THE FUCOSE-MANNOSE LIGAND–ELISA IN THE DIAGNOSIS AND PROGNOSIS OF CANINE VISCERAL LEISHMANIASIS IN BRAZIL

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Abstract. The fucose-mannose ligand (FML)–ELISA assay showed a sensitivity of 100% and a specificity of 100% in diagnosis of canine visceral leishmaniasis (CVL) (kala-azar) in sera from naturally infected dogs from São Gonçalo do Amarante, Rio Grande de Norte, Brazil. The overall prevalence of antibodies to *Leishmania* in the endemic area was 23% (79 of 343). Seroreactivity detected by a *Leishmania chagasi* immunofluorescent (IF) assay was much lower (2.9%) and similar to the percentage of dogs with kala-azar symptoms (2.6%). Twenty-one of 21 asymptomatic, FML-seropositive animals died of kala-azar in a period ranging from 0 to 6 months after diagnosis. The predictive value was 100% for the FML-ELISA, 43% for an *L. mexicana* ELISA, and 24% for the *L. mexicana* and *L. chagasi* IF assays, respectively. In experimentally infected dogs, all assays detected seropositivity between 90 and 120 days after infection. Since the current strategy for control of CVL is based on detection and destruction of infected dogs, the highly predictive, sensitive, and specific FML-ELISA represents a useful tool for field control of the disease.

Zoonotic visceral leishmaniasis (ZVL) is one of the most important emerging diseases. Its etiologic agent, (*Leishmania* chagasi or *L. infantum*) is introduced into the domestic cycle when infected foxes visit houses to scavenge poultry. Peridomestic sand flies acquire the parasite by feeding on the skin of foxes and transmitting it to dogs. The subsequent transmission to humans causes human visceral leishmaniasis (kala-azar), a severe disease that is lethal if not treated soon after the onset of symptoms. About 500,000 human cases of kala-azar are recorded annually worldwide. The current strategy for control of ZVL, as recommended by the World Health Organization, is based on detection and destruction or treatment of infected dogs and vector control.

The control of visceral leishmaniasis in northeastern Brazil involves the detection of infected dogs by serologic analysis using an immunofluorescent (IF) assay and elimination of seropositive dogs. Seropositivity detected by the IF assay usually shows a good correlation with parasitologic evidence of infection. However, due to its low sensitivity, this method underestimates the true prevalence of canine infection. More recently, an ELISA, a dot-ELISA, Western blotting, rapid immunochromatographic tests, and DNA molecular tools have also been compared with conventional serology and proposed as alternative methods. No information is available concerning the predictive value of these tests. However, it is known that in human and canine kala-azar, antibodies to *Leishmania* appear in the circulation soon after infection, leading to an early subclinical period. The early detection of infected dogs in the field could improve the control program since it would identify dogs that are still subclinical but already infective for sand flies.

We have recently tested the diagnostic and predictive value of the fucose-mannose ligand (FML)–ELISA for human kala-azar in Brazil. The FML is a surface glycoprotein complex isolated from *L. donovani* promastigotes that is species specific in *in vitro* interactions with macrophages. This ligand is also the antigen of the FML-ELISA, which has a sensitivity of 100% and a specificity of 96% in the diagnosis of human kala-azar in Brazilian patients infected by *L. chagasi*. Its GP36 glycoprotein is specifically recognized by sera of these patients. Therefore, in serodiagnosis, FML behaves as a complex-specific antigen, since although isolated from *L. donovani*, it is effective in the diagnosis and prognosis of human infection by *L. chagasi*. It also identifies subclinical infections with a potential for evolving into kala-azar in seroreactive asymptomatic subjects, and in asymptomatic blood donors from endemic areas.

Considering the need for new predictive, noninvasive tests for control of zoonotic visceral leishmaniasis, and the diagnostic and prognostic potential shown by the FML-ELISA for kala-azar in humans, we analyzed its potential use in a survey of canine visceral leishmaniasis. For this purpose, we analyzed the sera of dogs that were either naturally or experimentally infected with *Leishmania*, as well as dogs that had been vaccinated with FML, using the FML-ELISA, an IF assay for *L. chagasi* and *L. mexicana* promastigotes, and an ELISA with *L. mexicana* soluble antigen.

MATERIALS AND METHODS

**Dog sera.** A total of 467 samples were analyzed in this study. All samples were collected and conserved in serum: glycerol (1:1 [v:v]) at −20°C. For standardization of the FML-ELISA, sera of dogs from Rio de Janeiro with visceral leishmaniasis (n = 11), tegumentary leishmaniasis (n = 4), toxoplasmosis (n = 5), canine distemper (n = 3) demodesic mange (a dog-specific mange caused by the mite *Demodex canis*) (n = 1), dirofilariasis (n = 1), or infected with *Dipetalonema* (n = 1), *Babesia canis* (n = 2), *Ehrlichia canis* (n = 3), *Leptospira* sp. (n = 3), *Toxocara canis* (n = 1), *Trichuris vulpis* (n = 1), and *Ancylostoma caninum* (n = 7) were obtained from Dr. Vanda Coutinho Pandolpho (Instituto Municipal de Medicina Veterinária Jorge Vaitsman, Rio de Janeiro, Brazil). In addition, samples were collected from 27 dogs with kala-azar in São Gonçalo do Amarante, Brazil, a highly endemic area for human and canine visceral leish-
maliania. Thirteen samples from healthy dogs from Rio de Janeiro were also included.

A series of 343 sera samples were collected from domiciliary dogs during a kala-azar field screening performed in São Gonçalo do Amarante, Rio Grande do Norte by our personnel and by the Brazilian National Foundation of Health. Each dog serum sample was taken from the cephalic vein and analyzed for the presence of antibodies to *Leishmania* by the FML-ELISA. At the same time, blood was collected on filter paper and further analyzed by immunofluorescence using *L. chagasi* promastigote lysate.

Fifty-four samples were obtained from mongrel dogs treated with saline or vaccinated subcutaneously in our laboratory with three doses of FML (1.5 mg) and *Quillaja saponaria* (Quila) saponin (1 mg) (Superfos Biosector, Vedbaek, Denmark). The vaccination was performed on days 0, 20, and 40 and sera were collected on days 0, 7, 31, and 51. One month after the third dose of vaccine, animals were challenged with an intravenous injection of 10^6 *L. donovani* Sudan (LD 1S/MHOM/SD/00-strain 1S) amastigotes obtained from spleens of infected CB hamsters; additional serum samples were collected on days 30, 60, 90, 120, and 180 after infection. All sera were obtained, naturally and experimentally infected dogs were assayed for the presence of antibodies to *Leishmania* by the FML-ELISA, the *L. amazonensis* ELISA, and the *L. chagasi* and *L. amazonensis* IF assays.

**Fucose-mannose ligand-ELISA.** Isolation and chemical characterization of FML obtained from stationary growth phase promastigotes of *L. donovani* Sudan (LD 1S/MHOM/SD/00-strain 1S) were performed as previously described. The FML (125 ng or 2 µg/well) was solubilized in carbonate buffer (pH 9.6) and used to coat flat-bottom, 96-well plates (Haemobag, Ribeirão Preto, Brazil). Antibodies were detected with peroxidase-labeled protein A (Sigma, St. Louis, MO) in a 1:1,000 dilution; the reaction was developed as described elsewhere. The absorbance values at 492 nm were compared using a 1:100 dilution of the serum samples. Results are expressed as mean values of triplicate determinations. The cut-off value of the method for the analysis of canine sera was determined according to the Youden test. Briefly, sera from healthy dogs were assayed and the mean absorbance values + 1, 2 or 3 SD were determined. Using these values as cut-off values, all samples were analyzed and classified into the following groups: a = disease seronegative; b = disease seropositive; c = healthy seronegative; and d = healthy seropositive. The Youden index was then calculated using the formula j = [a/(a + b) + c/(c + d)] − 1. The Youden test circumvents the use of an arbitrary cut-off value by taking into account the possible errors that could lead to negative results for diseased individuals and positive results for healthy individuals. All serologic determinations in this report were done by double-blind tests.

**Indirect IF assay with *L. mexicana***. A modification of the method described by Camargo was used. Antigen for the assay was obtained from log phase promastigotes of *L. mexicana* (MHOM/BR/60/BH6) grown on a liver infusion tryptose (LIT)-based medium. Ten microliters of a 1.2 × 10^6 promastigotes/ml suspension were plated on slides, incubated (in triplicate) with diluted dog sera, and treated with a fluorescein-conjugated goat anti-dog IgG-specific conjugate (Biomanguinhos, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil) at a 1:40 dilution. For the indirect IF assay against *L. chagasi*, drops of dog blood were collected on squares of filter paper, dried, and stored at −4°C until analysis. Antibodies were eluted from the filter paper with phosphate-buffered saline and incubated with *L. chagasi* promastigotes (Biomanguinhos) plated on slides. In both assays, sera were considered positive if there was fluorescence at a 1:40 dilution. This is the usual limit value for diagnosis of leishmaniasis in IF assays in field control in Brazil considered as a reference by the Brazilian National Foundation for Health.

**Leishmania mexicana promastigote ELISA.** This assay was performed according to Voller and others. The antigen was obtained from stationary phase promastigotes of *L. mexicana* (MHOM/BR/60/BH6) grown in a LIT-based medium. Parasite suspensions were washed with 0.9% NaCl, sonicated, and centrifuged at 10,000 rpm for 10 min. Flat-bottom, 96-well plates (Haemobag) were coated with 2 µg of antigen/well, blocked with 2% casein in phosphate-buffered saline, pH 7.6, washed, and incubated with a 1:80 dilution of dog serum (in triplicate). Antibodies were detected using peroxidase-labeled, goat anti-dog IgG-specific conjugate (Sigma) at a 1:2,000 dilution. Reactions were developed with o-phenylenediamine (Sigma) and monitored at 492 nm. Results are expressed as mean values. The cut-off value of the method was the mean value + 2 SD absorbance of 20 sera samples from healthy dogs from a non-endemic area (0.150). Another series of 123 serum samples was analyzed. The mean + SD absorbance values fell beneath the previously determined cut-off value (0.102 ± 0.024).

**Parasitologic evaluation.** Thirty-nine dogs with clinical symptoms of kala-azar (loss of weight, cachexia, alopecia, onycogryphosis, apathy, ulcerative skin lesions) were anesthetized (intravenous thionembutal; Abbott Laboratories, São Paulo, Brazil) and subjected to bone marrow aspiration (sternal bone) and skin biopsy (distal edge of the ear). Parasitologic analysis based on Giemsa-stained smears confirmed the presence of *Leishmania* amastigotes. Animals were killed and the presence of parasites was determined in spleen, liver, ganglia, and bone marrow. All animals included in this investigation were treated following the guidelines for animal experimentation of the U.S. National Institutes of Health, and experiments were done in accordance with the institutional guidelines for the humane use of laboratory animals to reduce the animal suffering to a minimum.

**Statistical analysis.** Means were compared by a standard t-test. Chi-square and Fisher’s exact tests were used in comparing proportions.

**RESULTS**

The FML-ELISA was initially tested on sera of healthy dogs and of dogs with visceral leishmaniasis from Rio de Janeiro, where kala-azar is considered to be a diseases seen occasionally. We chose to work with 2 µg of FML/well, using the mean value + 3 SD (absorbance at 492 nm = 0.435) as the cut-off value (Table 1). This criterion provided the best separation (9.6 SD) between normal and kala-azar sera (Abs at 492 nm = 0.999). After standardization of the FML-ELISA, an additional 235 serum samples from healthy dogs
Results of the fucose-mannose ligand (FML)-ELISA in sera of healthy dogs and dogs with canine visceral leishmaniasis from a non-endemic area, with calculation of the cut-off values and the Youden index:

<table>
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<tr>
<th>FML/well</th>
<th>Interval</th>
<th>Cut-off value</th>
<th>False positive</th>
<th>False negative</th>
<th>j²</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 μg</td>
<td>Mean ± 2 SD</td>
<td>0.180 ± 0.170</td>
<td>0.350</td>
<td>0/11</td>
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</tr>
<tr>
<td></td>
<td>Mean ± 3 SD</td>
<td>0.180 ± 0.255</td>
<td>0.435</td>
<td>0/11</td>
<td>1.00</td>
</tr>
<tr>
<td>125 ng</td>
<td>Mean ± 2 SD</td>
<td>0.162 ± 0.146</td>
<td>0.308</td>
<td>0/13</td>
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<tr>
<td></td>
<td>Mean ± 3 SD</td>
<td>0.162 ± 0.219</td>
<td>0.381</td>
<td>1/11</td>
<td>0.917</td>
</tr>
</tbody>
</table>

* Results are expressed in absorbance values at 492 nm for sera diluted 1:100. The use of the mean ± 2 SD value for normal sera for 125 ng of FML and the use of the mean ± 2 or 3 SD value for 2 μg of FML yielded j² = 1, which corresponds to the absence of false-positive or false-negative results.

† The Youden Index (j²) measures the efficiency of diagnostic tests; values range from 0 to 1 (optimal).

with no evidence of any infections were analyzed; all showed negative results in the L. chagasi IF assay. The mean ± SD values of these sera fell below the cut-off value expected for normal dogs (0.311 ± 0.075). All positive serum samples used for standardization of the assay were from dogs with clinically confirmed visceral leishmaniasis. They had positive L. chagasi IF assay results and parasites were detected in Giemsa-stained smears from bone marrow, spleen, liver, or ganglia after necropsy. Only one of these gave a false-negative result in the FML-ELISA using 125 ng of antigen (Table 1). No false-positive results were detected. The healthy dogs used in this investigation were seronegative throughout the test period, showing no clinical signs of disease and no leishmanial DNA in blood (polymerase chain reaction–specific analysis).

The average absorbancy (0.802) of sera from 38 dogs with clinically, parasitologically, and serologically confirmed kala-azar (Figure 1) was significantly higher (P < 0.001) than that of sera from healthy dogs from a non-endemic area (0.180) (Figure 1). Twenty-four standard error values separated the two means. The reactivity of sera from dogs with other canine pathologies was almost zero, indicating that the FML-ELISA can be used for the diagnosis of canine visceral leishmaniasis with a sensitivity of 100% and a specificity of 100%. Furthermore, in the kala-azar endemic area, the FML-ELISA was a highly discriminating tool, distinguishing the nonreactive dog sera (264 of 343) from the group of individuals reactive with FML (79 of 343) and indicating a seropositivity of 23% (Figure 1). The seroreactivity revealed by the L. chagasi IF assay was much lower: 10 of 343 (2.9%) and very similar to the percentage of dogs that were symptomatic for kala-azar: 9 of 343 (2.6%). These results suggest a higher sensitivity of the FML-ELISA compared with the IF assay in detecting asymptomatic dogs that carry subclinical L. chagasi infections. This hypothesis proved to be correct in a sample of 27 seroreactive dogs from São Gonçalo do Amaranto (Table 2). The sensitivity for detection of canine visceral leishmaniasis was 100% for the FML-ELISA, 55.6% for the L. mexicana ELISA, and 33.3% for both IF assays. Only six dogs showed clinical signs of kala-azar at the time of serum collection, and all six were seropositive in both ELISAs. Both IF tests indicated that four of these six dogs were seropositive. All 27 dogs died of parasitologically, clinically, and serologically confirmed kala-azar within 6 months after the serologic screening. Among the 21 asymptomatic dogs, the predictive value was 100% for the FML-ELISA, 43% for the L. mexicana ELISA, and 24% for the two IF assays (Table 2). Differences between the predictive values of the FML-ELISA, the L. mexicana ELISA, and the two IF assays were highly significant (χ² = 14.117 and χ² = 22.716, P < 0.001, respectively).

The predictive value of the FML-ELISA assay for the development of canine kala-azar was assayed in experimentally infected dogs participating in a vaccination experiment (Table 3). In saline-treated control dogs, all the assays showed similar sensitivities. The L. mexicana IF assay detected an earlier positive reaction in one dog 90 days after infection. This suggests a slightly higher predictive value of the IF assay compared with the FML-ELISA in experimental infections. In FML-QuilA–vaccinated dogs, seropositivity was detected by the FML-ELISA 7 days after the first vac-
cine dose, by the IF assay after the second dose, and by *L. mexicana* ELISA only after the third dose. Thus, the sensitivity was 78% for the FML-ELISA, 70% for the two IF assays, and 22% for the *L. mexicana* ELISA.

**DISCUSSION**

The sensitivity of the IF assay in the field was much lower than that of the FML-ELISA and yielded data similar to the percentage of dogs with kala-azar symptoms. Based on IF assays for seroreactivity, an 8–12% prevalence of kala-azar infection in dogs is usual in Brazil, but such a low prevalence of infection could not explain the high prevalence of seroreactivity. Indeed, while crude promastigote preparations are known to include antigens shared by all *Leishmania* species, the FML antigen was specific for the *Leishmania* species causing visceral leishmaniasis.

In the laboratory, dogs were experimentally infected with *L. donovani Sudan*, the strain used for isolation of FML. Thus, if sensitivity or the predictive value were species-related, one would expect a distinctly better performance with the FML-ELISA. However, all methods showed similar efficacies. In this study, the differences between the natural and experimental infections are not restricted to the *Leishmania* species used for inoculum. While the parasitic stage involved in natural infection is the promastigote inoculated into dog skin after the bite of the sand fly, the infective challenge for vaccinated and control dogs (10⁶ amastigotes by the intravenous route) differs from the usual challenge in nature. For this reason, and although FML antigen has been found on the surface of both the promastigote and amastigote species, intravenous amastigote infection may elicit a different humoral response. In a previous report, we described the slow onset of the antibodies to the parasite after intravenous injection of a heavy amastigote load in both dogs (Genaro O and others, unpublished data) and mice. The parasitologic tests also show lower sensitivity, with positive results only in cases of advanced canine visceral leishmaniasis. An analysis of a cohort of 1,798 naturally infected dogs showed that sensitivity compared to parasitology was 99.2% for the IF assay and 92.1% and 85.8% for
a rapid test antibody for *L. donovani* (TRALd) when performed in the laboratory or in the field, respectively (Genaro O, unpublished data). If the field screening were performed only by analysis with TRALd (Corixa Corp., Seattle, WA), 14.2% of the dogs already parasitologically confirmed to have kala-azar would not be detected or removed from the area, and thus would survive as *Leishmania* reservoirs. Variations in sensitivity and specificity ranging from 90% to 100% are expected for immunochromatographic diagnostic tests, depending on the kind of antigen, nature and size of serum samples, and possible experimental error. We believe that the only factor that needs to be strictly improved when searching for a diagnostic tool for canine kala-azar control programs is the predictive value of the test. Thus, the highly predictive, sensitive, and specific FML-ELISA represents a useful tool for field control of canine visceral leishmaniasis in endemic areas.

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### Table 3

Comparison of the predictive values of the fucose-mannose ligand (FML)-ELISA and the *Leishmania mexicana* immunofluorescent (IF) assay, and ELISA in the diagnosis of kala-azar in FML-vaccinated and control dogs experimentally infected with *L. donovani*

<table>
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<tr>
<th>Time†</th>
<th>FML-ELISA</th>
<th>IF</th>
<th>L. mexicana ELISA</th>
<th>L. mexicana ELISA</th>
<th>Vaccinated dogs‡</th>
<th>FML-ELISA</th>
<th>IF</th>
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<td>Total</td>
<td>6/27 (23%)</td>
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*Control dogs were treated with saline.
† Dogs were vaccinated with three doses of FML (1.500 µg) and QuilA saponin (1 mg).
‡ Animals were vaccinated on days 0, 20, and 40 and infected on day 70. Sera were collected after the first (day 7), second (day 31) and third doses (day 51) of the vaccine and 30, 60, 90, 120, and 180 days after infection.
§ Days after infection with $10^6$ *L. (L.) donovani* amastigotes.

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