Leishmania major-Like Antigen for Specific and Sensitive Serodiagnosis of Human and Canine Visceral Leishmaniasis

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An antigen (LMS) prepared from Leishmania major-like promastigotes was used in an enzyme-linked immunosorbent assay (ELISA) for the diagnosis of human and dog visceral leishmaniasis. The results were compared with those from the indirect immunofluorescent antibody test (IFAT). A total of 1,822 canine sera were tested, including sera from dogs with visceral leishmaniasis, transmissible venereal tumors, ehrlichiosis, rickettsiosis, or Chagas' disease and sera from healthy dogs. The antigen was also tested with 227 samples of human sera, including sera from patients with visceral, cutaneous, or diffuse cutaneous leishmaniasis and from noninfected individuals, as well as sera from patients with Chagas' disease, toxoplasmosis, rickettsiosis, hepatitis B, schistosomiasis, ascariasis, malaria, rheumatoid factor, leprosy and rheumatoid factor, tuberculosis, or leprosy. All dogs and all human patients had a clinical and/or serological and/or parasitological diagnosis. For detecting antibodies in sera from dogs with leishmaniasis, the antigen showed a sensitivity of 98%, specificity of 95%, and concordance of 93% and when used for detecting antibodies in human sera presented a sensitivity of 92%, specificity of 100%, and concordance of 92%. Comparison between ELISA and IFAT demonstrated that ELISA using the LMS antigen yielded more reliable results than IFAT. The LMS antigen displayed no cross-reactivity with sera from patients or dogs that had any of the other diseases tested.

Leishmaniasis comprises a spectrum of diseases widely distributed in tropical and subtropical countries, ranging in severity from self-healing skin lesions to severely mutilating mucocutaneous involvement or visceral infections (kala-azar) caused by the protozoan hemoflagellate Leishmania. The leishmanial diseases, except for cutaneous leishmaniasis, have a lengthy incubation period, an insidious onset, and a chronic course. Kala-azar, or visceral leishmaniasis (VL), is characterized by irregular fever, progressive enlargement of the spleen and liver, leukopenia with marked neutropenia, anemia, emaciation, and discoloration of the skin. Leishmanial infections are accompanied by a dramatic humoral response against some forms of leishmania. A marked increase in immunoglobulin G (IgG) in serum and, to a lesser extent, IgM is the common feature in the majority of kala-azar patient sera (17).

The antigens used in immunodiagnosis consist of a repertoire of at least 30 somatic antigens and an unknown number of surface components, and the existence of both heterospecific antigens and specific parasite antigens has been established (23, 27). As a result, to date most immunodiagnostic methods have been hampered by the problem of cross-reactivities of species within a family as well as with phylogenetically distant microorganisms (4, 22, 23, 31, 35). The problem is further complicated in geographical areas where different forms of leishmaniasis and trypanosomal infections occur simultaneously (27).

The serological test most commonly used for diagnosis of leishmaniasis has been an indirect immunofluorescent antibody test (IFAT) (1, 9, 21). Although the major parasitoses infect millions of people in the world, technical expertise in the use of diagnostic tests is currently very limited. In addition, the IFAT is not readily adaptable to large-scale seroepidemiological studies. Conversely, the enzyme-linked immunosorbent assay (ELISA) has proved to be at least as sensitive and specific as immunofluorescence and is suitable for large-scale studies.

As shown by Martin et al. (26), previous attempts to use nonrecombinant antigens in serological tests for diagnosis of VL were hampered by low sensitivity and specificity and poor reproducibility. The reason for this limitation is unknown but could be related to antigen preparation. Alternatively, Andrade (2) described a kinetoplast DNA probe that seems to be a promising method for diagnosis of tegumentary leishmaniasis. Although this method can be viewed as satisfactory for epidemiological studies, it is unsuitable for the rapid diagnosis of leishmaniasis.

Leishmania chagasi is responsible for VL in periurban and urban areas of Latin America, with dogs being the major reservoir of the parasite (33). An outbreak of VL in the city of Belo Horizonte (metropolitan population, approximately 3 million) in the state of Minas Gerais, Brazil, started in 1988.
and spread throughout the whole city. There has been an increase in the number of cases of VL in the last few years in the metropolitan region of Belo Horizonte (24). According to the Brazilian Ministry of Health, about 3,500 human VL cases occur annually in Brazil. Since there is no vaccine for dogs and since drugs are often ineffective or too expensive for wide-spread use, the animals are sacrificed. In 1996, 30% of about 90,000 dog sera in Brazil tested positive for leishmaniasis. The routine method for diagnosis is immunofluorescence, but this method is expensive, demands highly trained personnel, and is time-consuming.

The aim of this study was to identify and purify an antigen(s) which could improve the immunodiagnosis of VL by ELISA. Antigens used in the design of immunodiagnostic tests for leishmaniasis have traditionally been derived from promastigotes that have been cultivated in vitro (11, 12, 37). Accordingly, in the present study, we developed a new antigen, Leishmania major soluble (LMS) antigen, from promastigotes of Leishmania major-like organism. When used for the diagnosis of VL by ELISA, the LMS antigen presented high sensitivity, specificity, and concordance for both human and canine sera and did not present cross-reactivity with sera from patients or dogs with several other diseases.

MATERIALS AND METHODS

Serum selection. Sera from 49 patients with established diagnosis of VL, 20 sera from individuals infected with cutaneous leishmaniasis, and specimens from 169 healthy individuals without a history of Chagas’ disease or VL for use as controls were provided by M. S. M. Michalik and O. Genaro, Department of Parasitology, Universidade Federal de Minas Gerais (UFMG), Belo Horizonte, Brazil. Sera from 29 patients with serological diagnosis of Chagas’ disease were provided by Lúcia M. C. Galvão, Department of Parasitology, UFMG. Sera from 18 patients with serological diagnosis of toxoplasmosis were provided by R. W. A. Vitór, Department of Parasitology, UFMG, and 22 sera from patients with serological diagnosis of leprosy were provided by L. F. M. Teixeira, Department of Clinical Analysis, Universidade Federal de Ouro Preto (UFOP), Ouro Preto, Brazil. The following sera were also used: 4 sera from patients with rheumatoid factor, 3 sera from patients with leprosy and rheumatoid factor, and 11 sera from patients with tuberculosis, provided by V. Toledo, Faculty of Pharmacy, UFMG; 10 sera from patients with rickettsiosis, 13 sera from patients with hepatitis B, 16 sera from patients with schistosomiasis, 9 sera from patients with ascariasis, and 16 sera from patients with malaria, provided by J. R. Lambertucci, Faculty of Medicine, UFMG; and 9 sera from patients with diffuse cutaneous leishmaniasis, provided by A. Barral, FIOCRUZ, Bahia, Brazil.

The sera from 1,582 healthy dogs, from 159 dogs with serological diagnosis of VL, and from 29 dogs with serological and parasitological diagnoses of VL were provided by the Ezequiel Dias Foundation, Belo Horizonte, M. S. M. Michalik, Department of Parasitology, UFMG, and W. Tafuri, Department of Pathology, UFMG. Sera from 33 dogs with transmissible canine venereal tumors (TVT) were provided by R. A. Carneiro, Veterinary Hospital, UFMG; 22 sera from dogs with Chagas’ disease were provided by T. Bahia, Department of Biological Sciences, UFOP; and sera from dogs with brucellosis and rickettsiosis were provided by C. Mafra, Department of Clinical Analysis, UFOP.

The sera were tested by ELISA and IFAT. All sera were stored at −20°C.

The micro-ELISA technique. ELISA was performed as described by A. Voller et al. (38) and M. Hommel et al. (20) and as optimized by several authors (7, 19, 26, 36). In preliminary checkerboard titrations, the antigen, in increasing concentrations (0.015625 to 1,000 μg/100 μl), was incubated in 0.1 M carbonate-bicarbonate buffer, pH 9.6, overnight at 4°C. The antigen was titrated against serial dilutions of known positive and negative sera (1:40 to 1:1,520). The following antigen-sera combination consistently gave the best differentiation between positive and negative sera and was used throughout the study: an antigen concentration of 0.25 μg of protein/well and a serum dilution of 1:640. The cutoff value was the mean of the absorbance value of all negative serum samples plus 2 standard deviations (SD) at a dilution of 1:640. The plates were coated overnight at 4°C with 0.1 ml of diluted antigen in each well. After washing twice with 0.01 M phosphate-buffered saline with 0.05% Tween 20 (PBS-Tween), pH 7.3, the plates were incubated for 1 h at room temperature with 0.1 ml of 1:640-diluted serum in each well. Sera and conjugates were diluted in 0.01 M PBS with 0.2% casein (pH 7.3). After washing six times, 0.1 ml of diluted conjugate was added and incubated for 1 h. The conjugates used were peroxidase-conjugated anti-human or anti-dog IgG (Sigma). The anti-human IgG conjugate was used at a dilution of 1:2,000, and the anti-dog conjugate was used at a dilution of 1:20,000, determined by checkerboard titration. After six washings, 0.1 ml of hydrogen peroxide-ortho-phenylenediamine in a pH 5, 0.03 M citrate buffer was added as a substrate and chromogen. Color development was obtained by incubation at room temperature for 5 to 10 min. The reaction was stopped by the addition of 0.02 ml of 4 N H2SO4. Spectrophotometric reading of the plates was done at 492 nm.

Antigen. Antigen for ELISA was obtained from L. major-like (MHOM/BR/71/BH121) promastigotes. The strain was isolated in the state of Minas Gerais and was maintained in liver infusion tryptose culture medium. L. major-like promastigotes were grown in liver infusion tryptose broth with 10% fetal calf serum. The parasites were harvested in late log phase and washed three times (800 × g, 4°C, 15 min) in PBS, pH 7.2. The parasites were disrupted by resuspension in sterile distilled water and three rapid freeze-thaw cycles (liquid nitrogen, 25°C). The suspension was centrifuged at 100,000 × g for 1 h at 4°C. After determination of the protein content, the supernatant LMS antigen was stored in aliquots at −20°C. Further purification led to a proprietary antigen preparation (R. Barbosa de Deus, C. A. Pereira Taveres, M. L. dos Mares Guia, patent application number PI – 97050830-3, 8 December 1998, Brazilian Patent Office).

IFATs. For antigen preparation, promastigotes of Leishmania braziliensis were washed five to six times in PBS and resuspended in PBS-buffered formalin. The parasites were washed twice in PBS, and the pellet resulting from the final centrifugation was resuspended in PBS so that 1 drop yielded 20 to 25 parasites per microscope field (×500). One drop of the antigen solution was placed on microscope slides and dried at room temperature. Serum samples were thawed and diluted from an initial dilution of 1:20. Each dilution of serum was placed over the antigen and incubated in a moist chamber at 37°C for 30 min. Doubling serum dilutions were used starting at 1:10 for human sera and 1:40 for canine sera. The slides were washed with PBS, covered with rabbit anti-human or rabbit anti-dig IgG serum conjugated to fluorescein isothiocyanate (19) (fluorescein/protein weight ratio = 9.6) diluted with PBS containing 0.2% Evans Blue, and incubated in a moist chamber at 37°C for 30 min. The slides were washed twice in PBS, covered with buffered glycerin (pH 8.0) and a coverslip, and then examined the same day on a fluorescent microscope. In all experiments normal and positive sera were included as negative and positive controls. For reactive serum samples, parasites displayed a bright-green peripheral stain with a dull fluorescence of the cytoplasm. For negative sera, parasites remained dim red spots (18).

Statistical methods. Data were analyzed for comparison of proportions and means in related samples by McNemar’s test (3, 25). Sensitivity (specificity) and specificity (concomitancy) indices were determined according to the methods of A. Buck and J. J. Gart (8), A. C. Ghose et al. (17), and G. L. Machado-Coelho et al. (25). Positive and negative predictive values were determined as described by T. J. Vecchio (37).

Comparison of absorbance values for all human and canine serum tests was done by an analysis of variance followed by Tukey’s test (13).

In this work IFAT was used as a reference for ELISA validation with our antigen.

RESULTS

For this study, 218 sera from humans (49 from patients with VL, 169 from uninfected individuals) and 1,741 sera from dogs (159 from dogs with VL and 1,582 from healthy dogs) were tested by ELISA using the antigen obtained from L. major-like promastigotes. The results were compared with those from IFAT.

Tables 1 and 2 show the results for dog and human sera with IFAT and ELISA. The cutoff of ELISA (mean of absorbance plus 2 SD of controls) for dog sera was 0.157 and for human sera was 0.115 at a dilution of 1:640.

When compared with IFAT, ELISA analysis of dog sera presented a sensitivity of 98%, a specificity of 95%, a positive predictive value of 66%, a negative predictive value of 100%,

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and a concordance rate of 93% (Table 1). ELISA analysis of human sera displayed a sensitivity of 92%, a specificity of 100%, a positive predictive value of 100%, a negative predictive value of 98%, and a concordance rate of 92% (Table 2). The LMS antigen used in ELISA did not show significant numbers of false positive or false negative reactions. The results show that the LMS antigen is very specific, able to detect low levels of anti-leishmaniasis immunoglobulins present in positive samples, and can discriminate between negative and positive sera.

In order to verify the specificity of our antigen, it was tested in ELISA with sera from dogs and patients having diseases reported to present antibodies that cross-react with leishmania antigens. We first examined sera from 17 patients with a diagnosis of acute VL and compared the results with sera from 31 healthy controls, 20 sera from patients with cutaneous leishmaniasis, 29 sera from patients with Chagas’ disease, 18 sera from patients with toxoplasmosis, 10 sera from patients with rickettsiosis, 13 sera from patients with hepatitis B, 16 sera from patients with schistosomiasis, 9 sera from patients with ascaridiasis, 16 sera from patients with malaria, 4 sera from patients with rhabdomyosarcoma factor, 3 sera from patients with leprosy and rhabdomyosarcoma factor, 11 sera from patients with tuberculosis, 22 sera from patients with leprosy, and 9 sera from patients with diffuse cutaneous leishmaniasis (Fig. 1). The results demonstrate a clear discrimination between VL sera, control sera, and sera from all other diseases tested, even when high dilutions of sera were compared. The LMS antigen did not show significant numbers of false positive or false negative reactions.

### Table 1. Cross distribution of positive and negative reactions for VL with dog sera tested by ELISA and IFAT (reference)

<table>
<thead>
<tr>
<th>ELISA(a) result</th>
<th>No. of sera with IFAT result that was:</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Positive</td>
<td>156</td>
<td>79</td>
</tr>
<tr>
<td>Negative</td>
<td>3</td>
<td>1,503</td>
</tr>
<tr>
<td>Total</td>
<td>159</td>
<td>1,582</td>
</tr>
</tbody>
</table>

\(a\) The ELISA had the following characteristics compared to IFAT: sensitivity (copositivity), 98%; specificity (conegativity), 95%; concordance, 93%; predicted positive value, 66%; predicted negative value, 100%; Youden value, 93%; \(\chi^2_{1df} = 66.8\) \((P \leq 0.05)\).

### Table 2. Cross distribution of positive and negative reactions for VL with human sera tested by ELISA and IFAT (reference)

<table>
<thead>
<tr>
<th>ELISA(a) result</th>
<th>No. of sera with IFAT result that was:</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Positive</td>
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</tr>
<tr>
<td>Total</td>
<td>49</td>
<td>169</td>
</tr>
</tbody>
</table>

\(a\) The ELISA had the following characteristics compared to IFAT: sensitivity (copositivity), 92%; specificity (conegativity), 100%; concordance, 92%; predicted positive value, 100%; predicted negative value, 98%; Youden value, 92%; \(\chi^2_{1df} = 6.25\) \((P \leq 0.05)\).
not cross-react with sera from any of the patients having other diseases reported to present antibodies that cross-react with leishmania antigens. The cutoff (mean + 2 SD of controls) was 0.126 for human sera at a dilution of 1:640.

Analysis of variance of the data in Fig. 1 shows that the absorbance values for the VL sera were significantly higher than those of the other groups ($P < 0.001$). There was no significant difference among the means of sera of other diseases.

Comparison among sera from different diseases was done by Tukey’s test (13). The means of absorbance values were compared at 1% probability. It was shown that the mean of absorbance values for VL sera is significantly different from the mean of sera of all other diseases tested ($P < 0.001$).

The same analysis of specificity was done for canine sera. Sera from dogs with serological and clinical diagnoses of VL and from dogs with clinical and parasitological diagnoses of VL were tested by ELISA with LMS antigen and compared with the reactivity of sera from healthy dogs and from dogs with TVT, Chagas’ disease, ehrlichiosis, or rickettsiosis. The results are shown in Fig. 2. In this plot there is again a clear difference between VL sera, control sera, TVT sera, and Chagas’ disease sera, even when high dilutions of sera were compared. LMS antigen did not cross-react with sera from dogs with clinical and serological diagnoses of TVT or from healthy dogs. The cutoff value was 0.140 for dog sera at a dilution of 1:640.

Analysis of variance of the data in Fig. 2 shows that the absorbance values for both groups of VL sera are significantly higher than the values observed for other diseases ($P < 0.001$).

Comparison among absorbance values from different diseases was done by Tukey’s test (13). The means of absorbance values were compared at 1% probability. There is no significant difference between the two groups of VL sera tested. However, the means of both groups of VL sera are different from the means of sera of all other groups tested ($P < 0.001$).

**DISCUSSION**

Previous attempts to use antigens in serological diagnostic tests for VL have been hampered by low sensitivity, specificity, and test reproducibility. No clear reason exists for this limitation. However, the techniques used to extract antigens from leishmania parasites may inadvertently alter the primary structure of the molecules and compromise function (26, 34).

ELISA and IFAT have been previously compared for the serodiagnosis of cutaneous and VL (15), and the levels of leishmanial antibody detected by *Leishmania donovani* antigens in both tests have been compared. From these results it was concluded that ELISA is slightly more sensitive than IFAT, but IFAT seems to be more specific.

Although the IFAT test is quite reliable for the diagnosis of VL (6, 14, 32), it is also known to have cross-reactivity with other trypanosomes (10, 28) and thus is rendered unreliable for use in field studies or population surveys (7). The ELISA has been shown to be at least as sensitive as the IFAT for the
diagnosis of leishmaniasis (11, 20). However, the diagnosis of VL remains problematic since most of the serological methods for diagnosis use antigen preparations that lack specificity. A cloned antigen (rK39) of Leishmania that is specific for all members of the L. donovani complex has been described (5). This antigen was very useful for the serodiagnosis by ELISA of both human and canine VL, and rK39 is an indicator of active disease.

The results presented here, using 1,741 dog serum samples and 218 human serum samples, demonstrate that our LMS antigen is highly satisfactory for use in ELISA for diagnosis of VL, when compared to IFAT. All steps of our ELISA were standardized in a manner similar to that described by R. Rajasekariah et al. (30).

Comparison between ELISA and IFAT using LMS antigen and dog sera gave excellent results, with a sensitivity of 92%, 100% specificity (conenagativity), and a concordance value of 92%. A χ² analysis (McNemar) of the data showed a difference in terms of accuracy between IFAT and ELISA ($\chi^2_{df} = 6.25$, where $df$ is degree of freedom; $P = 0.05$). Therefore, our LMS antigen presents better results when used in ELISA than when used in IFAT.

The calculated predictive value of 100% for human sera also indicates that our antigen is highly specific. Using ELISA to test human sera, we found a sensitivity (copositivity) of 98%, a specificity (conenagativity) of 95%, and a concordance of 93%. The χ² analysis (McNemar) showed a difference in terms of accuracy between IFAT and ELISA ($\chi^2_{df} = 66.8; P = 0.05$), indicating that the LMS antigen gives better results when used in ELISA than when used in IFAT.

For dog sera, the calculated predictive positive value was 66%, suggesting a relatively low specificity, even when the calculated value was 95%. The predictive positive value is related to the number of dog sera that gave a positive result in ELISA and a negative result in IFAT (false positives). It was observed, however, that new samples taken 2 months later from a smaller group of the same dogs gave positive results in both tests, thus demonstrating that the ELISA with our antigen detects the disease at an earlier stage.

The data presented suggest that our LMS antigen is a serological indicator of VL. The serological reactivity accompanies acute disease and, when dog serum is used, also detects subclinical cases that progress to VL.

The diagnosis for VL by use of ELISA with our antigen is better than that by use of IFAT. There are no requirements for highly trained personnel (who are in short supply in areas where VL is endemic) or for expensive equipment (which is also rare). The ELISA with our antigen makes this test reliable for use in field studies or population surveys.

A recombinant ORFF protein from Leishmania infantum, assessed by ELISA for diagnosis of VL, was developed by V. S. Raj et al. (29). The authors used a serum dilution of 1:20, with an optimal concentration of recombinant ORFF of 5 ng, and obtained high sensitivity but high cutoff values. In our assays the serum dilution used was 1:640 and the concentration of LMS was 250 ng. Under these conditions our method is able to detect antibodies against VL even in early stages of the disease.

An important factor in diagnostic tests is cross-reactivity with other diseases that often overlap, such as cutaneous leishmaniasis and Chagas’ disease. Variable degrees of cross-reactivity are observed for cutaneous leishmaniasis, VL, and Chagas’ disease, and there is a need for a serological test to distinguish between them (19). The ELISA results using our antigen were distinctive for VL and could be used to differentiate human VL sera from sera of patients with Chagas’ disease, cutaneous leishmaniasis, toxoplasmosis, rickettsiosis, hepatitis B, schistosomiasis, ascariasis, malaria, rheumatoid factor and leprosy, lepromat, and tuberculosis. It can also differentiate sera of dogs with VL from sera of dogs with Chagas’ disease, TVT, erlichiosis, and rickettsiosis and from sera of healthy dogs.

The diagnosis of VL is usually straightforward for patients with the characteristic hepatosplenomegaly, fever, wasting, and pancytopenia of kala-azar. However, the ability to detect asymptomatic infection or disease at an early subclinical stage is needed to improve intervention, to potentially direct chemotherapy to the appropriate individuals, and to better understand the population at risk and the determinants of disease.

The results presented here demonstrate that our LMS antigen is highly satisfactory for use in the ELISA diagnosis of VL and, when compared to IFAT, is a reliable test that can be safely used for leishmaniasis diagnosis.

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REFERENCES