

## Specific Serodiagnosis of Canine Visceral Leishmaniasis Using *Leishmania* Species Ribosomal Protein Extracts<sup>∇</sup>

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**In the present work, we have analyzed the antigenicity of *Leishmania* species ribosomal proteins (LRPs). To accomplish this, *Leishmania infantum* ribosomes were biochemically purified from promastigote cytosolic extracts, and their reactivities were analyzed by using the sera from dogs naturally infected with *L. infantum*. Since antibodies reacting against different ribosomal proteins were observed in all the serum samples obtained from dogs with symptomatic visceral leishmaniasis tested, we have analyzed the potential usefulness of the LRP extracts in the development of an enzyme-linked immunosorbent assay (ELISA) for the serodiagnosis of canine visceral leishmaniasis (CVL) in an area of Brazil where visceral leishmaniasis is endemic due to infection by *Leishmania chagasi*. A comparative ELISA with crude soluble *Leishmania chagasi* antigen (SLA) and *L. infantum* LRPs was performed. LRP- and SLA-based ELISAs gave similar sensitivities for the diagnosis of symptomatic CVL, but the LRP extract provided a very high sensitivity for the detection of oligosymptomatic and asymptomatic dogs. In addition, an LRP-based ELISA showed a higher specificity when the sera from dogs harboring other infections were included in the analysis. The LRP antigen displayed no cross-reactivity with sera from dogs that had any of the other diseases tested, notably, Chagas' disease. Our findings suggest that LRPs are a potential tool for the diagnosis of CVL and will be particularly useful for the diagnosis of asymptomatic CVL.**

Canine visceral leishmaniasis (CVL) is an important emerging zoonosis in countries around the Mediterranean Basin, the Middle East, and Latin America (26). This severe disease is caused by *Leishmania infantum* in the Mediterranean area and in the Middle East and Asian countries and is caused by *Leishmania chagasi* in Latin America (26, 27). Due to their genotypic relationships, both species causing CVL in different continents can be considered to be identical (32).

Upon infection, dogs can develop different forms of the disease: asymptomatic, oligosymptomatic, or symptomatic (5). Symptomatic infection results in death; and its clinical manifestations include cutaneous alterations, such as alopecia, dermatitis, and onychogryphosis (4, 16), and also visceral manifestations with splenic, renal, hepatic, and cerebral alterations (22, 37). However, some of the infected dogs remain asymptomatic or develop a few mild symptoms and are classified as oligosymptomatic (5). CVL cannot be considered only a veterinary disease, since infected dogs (even asymptomatic ones) are the main domestic reservoir of the parasite for human infection (2). Thus, to reduce the frequency of transmission of

*Leishmania* from dogs to humans, it is necessary to diagnose canine leishmaniasis as early as possible (35).

The presence of anti-*Leishmania*-specific antibodies in asymptomatic, oligosymptomatic, and symptomatic infected dogs (5, 12, 45) has allowed the development of serologic tests, including immunofluorescent antibody tests, Western blotting, immunochromatographic tests, and enzyme-linked immunosorbent assays (ELISAs) (for a review, see reference 29). The diagnosis of CVL by ELISAs based on crude soluble *Leishmania* antigens (SLAs) have shown a high degree of sensitivity but a low degree of specificity because of the antigenic relatedness of *Leishmania* and other pathogenic protozoa (21). As a strategy for the development of specific serodiagnostic tests for CVL, different parasite antigens were obtained as recombinant proteins (23, 30, 48). However, due to the high degree of variability in the humoral responses to different parasite antigens observed in infected dogs (24, 42), the efficient diagnosis of CVL based on recombinant proteins may require a mixture of recombinant proteins or the use of chimerical proteins containing several nonrelated parasite antigens (7, 42, 49). The specific diagnosis of CVL can also be developed by using preparations purified from the parasite (6, 9) or crude parasite fractions analyzed by Western blotting (1, 20).

Some of the parasite ribosomal constituents, such as the parasite acidic P proteins, induce strong humoral responses in dogs clinically infected with *L. infantum* (46). In addition,

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*Leishmania* ribosomal proteins (LRPs) seem to be immunologically relevant molecules during murine experimental cutaneous leishmaniasis because high titers of antibodies recognizing the parasite ribosomal proteins were detected in sera from BALB/c mice infected with *L. major* (28). Here, we show that sera from dogs naturally infected with *L. infantum* showed reactivity against different ribosomal proteins by Western blotting. In addition, a serological evaluation of LRPs by ELISA with these sera revealed that LRP-based assays have a sensitivity similar to that of SLA-based assays for the diagnosis of symptomatic CVL. Thus, the ELISA technique was used to evaluate the diagnostic potential of LRP extracts in comparison with that of SLAs by using sera from asymptomatic, oligosymptomatic, and symptomatic *L. chagasi*-infected dogs as well as dogs infected with other protozoan parasites. We conclude that the ELISAs with LRPs have a better sensitivity and a higher specificity than the SLA-based assays for the diagnosis of CVL.

#### MATERIALS AND METHODS

**Parasites.** *Leishmania chagasi* (MOM/BR/1970/BH46) and *L. infantum* (MCAN/ES/1996/BCN/150/MON-1) were grown at 24°C in Schneider's medium (Sigma, St. Louis, MO) supplemented with 20% heat-inactivated fetal bovine serum (Sigma), 20 mM L-glutamine, 200 U/ml penicillin, 100 µg/ml streptomycin, and 50 µg/ml gentamicin at pH 7.4.

**Antigen preparation.** SLA was prepared from stationary-phase promastigotes of *L. chagasi* and *L. infantum*, as described previously (17). Briefly,  $2 \times 10^8$  promastigotes per ml, in a volume of 5 ml, were washed three times in cold sterile phosphate-buffered saline (PBS). After six cycles of freezing and thawing followed by ultrasonication (GEX600 ultrasonic processor) by the use of five cycles of 30 s at 38 MHz, the suspension was centrifuged at  $8,000 \times g$  for 30 min at 4°C and the supernatant containing the SLA was collected. The protein concentration was estimated by the method of Bradford (8), and aliquots of 500 µl were stored at -70°C.

LRP was prepared from logarithmic-phase promastigotes of *L. infantum*, as described previously (28). Briefly,  $1 \times 10^9$  promastigotes were harvested, washed twice in prechilled PBS, resuspended in 1 ml Nonidet P-40 lysis buffer (10 mM Tris HCl, pH 8.0, 150 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.5% Nonidet P-40), and pipetted up and down 10 times. After lysis, the samples were centrifuged on a Microfuge at  $3,000 \times g$  for 2 min at 4°C to pellet the nuclei. The supernatant was twice centrifuged on a Microfuge at  $13,000 \times g$  for 15 min at 4°C. The purified cytosolic supernatant was submitted to high-speed centrifugation at 90,000 rpm for 30 min at 4°C in a Beckman TL100.3 rotor. The crude ribosomal pellet was resuspended in buffer A (20 mM Tris-HCl, pH 7.4, 500 mM ammonium acetate, 100 mM MgCl<sub>2</sub>, 5 mM β-mercaptoethanol) and centrifuged through a discontinuous sucrose gradient (20/40%) in buffer A at 90,000 rpm and 4°C in a TL100.3 rotor. The pellet of washed ribosomes was dissolved in PBS, sonicated, and stored at -70°C. The LRP concentration was estimated by the method of Bradford (8).

**Serum samples.** Serum samples were collected in Spain and Brazil. Serum samples were collected from 28 clinically symptomatic dogs with CVL in the Extremadura region of Spain. *L. infantum*-infected animals were clinically, immunologically, and parasitologically evaluated at the Department of Parasitology of the Veterinary School, Extremadura University, Cáceres, Spain. The animals were considered symptomatic when three or more of the following symptoms were present: loss of weight; alopecia; adenopathy; onychogryposis; hepatomegaly; conjunctivitis; and exfoliative dermatitis on the nose, tail, and ear tips. All serum samples were positive when they were tested by indirect immunofluorescence, as described previously (10). The presence of amastigote forms was confirmed by direct observation in popliteal and prescapular lymph nodes. Control sera were obtained from eight healthy animals that had been bred and maintained in an experimental kennel under defined conditions at the Department of Parasitology (Extremadura University).

Serum samples from 83 *L. chagasi*-infected dogs (44 clinically symptomatic, 17 oligosymptomatic, and 22 asymptomatic dogs) from the Belo Horizonte area, Minas Gerais, Brazil, were used. The animals were considered symptomatic when they presented with three or more of the clinical symptoms described above, were considered oligosymptomatic when only one or two symptoms were

present, and were considered asymptomatic when the dogs were free from clinical symptoms. Diagnosis of the disease was defined when amastigotes were seen in Giemsa-stained smears of bone marrow aspirates or promastigotes were identified on culture of peripheral blood or bone marrow aspirates. Fourteen serum samples from dogs with other parasite infections were used to analyze cross-reactivity, as follows: *Toxoplasma gondii* ( $n = 5$ ) and *Trypanosoma cruzi* ( $n = 9$ ). These sera were provided by Evaldo Nascimento and Maria Norma Melo (Department of Parasitology, Institute of Biological Sciences, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil). Serum samples from 47 dogs that were living in regions where visceral leishmaniasis (VL) is endemic but that had no clinical signs or suspicion of CVL and that were negative after parasitological and serological tests constituted the control group. Serum samples from healthy dogs and from dogs vaccinated with the Leishmune vaccine ( $n = 18$ ) or the Leishtec vaccine ( $n = 23$ ) were used to analyze their reactivity with LRPs and the SLA.

**ELISA.** Microtiter immunoassay plates (Falcon) were coated with *L. infantum* or *L. chagasi* SLA or with *L. infantum* LRPs (each one at 0.5 µg/well) in coating buffer (pH 9.6) for 18 h at 4°C. A titration curve was prepared to determine the best protein concentration (for LRPs, 4 µg to 0.063 µg; for SLA, 2 µg to 0.031 µg) and the best antibody dilution to be used. Free binding sites were blocked with a PBS-0.05% Tween 20 (PBST) and 3% casein solution for 2 h at 37°C. After three washes with PBST, the plates were incubated with 100 µl of canine serum for 1 h at 37°C. Serum samples were diluted 1:200 in PBST and 0.3% casein. The plates were then washed seven times and incubated with horseradish peroxidase-conjugated 1:10,000 anti-dog immunoglobulin G (IgG) antibody (Sigma). The reaction developed through incubation with H<sub>2</sub>O<sub>2</sub>, ortho-phenylenediamine, and citrate-phosphate buffer (pH 5.0) for 30 min in the dark and was stopped by addition of 2 N H<sub>2</sub>O<sub>2</sub>. The optical densities were read at 492 nm in an ELISA microplate spectrophotometer (Spectra Max Plus; Molecular Devices, Concord, Ontario, Canada).

**Western blotting.** For Western blot analysis, *L. infantum* SLA (15 µg) and LRPs (15 µg) were resuspended in Laemmli's buffer and resolved in a 10% (SLA) or a 10 to 14% gradient (LRP) sodium dodecyl sulfate (SDS)-polyacrylamide gel with preparative combs by using a Bio-Rad (Hercules, CA) protein electrophoresis minigel system. In both cases, the gels were transferred to nitrocellulose membranes (GE Healthcare). The blots were probed individually with the sera (1:200) from dogs infected with *L. infantum* (by SDS-polyacrylamide gel electrophoresis). As the secondary antibody, horseradish peroxidase-conjugated anti-dog IgG (1:2,000) purchased from Nordic Immunological Laboratories (Tilburg, The Netherlands) was used.

**Statistical analysis.** Statistical analysis was performed with GraphPad Prism software (version 4 for Windows). The reactivities of the serum samples collected in Spain against SLA and LRPs were tested for significance by using the Mann-Whitney test. The reactivities of the serum samples collected in Brazil against SLA and LRPs were evaluated by means of the one-way analysis of variance nonparametric test (Kuskal-Wallis by using the Dunn posttest for multiple comparisons of groups). *P* values of <0.05 were considered statistically significant.

#### RESULTS

**Antigenicity of LRPs during canine infection.** In order to analyze the antigenicity of the LRPs during canine infection, sera from 10 dogs clinically infected with *L. infantum* that recognized a large number of protein bands in the SLA extracts (Fig. 1A, SLA panel, lanes 4 to 13) were incubated with a nitrocellulose membrane containing the LRP extracts from this species. All serum samples from dogs with symptomatic CVL recognized the parasite ribosomal purified protein fraction (Fig. 1A, panel LRP, lanes 4 to 13). The sera from healthy dogs were negative, and only a few protein bands were slightly stained in the LRP and SLA preparations (Fig. 1A, lanes 1 to 3). Most of the serum samples from dogs with symptomatic CVL recognized several protein bands in both protein extracts, although the complexity and intensity of the recognition pattern were different between the serum samples from individual dogs. Despite the variability obtained, two immunodominant regions were observed in the LRP Western blot: 45- to 36-kDa and 25- to 22-kDa proteins. The immunoreactivities of sera

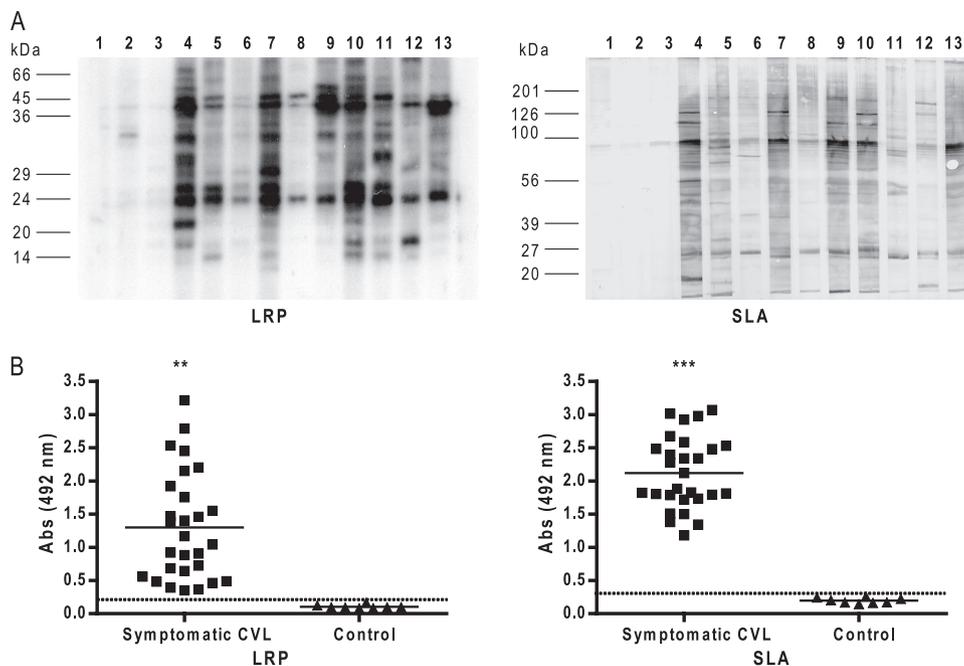


FIG. 1. (A) *Leishmania infantum* LRPs were electrophoresed on a linear 10 to 14% gradient SDS-polyacrylamide gel. *Leishmania infantum* SLA was electrophoresed on a 10% SDS-polyacrylamide gel. Both gels were transferred onto nitrocellulose membranes and incubated with sera from healthy dogs maintained in an experimental kennel under defined conditions (lanes 1 to 3) and sera from dogs naturally infected with *L. infantum* and with symptomatic CVL (lanes 4 to 13). The individual serum samples were used at a 1:200 dilution. A horseradish peroxidase-conjugated anti-dog IgG antibody was used as the secondary reagent. (B) ELISA reactivity of sera collected in Spain from dogs clinically infected with *L. infantum* and control sera with LRPs and SLA. Solid lines, mean values; dotted lines, cutoff value, defined as the mean optical density plus 3 standard deviations of the values obtained with sera from healthy controls. Abs, absorbance; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

from dogs clinically infected with *L. infantum* against LRP and SLA were measured by ELISA. Figure 1B shows the absorbance values for the sera from dogs with symptomatic CVL ( $n = 28$ ) and the controls ( $n = 8$ ) collected in Spain. For both protein preparations, sera from 100% of the dogs with symptomatic CVL showed absorbance values above the cutoff, and the differences in the mean value of the reactivity between the sera from dogs with symptomatic CVL and control sera were statistically significant.

#### Comparison of LRPs and SLA for serodiagnosis of CVL.

Since antibodies reacting against LRP were observed in the sera from dogs with symptomatic CVL due to *L. infantum* infection, we analyzed whether the *L. infantum* LRP extract could be considered a valuable tool for the serodiagnosis of CVL in other regions of the world. For that purpose, we analyzed the reactivity against *L. infantum* LRPs and *L. chagasi* SLA of 130 canine serum samples collected in a region of Brazil that is endemic for CVL due to infection by *Leishmania chagasi*. Those samples included sera from dogs infected with *L. chagasi* with symptomatic infections ( $n = 44$ ), oligosymptomatic infections ( $n = 17$ ), and asymptomatic infections ( $n = 22$ ), as well as 47 healthy dogs living in that area of endemicity. For both protein preparations, the differences between the mean of the absorbance values observed for sera from dogs with symptomatic CVL and healthy control sera were statistically significant ( $P < 0.001$ ) (Fig. 2). The spectrum of the absorbance values from the LRPs and SLA were different, as the reactivity of sera from dogs with CVL against SLA was higher (mean,  $1.58 \pm 0.10$ ) than that of sera from dogs with

CVL against LRP (mean,  $0.64 \pm 0.28$ ). The reactivity of the healthy sera was also higher against SLA (mean,  $0.41 \pm 0.1$ ) than against LRP (mean,  $0.09 \pm 0.04$ ). The cutoff values (defined as the mean reactivity value from healthy sera plus 3 standard deviations) were 0.213 for LRPs and 0.735 for SLA. These cutoff values allowed us to identify positive and negative sera and, consequently, to estimate the performance parameters of the ELISA for the diagnosis of symptomatic CVL (Table 1). When the sera from oligosymptomatic and asymptomatic dogs were included in the analysis, ELISA with LRPs gave the best results in terms of sensitivity: 100% for both groups ( $P < 0.001$ ; the mean of the absorbance values observed for sera from oligosymptomatic and asymptomatic dogs compared with the mean of the absorbance value for sera from healthy dogs) (Fig. 2). On the other hand, the SLA-based ELISA was only 59% sensitive (10/17) with sera from oligosymptomatic dogs ( $P < 0.01$ ; the mean of the absorbance values observed for sera from oligosymptomatic dogs compared with the mean of the absorbance value for sera from healthy dogs). In addition, the SLA-based ELISA detected considerably fewer asymptomatic cases (19%; 4/22) than the LRP-based ELISA, with no statistically significant differences in the mean of the absorbance values for these sera and the mean of the absorbance values for sera from healthy dogs being observed (Fig. 2).

**Cross-reactivity of LRPs and SLA.** Since LRP is composed of evolutive conserved proteins in *Leishmania*, we have analyzed the potential cross-reactions of the LRP extracts with the sera from dogs infected with other unicellular protozoa: *Toxoplasma gondii* ( $n = 5$ ) and *Trypanosoma cruzi* ( $n = 9$ ). In Fig.

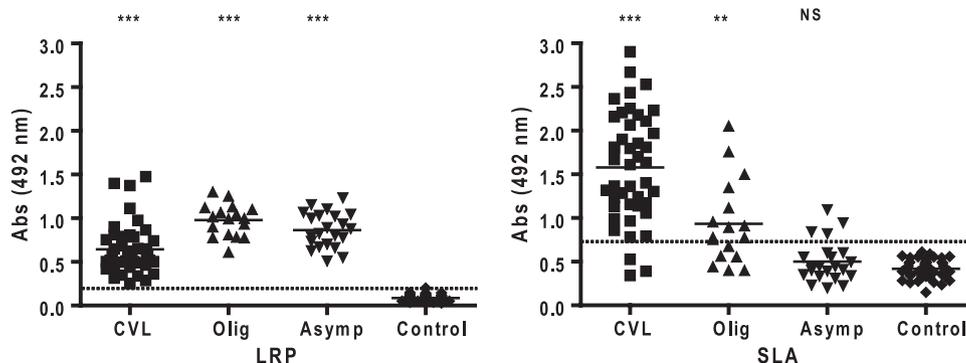


FIG. 2. Comparative evaluation of the diagnostic sensitivities of the *Leishmania infantum* LRPs and the *Leishmania chagasi* SLA. The reactivities of sera collected from dogs with symptomatic, oligosymptomatic, and asymptomatic CVL against LRPs and SLA were analyzed by ELISA. As a control, the reactivities of sera from healthy (uninfected) dogs living in regions where VL is endemic against LRPs and SLA were measured. Solid lines, mean values of reactivity for different groups of sera; dotted lines, cutoff value, defined as the mean optical density plus 3 standard deviations of the values obtained with sera from healthy controls. Abs, absorbance; Olig, oligosymptomatic CVL; Asymp, asymptomatic CVL; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; NS, not significant ( $P \geq 0.05$ ).

3A, the reactivity values of the individual sera for each group against LRPs are shown. None of the sera from the *T. gondii*-infected dogs (mean,  $0.1012 \pm 0.056$ ) or the *T. cruzi*-infected dogs (mean,  $0.101 \pm 0.06$ ) showed reactivity above the cutoff defined by the sera from healthy dogs. When the reactivities of these two groups of sera to LRPs were compared with the responses shown by the sera from the healthy dogs, there were no statistically significant differences (Fig. 3A). On the other hand, the mean reactivities of these sera against SLA ( $0.629 \pm 0.21$  for *T. gondii*-infected dogs and  $0.99 \pm 0.29$  for *T. cruzi*-infected dogs) were higher than those observed against LRPs, with some of the absorbance values being above the cutoff (two of five serum samples for *T. gondii*-infected dogs and eight of nine serum samples for *T. cruzi*-infected dogs). Yet the reactivity against SLA of the sera from the *T. cruzi*-infected dogs was statistically significantly different ( $P < 0.05$ ) compared with the reactivity against SLA of the sera from healthy dogs (Fig. 3A).

Finally, the reactivities of the sera from dogs vaccinated with two *Leishmania* prophylactic vaccines licensed in Brazil, Leish-

mune (39) and Leishtec (19), against the LRP and SLA extracts were assayed. Although there were no statistically significant differences between the sera from Leishmune- or Leishtec-vaccinated dogs and the sera from the healthy control dogs, we found that 22.2% (4/18) of the serum samples from dogs vaccinated with Leishmune showed absorbance values above the cutoff when the LRP extracts were used in the ELISAs (Fig. 3B). When the sera from the Leishmune-vaccinated dogs were analyzed in the SLA-based ELISAs, the same percentage (22.2%; 4/18) showed reactivity values above the cutoff, with 3 of these 4 serum samples being the same samples that showed cross-reactivity with LRPs. None of the 23 serum samples obtained from dogs vaccinated with Leishtec showed reactivity against SLA. Only one of these serum samples showed reactivity against LRPs and had an optical density value close to the cutoff defined by the negative sera from the healthy controls. When the reactivities of these two groups of serum samples to LRPs and SLA were compared, the mean absorbance values were higher for the SLA extracts ( $0.52 \pm 0.24$  for Leishmune and  $0.19 \pm 0.1$  for Leishtec) than for the LRP extracts ( $0.24 \pm 0.13$  for Leishmune and  $0.08 \pm 0.04$  for Leishtec).

TABLE 1. Sensitivities and specificities of ELISAs with LRPs and SLA for serodiagnosis of symptomatic dogs infected with *L. chagasi*

Antigen	Sensitivity <sup>a</sup> (%)	Specificity <sup>b</sup> (%)	PPV <sup>c</sup> (%)	NPV <sup>d</sup> (%)
LRP	100 (0/44)	98.2 (1/47)	97.70	100
SLA	96 (3/44)	100 (0/47)	100	94

<sup>a</sup> Sensitivity was calculated from the equation [number of true-positive samples/(number of true-positive samples + number of false-negative samples)] × 100. The number of samples with false-negative results/total number of samples tested is indicated in parentheses.

<sup>b</sup> Specificity was calculated from the equation [number of true-negative samples/(number of true-negative samples + number of false-positive samples)] × 100. The number of samples with false-positive results/total number of samples tested is indicated in parentheses.

<sup>c</sup> PPV, positive predictive value, which was calculated from the equation [number of true-positive samples/(number of true-positive samples + number of false-positive samples)] × 100.

<sup>d</sup> NPV, negative predictive value, which was calculated from the equation [number of true-negative samples/(number of true-negative samples + number of false-negative samples)] × 100.

DISCUSSION

As occurs with other *Leishmania* intracellular proteins, such as histones, cysteine proteinases, or kinesin (3, 14, 15, 41, 43, 46), we show in this work that during CVL many of the ribosome constituents are antigenic proteins. Since the antigenicity of the parasite ribosomal proteins was also demonstrated in mouse models of cutaneous leishmaniasis (28), it can be deduced that parasite ribosomes interact with the immune system of the vertebrate hosts during natural and experimental *Leishmania* infections. Parasite ribosomal proteins were employed as the source of antigen for ELISA, a precise and sensitive technique for the screening of a large number of samples for the diagnosis of VL (21, 44). A comparative analysis of the LRP extracts and total parasite proteins obtained from promastigote lysates was performed because the use of crude

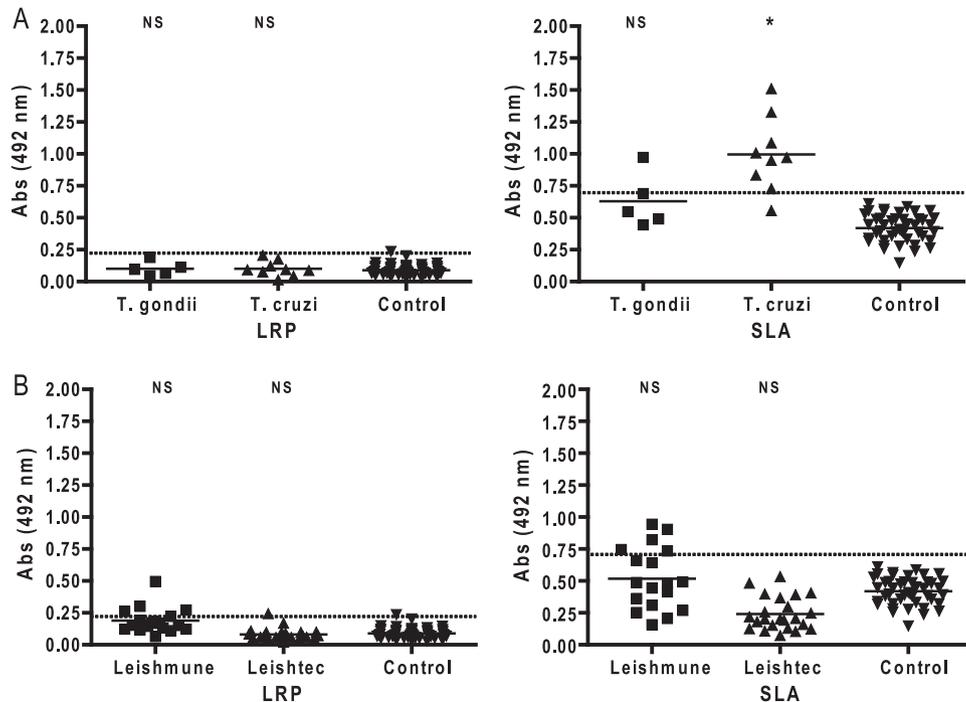


FIG. 3. Comparative evaluation of the diagnostic specificity of *Leishmania infantum* LRPs and *Leishmania chagasi* SLA. (A) ELISA reactivities of sera from dogs infected with *T. gondii* or *T. cruzi* with LRPs and SLA. (B) ELISA reactivities of sera from dogs vaccinated with the Leishmune or the Leishtec vaccine with LRPs and SLA. As a control, the reactivities of sera from healthy (uninfected) dogs living in regions where VL is endemic against LRPs and SLA were measured. Solid lines, mean value of the reactivity; dotted lines, cutoff value, defined as the mean optical density plus 3 standard deviations of the values obtained with sera from healthy controls. Abs, absorbance; \*,  $P < 0.05$ ; NS, not significant ( $P \geq 0.05$ ).

SLA-based ELISAs has usually provided a high degree of sensitivity for the diagnosis of VL (6, 31, 44).

By using sera from dogs with symptomatic CVL in a region of Brazil where VL is endemic, the sensitivity and specificity values obtained with the LRP extracts were similar to those shown by the use of SLA when the sera from healthy dogs obtained in the same region were employed as a control. A slight increase in sensitivity was obtained with LRP compared with that achieved with SLA (100% and 96%, respectively), and only one of the serum samples obtained from healthy dogs showed an absorbance value against LRP above the cutoff defined by the reactivity of the control sera. In addition, 100% of the serum samples from oligosymptomatic dogs assayed had a positive reaction against LRP, but only 59% were found to be positive when SLA was used. Thus, it can be concluded that the diagnostic performance of the LRP-based ELISA was similar to that of the SLA-based ELISA for the diagnosis of symptomatic CVL. In addition, the sensitivity of the LRP-based ELISA was higher than that of the SLA-based ELISA for the diagnosis of oligosymptomatic CVL.

Serodiagnosis is usually carried out to confirm the presence of CVL in dogs presenting with clinical manifestations (4, 26). However, the detection of asymptomatic dogs may be critical in epidemiological studies for controlling the spread of the disease among dogs and also between dogs and humans, since seropositive asymptomatic dogs have been implicated in the transmission of the parasite to the insect vector (18, 33, 36). Since the SLA-based ELISA failed to detect a large percentage of asymptomatic cases of CVL (34, 42), we analyzed the sen-

sitivity of the LRP extracts for the diagnosis of asymptomatic CVL. Whereas the LRP antigen mixture detected all the asymptomatic cases (100%), the assay with the SLA preparation detected only about 19% of the cases. Although the reactivity against LRP needs further confirmation by use of a larger number of serum samples from asymptomatic dogs, the use of LRP-based ELISAs in combination with other proposed parasitological, cellular, and serological tests may be useful for the detection of asymptomatic dogs in areas of endemicity (11, 38). In addition, we are analyzing the correlation between seroreactivity against LRP and the transmission of the parasites to the insect vector, as well as the clinical evolution of LRP-positive asymptomatic dogs.

The specificity of the ELISA with SLA largely depends on the antigen preparation, and some false-positive results were obtained with the sera collected from patients or dogs with diseases that are coendemic with VL or CVL, such as Chagas' disease, malaria, leprosy, and toxoplasmosis (21, 29, 42). For this reason, several parasitic recombinant proteins have been individually employed as the antigen in ELISAs for the development of a more specific diagnostic test (30). Comparative ELISAs generally revealed higher specificities but lower sensitivities when individual recombinant antigens instead of SLA were employed for the diagnosis of human VL (31) or CVL (42). Lower sensitivity values were related to the variability in the heterogeneous humoral response elicited against parasite proteins observed in each patient or infected dog. The use of a combination of nonrelated antigens (25, 42) or the production of polyproteins containing several parasite antigens (7, 49)

could further improve the performance of the ELISAs. Alternatively, purified parasite fractions containing different parasite antigens have been shown to be an alternative for the development of sensitive and specific tests for the diagnosis of CVL (6). Since the sera from *T. cruzi*- or *T. gondii*-infected dogs were not able to recognize LRP extracts, whereas some of these sera showed a high degree of reactivity against SLA, our data indicate that LRP can be employed as a more specific antigen than SLA for the differential diagnosis of CVL.

In order to define the composition of future diagnostic tests based on parasite ribosomal proteins, we are now characterizing the main antigenic components of the parasite ribosomes. The use of these molecules in combination with other parasite recombinant antigens recognized by sera from different percentages of symptomatic and asymptomatic dogs with CVL, such as rK39 (25), A2 (13), and trypanothione peroxidase (47), may promote the development of more specific and sensitive tests for the diagnosis of CVL.

The diagnostic specificity of the test should also be maintained when sera are obtained from vaccinated dogs. Due to the existence of licensed commercial vaccines in Brazil (19, 39), it would be desirable to differentiate infected dogs from vaccinated animals. Our results show that while some of the serum samples from animals vaccinated with Leishmune showed some reactivity against LRPs and SLA, the sera from none of the animals vaccinated with Leishtec reacted against these antigenic preparations. Palatnik-de-Sousa et al. (40) have described the protective effect of the fucose-mannose ligand vaccine, like the Leishmune vaccine, against CVL. As expected with the use of saponin adjuvants, the Leishmune vaccine induces a strong humoral immune response soon after the complete vaccination is provided (9). Therefore, an indistinct IgG-mediated anti-*L. chagasi* immune response is detected in Leishmune-vaccinated dogs, and that response is indistinct from that due to natural infection with *L. chagasi*. This fact can explain the reactivity of some serum samples obtained when *Leishmania* antigens (LRPs and SLA) were used in the ELISAs.

Taken together, the results presented here demonstrate that the LRP extracts may be considered an interesting alternative for use for the diagnosis of CVL by ELISA, mainly in asymptomatic animals, in epidemiological studies in areas of endemicity.

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