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Detection and molecular characterization of *Leishmania* in dogs from northeastern Brazil

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ABSTRACT

OBJECTIVE

The aim of the present study was to determine the current seroprevalence of canine leishmaniasis (CanL) among domiciled dogs, factors associated with seropositivity, circulating *Leishmania* species and spatial analysis, in six municipalities in the Sertão and São Francisco mesoregions, which are located in the state of Pernambuco, Brazil.

METHODS

Blood samples from 330 dogs were analyzed using serological and molecular assays: dual path platform (DPP®); enzyme-linked immunosorbent assay (ELISA), indirect fluorescent antibody test (IFAT) and polymerase chain reaction (PCR). The nucleotide sequences of four gene markers (kDNA, cathepsin L-like, SSU-rDNA and gGAPDH) were explored to perform a phylogenetic analysis.

RESULTS

The overall seroprevalence was 13% (43/330) in dogs that were simultaneously positive according to DPP® ELISA and IFAT, consisting of 13.9% (23/165) in Sertão and 12.1% (20/165) in São Francisco. The factors associated with high prevalence of *Leishmania infantum* antibodies in dogs comprised living in the municipality of Petrolina (P = 0.045) and presenting ocular lesions (P = 0.049) (P ≤ 0.05). Significant clusters of positive dogs were found in rural areas. The positivity values obtained through PCR based on the genes kDNA and cathepsin L-like were 6.7% (22/330) and 2.4% (8/330), respectively. In the phylogenetic analysis, it was observed that all the isolates obtained showed 100% similarity to *L. infantum*.

CONCLUSIONS

For the first time, *L. infantum* was confirmed as the etiological agent of CanL in this region. Thus, assessment of the genetic structure of populations of *Leishmania* spp. is important for understanding the patterns for transmission of CanL.

DESCRIPTORS

Canine leishmaniasis, Diagnosis, Geoprocessing, Phylogeny.

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INTRODUCTION

Visceral leishmaniasis (VL) is caused by the protozoan parasite *Leishmania infantum* (synonymous with *Leishmania chagasi*) in America and Europe, and is transmitted to humans through the bites of infected female phlebotomine sand flies[1]. This disease is considered to be a serious public health problem because of its rapid spread and global distribution. It has been estimated that approximately 200,000 to 400,000 new cases occur every year worldwide[2]. In South America, 97% of the reported cases are located in Brazil[3], where from 2007 to 2018 more than 40,000 cases were notified[4].

Over the last 30 years, northeastern Brazil has been considered the main endemic region of the country. The number of human cases has been increasing, since the improvements achieved in this region have been insufficient to mitigate the risk of the disease among the most affected populations[5]. Between 2010 and 2018, 21,621 human cases of VL were confirmed in this region, among which 5.1% occurred in the state of Pernambuco, with an average number of 107 cases per year and a mean annual incidence rate of 1.17 cases per 100,000 inhabitants[4].

VL has historically been endemic in Pernambuco, with cases widely distributed across the state and recorded in all mesoregions (i.e. the metropolitan region of Recife, Atlantic Forest region, Agreste, Sertão and SãoFrancisco)[6-7], in spite of their different social, economic and geographical characteristics8. The epidemiological data obtained over the last twelve years (2007-2018) through the National Information System for Notifiable Disease (SINAN) show that there were increases in the numbers of VL cases in the São Francisco and Sertão mesoregions of Pernambuco. Some municipalities in these regions (i.e. Lagoa Grande, Ouricuri, Petrolina, Santa Maria da Boa Vista, Salgueiro and Serra Talhada) have been highlighted as priorities for controlling this disease. These areas are considered to present moderate to severe risk of transmission[9].

Domestic dogs are considered to be the main reservoir hosts for *L. infantum* in urban areas, which causes canine leishmaniasis (CanL)[10]. Canine infection usually precedes human infection, given the bond between dogs and humans and the high capacity of infected dogs to transmit the parasite to the vector[10,11].

Until 2011, the strategies proposed through the control program guidelines created by the Brazilian Ministry of Health (BMH) were based on identification of infected dogs from the titer of positivity for serum anti-*Leishmania* IgG antibodies through an indirect fluorescent antibody test (IFAT) for screening and ELISA (enzyme-linked immunosorbent assay) as the confirmatory test[12]. Despite the high specificity of IFAT, in 2012 it was replaced by a rapid immunochromatographic test (TR DPP®; dual-path platform) that was fast and easily performed[13]. However, some studies have reported that this test shows low sensitivity, especially among dogs that are clinically normal, and ross-reactions with other *Leishmania* spp. (e.g. *Leishmania braziliensis*) and *Trypanosoma* spp.[13].

Indeed, the canine population may be infected with species other than *Leishmania infantum*[14], and cross-reactions with other strains of the agent might be overestimating the prevalence of *Leishmania* sp. in many regions of Brazil. Hence, in order to classify *Leishmania* parasites at species level, through phylogenetic analysis, it is essential to control and prevent outbreaks due to other forms of leishmaniasis[15-16].

In epidemiological studies, important techniques for finding and characterizing the main areas of high incidence of VL have been used[17]. Spatial analysis is one of these: this technique is widely used and can also indicate the distribution of vector populations and identify the main areas that show spatial dependency between CanL and human VL, thus contributing towards disease control[18].

In this context, the objective of the present study was to determine the level of *Leishmania* infection among domiciled dogs and perform spatial analysis on canine VL in six municipalities in the São Francisco and Sertão mesoregions. In addition, the aim was to evaluate the factors that may be associated with canine seropositivity in these areas and to identify, for the first time, the *Leishmania* species that infect the canine population of these regions.

METHODS

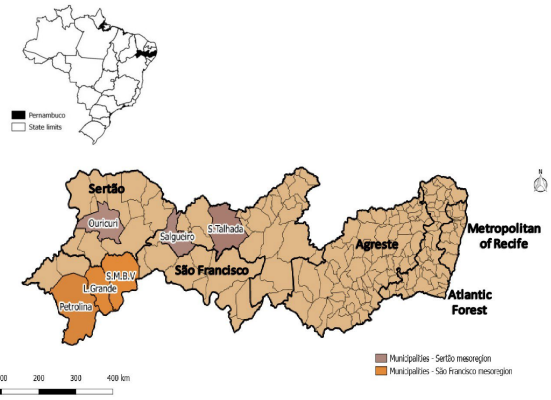
Ethical considerations

This study was approved by the Research Ethics Committee of the Federal University of Vale do São Francisco, under protocol no. 0010/120215.

Study area

The state of Pernambuco is divided into five mesoregions (metropolitan region of Recife, Atlantic Forest, Agreste, Sertão and São Francisco) (Figure 1). This study was conducted in three municipalities located in the Sertão mesoregion (Salgueiro, Serra Talhada and Ouricuri) and three in the São Francisco mesoregion (Petrolina, Lagoa Grande and Santa Maria da Boa Vista) (Figure 1). This area is situated in the semi-arid region, at an altitude of 370-400 meters, within the Caatinga biome and also presenting stretches of hyperxerophilic deciduous forest[19].

Figure 1. Mesoregions of state of Pernambuco and localization of the municipalities from the study in São Francisco mesoregion (Petrolina, Lagoa Grande and Santa Maria da Boa Vista) and Sertão mesoregion (Ouricuri, Salgueiro and Serra Talhada), located in northeast of Brazil



Sampling procedures and clinical examination

This study was conducted from August 2016 to January 2017. The sample size was determined using the Epi Info software, version 7.1, with a 95% confidence interval, 2% margin of error and estimated prevalence of 11.2%[20]. Considering an infinite population, the calculated sample size required for our study was approximately 330 dogs, comprising 55 animals per municipality. Sampling points were selected randomly, and blood samples were collected only from dogs aged six months or older that had not been vaccinated against leishmaniasis.

The blood samples were obtained from each animal by means of cephalic or jugular venipuncture, and were placed in EDTA tubes. After centrifugation, the whole blood samples were separated from pellet of blood and the supernatant of plasma, and then were stored at −20°C until the time of serological analysis.

Each dog was examined physically by a veterinarian to identify the main clinical signs of CanL. These signs could include emaciation, epistaxis, icterus, pale mucous membranes (ocular

and oral), apathy, lymphadenomegaly (evaluation of the main popliteal, prescapular and submandibular lymph nodes),hepatosplenomegaly (by means of abdominal palpation), ocular lesions, cutaneous alterations (alopecia, dermatitis, ulcers or lesions on the ears, face and limbs), cachexia, onychogryphosis and conjunctivitis[21].

Serological diagnosis

The plasma samples thus obtained were tested using three different serological tests to detect the presence of anti-*Leishmania* IgG antibodies. Initially, the serum samples were qualitatively screened using DPP®, produced by Fiocruz (Bio-Manguinhos Unit, Rio de Janeiro, Brazil), and then they were tested using a commercial ELISA kit (IMUNODOT® *Leishmania*; Imunodot, Jaboticabal, São Paulo, Brazil). All procedures for the DPP® and ELISA assays were performed as instructed by the manufacturer. Lastly, the serum samples were tested by means of IFAT, as previously described by Oliveira et al[22]. *Leishmania infantum* promastigotes (CBT 153 strain), which had originally been isolated from the popliteal lymph nodes of a naturally infected dog and had then been deposited in the Brazilian Trypanosomatid Collection (Coleção Brasileira de Tripanossomatídeos), were used as antigens[15]. Positive and negative canine serum samples were used as controls. For the prevalence calculations, only dogs that were seropositive in all three tests (DPP®, IFAT and ELISA) were considered.

Factors associated with seropositivity

For each dog sampled, a comprehensive questionnaire was administered to the owner, in order to obtain information about independent variables that were possibly associated with seroreactivity for *L. infantum* (dependent variables); to describe the general and individual characteristics of the canine population and their environment; and to determine the factors associated with CanL in the six municipalities of this study.

The following variables were considered: breed (mongrel or purebred); sex (male or female); age (up to 12, 13 to 84 or > 84 months); dog pelage (light or dark); size (small, medium or large); presence of green area/trees (yes or no); urban area (yes or no); rural area (yes or no); street access (yes or no); contact with other animals (yes or no); veterinary care (yes or no); contact with forest/Caatinga (yes or no); interaction with wildlife (yes or no); presence of a chicken coop (yes or no); and human cases of leishmaniasis (in the household and/or neighborhood).

Spatial analysis

The geographical coordinates of the sampled households were determined by means of the global positioning system (GPS). The data were combined using QGIS® v. 2.18 software, to create spatial maps.

Based on the average flight radius of the VL vector, of 250 m[23], buffer zones of 250 m were simulated around seropositive dogs in order to identify areas of greater risk for the presence of infected vectors, and therefore for the disease in dogs and humans.

Statistical analysis

To conduct the analysis on factors associated with seroprevalence, univariable analysis was initially performed, in which each independent variable underwent an association analysis in relation to the dependent variable (positivity in serological tests). The univariable analysis was performed using the chi-square test or Fisher's exact test[24]. The variables that presented *P*- values ≤ 0.2 in these tests were selected for multivariable analysis using a logistic regression model[25]. Collinearity between independent variables was verified through a correlation analysis; for variables with strong collinearity (correlation coefficient > 0.9), one of the two variables was excluded from the multiple analyses, according to their relative biological plausibility[26]. The significance level in the multiple analysis was 5%. The tests were performed using the SPSS 20.0 statistical analysis software for Windows.

Conventional Polymerase chain reaction (cPCR) and DNA sequencing

The blood samples were subjected to DNA extraction using the Wizard® genomic DNA purification kit (Promega, Madison, USA), following the manufacturer's instructions. DNA from *Leishmania* isolates was extracted from culture pellets using the phenol-chloroform method[15].

To screen for positive samples, the primers RV1 and RV2 were used to amplify a fragment of 145 base pairs (bp) of *Leishmania* spp. kDNA[27], using reagents and thermal conditions that have been described elsewhere[28]. To amplify a fragment of 223 bp that corresponded to cathepsin L-like with a specific diagnosis of *L. infantum*, the primers CatLeishF and CatLeishR were used, using conditions that have been described elsewhere[29].

The conventional PCR (cPCR) assays were run in a Biocycler® thermal cycler, and the amplification products were analyzed by means of electrophoresis on 1.5% agarose gel. The bands were stained with ethidium bromide and viewed under ultraviolet transillumination[28].

Phylogenetic analysis

The sequences obtained through cPCR were aligned with sequences retrieved from GenBank, using Clustal X[30], and were adjusted manually using GeneDoc v.2.6.01[31]. The phylogenetic tree was used with maximum parsimony and Bayesian analysis. Maximum parsimony was implemented in PAUP version 4.0b10[32] with 500 bootstrap replicates. Bayesian analysis was performed using MrBayes v.3.1.2[33] with 1,000,000 replicates. The first 25% of the trees were taken to be burn-in, and the remaining trees were used to calculate Bayesian posterior probabilities.

RESULTS

The serological tests to detect the presence of antibodies to *L. infantum* 266 revealed positivity levels of 27.9% (46/165), 37.6% (62/165) and 23.6% (39/165) in the Sertão mesoregion; and 32.7% (54/165), 36.4% (60/165) and 23.6% (39/165) in the São Francisco mesoregion; by means of DPP, ELISA and IFAT, respectively. In comparing the results between the three diagnostic tests used, different seroprevalence rates were observed: DPP, 30.3% (100/330);ELISA, 37.0% (122/330); and IFAT, 23.6% (78/330).

The overall seroprevalence was 13% (43/330), comprising 13.9% (23/165) in the Sertão mesoregion and 12.1% (20/165) in the São Francisco mesoregion. The highest positivity rates were observed in the municipalities of Petrolina (25.4%), Serra Talhada (21.8%) and Ouricuri (12.7%) (Table 1).

Regarding possible associations between independent variables ($P \leq 0.20$) and seropositivity among the dogs (Table 2), the variable of living in the municipality of Petrolina was selected for multivariate analysis. This showed that there was a statistically significant difference ($P \leq 0.05$) (Table 3).

Table 1. Seroprevalence of canine leishmaniasis (CanL) of the municipalities from São Francisco and Sertão mesoregions. Only dogs with positive results in all three tests (DPP®, IFAT and ELISA) were considered seropositive.

Municipality	Dogs		Serology		Positivity rate
	(n)	(%)	Positive	Negative	(%)
Petrolina	55	16,7	14	41	25,4
L.Grande	55	16,7	5	50	9,1
S.M.B.V	55	16,7	1	54	1,8
Salgueiro	55	16,7	4	51	7,2
Serra Talhada	55	16,7	12	43	21,8
Ouricuri	55	16,7	7	48	12,7
Total	330	100	43	287	13,0

* L. = (Lagoa), S.M.B.V = Santa Maria da Boa Vista, (n) = number of dogs.

Approximately 48.5% (160/330) of the dogs showed clinical signs compatible with CanL and 62.8% (27/43) of the seropositive animals presented clinical signs. These included the following: ulcers (P = 0.019), onychogryphosis (P = 0.084), lymphadenomegaly in popliteal lymph nodes (P = 0.017), cachexia (P = 0.145), apathy (P = 0.119), hepatosplenomegaly (P = 0.000) and ocular lesions (P = 0.000). All of these were significantly associated with the presence of antibodies against *L. infantum* ($P \leq 0.20$) (Table 2). Among these independent variables, ocular lesions showed a significant difference in the multivariate analysis (P = 0.049) and were found to be significantly associated with seropositivity for *L. infantum* (Table 3).

Table 2. Univariate analysis with the distribution of the variables associated with *Leishmania* spp. in dogs from São Francisco region and Sertão mesoregions, northeast, Brazil.

Variable	Category	N° total of animals	Positives (%)	OR[a]	IC 95%[b]	*P*-value*
Lymp node Popliteal	No	235	24 (10.2)	2.19	1.14 -	0.017
	Yes	95	19 (20)		4.23	
Cachexia	No	314	39 (12.4)	2.35	0.72 -	0.145
	Yes	16	4 (25)		7.65	
Apathy	No	328	42 (12,8)	6.81	0.41 -	0.119
	Yes	2	1 (50)		110.94	
Hepatosplenomegaly	No	328	41 (12.5)	1.04	0.98 -	0.000
	Yes	2	2 (100)		1.12	
Ulcers	No	319	39 (12.2)	4.10	1.14 -	0.019
	Yes	11	4 (36.4)		14.65	
Onychogryphosis	No	288	34 (11.8)	2.03	0.89 -	0.084
	Yes	42	9 (21.4)		4.62	
Ocular lesions	No	326	40 (12.3)	21.45	2.17 -	0.000
	Yes	4	3 (75)		211.23	
Municipalities	Lagoa Grande	55	5 (9.1)	-	-	0.001
	Ouricuri	55	7 (12.7)			
	Petrolina	55	14 (25.4)			
	Salgueiro	55	4 (7.3)			
	S. M. Boa Vista	55	1 (1.8)			
	Serra Talhada	55	12 (21.8)			

S.M. Boa Vista: Santa Maria da Boa Vista
[a]Odds Ratio; [b]confidence interval 95%; *$P \leq 0.2$

Table 3. Multivariable analysis for associated factors with *Leishmania* infection in the municipalities of the study.

Variable category	Coefficient estimates	Standard error	Wald Chi-square	Prevalence ratio	95% confidence interval	*P*-value*
Municipality of Petrolina	1.313	0.656	4.003	3.718	1.027-13.458	0.045*
Ocular lesions	2.577	1.310	3.867	0.076	0.006-0.991	0.049*

*$P \leq 0.05$

The positive and negative dogs in each household were widely distributed in the six municipalities of this study (Figure 2). However, through buffer zones analysis, significant agglomerations of positive dogs were observed in the rural areas of each municipality (Figure 3).

Figure 2. Seropositive dogs for the presence of anti-*Leishmania* antibodies in the municipalities of the study.

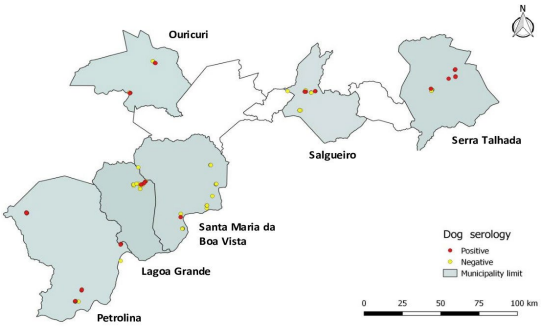
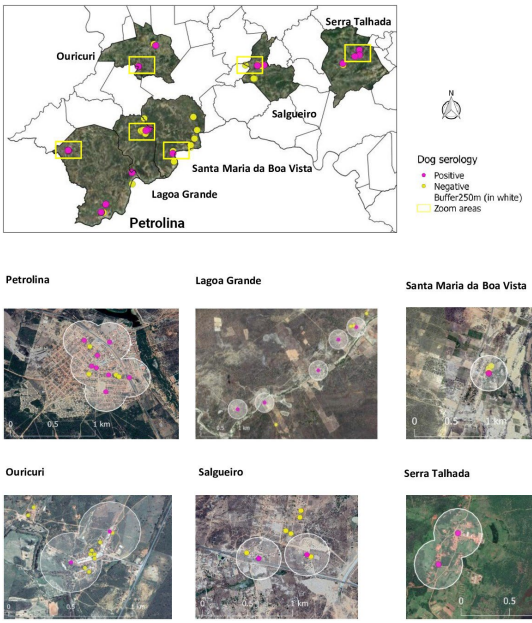


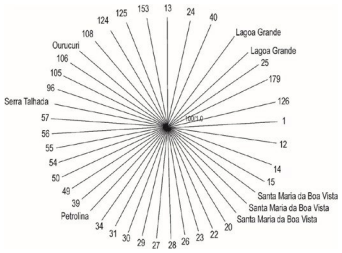
Figure 3. Location of the Buffer with a 250 m radius around seropositive dogs for *Leishmania* spp. in the municipalities of study. Clusters of positive dogs were observed in rural area of the municipalities



cPCR products of the expected size were amplified from 22 dog samples (6.7%; n = 330) using genus-specific primers targeting *Leishmania* spp. by using the genetic marker kDNA. In addition, eight dog samples (2.4%; n = 330) were revealed through PCR, with the presence of *L. infantum* detected using the cathepsin L-like gene.

Eight sequences [Lagoa Grande (2), Ouricuri (1), Petrolina (1), Santa Maria da Boa Vista (3) and Serra Talhada (1)] were obtained through nucleotide sequencing from the fragments amplified (Figure 4). In the phylogenetic analysis, it was observed that all the sequences of cathepsin L-*like* from *L. infantum* were identical to each other (Figure 4). There was no segregation of the isolates according to the geographical origin of the sequences obtained in each region sampled.

Figure 4. Dendogram based on Cathepsin L-like sequences of the 36 isolates of L. infantum and 8 sequences obtained in study and used in the maximum parsimony and bayesian inference analysis. Support values are shown in the branches (bootstrap/ a posteriori probability).



DISCUSSION

Dogs play an important role as *L. infantum* domestic reservoirs and are able to present intense cutaneous parasitism, thereby contributing towards maintaining the VL transmission cycle in humans. Thus, detection of infection in dogs is the main means for ascertaining the current situation of VL in any given municipality[34].

The overall prevalence of CanL (13%, comprising 13.9%, i.e. 23/165, in Sertão; and 12.1%, i.e. 20/165, in São Francisco) in the present survey was higher than had previously been detected in other endemic areas in znortheastern Brazil[35-36].

Indeed, over the past few years, there has been an increase in the average number of cases of human VL in the regions of the present study. The municipality of Petrolina shows the greatest average number of cases per 100,000 inhabitants (14.8), followed by Salgueiro (8.4), Santa Maria da Boa Vista (5.6), Ouricuri (5.2), Serra Talhada (4.5) and Lagoa Grande (2.3)[4].

Epidemiological studies conducted over the last years in Petrolina have shown prevalences of CanL ranging from 11.2% to 34%[15-20]. This is considered to be an area of intense transmission of VL[6]. These data support the findings of the present study, because the highest seroprevalence rate (25.4%) in the São Francisco mesoregion was found in this municipality. This also highlights the fact that Petrolina often remains a municipality with higher infection rates[20].

Even though the prevalences in Lagoa Grande (9.1%) and Santa Maria da Boa Vista (1.8%) were the lowest in the São Francisco mesoregion, Evaristo t al[38]. observed, in an epidemiological study performed in these regions, that these municipalities are endemic areas with a high risk of transmission. However, it is important to highlight that prevalence studies depend on the type of serological test used and the number of dogs evaluated[34].

In the Sertão mesoregion, the prevalence of anti-*L. infantum* antibodies in the canine population was highest in the municipality of Serra Talhada (21.8%), followed by Ouricuri (12.7%) and Salgueiro (7.2%). In spite of the prevalences observed in these municipalities, no seroepidemiological studies on CanL had previously been conducted in these areas, which may impair deeper analysis on the epidemiology of this disease and enable its silent spread[11].

The variation in the seroprevalence of CanL between the areas studied here may have been associated with their economic status and their environmental and geographical characteristics[40].

In our study, 21.8% (72/330) of the dogs were seropositive in both the DPP and ELISA tests. Moreover, when the results from the three tests (DPP, ELISA and IFAT) were considered simultaneously, the seropositivity decreased to 13.0% (43/330). This indicated that combined use of two tests with high sensitivity, such as DPP and ELISA[41], without using a test with greater specificity, such as IFAT, may elevate the sensitivity of the result and generate false positives[13].

The presence of clinical manifestations contributes towards achieving early diagnosis of the disease and can indicate the phase of the infection[42]. In the present study, 62.8% of the seropositive dogs showed clinical signs of CanL. Ulcers, onychogryphosis, lymphadenomegaly and cachexia were the most prevalent clinical signs, similar to what was seen by Assis et al[43]. Furthermore, the presence of ocular lesions was significantly associated with seropositivity for *Leishmania*. Clinically infected dogs show a greater humoral response against parasite[44]. They are more easily detected in tests with higher sensitivity, which improves the diagnosing of clinically suspected cases. However, to diagnosis infected dogs with absence of typical clinical signs of CanL is still an important critical point in diagnosing the disease[45].

In this study, it was observed that living in the municipality of Petrolina was a risk factor for infection with *Leishmania* spp., among dogs. This finding, together with the high seroprevalence found in this municipality, emphasizes that there is a need for more surveillance and control actions against thisdisease. The aim needs to be to decrease the infection rate among dogs and, consequently, avoid the emergence of new human cases[20].

Regarding the 250 m buffer zones that were formed around positive dogs, clusters of infected dogs were observed in the rural areas of the municipalities, thus indicating that the infected vector population may be present at higher frequency in these areas[46]. Through this finding, health education activities aimed towards reducing the sand fly population can be focused on specific areas of distribution of the vector. This enables more effective disease control and prevention and decreases the emergence of new canine and human cases[47].

Detection of species-specific primers through use of DNA has commonly been applied to classification of *Leishmania* parasites in clinical cases of canine infection and in epidemiological studies[48-29].

Regarding molecular testing, the frequency of positive dogs was found to be 6.7% using primers for the kDNA gene that were specific for *Leishmania* spp. However, only 2.4% were positive through use of primers for the cathepsin L-*like* gene that were specific for *Leishmania infantum*. Cathepsin L-*like* is a good marker for phylogenetic analysis on the genus *Leishmania*. It enables a better clinical diagnosis through differentiation of the clinical forms of the disease, thereby improving the clinical prognosis for the animals[49]. Although the kDNA gene showed greater positivity than the cathepsin gene, it was not specific for *Leishmania infantum*. Thus, it is possible to suppose that other species may exist in the region or that there may be nonspecific reactions with other agents[15].

Markers based on the SSU-rDNA and gGAPDH genes did not show any amplification. This may indicate that they have different sensitivity and specificity. These observations emphasize the importance of, whenever possible, performing PCR assays with primers targeting various genes, especially in samples such as blood, in which the parasite load tends to be low[50].

Identification of L. infantum as the canine strain in the municipalities of the present study contributes new insights into the epidemiology of CanL[48]. Therefore, in areas that are endemic for VL, it is essential to keep the epidemiological approaches up-to-date and to identify new strains of the agent, thus contributing towards proper diagnosing of the disease[29]. Presence of the agent could be seen in the São Francisco and Sertão mesoregions, with high serological prevalence among domiciled dogs and clusters of positive dogs in rural area of the municipalities. For the first time, presence of *Leishmania infantum* was characterized in this region. However, no phylogenetic difference was observed between the strains obtained. The findings from this study indicate that there is a need for more studies on the *Leishmania* species identified in this region, in order to obtain better understanding of the epidemiology of VL and improve the measures for controlling and preventing this disease.

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