagnosis, communicants, inhabitants of a non-endemic area and carriers of other etiologies. Two antigens were prepared from promastigotes of *Leishmania (Viannia) braziliensis* and *Leishmania (Leishmania) amazonensis* for indirect immunofluorescence assay, ELISA and immunoblotting. The parasitological approaches detected 79.3% of the patients with a clinical diagnosis; the parasites were identified by PCR as *L. (V.) braziliensis.* Serological methods showed 95% sensitivity for homologous antigens. Immunoblotting revealed specific proteins for diagnosis of CL and detected 96.6% of the patients when *L. (V.) braziliensis* was used as an antigen, and 83.3% with *L. (L.) amazonensis*. This study demonstrated the importance of differential diagnosis for leishmaniasis; the association of two or more indirect methods increased diagnosis sensitivity.
Key-Words: Cutaneous leishmaniasis, immunodiagnosis, antigen, western blotting.

Leishmaniasis is endemic in Latin America and is present in 21 countries, presenting both visceral and cutaneous clinical forms [1]. In Brazil, the disease is found in 17 out of the 27 states, with 28,000 cases of cutaneous leishmaniasis (CL) [2]. This disease affects people of all ages and of both sexes [3]. Early diagnosis of the disease is the best preventive measure, so it is essential to adopt low-cost, efficient methods that can be applied in endemic and epidemic transmission areas.

Diagnosis of CL can be made by clinical, epidemiological, parasitological, serological or molecular methods. Direct exams or culture should be the method of choice to confirm a clinical suspect, because they are reliable. However, these tests are time consuming and labor intensive and have low sensitivity. Consequently, we need to search for other, complementary, methods of diagnosis to be employed in outbreaks, or to carry out epidemiological studies to analyze a large number of patients at the same time and give a faster diagnosis. In addition, more than one diagnostic method should be available at public health services [1,2].

The antigenic diversity associated with this disease accounts for the difficulties of serological diagnosis, as it is caused by various different species of *Leishmania*. The antigenic diversity due to molecular and biochemical differences of each complex or species has been characterized recently with modern molecular biology tools [4-6]. Some questions that remain are: Is the sensitivity the same when we use a homologous or a heterologous antigen for CL diagnosis? Which serological method (IFA, ELISA, or immunoblotting) gives the best sensitivity?

We standardized and compared the sensitivity of three immunological methods using two antigens: *Leishmania (Leishmania) amazonensis* and *Leishmania (Viannia) braziliensis*. These species were chosen due to their widespread geographical distribution and incidence in Brazil.

After standardization, these three diagnostic methods were applied to five groups of patients, including those with clinical, parasitological and epidemiological confirmation, and to carriers of other pathologies, in order to determine the sensitivity and specificity of the serological methods.

Material and Methods

Population

We screened 100 patients, through active search, followed by clinical evaluation. Thirteen patients were excluded from the study because they presented other pathologies, such as varicose ulcers and basocellular carcinoma. Eighty-seven patients presented characteristic lesions and were included in the parasitological and serological exams (Group I). Sixty-nine patients of the Group I had their diagnosis confirmed by parasitological exams (Group II).

Another three groups were included in the study. Group III consisted of 13 people living in the transmission area and who were submitted to the same conditions as those of the CL patients, named communicants. Group IV consisted of 13 inhabitants of the non-endemic area, without clinical history of leishmaniasis. Group V consisted of patients with other diseases (Group Va: 30 paracoccidioidomycosis patients; Group Vb: 10 patients with Chagas' disease; Group Vc: eight patients with Toxoplasmosis).

Suspicious cases of leishmaniasis were evaluated using parasitological, and serological diagnostic techniques. Two or more methods were applied in all cases from all groups to confirm the diagnosis.

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Parasitological Diagnosis

The parasitological exams were conducted on smears (stained by May-Grunwald-Giemsa, observed at 1,000 X); sample sections were macerated and inoculated into Novy-McNeal-Nicolle and Tobbie & Evans media, incubated at 24°C, and were examined and subcultured every week.

Human Sera

Serum samples were obtained by venous puncture, incubated at 37°C for two hours and centrifuged at 800xg for three minutes. Aliquots of 300 μL were identified and stored at -20°C, until use.

Serological Diagnosis

Three approaches were used: indirect immunofluorescence assay (IFA), immunoenzymatic assay (ELISA), and immunoblotting (Western Blot). The antigens were suspensions of *Leishmania (Leishmania) amazonensis* (MHOM/BR/78/PH8) and *Leishmania (Viannia) braziliensis* (MHOM/BR/75/M2903) promastigotes. The strains, characterized by isoenzymes, were obtained from the Cryobank of the *Laboratório de Parasitologia Molecular, Departamento de Patologia Básica,* UFPR. After thawing, strains were grown in Tobbie & Evans medium, incubated at 24°C. Scale-up was done in Erlenmeyer flasks (250mL) and Roux bottles (1L) with brain-heart-blood agar biphasic medium (CCS), and subcultured every five (*L. amazonensis*) or seven days (*L. braziliensis*), until enough biomass was obtained.

For IFA antigen production, promastigote forms were cultivated in CCS nutritive medium at 24°C for five days. In the exponential phase, promastigotes were collected and washed by successive centrigugations, at 4°C with sterile saline (0.9% and 0.3%) and PBS pH 7.2. The final sediment was diluted into PBS at pH 7.2. For inactivation and conservation of the protozoa, the product was maintained at 55°C for eight minutes [7]. The prepared and inactivated antigens were diluted to a concentration of 10^7 promastigotes/mL.

Production of soluble antigens for ELISA assay and immunoblotting was carried out as described by Castro et al. [8].

Indirect Immunofluorescence Assay (IFA)

The procedure was carried out according to Chiari et al. [9]. The indirect immunofluorescence assay was standardized against *L. (L.) amazonensis* and *L. (V.) braziliensis* antigens. Standardization of the technique was made with progressive dilutions of positive and negative control serum (1:20 to 1:320) against progressive dilutions of the conjugate (1:50 to 1:200), on five slides. The serum tests were screened in 1:20 and 1:40 dilutions.

The reactive sera were serially titrated up to a 1:640 dilution. The IgG conjugate (*Fundação Oswaldo Cruz, Bio-Manguinhos*) was used at 1:150 dilution in 10% Evans Blue, from the previous titration. Each series of serum samples was run with a negative and a positive control. The IFA was analyzed using fluorescence microscopy (HBO 200 bulb and BG 12 filter).

Immunoenzymatic Assay (ELISA)

The ELISA approach was carried out following Engval & Perlamann [10]. The technique was applied with some modifications in incubation time and sera dilution.

Standardization was carried out with tests of various antigen concentrations (500 to 62.5 ng/well) against progressive dilutions of the positive and negative serum controls (1:50 to 1:400), and progressive dilutions of the conjugate (1:500 to 1:4,000). The ideal combination of antigen concentration, serum and conjugate dilutions, offering the best differentiation between positive and negative was chosen for the test. To apply this technique, *L. (V.) braziliensis* and *L. (L.) amazonen*sis soluble antigens were added at 500ng/well in 96-well microtiter plates (Cornig®). The serum samples were diluted at 1:100 and the anti-human immunoglobulin G peroxidase conjugate (Sigma®, code A-170) was added at a dilution determined by titration at 1/1,000. The OPD was used as a substrate (0.38 mg/mL citrate buffer) and hydrogen peroxide as cromogen (4μL in 10.5 mL citrate buffer). In all tests, the serum samples were evaluated in duplicate with a plate control, and positive and negative controls. Absorbance was read at 490nm. The cut-off was calculated by absorbance mean values (ω) of the serum from 11 samples from non-endemic areas for CL, plus three standard deviations.

Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Leishmania (L.) amazonensis and L. (V.) braziliensis antigens were solubilized in an equal volume of sample buffer (Tris pH 6.8, 10% SDS, Glycerol, 0.1M EDTA, bromophenol blue and the reducing agent 2-mercaptoethanol) and denatured by heating to 100°C. A sample containing 400μg protein/mL of the antigens was submitted to SDS gel electrophoresis in 15% polyacrylamide separating gel and 5% stacking gel. Electrophoresis was performed in a vertical system using TRIS pH 8.3 as the running buffer, at room temperature and under constant amperage (30mÅ). Either the gel was removed and stained with Coomassie Bright Blue (R250) for 30 minutes or the proteins were transferred out of the gel onto a nitrocellulose membrane (Hybond ECL – code rpn 303D/ Amersham®).

Transfer

The peptides were transferred to a nitrocellulose membrane using an electric current at constant 24V voltage and variable amperage for 14 hours, and subsequently for 60 minutes at 48V. The membrane with transferred proteins was stained with Ponceau S-Solution for 30 minutes and washed in distilled water until band development. After being air-dried, the membranes were sliced into 5mm-wide strips.

Immunoblot

The technique that we used followed Towbin et al. [11], but with some modifications in the choice of dyes and periods of serum and incubation time for serum and conjugate. The strips were soaked with blocking buffer (3% skimmed milk in

PBS pH 7.4) for 60 minutes at room temperature in a platform-like agitator. The strips were washed twice for five minutes with PBS pH 7.4 and once with blocking buffer. Next, the strips were incubated with the serum samples diluted into blocking buffer at 1:75 (defined by titration) for 12 hours under slow agitation. The strips were washed as described above and incubated for 120 minutes with anti-human immunoglobulin G peroxidase conjugate (Sigma®, code A-170) diluted at 1:1,000 into blocking solution. They were washed five times as described above and once with substrate and cromogen buffer (0.1M sodium acetate pH 5.2). The antigens were detected with a solution of 0.4% 3-amino-9-ethilcarbazole in dimethylformamide diluted in substrate and cromogen buffer and 30% hydrogen peroxide. The reaction was interrupted with addition of PBS pH 7.4.

Statistical Analysis

For estimating the indexes that validate the results obtained by serology, a positive result was established by comparison with the certainty diagnosis given by the parasitological exams (smear and/or culture). The sensitivity and specificity indexes were estimated according to Castro et al. [8]. Sensitivity was established as the ratio between the number of positive tests in confirmed Group II and the sum of these and the negative tests in this group. Specificity was established as the ratio between the number of tests confirming the group condition of being not-IV and this number added to the reagent tests in the group. The Positive Predicitive Value (PPV) was calculated as the ratio between the number of positive tests in the confirmed Group II and the sum of positive tests. The Negative Predictive Value (NPV) was calculated as the ratio between the number of negative tests in the confirmed Group II and the sum of negative tests. The Younden Index (YI) was calculated as the number of positive sera in confirmed Group II plus the number of tests confirming the group condition of being not-IV minus one.

Results

Parasitological Diagnosis

Out of 87 patients that had their clinical diagnosis confirmed for leishmaniasis (Group I), 69 (79.3%) had their diagnosis confirmed by the parasitological analysis (smear and/or in culture after 7, 14, or 21 days of successive subcultures - Group II). All strains isolated in culture media were identified by PCR as *L. (V.) braziliensis* [12].

Serological Diagnosis

Out of 87 patients, 81were serologically tested with IFA and ELISA, including the 69 who had positive parasitological results by smear and/or in culture. For the IFA technique, the patient was considered reactive if the titrations were equal to or higher than 40. The reactivity rate for *L. (V.) braziliensis* antigen was 80.2%, with titrations until 640, and the rate for *L. (L.) amazonensis* was 54.3%, with titrations until 80. For the ELISA test, the absorbance cut-offs were 0.11 and 0.22 for *L. (V.) braziliensis* and *L. (L.) amazonensis,* respectively. For the first antigen, the positivity rate was 88.9%, while for the second one, it was 70.4%. Among the 13 patients without CL clinical signs (Group III), three were serum reactive to *L. (L.) amazonensis* antigen in ELISA. Based on the IFA, two sera were reactive to *L. (L.) amazonensis* and four to *L. (V.) braziliensis.* When we focused on antibodies in the sera of 13 individuals from the non-endemic area (Group IV) with no clinical signs of cutaneous Leishmaniasis, there was no reaction to any of the antigens in the IFA, and only one serum was reactive to both antigens in the immunoenzymatic assay.

Among 30 patients with paracoccidioidomycosis (Group Va), 70% were reactive to *L. (L.) amazonensis* antigen and 43% to *L. (V.) braziliensis* in the ELISA. Using IFA, 23% were serum reactive to both antigens. Among the 10 patients with Chagas' disease (Group Vb), nine were reactive to *L. (L.) amazonensis* and eight to *L. (V.) braziliensis* by ELISA assay. Using IFA, seven sera were reactive to *L. (L.) amazonensis* and eight to *L. (V.) braziliensis.* Among the toxoplasmosis patients (Group Vc), only one was reactive to *L. (L.) amazonensis* antigen by ELISA and IFA tests.

Immunoblotting

Leishmania (V.) braziliensis

Sixty serum samples with confirmed diagnosis by parasitological exam (Group II) revealed 20 proteins with molecular weights ranging from 16.6 to 218.8 kDa. The 177.8 and 169.8 kDa proteins were prevalent and were recognized by 58 (96.6%) and 57 (95.0%) of the sera, respectively, followed by proteins of 112, 61.6, 120, and 134.8 kDa. Proteins of molecular weights 218.8, 85, 70.8, 56, 53.7, and 17.4 kDa were not detected in serum samples of the communicant group (Group III). Proteins with molecular weights 177.8 kDa (eight sera), 169.8 kDa (seven sera), and 199.5 kDa (three sera) were the most prevalent. The 48.9 and 46.6 kDa proteins were present only in this group. The proteins detected in serum samples of individuals with no history of leishmaniasis and coming from an endemic area (Group IV) were different from those found in the sera of patients with CL. The serum samples of patients with other pathologies (Group V) revealed nine proteins with molecular weights between 58.9 and 151.0 kDa. There was a cross reaction between the sera samples of patients with CL and the Chagas' disease sera (Group Vb), with 135.0 and 148.0 kDa proteins. A 70.8 kDa protein was recognized by the serum samples of patients with PCM (Group Va) and from the leishmaniasis patients (Figure 1A).

Leishmania (L.) amazonensis

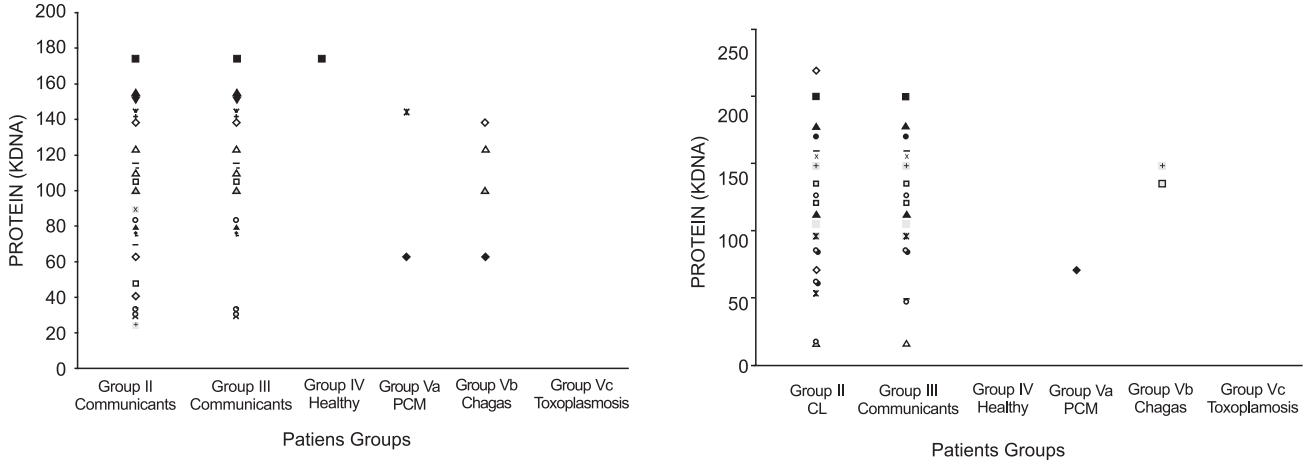
Twenty-six proteins with molecular weights between 23.9 and 173.7 kDa were detected in the 60 serum samples (Group II). The proteins with 83.1 and 79.4 kDa molecular weights were prevalent and recognized in 83.3% of the cases (50/60), followed by the proteins of 75.8, 74.1, 33.9, 33.1, 30.9, and 29.5 kDa. Twenty proteins with molecular weights between 29.5 and 173.7 kDa were detected in the serum samples of people

Table 1. Evaluation of serological diagnosis by IFA, ELISA and Immunoblot using *Leishmania (Viannia) braziliensis* and *Leishmania (Leishmania) amazonensis* as antigens.

	IFA		ELISA		Immunoblot	
	L. amazonensis	*L. braziliensis*	*L. amazonensis*	*L. braziliensis*	*L. amazonensis*	*L. braziliensis*
Sensitivity	56.7% (34/60)	91.7% (55/60)	71.7% (43/60)	95% (57/60)	83.3% (50/60)	96.6% (58/60)
Specificity *Healthy*	100% (13/13)	100% (13/13)	84.6% (11/13)	92.3% (12/13)	92.3% (12/13)	100% (13/13)
PPV	100%	100%	95.5%	98%	100%	100%
NPV	33.3%	72.2%	39.3%	80%	100%	100%
Youden Index	0.57	0.92	0.56	0.87	0.76	0.97
Specificity *PCM*	76.6% (23/30)	76.6% (23/30)	30% (9/30)	56.6% (17/30)	93.3% (28/30)	96.6% (29/30)
Specificity *Chagas*	30% (3/10)	20% (2/10)	10% (1/10)	20% (2/10)	60% (6/10)	80% (8/10)
Specificity *Toxoplasmosis*	100% (8/8)	87.5% (7/8)	87.5% (7/8)	50% (4/8)	100% (8/8)	100% (8/8)

Sensitivity= number positive/(totalx100); Specificity= number negative/(totalx100); PPV (Positive Predictive Value)= number positive/(total positivex100); NPV (Negative Predictive Value)= number negative/ (total negativex100); Youden Index= (number positive/total) + (number negative/total) – 1.

Figure 1. Proteins (kDa) recognized by immunoblotting in serum samples of six groups of patients using antigens of *Leishmania (Leishmania) amazonensis* (A) and *Leishmania (Viannia) braziliensis* (B).



from the communicant group (Group III). Two sets of proteins were the most frequently recognized: 74.1, 75.8, 79.4, and 83.1 kDa; and 33.9, 33.1, 30.9 and 29.5 kDa. Five proteins with molecular weights 30.9, 33.9, 75.8, 74.1, and 173.7 kDa were the same as those detected by serum samples of patients with CL and by 13 sera of people without leishmaniasis (Group IV). Eighteen proteins between 19.0 and 170.0 kDa were detected in the reactions of sera of patients carrying other etiologies (Group V). Antibodies of patients with Chagas' disease (Group Vb) reacted against 63.0, 100.0, 123.0, and 138.0 kDa proteins. Among the proteins reactive to sera of PCM patients (Group Va), those of 63.0 kDa and 145.5 kDa were the same as those detected in the control group (cases confirmed by the parasitological test, Figure 1B).

Sensitivity and Specificity

To estimate sensitivity, the sera of 60 patients with positive parasitological diagnosis for CL (included in the Group II) were considered; and for specificity, the sera of 13 people coming from the non-endemic area were considered-healthy

(Group IV), along with the sera of patients carrying other etiologies (Group V), 30 paracoccidioidomycosis patients, 10 patients with Chagas' disease and eight with toxoplasmosis (Table 1).

Discussion

We evaluated parasitological and immunological techniques for CL diagnosis in transmission areas of the state of Paraná in southern Brazil. Parasitological diagnosis showed 79.3% sensitivity for both techniques, smear and culture. Parasitological exams are the main diagnosis methods; they are efficient and very important in eco-epidemiological studies, when identification of the circulating species is possible. The differences observed among the different studies could be related to differences in methodologies and, above all, to the stage of the disease, since isolation rates are inversely proportional to time since infection.

Immunological methods can reach 95% sensitivity when using the homologous antigen *(L. (V.) braziliensis*). However, when *L. (L.) amazonensis* antigen was used, cross-reactions with other pathologies were frequent, mainly with Chagas disease and paracoccidioidomycosis. This result was also found by others [13-15]. Some authors [13,15] report mixed infections in areas where these protozoa coexist. Cross-reactivity was also observed among antigens of *Leishmania* and antibodies of people bearing deep mycoses, when these diseases share the same transmission area [16,17]. There were unspecified reactions with antibodies of people without leishmaniasis or carriers of other pathologies.

The communicant group showed reactivity in immunological tests against *L. (V.) braziliensis* (30.7% IFA and 84.6% with ELISA) suggesting that those patients either became immune or were hosting the parasite (asymptomatic). In case of decreased immunity, those people would develop the disease, as occurred among HIV patients who developed late clinical manifestations [18]. Monroy-Ostria et al. [19] reported that 30% of the patients had titrations similar to those of healthy people in the endemic area, similar to what we found. The immunoblot method proved to be a highly sensitive tool and thus useful for differential diagnosis of leishmaniasis; it allows identification and exclusion of proteins responsible for cross-reactions. Another advantage is the possibility of detecting asymptomatic cases, when other serological tests cannot establish a reliable result. Detection of asymptomatic and cross-reacting cases is important in the control of leishmaniasis because it allows establishing strategies for monitoring these patients as well as for planning vigilance actions for fast diagnosis and early treatment.

In our study, the high molecular weight proteins were the most frequently recognized for both antigens. Although we found a smaller number of molecules among the *L. (V.) braziliensis* antigens, the high molecular weight proteins were more expressive and conferred specificity to our testing. Reed et al. [20] suggested that the high molecular weight glycoproteins would be found mainly in *Leishmania* sp. flagella. In our communicants group, the 177.8 kDa and 169.8 kDa proteins coincided with what was observed in the CL patients group. They would indicate contact of those people with the antigen without developing symptoms. One serum sample from the asymptomatic group showed reactivity to 14 proteins, with values ranging from 46.5 to 177.8 kDa. This could be at a stage that precedes the development of disease, since these proteins only showed up in the group of people in which leishmaniasis was detected. This deserves more attention; analysis of new samples at different intervals of time would be useful.

Several proteins from *(L. (V.) braziliensis*) and *L. (L.) amazonensis* were recognized by sera from patients with different diseases. This reaction could be an unspecific response, and not just a co-infection. The 70.8 kDa protein showed reactivity with serum samples of patients with paracoccidioidomycosis and serum samples of patients with leishmaniasis, although at a lower frequency. In some analyses, the 72 kDa protein has been recognized as an important surface antigenic component, specific for *L. braziliensis* [21-23]. However, other researchers have identified the 70-72 kDa proteins as members of protein families from highly-preserved areas of DNA, and have also been recognized by sera of patients with visceral leishmaniasis, along with other illnesses, such as tuberculosis, toxoplasmosis and hydatidosis [24].

In the immunoblot analysis for *L. (L.) amazonensis* antigen, the two groups of prevalent proteins 74.1-83.1 kDa and 29.5-33.9 kDa that were always found in CL patients were also detected in serum samples of asymptomatic patients. These proteins would not necessarily be indicative of disease, but indicative of previous contact of those people with those antigens, because many proteins are common to both species of *Leishmania* and are detected in the sera of CL patients in *L. braziliensis* transmission areas.

High rates of cross-reaction were detected between the *L. (L.) amazonensis* antigen and sera of patients with Chagas' disease. The 63 kDa glycoprotein, an important surface protein and common to all species of *Leishmania* in both forms, amastigote and promastigote [25-27], though considered species specific, also reacted against sera of patients with paracoccidioidomycosis.

Based on the comparative analysis of the various methodologies in our study, we conclude that: the parasitological techniques of diagnosis (smear and culture) are equally sensitive and when associated, they constitute a highly-specific diagnostic tool, reinforcing the importance of confirmation of the serum diagnosis by parasitological tests. In the serological diagnosis of leishmaniasis, using either IFA or ELISA assays, the homologous antigen gives higher sensitivity when compared to the heterologous antigen, which gives unspecific reactions with antibodies of people without leishmaniasis and with carriers of other pathologies. Thus, the importance of determining a differential diagnosis is evident. The association of two or more complementary

techniques is recommended when serology is the main methodology used for diagnosis.

The immunoblot approach was 100% sensitive and proved to be useful in the identification of asymptomatic carriers as well as to establish a differential diagnosis between CL and other diseases frequently reported as responsible for cross-reactions. Asymptomatic patients and those with positive serology deserve some attention because they may either develop the illness or immunity without presenting clinical signs. The most concrete evidence of the existence of such phenomena is visceral leishmaniasis cases in HIV-contaminated individuals, those without any previous history of visceral leishmaniasis [28]. We believe that this immune balance, resulting from years of parasite-host-vector co-evolution, is the most common pattern in endemic regions. However, these cases are rarely studied. Such markers could be used to help develop vaccines. On the other hand, reducing cross-reactions with other illnesses is important as it improves the diagnosis of leishmaniasis and allows for early treatment.

As the proteins of both antigens exclusively recognized CL patients, they could be isolated and analyzed against anti-sera specific for the evaluation of its reactivity (possible candidates for vaccines).

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