EVALUATION OF ANTIGENS FROM VARIOUS LEISHMANIA SPECIES IN A WESTERN BLOT FOR DIAGNOSIS OF AMERICAN TEGUMENTARY LEISHMANIASIS

CELY CRISTINA MARTINS GONÇALVES, EDNA MARIA VISSOCI REICHE, BENÍCIO ALVES DE ABREU FILHO, THAIS GOMES VERZIGNASSI SILVEIRA, TÂNIA CRISTINA FELIZARDO, KAROLINE ROCHA MAIA, RAFAEL COSTACURTA, EVANDRO JOSÉ PADOVESI, BENEDITO PRADO DIAS FILHO, SHIDUCA ITOW JANEKIVIC, AND JOSÉ VITOR JANEKIVIC

Laboratório de Tripanosomatideos, Centro de Ciências Biológicas, Universidade Estadual de Londrina, Londrina, Paraná, Brazil; Hospital Universitário Regional Norte do Paraná, Centro de Ciências da Saúde, Universidade Estadual de Londrina, Londrina, Paraná, Brazil; Departamento de Análises Clínicas da Universidade Estadual de Maringá, Maringá, Paraná, Brazil; Ambulatório do Hospital da Sociedade Filantrópica Humanitas, Sao Jeronimo da Serra, Parana, Brazil

Abstract. A Western blot method that uses antigens from culture promastigote forms of Leishmania (Viannia) braziliensis, L. (Leishmania) amazonensis, L. (Leishmania) tropica, and a trypanosomatid (strain 268T) isolated from naturally infected tomatoes was evaluated for laboratory diagnosis of American tegumentary leishmaniasis (ATL). Serum samples were obtained from 108 patients with ATL (group I), 23 chagasic patients (group II), 32 patients with other diseases (group III), and 78 healthy individuals (controls). The overall analysis showed a sensitivity of 76.90%, 90.40%, 78.50%, and 87.90%, a specificity of 100%, 93.80%, 87.80%, and 77.10%, a positive predictive value of 100%, 94.00%, 89.50%, and 72.50%, and a negative predictive value of 75.70%, 90.00%, 75.40%, and 90.20%, and a concordance coefficient kappa of 0.7358, 0.8400, 0.6491, and 0.6287 for L. (V.) braziliensis, L. (L.) amazonensis, L. (L.) tropica, and strain 268T antigens, respectively. The antigenic profile recognized by serum samples from patients with ATL and with Chagas’ disease permits serologic distinction between these infections.

INTRODUCTION

The flagellated protozoa of the Trypanosomatidae family comprises important human parasites, mainly Trypanosoma cruzi, the pathogen that causes Chagas’ disease and various species of Leishmania, which are the etiologic agents of a spectrum of diseases known as cutaneous, tegumentary, and visceral leishmaniasis.1 They are all endemic in many countries in South America and their geographic distribution overlaps in many areas. American tegumentary leishmaniasis (ATL) is transmitted by the sand fly vector (Phlebotominae of the genus Lutzomyia) to a suitable host in which Leishmania invades host macrophages, starting a localized chronic granulomatous lesion at the sand fly bite site.2 The diagnosis of ATL is based mainly on the clinical picture and the identification of the parasite from skin or bucco-pharyngeal lesions.3 The parasitologic methods have always been considered first-choice procedures for the diagnosis of leishmaniasis due to their specificity of 100%, although with variable sensitivities.4-6 However, in some instances it is very difficult to demonstrate the presence of parasites7,8 and immunodiagnosis then becomes an important alternative for demonstrating the presence of the parasite.9,10 The main line of defense against infection in ATL is the cell immune response. The delayed intradermal Montenegro test is the best method for evaluation,11,12 but present infections are not distinguished from past infections.13 The humoral immune response occurs only during the active phase of infection, with the appearance of low titers of antibodies, representing a temporary response.14,15

Several techniques have been developed for the serologic diagnosis of leishmaniasis. These include an immunofluorescence assay (IFA), an enzyme-linked immunosorbent assay (ELISA), and the Western blot.16-18 The Western blot technique is highly sensitive and specific, and provides more information about the parasite antigenic profile.19 Crude extracts of cultured promastigote forms of Leishmania species are the current source of antigen for seroimmunologic assays in the clinical laboratory.20-22 The parasites Trypanosoma cruzi and Leishmania species share antigenic determinants that cause significant cross-reactions in serologic tests, thus impairing accurate laboratory diagnosis of the specific infections.23,24 Other trypanosomatid-derived antigens, which also share antigenic determinants with Leishmania, are easily produced and contain epitopes recognized by human antibodies. They could be an alternative source of antigens for studying the humoral immune response in leishmaniasis and, perhaps, an alternative laboratory diagnostic method with minimal expense and no risk of laboratory infection.25 We report the results of Western blot studies with antigens derived from Leishmania (Viannia) braziliensis, L. (Leishmania) amazonensis, L. (Leishmania) tropica, and a trypanosomatid (strain 268T) isolated from naturally infected tomatoes and serum samples from humans with ATL, those with other diseases, and healthy individuals (controls).

MATERIALS AND METHODS

This study was reviewed and approved by the Internal Scientific Commission and the Bioethics in Research Committee of the Universidade Estadual de Londrina (UEL), Londrina, Paraná, Brazil.

Human sera. The serum samples used were divided into four groups.

Group I. One hundred eight serum samples were obtained from individuals with a clinical diagnosis of ATL defined by an active lesion and coming from endemic areas. Twenty-nine serum samples were from the Universidade Estadual de Maringá (UEM) (Maringá, Paraná, Brazil) from patients reactive by an IFA, and positive by the Montenegro skin test and microscopy. The other 79 serum samples were obtained from patients reactive in at least one conventional laboratory test for ATL, such as the IFA, the Montenegro skin test, or microscopy: 74 from patients seen at the Outpatient Department (Ambulatório) of the Hospital das Clínicas, Universidade Estadual de Londrina (UEL) (Londrina, Paraná, Brazil)
and five from the Outpatient Department (Ambulatório) of the Hospital da Sociedade Filantrópica Humanitas (HSFH) (São Jérônimo da Serra, Paraná, Brazil).

**Group II.** Twenty-three serum samples were obtained from patients seen at the Hospital Universitário Regional Norte do Paraná (HURNP) with a serologic diagnosis of Chagas’ disease (by IFA and ELISA) and without lesions suggestive of leishmaniasis.

**Group III.** Thirty-two serum samples were obtained from patients seen at HURNP with a laboratory diagnosis of other infectious or autoimmune diseases: toxoplasmosis (3), syphilis (5), antinuclear antibodies (8), microsomal antibodies (5), streptococci infection (5), anti-dsDNA antibodies (2), and leprosy (4). None of these patients had any lesions suggestive of leishmaniasis.

**Group IV.** Seventy-eight serum samples were obtained from donors at the hemotherapy unit of the HURNP. They were seronegative for Chagas’ disease, antibodies to hepatitis C, hepatitis B surface antigen, hepatitis B virus core antigen, antibodies to human immunodeficiency virus, and antibodies to human virus T cell lymphotropic virus types 1/2, and had negative Venereal Disease Research Laboratory test results and normal levels of alanine aminotransferase. They were used as negative controls.

**Serologic tests for leishmaniasis.** Indirect immunofluorescence assay. This test was performed according the standards used by each institution (UEL, UEM, or HSFH). In the serum samples from HURNP and HSFH, the titers of antibodies against *Leishmania* were measured using fixed *L. braziliensis* or *L. donovani* promastigotes on commercially available slides (Lio Serum; Indústria e Comércio de Equipamentos e Produtos para Laboratório Ltda., Ribeirão Preto, Brazil and Cecon, São Paulo, Brazil) and fluorescein-conjugated goat anti-human IgG (Laborclin; Produtos para Laboratório Ltd., Birmingham, United Kingdom) as a second antibody at an initial serum dilution of 1:20. The serum samples that were used as negative controls.

The Montenegro skin test. In the patients seen at HURNP and HSFH, the Montenegro skin test was performed using commercially available antigens of *L. braziliensis* and *L. donovani* promastigotes (Reagentes Biológicos Pimenta Abreu Ltda., São Paulo, Brazil). In the patients seen at UEM, antigens from *L. (V.) braziliensis* (M11272) were used. The reactions were performed by injecting intradermally 0.1 ml of antigen into the forearm. The test was read 48–72 hr later and induration > 5 mm in diameter was considered positive.

**Parasites.** *Leishmania* (V.) *braziliensis* strain M11272 (serodeme 1) was originally isolated at UEM from a patient with cutaneous leishmaniasis and identified as *L. (V.) braziliensis* by monoclonal antibodies and isoenzyme analysis by Dr. J. J. Shaw (Instituto Evandro Chagas, Belém, Pará, Brazil). This strain was maintained by continuous passages in hamsters by UEM. *Leishmania* (L.) *amazonensis* was maintained by successive passages into biphasic medium at the Laboratório de Tripanossomatídeos of UEL. *Leishmania* (L.) *tropica* (strain ATCC 30012) was maintained by successive passages into biphasic medium at the Laboratório de Tripanossomatídeos of UEL. A trypanosomatid (strain 268T) was isolated from tomatoes (*Lycopersicon esculentum*) naturally infected in the Londrina region (Paraná, Brazil) by the Laboratório de Tripanossomatídeos of UEL in 1989. This parasite was maintained by successive passages into glucose yeast peptone meat infusion (GYPMI) medium.

**Preparation of Leishmania and trypanosomatid isolated from tomato (strain 268T) antigens.** The leishmanial and strain 268T antigens were derived from culture promastigotes forms. *L. (L.) tropica* and *L. (L.) amazonensis* were grown in liver infusion tryptose medium (LIT) containing 10% fetal calf serum (Sigma Chemical Co., St. Louis, MO) at 25°C without shaking. *Leishmania* (V.) *braziliensis* was grown in the BAB (blood agar base; Oxoid USA Inc, Columbia, MD) biphasic medium with 15% rabbit defibrinated blood in a tissue culture flask with an LIT overlay. The cells were cultured at 25°C without shaking. Strain 268T was cultured in GYPMI medium at 28°C without shaking. The cells were harvested in the late log phase. Antigens for the Western blot test were prepared by previously published methods. The cells were pelleted by centrifugation at 3,200 × g for 10 min at 4°C, and washed four times with 150 mM phosphate-buffered saline (PBS), pH 7.2, by centrifugation at 3,200 × g for 10 min at 4°C. The sediment was resuspended in sample buffer (0.125 M Tris-HCl 4% sodium dodecyl sulfate [SDS], 20% glycerol, 5% β-mercaptoethanol, 0.042% bromophenol blue, pH 6.8) and boiled for 5 min. The suspension was centrifuged at 3,200 × g for 30 min at 4°C, and the supernatant was divided into aliquots and stored at −20°C up to the time of use. Protein concentration in the antigenic suspension was determined by the Bradford method using bovine serum albumin (1,000 μg/ml) as a standard.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).** The discontinuous SDS buffer system used with 0.8 mm-thick slab gels with a 7.5–15.0% polyacrylamide gradient in the running gel. The stacking gel was 5% acrylamide and had one narrow (1 cm wide) lane and one wide (8.6 cm wide) lane. The narrow lane was used for the molecular weight markers and the wide lane was used for 200–300 μl of freshly solubilized parasite antigens containing 1 mg of protein equivalent per gel. Carbonic anhydrase (29 kD), egg albumin (45 kD), and bovine serum albumin (66 kD) (SDS-200 Kit; Sigma Chemical Co.) were used as molecular weight markers. The molecular weight markers were diluted in the same sample buffer as the parasite antigens, boiled for 5 min, and applied to the gel. Running buffer (0.025 M Tris-base, 0.92 M glycine, 0.1% SDS, pH 8.8) was added and the samples were subjected to electrophoresis at 110 V (25 mA) until the stain front reached the bottom of the gel. The gel was then stained with Coomaissie brilliant blue R 250 for proteins at room temperature overnight. The gel was dried between two wet cellophane sheets and 10% gelatin (w/v) for 72 hr at room temperature. The relative molecular weight (MW) of each *Leishmania* strain 268T electrophoresed protein fraction was determined. The gel was also mounted in a protein transfer apparatus and subjected to electroimmunotransfer blotting.
FIGURE 1. Western blot analysis of sodium dodecyl sulfate–polyacrylamide gel electrophoresis of promastigote forms of the Leishmania (Viannia) braziliensis strain recognized by A, serum samples from patients with American tegumentary leishmaniasis (ATL) (group I, lanes 1–10); B, serum samples from patients with Chagas’ disease (group II, lanes 10–13), patients with other diseases (group III, lanes 6–9), and healthy individuals (group IV, lanes 1–5). MW = molecular weight markers; P = positive control serum; N = negative controls serum; kDa = kilodaltons.

Blot. The polypeptides in the gel were transferred to nitrocellulose sheets (pore size = 0.45 μm, Probind 45 membrane roll, Pharmacia Biotech, San Francisco, CA) at 400 mA (48 V) at 4°C for 3 hr in a Trans Blot apparatus (Electrophoresis Power Supply EPS-600; Pharmacia Biotech). This method was performed according to the procedure of Towbin and others with some modifications. The nitrocellulose sheet was cut into vertical 5-mm strips. The strips were blocked with a solution of 5% defatted (skim) milk (Molico, Nestlé, São Paulo, Brazil), 0.1% Tween 20 in PBS, pH 7.2, for 2 hr at room temperature with constant shaking, rinsed with PBS, and treated for 1 hr at room temperature with constant shaking with serum samples diluted 1:50 in PBS containing 5% defatted milk and 0.1% Tween 20. The strips were then washed three times (10 min/wash) with PBS containing 0.1% Tween 20, and treated for 1 hr at room temperature with a peroxidase-labeled, affinity-purified rabbit anti-human IgG conjugate (Sigma Chemical Co.) diluted 1:2,000 in PBS, pH 7.2, containing 5% defatted milk and 0.1% Tween 20. After three additional washes (10 min/wash), the substrate (30% H₂O₂, 0.3% 4-chloro-1-naphthol in methanol dissolved in PBS) was added. The color was allowed to develop for 30 min at room temperature in the dark. The strips were then rinsed with distilled water, dried, and photographed.

The reactivity criterion used for the interpretation of the Western Blot results was based on the antigenic bands that were recognized most frequently by the serum samples assayed. A serum sample was considered positive when it recognized at least one antigenic band from a group of three with the highest frequency of recognition. The result was considered negative when the serum sample showed no reactivity with these diagnostic antigenic bands.

Statistical analysis. Evaluation of Western blot results was based on sensitivity, specificity, positive and negative predictive values, and the kappa index concordance. Sensitivity was defined as the number of samples with a Western blot-positive result/the number of samples with a positive diagnosis by conventional methodology (group I) × 100. Specificity was defined as the number of samples with a Western blot-negative result/the number of samples with a negative diagnosis for leishmaniasis (groups II, III, and IV) × 100. The Epil-Info program (version 6.04 b) (Centers for Disease Control and Prevention, Atlanta, GA) was used for statistical analysis.

RESULTS

Gel electrophoresis. The soluble protein profiles of the parasites obtained by SDS-PAGE showed at least 41 major protein bands to L. (V.) braziliensis with relative MW, values ranging from 13 kD to 150 kD, 39 bands to L. (L.) amazonensis with MW, values from 13 kD to 140 kD 39 bands to L. (L.) tropica with MW, values ranging from 13 kD to 120 kD, and 44 bands to strain 268T with MW, values from 16 kD to 170 kD.

Immunologic detection of antigens from various Leishmania species and strain 268T by Western blot. Several antigenic bands of the various Leishmania species and strain 268T were recognized by the serum samples from groups I, II, III, and IV at different frequencies and intensities of reaction and exhibit a complex pattern of reactivity (Figures 1–4).

The average number of bands recognized by the serum samples from groups I, II, III, and IV of the Leishmania (V.)
FIGURE 2. Western blot analysis of sodium dodecyl sulfate–polyacrylamide gel electrophoresis of promastigote forms of the Leishmania (Leishmania) amazonensis strain recognized by A, serum samples from patients with American tegumentary leishmaniasis (ATL) (group I, lanes 1–14); B, serum samples from patients with Chagas’ disease (group II, lanes 4–8), patients with other diseases, (group III, lanes 1–3), and healthy individuals (group IV, lanes 9–13). P = positive control serum; N = negative control serum; MW = molecular weight markers; kDa = kilodaltons.

braziliensis, L. (L.) amazonensis, L. (L.) tropica, and strain 268T antigens in Western blots are shown in Table 1.

Leishmania (V.) braziliensis. All 41 L. (V.) braziliensis polypeptides stained with Coomassie brilliant blue in SDS-PAGE showed reactivity with at least one serum sample from groups I, II, III, and/or IV in Western blots.

The serum samples from patients with ATL (group I) recognized 34 antigenic bands of L. (V.) braziliensis and the most frequently recognized bands (diagnostic for ATL) are shown in Table 2. In this group, 5.60% of the serum samples recognized no band, 62.80% recognized between one and seven bands, and the remainder recognized more than seven bands. Serum samples from patients with Chagas’ disease (group II) showed a strong intensity of reaction to several antigen bands of L. (V.) braziliensis, with 73.90% recognizing between eight and 14 bands and only 4.35% showing no reactivity. In this group, Western blotting detected antibodies that recognized bands with MW, values ranging from 13 kD

FIGURE 3. Western blot analysis of sodium dodecyl sulfate–polyacrylamide gel electrophoresis of promastigote forms of the Leishmania tropica strain recognized by A, serum samples from patients with American tegumentary leishmaniasis (ATL) (group I, lanes 1–13); B, serum samples from patients with Chagas’ disease (group II, lanes 4–8), patients with other diseases (group III, lanes 1–3), and healthy individuals (group IV, lanes 9–15). P = positive control serum; N = negative control serum; MW = molecular weight markers; kDa = kilodaltons.
FIGURE 4. Western blot analysis of sodium dodecyl sulfate–polyacrylamide gel electrophoresis of promastigotes forms of the 268T strain recognized by A, serum samples from patients with American tegumentary leishmaniasis (ATL) (group I, lanes 1–12); lane 13 = whole cell proteins stained with Ponceau, and patients with other diseases (group III, lanes 14–16); B, serum samples from patients with Chagas’ disease (group II, lanes 1–6), and healthy individuals (group IV, lanes 7–11). P = positive control serum; N = negative control serum; MW = molecular weight markers; kDa = kilodaltons.

TABLE 1
Mean number of antigenic bands recognized by human sera from groups I, II, III, and IV in a Western blot with antigens of various Leishmania species and the 268T strain

<table>
<thead>
<tr>
<th>Antigen extract</th>
<th>Mean number of bands recognized by sera of</th>
<th>Group I*</th>
<th>Group II†</th>
<th>Group III‡</th>
<th>Group IV§</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. (V.) braziliensis</td>
<td>6.00</td>
<td>7.94</td>
<td>3.15</td>
<td>1.53</td>
<td></td>
</tr>
<tr>
<td>L. (L.) amazonensis</td>
<td>3.80</td>
<td>9.20</td>
<td>0.53</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>L. (L.) tropica</td>
<td>5.60</td>
<td>6.60</td>
<td>2.50</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>268T</td>
<td>7.10</td>
<td>13.20</td>
<td>4.00</td>
<td>1.00</td>
<td></td>
</tr>
</tbody>
</table>

* Samples from patients with a diagnosis of American tegumentary leishmaniasis (ATL).
† Samples from patients with Chagas’ disease.
‡ Samples from patients with other diseases.
§ Samples from healthy individuals.

TABLE 2
Leishmanial antigenic bands most frequently reactive with human sera from groups I and II defined as diagnostic antigenic bands

<table>
<thead>
<tr>
<th>Antigen extract</th>
<th>Molecular weights of bands (% recognition)</th>
<th>Group I*</th>
<th>Group II†</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. (V.) braziliensis</td>
<td>42 kD (53.70%)</td>
<td>30 kD (78.30%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>58 kD (39.80%)</td>
<td>38 kD (73.90%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>63 kD (63.90%)</td>
<td>72 kD (73.90%)</td>
<td></td>
</tr>
<tr>
<td>L. (L.) amazonensis</td>
<td>40 kD (75.50%)</td>
<td>28 kD (90.00%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 kD (72.00%)</td>
<td>35 kD (90.00%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>74 kD (75.50%)</td>
<td>66 kD (90.00%)</td>
<td></td>
</tr>
<tr>
<td>L. (L.) tropica</td>
<td>44 kD (53.70%)</td>
<td>28 kD (90.00%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 kD (49.30%)</td>
<td>38 kD (80.00%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>58 kD (47.80%)</td>
<td>40 kD (80.00%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>32 kD (44.00%)</td>
<td>28 kD (80.00%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>38 kD (53.00%)</td>
<td>32 kD (60.00%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50 kD (79.40%)</td>
<td>40 kD (70.00%)</td>
<td></td>
</tr>
</tbody>
</table>

* Samples from patients with a diagnosis of American tegumentary leishmaniasis.
† Samples from patients with Chagas’ disease.

to 120 kD. The most reactive bands (diagnostic for Chagas’ disease) are shown in Table 2. In serum samples from patients with other diseases (group III), no band was recognized by 31.30%, while 53.10% recognized between one and seven bands by Western blotting. The serum samples from patients with a positive diagnosis for leprosy recognized a smaller number of components, with MW, values between 58 kD and 120 kD. Serum samples from healthy persons (group IV) reacted faintly with bands with MW, values between 13 kD and 150 kD. Thirty-two percent of the samples showed no reactivity, 56.40% recognized between one and three bands, and the remaining 11.60% recognized up to six bands.

The recognition of at least one of the ATL diagnostic antigenic bands was considered a positive result. In the 108 serum samples from patients with ATL, 79.63% had positive results for ATL and 20.37% had negative results in the L. (V.) braziliensis Western blot (Table 3). Cross-reactivity was found in 30.43% of the serum samples from patients with Chagas’ disease (group II) in which the ATL diagnostic bands (63 kD and 58 kD) were recognized. Among the serum samples from patients with other diseases (group III), 15.63% had positive results for ATL and 84.37% had negative results. Serologic cross-reactions seen in 10.00% of the serum samples from patients with autoimmune diseases occurred with the ATL diagnostic band (63 kD). Serum samples from patients with a positive diagnosis for leprosy showed faint reactivity with the ATL bands (63 kD and 58 kD) (Table 4). None of the serum samples from healthy individuals showed positive results for ATL by Western blotting.

Leishmania (L.) amazonensis. Twenty-nine of the 39 L. (L.) amazonensis polypeptides stained with Coomassie brilliant blue showed reactivity with serum samples from groups I, II, III, and/or IV. Fourteen of these showed reactivity with group I sera; 7.60% of the serum samples showed no reactivity with any band, 71.60% recognized between one and
five bands, and 20.80% recognized between six and eight bands. The major bands recognized most frequently (diagnostic for ATL) are shown in Table 2. In the serum samples from group II, Western blotting detected antibodies that showed intense reactivity with bands with MW, values ranging from 13 kD to 170 kD. Among these serum samples, 80.00% recognized between six and 10 bands and 20.00% recognized between 13 and 15 bands. The most frequently recognized bands (diagnostic for Chagas’ disease) are shown in Table 2. From serum samples of group III, 76.50% did not recognize any band, 17.60% recognized one band, and 5.90% recognized six bands. The serum samples with a positive reactivity for syphilis showed no reactivity. Of the serum samples from group IV, 55.00% did not recognize any band and the remaining 45.00% recognized a few antigens (a maximum of two bands). Cross-reactivity was found in 30.00% of the serum samples from patients with Chagas’ disease, which recognized an ATL diagnostic band (40 kD) (Table 4). Based on the positivity criterion established, 88.70% of group I sera had positive results and 11.30% had negative results (Table 5). Although serum samples from patients in groups III and IV recognized some bands, these

| Table 3 |

Results obtained in the Western blot with Leishmania (Viannia) braziliensis for the diagnosis of American tegumentary leishmaniasis (ATL) with serum samples from groups I, II, III, and IV

<table>
<thead>
<tr>
<th>Serum samples</th>
<th>Laboratory diagnosis</th>
<th>No. of serum samples</th>
<th>Western blot*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Group I*</td>
<td>ATL</td>
<td>108</td>
<td>86</td>
</tr>
<tr>
<td>Group II†</td>
<td>Chagas’ disease</td>
<td>23</td>
<td>7</td>
</tr>
<tr>
<td>Group III‡</td>
<td>Antinuclear antibodies</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Microsomal antibodies</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Anti-streptolysin O</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Antinuclear + anti-dsDNA</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Toxoplasmosis</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Syphilis</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Leprosy</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Group IV§</td>
<td>Healthy individuals</td>
<td>78</td>
<td>0</td>
</tr>
</tbody>
</table>

* = positive when the sample recognized at least one antigenic band from a group of three with the highest frequency; N = negative when the sample showed no reactivity.
† = Samples from patients with a diagnosis of American tegumentary leishmaniasis (ATL).
‡ = Samples from patients with Chagas’ disease.
§ = Samples from healthy individuals.

| Table 4 |

Results obtained in the Western blot with the diagnostic antigenic bands and serum samples from groups I, II, III, and IV

<table>
<thead>
<tr>
<th>Antigen extract</th>
<th>Protein fraction</th>
<th>Group I*</th>
<th>Group II†</th>
<th>Group III‡</th>
<th>Group IV§</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leishmania (Viannia) braziliensis</td>
<td>30 kD</td>
<td>35.20%</td>
<td>78.30%</td>
<td>9.40%</td>
<td>0.00%</td>
</tr>
<tr>
<td></td>
<td>38 kD</td>
<td>28.70%</td>
<td>73.90%</td>
<td>6.30%</td>
<td>0.00%</td>
</tr>
<tr>
<td></td>
<td>42 kD</td>
<td>53.70%</td>
<td>0.00%</td>
<td>0.00%</td>
<td>0.00%</td>
</tr>
<tr>
<td></td>
<td>58 kD</td>
<td>39.80%</td>
<td>4.30%</td>
<td>6.30%</td>
<td>0.00%</td>
</tr>
<tr>
<td></td>
<td>63 kD</td>
<td>63.90%</td>
<td>26.00%</td>
<td>15.60%</td>
<td>0.00%</td>
</tr>
<tr>
<td></td>
<td>72 kD</td>
<td>34.30%</td>
<td>73.90%</td>
<td>3.10%</td>
<td>0.00%</td>
</tr>
<tr>
<td>Leishmania (Leishmania) amazonensis</td>
<td>28 kD</td>
<td>0.00%</td>
<td>90.00%</td>
<td>0.00%</td>
<td>0.00%</td>
</tr>
<tr>
<td></td>
<td>30 kD</td>
<td>0.00%</td>
<td>80.00%</td>
<td>0.00%</td>
<td>0.00%</td>
</tr>
<tr>
<td></td>
<td>35 kD</td>
<td>0.00%</td>
<td>90.00%</td>
<td>0.00%</td>
<td>0.00%</td>
</tr>
<tr>
<td></td>
<td>40 kD</td>
<td>75.50%</td>
<td>0.00%</td>
<td>0.00%</td>
<td>0.00%</td>
</tr>
<tr>
<td></td>
<td>50 kD</td>
<td>72.00%</td>
<td>0.00%</td>
<td>0.00%</td>
<td>0.00%</td>
</tr>
<tr>
<td></td>
<td>74 kD</td>
<td>75.50%</td>
<td>0.00%</td>
<td>0.00%</td>
<td>0.00%</td>
</tr>
<tr>
<td>Leishmania (Leishmania) tropica</td>
<td>28 kD</td>
<td>0.00%</td>
<td>90.00%</td>
<td>11.80%</td>
<td>0.00%</td>
</tr>
<tr>
<td></td>
<td>30 kD</td>
<td>41.80%</td>
<td>70.00%</td>
<td>29.40%</td>
<td>0.00%</td>
</tr>
<tr>
<td></td>
<td>38 kD</td>
<td>7.50%</td>
<td>80.00%</td>
<td>29.40%</td>
<td>0.00%</td>
</tr>
<tr>
<td></td>
<td>44 kD</td>
<td>53.70%</td>
<td>0.00%</td>
<td>5.90%</td>
<td>0.00%</td>
</tr>
<tr>
<td></td>
<td>50 kD</td>
<td>49.30%</td>
<td>30.00%</td>
<td>5.90%</td>
<td>0.00%</td>
</tr>
<tr>
<td></td>
<td>58 kD</td>
<td>47.80%</td>
<td>0.00%</td>
<td>0.00%</td>
<td>0.00%</td>
</tr>
<tr>
<td>268T</td>
<td>28 kD</td>
<td>11.80%</td>
<td>80.00%</td>
<td>17.60%</td>
<td>15.00%</td>
</tr>
<tr>
<td></td>
<td>32 kD</td>
<td>44.00%</td>
<td>60.00%</td>
<td>0.00%</td>
<td>0.00%</td>
</tr>
<tr>
<td></td>
<td>38 kD</td>
<td>53.00%</td>
<td>40.00%</td>
<td>5.90%</td>
<td>0.00%</td>
</tr>
<tr>
<td></td>
<td>40 kD</td>
<td>14.70%</td>
<td>70.00%</td>
<td>5.90%</td>
<td>10.00%</td>
</tr>
<tr>
<td></td>
<td>50 kD</td>
<td>79.40%</td>
<td>50.00%</td>
<td>0.00%</td>
<td>5.00%</td>
</tr>
<tr>
<td></td>
<td>66 kD</td>
<td>90.00%</td>
<td>50.00%</td>
<td>17.60%</td>
<td>0.00%</td>
</tr>
</tbody>
</table>

* = Samples from patients with a diagnosis of American tegumentary leishmaniasis (ATL).
† = Samples from patients with Chagas’ disease.
‡ = Samples from patients with other diseases.
§ = Samples from healthy individuals.
The percentages in bold represent the fractions with the highest recognition for the sera ATL (group I) and Chagas’ disease (group II).
Results obtained in the Western blot with *Leishmania (Leishmania) amazonensis* for the diagnosis of American tegumentary leishmaniasis (ATL) with serum samples from groups I, II, III, and IV

<table>
<thead>
<tr>
<th>Serum samples</th>
<th>Laboratory diagnosis</th>
<th>No. of serum samples</th>
<th>Western blot*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Group I†</td>
<td>ATL</td>
<td>53</td>
<td>47</td>
</tr>
<tr>
<td>Group II‡</td>
<td>Chagas’ disease</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>Group III§</td>
<td>Antinuclear antibodies</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Group I†</td>
<td>Microsomal antibodies</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Group III§</td>
<td>Antinuclear + anti-dsDNA</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Group II‡</td>
<td>Syphilis</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Group II‡</td>
<td>Leptosy</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Group IV¶</td>
<td>Healthy individuals</td>
<td>20</td>
<td>0</td>
</tr>
</tbody>
</table>

* P = positive when the sample recognized at least one antigenic bands from a group of three with the highest frequency; N = negative when the sample showed no reactivity.
† Samples from patients with a diagnosis of American tegumentary leishmaniasis (ATL).
‡ Samples from patients with Chagas’ disease.
§ Samples from patients with other diseases.
¶ Samples from healthy individuals.

results obtained in the Western blot with *Leishmania (Leishmania) amazonensis* and the test was considered 100.00% negative for both of these groups.

**Leishmania (L.) tropica.** Thirty-six of the 39 *Leishmania (L.) tropica* polypeptides stained with Coomassie brilliant blue showed reactivity by Western blotting with at least one of the serum samples from groups I, II, III, and/or IV; 34 of these were recognized by serum samples from patients in group I (ATL). A total of 13.40% of the serum samples in group I recognized no band, while 59.60% recognized between one and seven bands. The most frequently recognized bands (diagnostic for ATL) are shown in Table 2. In group II, several antigenic bands of *L. (L.) tropica* between 15 kD and 74 kD were strongly recognized. Eighty percent of the serum samples from group IV showed no reactivity and 20.00% recognized up to two antigenic bands of *L. (L.) tropica* and had weak immune reactivity. Cross-reactivity was found in 30.00% of the serum samples from patients with Chagas’ disease (group II), which recognized an ATL band with an MW, value of 50 kD (Table 4). From patients with other diseases (group III), 5.90% had positive results and 94.10% had negative results for ATL. Serologic cross-reactions occurred with the same band (50 kD) in 20.00% of the serum samples from patients with a positive diagnosis for syphilis. No serum samples from healthy individuals showed positive results by Western blotting for ATL. Based on the positivity criterion, 77.61% of the serum samples from patients with ATL had positive results and 22.39% had negative results by Western blotting (Table 6).

**Strain 268T.** Forty-one of the 44 polypeptides detected in strain 268T were recognized by at least one serum from groups I, II, III, and/or IV. The samples from group I recognized 31 antigenic bands, with 2.90% showing no reactivity, 56.00% recognizing between one and seven bands, 38.20% recognizing between eight and 13 bands, and 2.90% recognizing 13 bands. The most reactive bands (diagnostic for ATL) are shown in Table 2. In group II, Western blotting detected bands with MW, values ranging from 13 kD to 150 kD that showed high intensity of reactivity; 10.00% of the

<table>
<thead>
<tr>
<th>Serum samples</th>
<th>Laboratory diagnosis</th>
<th>No. of serum samples</th>
<th>Western blot*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Group I†</td>
<td>ATL</td>
<td>67</td>
<td>52</td>
</tr>
<tr>
<td>Group II‡</td>
<td>Chagas’ disease</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>Group III§</td>
<td>Antinuclear antibodies</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Group IV¶</td>
<td>Microsomal antibodies</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Group III§</td>
<td>Antinuclear + anti-dsDNA</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Group IV¶</td>
<td>Syphilis</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Group IV¶</td>
<td>Leprosy</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Group IV¶</td>
<td>Healthy individuals</td>
<td>20</td>
<td>0</td>
</tr>
</tbody>
</table>

* P = positive when the sample recognized at least one antigenic bands from a group of three with the highest frequency; N = negative when the sample showed no reactivity with the antigenic bands.
† Samples from patients with a diagnosis of American tegumentary leishmaniasis (ATL).
‡ Samples from patients with Chagas’ disease.
§ Samples from patients with other diseases.
¶ Samples from healthy individuals.
Results obtained in the Western blot with the 268T strain for the diagnosis of American tegumentary leishmaniasis (ATL) with serum samples from groups I, II, III, and IV

<table>
<thead>
<tr>
<th>Serum samples</th>
<th>Laboratory diagnosis</th>
<th>Number of serum samples</th>
<th>Western blot*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>P</td>
</tr>
<tr>
<td>Group I†</td>
<td>ATL</td>
<td>34</td>
<td>30</td>
</tr>
<tr>
<td>Group II‡</td>
<td>Chagas’ disease</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>Group III§</td>
<td>Antinuclear antibodies</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Microsomal antibodies</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Antinuclear + anti dsDNA</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Syphilis</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Leptosy</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Group IV¶</td>
<td>Healthy individuals</td>
<td>20</td>
<td>1</td>
</tr>
</tbody>
</table>

* P = positive when the sample recognized at least one antigenic bands from a group of three with the highest frequency; N = negative when the sample showed no reactivity.
† Samples from patients with a diagnosis of American tegumentary leishmaniasis (ATL).
‡ Samples from patients with Chagas’ disease.
§ Samples from patients with other diseases.
¶ Samples from healthy individuals.

serum samples recognized five bands, 50.00% recognized between eight and 13 bands, and the remaining 40.00% recognized between 16 and 18 bands. The most frequently recognized bands (diagnostic for Chagas’ disease) are shown in Table 2. In group III, 47.00% of the serum samples did not recognize any bands, 35.60% recognized between one and five bands, 11.80% recognized between 10 and 11 bands, and 5.90% recognized 32 bands. Samples from patients with a positive diagnosis for syphilis showed no reactivity. Sera from healthy persons (group IV) reacted with polypeptides with MW, values ranging from 13 kD to 105 kD; 45.00% of the samples showed no reactivity, 20.00% recognized between one and three bands, and 35.00% recognized between four and six bands. Extensive cross-reactivity was found in the serum samples from patients with Chagas’ disease, in which 80.0% had a positive result (recognizing all the ATL diagnostic bands of 50 kD, 38 kD, and 32 kD). In the group III, a total of 5.90% of sera from patients with autoimmune diseases showed cross-reactivity with an ATL band (38 kD) and the sera from patients with a positive diagnosis for syphilis or leprosy showed no reactivity. Five percent of sera from healthy individuals (group IV) recognized an ATL band (50 kD) (Table 4). In accordance with the positivity criterion, 88.20% of the serum samples from patients with ATL had positive results and 11.80% had negative results (Table 7).

Table 8 shows the sensitivity (95% confidence interval), specificity, positive and negative predictive values, and the kappa concordance coefficient of the antigen extracts from L. (V.) braziliensis, Leishmania (L.) amazonensis, Leishmania (L.) tropica, and strain 268T. The results showed that no band recognized by 100% of the sera from patients with ATL was found using the four antigens analyzed by Western blotting.

**DISCUSSION**

Western blotting can provide detailed information about a parasite’s antigenic structure, which is very useful in the standardization of more sensitive and specific diagnostic procedures. This method has used soluble extracts,15,18,20,21,44 purified membrane proteins,45 or recombinant proteins as antigens,46–48

The immunoblot results obtained with soluble protein extracts of Leishmania species and strain 268T showed a complex reactivity pattern (Figures 1–4), in which most (93%) of the polypeptide fractions of parasites were recognized by at least one serum sample from the individuals tests. Similarly complex profiles were found15,49 when the human antibody specificity to antigens of L. (V.) braziliensis and L. infantum was analyzed by Western blotting.

The protein fractions recognized by serum samples varied with the different parasite strains and different individuals recognized different protein fractions of the same parasite. Variability has been observed50 in T cell stimulation in sera from patients with ATL. This could be explained in part by the complexity of the antigenic constitution of each parasite.
and by the genetic background of each individual, which together influence the immune response.\textsuperscript{22,51}

Western blotting showed that serum samples from patients with ATL recognized 31 bands (13–150 kD) from \textit{L. (V.) braziliensis}, 34 bands (13–150 kD) from \textit{L. tropica}, and 31 bands (17–170 kD) from the 268T strain. Similar results were observed\textsuperscript{52} with serum samples from patients with American visceral leishmaniasis, which recognized 36 antigenic components (14.5–123 kD) and with serum samples from patients with cutaneous leishmaniasis, which recognized 33 soluble proteins (18.5–115 kD) from a \textit{L. donovani chagasi} extract.

Only 14 of 39 polypeptide fractions from \textit{L. (L.) amazonensis} that stained with Coomassie brilliant blue were recognized by serum samples from patients with ATL. The reactivity (immune staining) of the serum samples with \textit{L. (L.) amazonensis} seemed less intense but more homogeneous, with the majority (75.50%) of the serum samples from group I recognizing the same fractions (Figure 2). The low intensity of immunostaining of fractions from \textit{L. (L.) amazonensis} could be explained by the long period (more than four years) of maintenance in artificial culture medium, leading to lower virulence as demonstrated in animal models,\textsuperscript{53} less \textit{L. donovani chagasi} antigen, and a concomitant decrease in the gp 63 content of the promastigote form. However, this was not observed in \textit{L. tropica} that was also maintained for many years \textit{in vitro}, in which serum samples from patients with ATL recognized various fractions of the protein extract of this strain (Figure 3).

The 63-kD protein fraction of \textit{L. (L.) amazonensis} was not recognized by sera from group I (ATL). Similarly, only 19.4% of these sera recognized the 63-kD antigen of \textit{L. tropica}. The majority (63.89%) of ATL-positive sera recognized the 63-kD protein of \textit{L. (V.) braziliensis}. This protein was identified as a glycoprotein by staining with periodic acid-Schiff, indicating that it could be the main promastigote surface molecule (MW = 60–65 kD) common to all \textit{Leishmania} species. This surface antigen may be involved in the development of the infective stage\textsuperscript{31} in promastigote forms of \textit{L. (V.) braziliensis}, in which there is a progressive increase in the expression of a 65-kD surface antigen (gp 63) in the stationary growth phase.\textsuperscript{31}

A comparison of the MW values of protein fractions is a complex process, and many factors such as composition of the culture media, parasite growth phase, concentration of SDS and 2-mercaptoethanol in the sample buffer, separation of soluble fractions from pellets, SDS-PAGE gel size, polyacrylamide concentration, and the molecular weight markers used can influence the fraction migration and determination of the MW values.\textsuperscript{34} Thus, direct comparisons of molecular weights of antigens cited in the literature are always very difficult and must be done with caution.

In spite of the complex recognition pattern, the protein fractions most reactive with ATL-positive sera (Table 4) have relatively low molecular weights (28–74 kD). This is similar to results found for \textit{L. infantum} (14–90 kD)\textsuperscript{40} and \textit{L. chagasi} (26–116 kD).\textsuperscript{38} Relatively high molecular weights were found for the most frequently reacting protein fractions of \textit{L. braziliensis panamensis} (78–120 kD)\textsuperscript{18} and \textit{L. (V.) braziliensis} (69–250 kD).\textsuperscript{20}

When we compared individual reactive protein fractions, a 60-kD protein from \textit{L. (V.) braziliensis} had been previously identified by human sera from patients with ATL,\textsuperscript{13} whereas we detected a 63-kD antigen (Table 4). In the \textit{L. (L.) amazonensis} extract, a 74-kD fraction was recognized by 75.47% of the ATL-positive sera, whereas in \textit{L. tropica} and strain 268T, the recognition was much lower (Table 4). Similar results were obtained with a fraction of 70–73 kD from \textit{L. donovani chagasi} extracts that was recognized by 89.47% of sera from patients with visceral leishmaniasis.\textsuperscript{30}

Fractions of 40 kD, 42 kD, 44 kD, and 38 kD were detected in extracts of \textit{L. (L.) amazonensis}, \textit{L. (V.) braziliensis}, \textit{L. tropica}, and strain 268T, respectively (Table 4). All were recognized by more than 50.00% of the ATL-positive sera. A 42-kD protein was purified from the membrane of \textit{L. amazonensis}, and it was shown that this protein (La gp 42) was recognized by sera from patients with different forms of leishmaniasis caused by \textit{L. major}, \textit{L. donovani}, and \textit{L. chagasi}, and was also recognized by sera from patients with Chagas’ disease.\textsuperscript{45} Analysis of the N-terminal sequence of La gp 42 showed homology with gp 46/M-2.\textsuperscript{54} This protein belongs to the promastigote surface antigen complex-2, which has been found in all \textit{Leishmania} species studied to date, except for \textit{L. braziliensis}.\textsuperscript{55} A 42-kD antigen was identified by sera from patients with cutaneous and visceral leishmaniasis in an \textit{L. major} extract.\textsuperscript{31} The immune response induced by a recombinant protein from \textit{L. (L.) amazonensis} (33 kD [Larp33]) was also evaluated. Western blotting showed that Larp33 was a 40-kD protein expressed in \textit{L. (L.) amazonensis} and \textit{L. (V.) braziliensis}.\textsuperscript{57}

A protein with an MW, of 50 kD, which was recognized by more than 50.00% of the ATL-positive sera, was detected in extracts from \textit{L. (L.) amazonensis}, \textit{L. tropica}, and strain 268T (Table 4). However, only 23.00% of the ATL-positive sera recognized the 50-kD fraction in the \textit{L. (V.) braziliensis} extract. In cutaneous leishmaniasis caused by \textit{L. major}, more than 90.00% of positive sera recognized a fraction of 50 kD.\textsuperscript{56} The investigators suggest that this fraction could be a promastigote surface protein (gp 63) with an MW of 50,000 (determined under non-reducing conditions). It is well known that some of the surface antigens are active proteases (gp 63). The proteolytic activity of a wide range of trypanosomatids was determined and many trypanosomatids (7 genera and 11 species) have various proteolytic activities, including some cross-reactive promastigote surface protein (gp 63).\textsuperscript{57}

An antigenic profile that enables one to discriminate positive from negative serologic results with regard to ATL, Chagas’ disease, other diseases, and healthy controls is urgently needed. A diagnostic antigen recognized by 100% of positive sera is not available. The principal antigen used depends on the expression of this diagnostic antigen because even a highly immunogenic antigen, if poorly expressed, will react as a weak band.\textsuperscript{58} The complex molecular constitution of the parasite, associated with limitations in the analysis of the results obtained by different techniques, lead to a lack of consensus in the definition of the principal antigens in \textit{Leishmania}. It was possible to correlate the presence of the parasite in dogs with the recognition of different antigenic fractions detected; statistical analysis was used to select the bands with a stronger correlation and define a criterion of positivity based on the recognition of these bands.\textsuperscript{34}
Based on these criteria, five bands were selected and the test result was considered positive when at least one band was recognized. A sensitivity of 95.80% and a specificity of 100.00% were obtained with this system. However, it is not always possible to correlate parasitologic and serologic results. Another approach is to define a specific antigenic component (e.g., a 94-kD L. infantum antigen) whose detection with sera from a patient with visceral leishmaniasis gave a sensitivity of 95.45% and a specificity of 100%. In the same L. infantum extract, 14-kD and 16-kD fractions were detected by sera from a patient with visceral leishmaniasis sera showed recognition frequencies of 92.00% and 95.00%, respectively. When combined, they gave a recognition frequency of 100.00%, a sensitivity of 100.00%, and a specificity of 98.00%. However, such a level of a recognition of antigenic fractions is not always achieved.

The average number of recognized antigenic fractions from the different strains was always higher for serum samples from group II (Chagas’ disease) than from group I (ATL) (Table 1). Analysis of cross-reactivity in human infections caused by L. (V.) braziliensis, T. cruzi, and L. chagasi showed that sera from chagasic patients recognized 24 bands with homologous antigen, 13 bands with L. chagasi antigen, and 17 with Leishmania (V.) braziliensis antigen. The sera from patients with visceral leishmaniasis recognized 29 bands with homologous antigen, 14 with T. cruzi antigen, and 10 bands with Leishmania (V.) braziliensis antigen. Sera from individuals with ATL recognized 17 bands with homologous antigen, 10 bands with T. cruzi antigen, and four bands with L. chagasi antigen. A serum sample was considered positive for ATL by Western blotting when it recognized at least one band among the three with the highest recognition frequency in each strain analyzed. Western blotting showed a sensitivity of 84.90% and a specificity of 91.10% with L. (V.) braziliensis, a sensitivity of 90.40% and a specificity of 93.80% with L. (L.) amazonensis, and a sensitivity of 78.50% and a specificity of 87.80% with L. (L.) tropica. A specificity of 77.10% was obtained by Western blotting with a distant trypanosomatid parasite of tomato strain 268T. Our results showed that the L. (L.) amazonensis soluble antigen is more adequate for the diagnosis of ATL by Western blotting test than the antigen from L. (V.) braziliensis (Table 8).

Cross-reactivity is still one of the biggest problems in clinical serology. With the L. (V.) braziliensis strain, the sera from chagasic patients showed cross-reactivity with the 63-kD fraction. This findings differs from that of another study, which reported that the 60-kD fraction is specific for Leishmania. Other sera (autoimmune diseases and leprosy) also recognized the gp 63 antigen. Another 22 L. donovani chagasi antigens (18.5–112 kD) were recognized by sera from Chagas’ disease patients and a small number of antigens (32–110 kD) were reactive with sera from patients with leprosy and tuberculosis.

We observed cross-reactivity with the 40-kD L. (L.) amazonensis fractions with sera from chagasic patients, as previously described. Studies of human IgG reactivity with antigens of T. cruzi and Leishmania revealed cross-reactivity of serum samples from chagasic patients with a 38-kD antigen of L. b. panamensis, but the profiles of the sera detected with the T. cruzi and Leishmania antigens permit clear serologic differentiation of these parasitoses. Cross-reactivity of sera from patients with ATL and Chagas’ disease in the diagnosis of visceral leishmaniasis, mainly with the 70-kD antigen, and the serologic differentiation of these infections, has also been reported. Conversely, an L. braziliensis braziliensis 72-kD antigen was described that had no cross-reactivity with sera from patients with Chagas’ disease. Extensive cross-reactivity was observed among the strain 268T antigens and serum samples from patients with Chagas’ disease, and at a lower frequency among patients with autoimmune diseases and sera from healthy individuals. A comparison of indirect immunofluorescence and the intradermal reaction in the diagnosis of leishmaniasis give a statistically significant agreement value (Z = 9.72, P < 0.01) but a poor kappa index (0.32). An epidemiologic study of visceral leishmaniasis examined 50 unselected subjects living in a high risk area for visceral leishmaniasis simultaneously by means of a skin test and Western blotting. The criterion of positivity in the Western blot used was based on the presence of antibodies directed against the L. infantum 14-kD and/or 16-kD antigens. The agreement of the two techniques (agreement percentage = 82.00%) does not consider the influence of agreement purely by chance. We analyzed the agreement of Western blotting with a positive diagnosis for ATL by means of clinical signs and classical laboratory tests (parasitologic, skin test, and IFA) using the kappa statistic, which corrects for the agreement expected by chance. The overall agreement between the Western blotting and these other techniques results in a kappa index of 0.840 for L. (L.) amazonensis, 0.7358 for L. (V.) braziliensis, 0.6491 for L. (L.) tropica, and 0.6287 for 268T (Table 8).

Our results showed that all strains studied showed cross-reactivity with serum samples from patients with Chagas’ disease, which is consistent with the findings of previously published reports and indicates the presence of common epitopes between Leishmania and T. cruzi. The overall cross-reactivity observed in serum samples from patients with positive diagnosis for ATL and in non-related microorganisms and other conditions indicates antigenic similarity between Leishmania and bacteria such as Mycobacterium species, as demonstrated by significant cross-reactivity in serologic tests.

We did not find any common antigenic band with 100% recognition that was diagnostic for all sera from patients with ATL. The more specific fractions for Leishmania antigens were 42 kD for L. (V.) braziliensis, 50 kD for L. (V.) amazonensis, and 58 kD for L. (L.) tropica with a frequency of recognition of 53.70%, 72%, and 47.80%, respectively. For strain 268T, none of the most reactive fractions were specific and there was cross-reactivity in at least one of the serum samples from groups II, III, or IV, albeit at a lower frequency (Table 4).

Western blotting is a sensitive test for detecting antibodies to Leishmania. It can also confirm false-positive results obtained by an ELISA. It allows specific serodiagnosis of visceral and mucocutaneous leishmaniasis in patients living in nonendemic areas, and appears to be superior to the classic IFA. The use of this test has been assayed in following chemotherapy and cure. Our results showed that some proteins were specific for the serum samples from patients with Chagas’ disease (Table 2). The major bands with apparent
molecular weight of 30 kD for \( L. \) (V.) \textit{braziliensis}, 35 kD for \( L. \) (L.) \textit{amazonensis}, 28 kD for \( L. \) (L.) \textit{tropica}, and 28 kD for strain 268T were recognized by these sera at a high frequency of 78.30\%, 90.00\%, 90.00\% and 80.00\%, respectively (Table 4). These fractions permit clear differentiation of leishmaniasis from Chagas’ disease, mainly with \( L. \) (L.) \textit{amazonensis} extracts. It has also been reported that sera from chagasic patients recognized \textit{Leishmania} antigens with MW, values of 124, 107, 92, 59, and 32 kD.\(^4\)

Our results show that it is possible to obtain recognition profiles that enable one to diagnose ATL and differentiate leishmaniasis from Chagas’ disease. Thus, a mixed human infection of \textit{T. cruzi} and \textit{Leishmania} species can be detected by Western blotting. This method should benefit from the use of recombinant antigens.

Acknowledgments: We thank J. C. Dalmas and T. Matsuo for statistical analysis.

Financial support: This investigation received financial support from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenadoria de Pós Graduação da Universidade Estadual de Londrina.

Authors’ addresses: Cely Cristina Martins Gonçalves, Rua Garibaldi Deliberador 483/33, Londrina CEP 86050-170, Paraná, Brazil. Edna Maria Vissoci Reiche, Benício Alves De Abreu Filho, Thais Gomes Verzignassi Silveira, Tânia Cristina Felizardo, Karoline Rocha Maia, Rafael Costacurta, Evandro José Padovesi, Benedito Prado Dias Filho, Shiduca Itow Jankevicius, and José Vitor Jankevicius, Centro de Ciências Biológicas, Universidade Estadual de Londrina, Campus Universitário, Caixa Postal 6001, CEP 86051, Londrina, Paraná, Brazil.

\[ \text{REFERENCES} \]


34. Allen Press
Friday Feb 01 2002 02:58 PM