

Serological tests fail to discriminate dogs with visceral leishmaniasis that transmit *Leishmania infantum* to the vector *Lutzomyia longipalpis*

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Abstract

Introduction: The control of reservoirs for *Leishmania infantum*-induced zoonotic visceral leishmaniasis requires the identification of dogs posing a population risk. Here, we assessed the performance of several assays to identify *Lutzomyia longipalpis* infectious dogs. **Methods:** We evaluated 99 dogs that were positive for visceral leishmaniasis based on parasite identification. Serological analyses were performed using an enzyme-linked immunosorbent assay, immunofluorescence antibody tests in 1:40 and 1:80 dilutions, rapid dual path platform tests, immunochromatographic assay with a recombinant rK39 antigen, fast agglutination screening tests, and direct agglutination tests. We also performed PCR to analyze peripheral blood and xenodiagnosis. **Results:** Forty-six dogs infected at least one *L. longipalpis* specimen. Although the serological test sensitivities were above 85% for detecting *L. longipalpis* infectious dogs, none showed a satisfactory performance, as both specificity (0.06 to 13%) and the area under the receiver operating characteristic curve (45 to 53%) were low. The PCR results were also weak, with a sensitivity of 30%, specificity of 72%, and an area under the receiver operating characteristic curve of 51%. The infected *L. longipalpis* proportion was higher among asymptomatic dogs than symptomatic dogs. Among the symptomatic dogs, those with ulceration-free skin diseases were more infectious, with an odds ratio of 9.3 (confidence interval of 1.10 - 428.5). The larger the number of insects fed, the greater the detected infectiousness. **Conclusions:** Our study supports the imperative to develop novel technologies for identifying the infectious dogs that transmit *L. infantum* for the benefit of public health.

Keywords: Visceral leishmaniasis. *Leishmania infantum*. *Lutzomyia longipalpis*.

INTRODUCTION

Visceral leishmaniasis (VL), also known as kala-azar, is responsible for approximately 60 thousand deaths yearly worldwide. The Phlebotominae sub-family of sandflies transmit VL¹, with an estimated 200,000 to 400,000 new victims each year². *Lutzomyia longipalpis* sandflies transmit the protozoa *Leishmania infantum* to spread the zoonotic form of VL across Central Asia, the Middle East, the Caucasus and Mediterranean regions, West Africa, and the Americas^{3,4}. The main *L. infantum* reservoirs are wild dogs, marsupials, the domestic dog, and humans⁵⁻⁷.

Zoonotic VL most commonly occurs in Brazil. Moreover, Brazil likely has the only zoonotic VL transmission control

program, which is based on the use of insecticide and culling infected dogs^{8,9}. However, a systematic review of the use of these strategies, especially the canine reservoir control program, has revealed little if any impact on the prevalence of zoonotic VL¹⁰. Notably, these programs have not been successful in limiting the urbanization and territorial expansion of the disease in Brazil after more than 30 years of implementation and despite the efforts of sanitation authorities^{11,12}.

The failure of the canine reservoir control program is in part due to its inability to correctly identify infected dogs^{10,13}. Serosurveys have been performed in areas with a low zoonotic VL prevalence; thus, many non-infected dogs have been culled. Moreover, many infected dogs remain in communities with a high zoonotic VL prevalence. The acknowledgment of such flaws has led some to question the program on both economic and moral grounds^{14,15}. Methods to precisely identify not only the infected animals but also those that represent a greater risk of transmitting *L. infantum* to the vector *L. longipalpis* are needed immediately.

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METHODS

Animals

The present study involved 99 domestic dogs (58 males and 41 females) of different breeds and ages who were parasitologically confirmed for canine visceral leishmaniasis (CVL) and diagnosed at the Animal Sanitary Laboratory at the Federal University of Piauí (UFPI) in Brazil. After a clinical evaluation, the dogs were administered CVL diagnostic exams. None of the dogs were serologically screened, but all were destined to be culled.

Ethical considerations

The study was approved by Ethics Committee on Animal Experimentation at the UFPI, under review number 053/2008.

Clinical evaluation and sample collection

All dogs were submitted to careful clinical evaluation from a veterinary doctor and diagnosed as symptomatic or asymptomatic. Dogs were considered symptomatic if they had at least one clinical sign suggestive of CVL, while asymptomatic dogs appeared completely healthy at the clinical examination. Skin features such as periocular and generalized alopecia, hair loss, seborrhea, and depigmentation in the muzzle were recorded to note the presence of skin disease without ulceration. Afterward, 10 mL of blood was collected from the jugular vein for serology and 40 µL of blood was collected from the ear onto filter paper for deoxyribonucleic acid (DNA) extraction. To obtain samples for parasitological examination, bone marrow and popliteous lymph node aspiration were performed, in addition to scraping healthy skin or lesions in the ear or muzzle. The samples were dyed with Giemsa and observed at 100x magnification to visualize parasite amastigote forms. After confirming the infection, the dogs were humanely sacrificed and an aspiration puncture of the liver and spleen were performed. Culling was performed using an endovenous administration of a combination of ketamine, acepromazine, and diazepam. After 15 minutes of sedation, 20 mL of 10% potassium chloride was administered endovenously.

Bone marrow, popliteous lymph node, liver, and spleen samples were cultured in NNN medium enriched with Schneider medium (Sigma-Aldrich, St Louis, MI, USA) and incubated at 26°C in a low-temperature biological oxygen demand (BOD) refrigerated incubator. Every 5 days, the cultures were examined under a microscope at 40x to control the *Leishmania* promastigote forms, repeating this procedure until the 30th incubation day or until a positive result was obtained. The cultures were considered negative after one month of testing.

Diagnostic tests

As previously reported¹⁶, we performed an enzyme-linked immunosorbent assay (ELISA), immunofluorescence antibody tests in 1:40 and 1:80 dilutions (IFAT40 and IFAT80), rapid dual path platform tests (RT-DPP), immunochromatographic assay with a recombinant rK39 antigen (ICrK39), fast agglutination screening tests (FAST), and direct agglutination tests (DAT), and polymerase chain reaction (PCR).

Infectiousness evaluation

To study infectiousness, a xenodiagnosis was performed with dogs that were sedated using 0.2% acepromazine. *L. longipalpis* females were obtained from a sandfly replenished colony that was initially established in 1995. First generation *L. longipalpis* females were kept at 26°C and 92% humidity, used five days after completely hatching, and deprived of any food source. Sixteen to 120 *L. longipalpis* females per dog were used, with a mean of 55 and median of 60 sandflies per animal. The sandflies were placed inside darkened plastic boxes with an approximate 12cm diameter and 5cm height. The boxes were open on one side, covered with organza tissue, and placed over the internal ear skin for 45 min. The xenodiagnosis was performed after 4:00pm.

Next, the insects were placed inside a BOD incubator with cotton embedded in a 50% sugar solution. After the fifth day of blood repast, the females were dissected on sterile slides for promastigote count under 40x magnification.

Statistical analysis

To verify the accuracy of the tests that identified the dogs that were infectious to the vector, we calculated sensitivity, specificity, and the area under the receiver operating characteristic (ROC) curve for each test and the respective 95% confidence intervals. A Fisher's exact test was performed to evaluate the possible relationship between the results of xenodiagnoses, as a dichotomic variable (positive and negative), and symptomatology, also as a dichotomic variable (symptomatic and asymptomatic). To analyze of the proportion of infected insects in the asymptomatic and symptomatic groups, a chi-square test was performed. The association between clinical signs and vector infectiousness was also evaluated by calculating the odds ratios (using univariate and multivariate analyses) and the Cornfield approximation 95% confidence interval. All analyses were conducted using the statistical package Stata® (College Station, TX, USA).

RESULTS

All tests diagnosing infectiousness performed poorly. Very low areas under the ROC curve were found, i.e., 53% for FAST, 51% for ICrK39 and PCR, 50% for RT-DPP, 48% for IFAT40 and ELISA, 46% for DAT, and 45% for IFAT180. Sensitivity was 98% for RT-DPP, 96% for FAST, 93% for IFAT40, 91% for DAT, 89% for ICrK39 and ELISA, and 67% for PCR. Most dogs with negative xenodiagnosis had reagent tests, which led to very low specificity (the highest value for PCRsp (28%); **Table 1**).

Of the 99 dogs investigated, 88 were symptomatic and 11 were completely asymptomatic. Xenodiagnosis identified 46 dogs that infected at least one *L. longipalpis* specimen. Of the symptomatic dogs, 39 (44%) had a positive xenodiagnosis result, while seven (64%) asymptomatic dogs transmitted to the vector; however, this difference was not statistically significant (p -value = 0.34, Fisher's exact test; **Table 2**).

The proportion of infected sandflies among all 2,646 examined was 5.8%. The proportion was higher among those that fed on an asymptomatic dog (17.6%) compared to those

TABLE 1

Sensitivity, specificity, and the area under the receiver operating characteristic curve for xenodiagnosis of canine visceral leishmaniasis in an endemic region.

Tests	Sensitivity (%) (95% CI) ^a	Specificity (%) (95% CI)	Area under the ROC curve (%) (95% CI)
IFAT 40 ^b	93 (86 – 100.0)	2 (0 – 6.0)	47 (44 - 52.0)
IFAT 80 ^c	85 (74 – 96.0)	0.06 (0 – 12.0)	45 (39 - 51.0)
DAT ^d	91 (83 – 100.0)	0 (0 – 0.0)	46 (42 - 50.0)
FAST ^e	96 (90 – 100.0)	9 (1 – 18.0)	53 (48 - 58.0)
ICrK39 ^f	89 (80 – 98.0)	13 (4 – 23.0)	51 (45 - 58.0)
RT DPP® ^g	98 (93 – 100.0)	2 (0 – 6.0)	50 (47 – 53.0)
ELISA ^h	89 (80 – 98.0)	8 (0 – 15.0)	48 (43 – 54.0)
PCRsp ⁱ	30 (17 – 44.0)	72 (17 – 59.0)	51 (42 - 60.0)

^a95% confidence interval. ^bindirect immunofluorescence reaction in a 1:40 dilution. ^cindirect immunofluorescence reaction in a 1:80 dilution. ^ddirect agglutination tests. ^efast agglutination screening tests. ^fimmunochromatographic test with rK39 antigen. ^grapid dual path platform tests. ^henzyme-linked immunosorbent assay. ⁱperipheral blood polymerase chain reaction.

TABLE 2

Number and proportion of infected dogs with a positive xenodiagnosis result and the number and proportion of infected insects among all the insects examined in the asymptomatic and symptomatic groups.

Symptomatology	Infected dogs	Number and proportion of animals with positive xenodiagnosis (%) [*]	Number of examined insects	Number and proportion of infected insects (%) ^{**}
Asymptomatic	11	7 (63.6)	267	47 (17.6)
Symptomatic	88	39 (44.3)	2,379	106 (4.5)
All	99	46 (46.5)	2,646	153 (5.8)

^{*}*p*-value = 0.34, Fisher's exact test. ^{**}*p*-value = 0.00, chi-square test.

that fed on a symptomatic dog (4.5%; *p*-value = 0.00, chi-square test; **Table 2**).

Of all the annotated clinical signs, only skin disease without ulceration was significantly associated with the likelihood of infectiousness in dogs (i.e., infecting at least one sandfly), with an odds ratio of 9.3 (1.10 - 428.5 confidence interval; **Table 3**). Additionally, in the multivariate analysis, skin disease without ulceration was significantly associated with infectiousness to the vector (**Table 3**).

Mortality until insect dissection was 49.8% and ranged from 4 to 100%, with a mean of 26.7 and a median of 25 dissected insects per dog that ranged from 3 to 81. The greater the number of insects used or dissected insects for xenodiagnoses, the greater the chance of at least one being infected; **Table 4**).

DISCUSSION

For almost 30 years, serological tests for CVL diagnosis have been used extensively for disease control programs in Brazil years¹⁷. These tests are antibody-based and do not effectively distinguish infected dogs from healthy dogs with an immunological memory of a previous infection, as we have

previously shown^{16,18}. Therefore, a large number of seropositive, not infective dogs have been culled in Brazil. This policy has high economical and moral costs, now facing increasing community resistance due to its inefficiency^{10,19,20}. In addition, dogs correctly found infected may not be actually infective to sandflies and place no risk for the population.

Studies inconsistently report the proportion of dogs with VL that are infectious to the vector *L. longipalpis*. In Brazil, Deane and Deane²¹ observed that 75% of 16 dogs transmitted *L. infantum* to 24.8% of 238 *L. longipalpis* specimens, with a mean of 14.9 insects per dog. Sherlock²² found that 65% of 20 dogs infected 29% of 368 *L. longipalpis* specimens, with a mean of 18.4 insects per dog. In Europe, Rioux et al.²³ reported having infected all 17 *Phlebotomus ariasi* that fed on a sick dog. Molina et al.²⁴ in Spain found that 13/16 dogs were infectious for *Phlebotomus perniciosus*, with only three dogs infecting over 70% of the sandflies evaluated. Differences in the infectiousness rate could have been due to a number of parasites acquired after an infectious repast (which depends on the infectiousness level of the dog), the type of maintenance diet sugars in the laboratory after repast²⁵, temperature and humidity conditions, the sandfly species, and mainly, the time elapsed until dissection²⁶. As a

TABLE 3
Symptoms associated with dog infectiousness to *Lutzomyia longipalpis*.

Clinical signs	Odds ratio (univariate analysis)	95% confidence interval	Odds ratio (multivariate analysis)	95% confidence interval
Skin disease with no ulceration	9.3	1.10 - 428.5	39.9	1.50 - 1080.9
Snout lesion	0.4	0.15 - 1.20	0.3	0.07 - 1.09
Apathy	6.3	0.70 - 305.5	2.1	0.10 - 44.3
Enlarged popliteal lymph node	1.2	0.5 0 - 2.80	0.9	0.31 - 2.73
Lymphadenomegaly	0.7	0.9 0 - 3.70	0.1	0.01 - 1.50
Snout depigmentation	0.5	0.13 - 1.90	0.6	0.15 - 2.90
Lip lesion	0.7	0.06 - 7.00	2.7	0.34 - 22.3
Shedding	0.5	0.04 - 4.20	0.6	0.06 - 5.39
Onicogriphosis	1.0	0.25 - 3.80	0.4	0.06 - 2.39
Ear lesion	1.1	0.08 - 16.5	3.2	0.24 - 42.2
Thinnes	1.3	0.40 - 4.20	0.7	0.17 - 3.10
Cachexia	3.7	0.30 - 197.7	5.0	0.06 - 461.8
Body lesions	1.2	0.30 - 4.80	0.9	0.19 - 4.43
Conjunctivitis	0.4	0.04 - 2.80	0.1	0.00 - 1.01
Pale mucous tissues	2.4	0.12 - 142.0	20.9	0.27 - 1617.1
Cornea opacity	1.1	0.08 - 16.5	0.01	0.00 - 1.85
Epistaxis	2.4	0.12 - 142.0	5.7	0.17 - 196.5
Periocular alopecia	2.0	0.35 - 14.0	11.7	0.83 - 165.3
Blepharitis	1.1	0.01 - 93.0	2.3	0.07 - 68.7
Asymptomatic	2.1	0.50 - 9.10	1.3	0.25 - 7.11

TABLE 4

Effect of increasing the number of *Lutzomyia longipalpis* females examined or used in xenodiagnosis under the presence of at least one infected female (positive xenodiagnosis).

Number of used <i>Lutzomyia</i> <i>longipalpis</i>	Number of positive xenodiagnosis/no. of xenodiagnosis dogs (%)	Number of examined <i>Lutzomyia longipalpis</i>	Number of positive xenodiagnosis/no. of examined dogs (%)
≥ 10	46/99 (46.5)	≥ 1	46/99 (46.5)
≥ 20	45/98 (45.9)	≥ 5	44/96 (45.8)
≥ 30	45/95 (47.9)	≥ 10	40/88 (45.5)
≥ 40	40/82 (48.8)	≥ 20	28/61 (45.9)
≥ 50	35/70 (50.0)	≥ 30	18/37 (48.7)
≥ 60	30/62 (48.4)	≥ 40	9/18 (50.0)
≥ 70	5/6 (83.3)	≥ 50	5/7 (71.4)
≥ 80	3/3 (100.0)	≥ 60	2/2 (100.0)

result, insects with a high infectiousness load may have died before being examined, while insects that acquired milder infections could have survived until the dissection day. However, this possibility is unlikely due to the short period (5 to 6 days) between feeding on the dogs and insect examination, as there was not enough time for the sandflies to develop high infection loads.

The present study was most concerned with whether every infected dog is actually infectious. From this perspective, infectiousness would depend on a high infectiousness load above a certain threshold in the insects used for xenodiagnosis, as our study suggested when a high number of insects were examined. Our study was not conclusive because only a small number of xenodiagnostic examinations were performed using a high number of insects. Nonetheless, under natural conditions, animals may be exposed to a high number of *L. longipalpis* bites when they are infectious; therefore, all infected dogs may indeed be infectious to some degree and for some period time, depending on an exposure to a critical amount of sandflies. Notably, xenodiagnostic studies^{21,27} have shown that some more infectious animals can be discriminated, while less infectious animals cannot. However, transmission is an exponential function depending on vector daily mortality²⁸, which is high, from 15 to 20%²⁹; thus, poorly infectious dogs may not contribute to transmission. These studies suggest that diagnostic tests can distinguish more infectious dogs, which are the actual sources of infection under natural conditions, from less infectious or non-infectious dogs. An elegant study in Ethiopia showed that in fact only a few individuals contribute to transmission³⁰. A control program that discriminates infectiousness would identify such animals.

Our study showed that no test could be used to discriminate infectious dogs from those that were previously infected. These serological tests identify antibodies but not parasites, although antibody titers may be related to infectiousness³¹. Our results show that only ICrK39 and FAST could indicate infectiousness. RT-DPP and ELISA, which are used in the Brazilian VL control program³², were unable to discriminate the most infectious dogs. Negative DAT, FAST, and ICrK39 results can be used to distinguish some portion of the most infectious dogs, but a significant proportion of these animals will remain in the community. Consequently, although some diagnostic tests can reveal the infectiousness status of the dog population, they are unable to distinguish the infected dogs that actually represent a population risk.

Serological tests cannot be used to indicate infectiousness because they only measure antibodies. PCR could be more useful in this regard. However, PCR yielded some of the worse results. We analyzed only a very small volume of blood, equivalent to what 20 sandflies ingest³³; thus, our approach may explain our results. As a result, with a low circulating parasite density³⁴, PCR and xenodiagnosis would discriminate different animals, as skin but not blood appears to be the main source of *L. infantum* transmission to vectors³⁵.

In our study, asymptomatic dogs had a greater probability of infecting *L. longipalpis* than symptomatic dogs, making the

correct identification of dogs representing a population risk even more difficult, although the literature is conflicting^{31,36-40}. In contrast, dogs with dermatitis were much more infectious, and this feature was the only clinical sign that had any discriminatory value. Although dermatitis is a low-frequency clinical sign, this information could be useful given the difficulty of identifying infectiousness-related signs. Quantitative buffy coat⁴¹ and molecular tests⁴⁰ are potentially useful approaches to identify infectious dogs, as they can directly identify circulating parasites. However, given that VL transmission from dogs likely occurs via the skin^{39,40,42}, new approaches to identify infectiousness should focus on skin rather than blood samples.

Vertebrate host infectiousness data vary widely, indicating that this elusive information is unstable, uncertain, complex, and dependent on multivariate factors. In addition, directly measuring infectiousness based on xenodiagnosis is complex, limiting its use on a large scale and for long periods of time. Therefore, to better correctly identify infectious individuals, as we attempted, further investigation is necessary. Such experiments should focus on identifying and controlling the variables that determine the complexity of infectiousness and the development of simple technological alternatives to xenodiagnosis. Until then, the present investigation and any similar future studies will only substantiate the fact that the available methods are inefficient.

Conflict of interest

The authors declare that there is no conflict of interest.

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