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Original Article

Exposure to and infection by *Leishmania infantum* among domestic dogs in an area of the Cerrado biome, Maranhão, Brazil

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A R T I C L E I N F O

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A B S T R A C T

Visceral Leishmaniasis is a serious public health problem and dogs are considered to be the main source of infection in urban areas. In Brazil, this disease is present in all regions, but with high concentration of cases in the Northeast, and the state of Maranhão is considered to be an endemic region. The aim of this study was to conduct an epidemiological, spatial, molecular and serological survey on *Leishmania infantum* among domestic dogs in the municipality of Belágua, Maranhão. Blood samples were collected from dogs and questionnaires were applied to their owners to obtain epidemiological data and risk factors relating to this zoonosis in the region. The coordinates of the dogs' homes were obtained to produce a disease risk map. Serological diagnoses were made using the indirect immunofluorescence reaction (IFAT) and the dual-path platform chromatographic immunoassay test (DPP®) (Bio-Manguinhos/FIOCRUZ, Brazil). A molecular investigation was undertaken using the polymerase chain reaction (PCR). Georeferencing was performed using the global positioning system (GPS) and cases of canine visceral leishmaniasis in the municipality were spatially represented and analyzed using QGIS version 3.16.6 (QGIS Development Team, 2021). A total of 205 blood samples were collected, of which 122 (59.51%) were seroreactive for L. *infantum* through IFAT, while the DPP test showed 84 reactive samples (40.97%). IFAT and DPP detected 16 positive animals simultaneously. One sample that was seroreactive through IFAT was also positive through PCR. In the clinical evaluation, it was observed that among the seropositive dogs, 112 (91.80%) were symptomatic and 10 (8.20%) were asymptomatic. In the spatial analysis, the Kernel density estimator enabled determination of the place at greatest risk of occurrence of the disease. The areas with the highest concentrations of cases were in districts with large quantities of precarious housing and lack of basic sanitation. This was the first report on the occurrence of L. *infantum* among dogs in the municipality of Belágua. The results show that canine visceral leishmaniasis is well dispersed in this municipality, thus putting at risk the human population.

1. Introduction

Canine visceral leishmaniasis (CVL) is considered to be a chronic parasitic zoonosis. It is caused by the protozoon *Leishmania infantum* (Dias et al., 2018) and is transmitted mainly by the phlebotomine (sandfly) *Lutzomyia longipalpis* in Brazil (De Oliveira et al., 2013), but *Lutzomyia cruzi* has also been implicated as vector of this parasite (Galati et al., 1997; Santos et al., 1998; Missawa et al., 2011). Dogs (*Canis familiaris*) are the main reservoir in urban areas (Werneck, 2014).

The prevalence of canine infection was found to differ between the five geopolitical regions of Brazil between 2014 and 2018, and its occurrence was correlated with the risk of the disease in humans (Brasil, 2006, 2017; Marcondes and Day, 2019). The largest number of human cases was reported from the northeastern region, especially in the state of Maranhão, which had the highest frequency of occurrence of cases. This state was thus found to be an endemic area for both human visceral leishmaniasis (HVL) and CVL (Sales et al., 2017).

In this area of the Amazon basin, Sales et al. (2017) conducted a

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retrospective study on the epidemiological aspects of CVL and HVL over the period 2009–2012. They found that 24.96% of the dogs and 57.24% of the humans surveyed were seropositive for anti-*Leishmania* antibodies.

It should be noted that controlling CVL is based on strategies that involve identification and stratification of at-risk areas through canine and entomological serological surveys and epidemiological investigations of human cases and adverse environmental conditions (Brasil, 2006). This is mainly done in regions classified by the Ministry of Health as presenting intense transmission of visceral leishmaniasis, with low sociocultural conditions in line with these communities' human development index (HDI).

The objective of this study was to conduct an epidemiological, spatial, molecular and serological survey of *Leishmania infantum* among domestic dogs in the municipality of Belágua, in the Cerrado Maranhense region of the state of Maranhão, considering that studies in different regions of Brazil have demonstrated an association between canine and human leishmaniasis. Thus, it is necessary to conduct studies that reveal the real situation of this disease among dogs, in municipalities with low HDI, and where studies of this nature are scarce, in order to understand the dispersion of the disease and to support the planning of appropriate epidemiological surveillance actions.

2. Material and methods

2.1. Ethical considerations

This research was approved by the Ethics Committee on Animal Experimentation (CEEA) of the State University of Maranhão (UEMA), under protocol no. 042/2019.

2.2. Study area

Belágua is a municipality in the state of Maranhão that lies within the Eastern Maranhão mesoregion, in the Chapadinha microregion. It covers an area of 499 km^2 and has a population of approximately 7528 inhabitants (IBGE, 2020).

2.3. Epidemiological survey

The number of dogs sampled in the urban area of Belágua was calculated considering a population of 1000 dogs (Health Department of the municipality of Belágua), with an expected frequency of 11.14% (Costa et al., 2015) and with a 95% confidence interval (CI).

For this study, domestic dogs of both sexes, aged 6 months or over, were selected through an active search that was conducted from November 2019 to February 2020. During blood sample collection, a questionnaire was applied to the dogs' keepers that asked for information about the animal (sex, age, environment, habits and clinical signs). For participation in the research, a free and informed consent statement was signed by the dogs' keepers. The animals were subjected to a clinical examination to verify any presence of clinical signs of leishmaniasis.

2.4. Obtaining biological samples

Blood samples of approximately 5 mL were collected by means of jugular venipuncture. These were divided between two tubes: one containing EDTA (BD Microtainer®) and the other without this (BD Microtainer®). One milliliter of whole blood with EDTA per sample was then divided into aliquots, labelled and frozen at −20 °C for later use in molecular diagnosis. The samples in tubes without EDTA were centrifuged at 2000 *x*g for 10 min for serum separation, and this serum was then transferred to 1.5 mL microtubes, labelled and stored at −20 °C until serological assays were performed.

2.5. Serological tests

The serum samples thus obtained were tested using the indirect immunofluorescence reaction (IFAT), with a dilution cutoff point of 1:80, and serial titrations were performed. The promastigote forms that were used as the L. *infantum* antigen were made available by the University of São Paulo (USP) (strain CBT 153) and by the Oswaldo Cruz Institute (IOC/FIOCRUZ) (strain IOC 579). The IFAT test was performed as described by Oliveira et al. (2008).

The dual-path platform chromatographic immunoassay (DPP®) test (Bio-Manguinhos/FIOCRUZ Brazil) was performed in accordance with the manufacturer's instructions.

2.6. Molecular tests

DNA from the blood samples was extracted using the Bio-Rad InstaGene™ matrix kit, in accordance with the manufacturer's instructions. The DNA samples were subjected to a polymerase chain reaction (PCR), with amplification of 223 bp of genomic DNA of L. *infantum*, using the CatLeishF primer (5′ GACAACGGCACTCGGCCAAAATAAAAG 3′) and CatLeishR primer (5′ CAGTACGGCGGTTTCGTCTGTGTGTTGAGcAGC 3′) (Silva et al., 2019). The PCR products were subjected to horizontal electrophoresis on 1.5% agarose gel and the bands were viewed and photographed using a UV light transilluminator.

2.7. Georeferencing and spatial analysis

Georeferencing was performed using the global positioning system (GPS), to position the homes of the dogs that were sampled in relation to the recorded latitude and longitude. Cases of canine visceral leishmaniasis in the municipality were spatially represented and statistically analyzed using QGIS version 3.16.6 (QGIS Development Team, 2021). The georeferenced data were inserted in the digitized cartographic base of the municipality, obtained from the IBGE website, using the SIRGAS 2000 geodesic reference system and the UTM coordinate system for zone 23 S.

2.8. Statistical analysis

The analyses on the descriptive data were undertaken through the information from the epidemiological questionnaires. Associations between risk factors and the presence or absence of L. *infantum* according to serological tests among the dogs were tested using Pearson's chi-square test (χ^2). The significance level adopted was $p < 0.05$. Statistical analyses were performed using the Epi Info™ software, version 7.2, developed by the Centers for Disease Control and Prevention (CDC).

3. Results and discussion

A total of 205 blood samples from dogs were collected for the IFAT, DPP and PCR tests.

In IFAT, 59.51% of the samples (122/205) were seroreactive for L. *infantum*, with a titer of 1:80. Of these, 81.97% (100/122) reached a titer of 1:160, 36.89% (45/122) reached 1:320, 15.57% (19/122) reached 1:640 and 1.64% (2/122) reached 1:1280.

The results showed that the DPP test presented 83 reactive samples (40.48%). IFAT and DPP detected 16 positive animals simultaneously.

IFAT is used in diagnosing canine visceral leishmaniasis and is one of the most used tests in canine surveys. However, the Ministry of Health has started to recommend use of the rapid immunochromatographic dual-path platform test (DPP; BioManguinhos/FIOCRUZ, Rio de Janeiro, Brazil), to identify infected dogs; and use of ELISA to confirm positive results (Paz et al., 2018).

IFAT is a qualitative serological test for diagnosing CVL (Paltrinieri et al., 2016). Among symptomatic animals, its specificity and sensitivity

Table 1
Univariate analysis (chi-square test) on the associations between independent variables and presence or absence of *Leishmania infantum* in samples ($n = 16$) positive to both IFAT tests and DPP tests, among dogs living in the municipality of Belágua, Maranhão, Brazil.

Variable	Category	Positive/exposed	%	Negative/exposed	%	*P* value
Sex	Male	9/120	7.5	111/120	92.5	0.52
	Female	7/85	8.2	78/85	91.8	
Collection of feces	Yes	14/170	8.2	156/170	91.8	0.46
	No	2/35	5.8	33/35	94.2	
Place where the dogs slept	Away from home	13/178	7.3	165/178	92.7	0.35
	Inside the house	3/27	11.2	24/27	88.8	
Movement restrictions	Allowed to go out on the streets	13/142	9.2	129/142	90.8	0.21
	Not allowed to go out on the streets	3/63	4.8	60/63	95.2	
Animals in peridomestic areas	Yes	12/151	8	139/151	92	0.58
	No	4/54	7.5	50/54	92.5	
Organic matter	Yes	14/163	8.5	149/163	91.5	0.32
	No	2/42	4.8	40/42	95.2	
Clinical signs	Yes	16/182	8.8	166/182	91.2	0.13
	No	0/23	0	23/23	100	

$P < 0.05^*$ = statistically significant value.

are close to 100%. However, cross-reactions with other pathogens and low sensitivity in identifying asymptomatic animals, compared with the ELISA test, are limitations of this technique (Solano-Gallego et al., 2014; Paltrinieri et al., 2016).

The serological method can also provide false negative results, especially from animals in the parasite incubation period or in the serum conversion period, since during these phases, antibody levels may vary (Lima et al., 2013). False-negative dogs constitute a public health problem because they remain viable as potential reservoirs for the vector (Evaristo et al., 2021).

Silva et al. (2016) analyzed the diagnostic techniques for canine leishmaniasis and observed that DPP presented moderate agreement with ELISA, while IFAT had almost perfect agreement. Thus, it is understood that IFAT is a valid test for use in combination with other techniques for carrying out serological surveys.

All samples were tested by PCR and L. *infantum* DNA was detected in one blood sample in the urban area. The source of the sample has a significant influence on the ability of molecular tests to identify infected dogs (Solano-Gallego et al., 2016). However, although peripheral blood is not the most sensitive type of sample, it is the most useful type for field research because obtaining these samples is less invasive (Lachaud et al., 2002a; Lachaud et al., 2002b). PCR sensitivity is higher for DNA samples extracted from bone marrow, spleen and lymph nodes, but collection of these samples is very invasive (Cabral, 2007; Dantas-Torres et al., 2017). Nonetheless, in performing PCR on blood samples from serologically positive dogs, Silva et al. (2016) found that the parasite was present in 43.9% (18/41) of the samples tested and that this positivity rate was 73.17% (30/41) through use of the real-time polymerase chain reaction (qPCR).

The serological tests showed more animals that are positive while molecular test showed just one positive sample, which had title 1:160 IFAT. None of the samples was positive in the three tests used. However, it is expected, because positive serological results did not necessarily indicate a current infection, but rather that the host presented an immunological reaction against the parasite (Maia et al., 2010).

In a review article on methods for diagnosing canine leishmaniasis, Costa et al. (2021) warned that, because this is a complex disease, with symptoms similar to those of other pathological conditions, and because of the large number of asymptomatic carrier animals, it is necessary to use combinations of various diagnostic techniques, since none of them is 100% reliable. In this regard, Evaristo et al. (2021) used serological tests (IFAT, ELISA and DPP) and molecular tests (conventional and quantitative PCR). They noted that, overall, 87.1% (155/178) of their samples were positive for anti-*Leishmania* antibodies and/or *Leishmania* spp. genetic material, and concluded that combining the tests increased the likelihood of detecting positive animals.

The results from the questionnaires relating to sex, feces collection, places where the dogs slept, movement restrictions, presence of animals in peridomestic areas, presence of organic matter and presence of clinical signs are summarized in Table 1.

Table 2
Univariate analysis (chi-square test) on the associations between clinical signs and presence or absence of *Leishmania infantum* according to serological tests among dogs living in the municipality of Belágua, Maranhão, Brazil.

Variable	Category	Positive/exposed	%	Negative/exposed	%	P value
Progressive weight loss	Yes	60/99	60.61	39/99	39.39	0.38
	No	62/106	58.49	44/106	41.51	
Onychogryphosis	Yes	59/99	59.60	40/99	40.40	0.49
	No	63/106	59.43	43/106	40.57	
Eye secretion	Yes	39/61	63.39	22/61	36.07	0.20
	No	83/144	57.64	61/144	42.36	
Alopecia	Yes	53/92	57.61	39/92	42.39	0.30
	No	69/113	61.06	44/113	38.94	
Opaque and dull fur	Yes	53/105	50.48	52/105	49.52	0.003*
	No	69/100	69	31/100	31	
Skin wound	Yes	48/77	62.34	29/77	37.66	0.26
	No	74/128	57.81	54/128	42.19	
Lesions on ear edge	Yes	68/98	69.39	30/98	30.61	0.003*
	No	54/107	50.47	53/107	49.53	
Lesions on snout	Yes	60/86	69.77	26/86	30.23	0.005*
	No	62/119	52.10	57/119	47.90	
Lesions on body	Yes	58/72	80.56	14/72	19.44	0.000002*
	No	64/133	48.12	69/133	51.88	

$P < 0.05^*$ = statistically significant value.

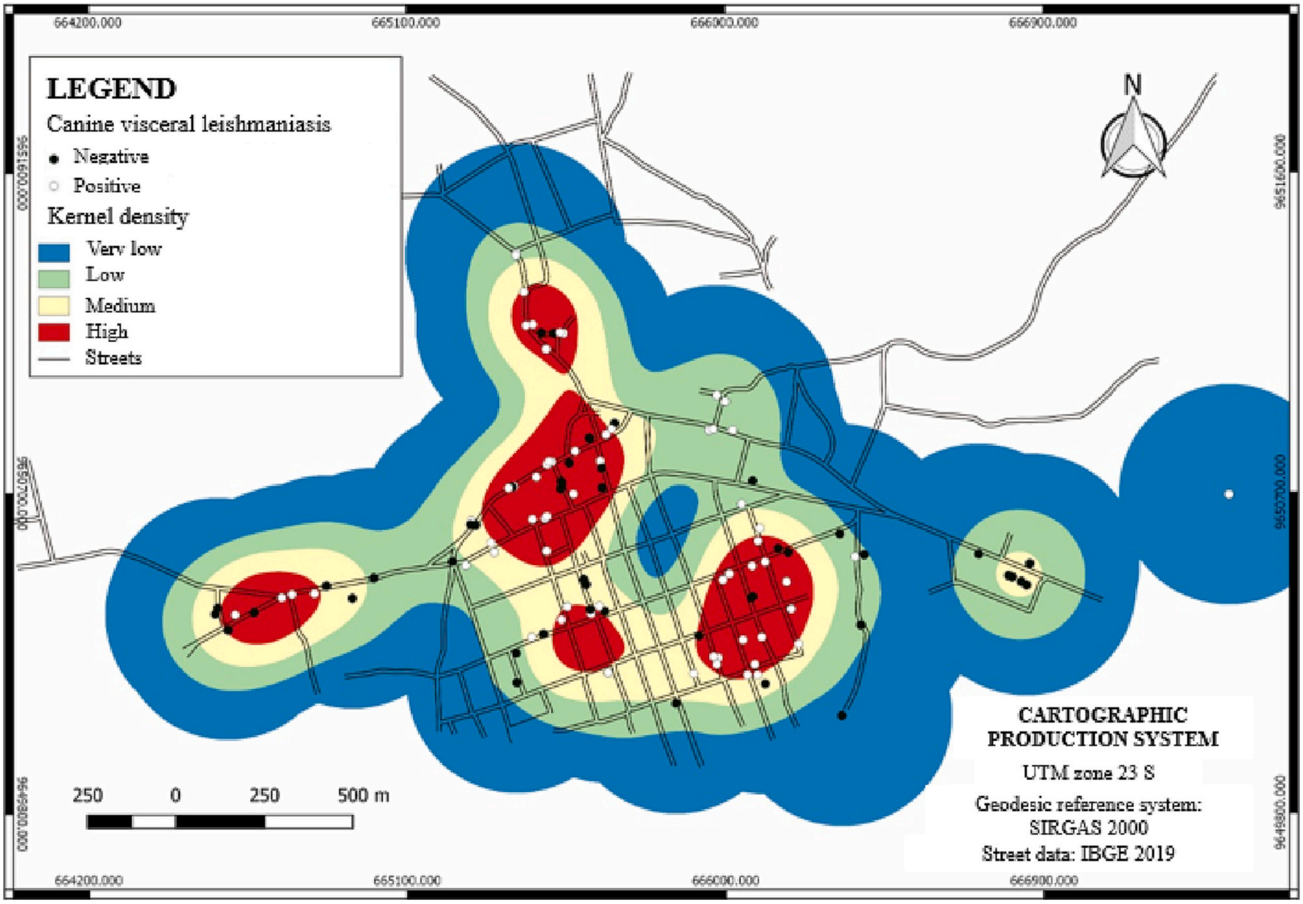

Fig. 1. Kernel density map with distribution of cases confirmed through serological tests for canine visceral leishmaniasis in the municipality of Belágua, Maranhão, Brazil.

There was no statistically significant difference ($p < 0.05$) between positive samples to both IFAT and DPP tests with regard to associations with the variables of the sex of the dog, feces collection, places where the dogs slept, movement restrictions, presence of animals in peridomestic areas, organic matter in the environment, clinical signs shown by the dogs or presence or absence of anti-*L. infantum* antibodies (Table 1).

Regarding sex, Silva et al. (2016) found that the risk of infection was higher among male dogs. This can be explained by their greater use for hunting and as guard dogs: both of these activities can lead to increased exposure to phlebotomines (sandflies). However, Azevedo et al. (2008), Silva et al. (2013) and Figueiredo et al. (2014) found that males and females were equally exposed to the risk of L. *infantum* infection, as also observed in the present study.

Although there were no statistically significant differences in the present study, in relation to the places where the dogs slept, movement restrictions, presence of animals in peridomestic areas or organic matter in the environment, Gálvez et al. (2010) and Cortes et al. (2012) observed that dogs living outside the home or with frequent access to the external environment presented higher risk of becoming infected with the disease. This was mainly due to greater exposure to phlebotomines (sandflies), given that these vectors become active from dusk onwards. Studies have shown that exposure to these vector insects is also associated with the environmental characteristics of peridomestic areas, i.e. whether the dogs live near green areas, whether other animals are present in these areas (such as chickens) and whether organic debris is present. These characteristics may favor proliferation of phlebotomines (sandflies) and form risk factors for both dogs and humans (Coura-Vital et al., 2011; Brito et al., 2016).

The clinical signs identified in this study were those commonly observed among dogs that are naturally infected by L. *infantum* in areas endemic for CVL and HVL. Among the 122 animals seropositive for IFAT with clinical signs for canine visceral leishmaniasis, the following results were obtained: progressive weight loss in 81.14% (99/122); onychogryphosis in 81.14% (99/122); eye secretion 50% (61/122); alopecia in 50.81% (92/122); opaque and dull fur in 86.06% (105/122); skin wounds in 63.11% (77/122); lesions on ear edge in 80.32% (98/122); lesions on the snout 70.49% (86/122); lesions on the body 59.01% (72/122). There were statistically significant differences in the univariate analysis for the variables of opaque and dull fur, lesions on the ear edge, lesions on the snout and lesions on the body (Table 2).

These clinical manifestations corroborate the findings of Baneth et al. (2008), Ramsey et al. (2010) and Evaristo et al. (2021). Moreover, according to Salzo (2008), skin lesions are present on 90% of dogs with canine visceral leishmaniasis. These lesions mainly appear on the snout or ears and in areas of alopecia because of multiplication of the parasite in macrophages (Marinho et al., 2017).

In the clinical evaluation of the present study, it was observed that among the seropositive dogs, 91.80% (112/122) were symptomatic, presenting the following titles in the IFAT: 83.92% (94/112) of 1:80; 72.32% (81/112) for 1:160; 36.60% (41/112) at the 1:320; 16.67% (18/122) for 1:640 and 1.68% (2/112) for 1:1280. However, 8.2% (10/122) were asymptomatic seropositive dogs.

In fact, according to Figueiredo et al. (2014), symptomatic dogs are about three times more likely to be positive in serological tests than are asymptomatic dogs. Furthermore, despite the low number of asymptomatic dogs, their presence has great importance in the epidemiological context, since these animals are able to infect the vectors and maintain the transmission cycle, thus disseminating the disease (Coura-Vital et al., 2011; Abrantes et al., 2018).

Regarding spatial analysis, the Kernel density estimator made it possible to determine the site of greatest risk of occurrence of the disease. In Fig. 1, the areas with the highest concentrations of cases are highlighted in red: these were located on the urban perimeter of the municipality of Belágua, while in the vicinity of the red areas there was average occurrence in yellow and densities ranging from low to very low, in light green and blue respectively (Fig. 1). The maps of the geographical distribution of canine visceral leishmaniasis demonstrated heterogeneous distribution of the disease in relation to the methods of investigation used, with intense agglomeration of cases in some areas. Oliveira et al. (2001) built Kernel maps for seropositive dogs and overlapped these occurrences with human cases. They noted that the results pointed to a correlation between human and canine cases.

The census tract of Belágua includes neighborhoods with large numbers of homes in a precarious condition, with lack of basic sanitation. This corroborates the data of Cerbino Neto (2003) and Guimarães et al. (2005), who stated that the highest numbers of cases occurred in areas of disorderly occupation and low levels of infrastructure, near forests, with the presence of wild animals, thus causing an abundance of vectors and reservoirs, which would contribute to high canine seroprevalence. In the same census tract, there are also sheds for poultry-rearing, which serve as breeding grounds and food sources for the vectors (Silva et al., 2021).

It is important to note that in a review article by Silva et al. (2021), they pointed out that there is higher occurrence of leishmaniasis in regions with low HDI, as is the case of the municipality of Belágua, whose HDI is considered very low (0.495). Thus, interventions should be carried out in this municipality with the aim of improving its infrastructure and income.

Georeferencing research is important because this provides support for identifying and visualizing areas that require priority intervention for disease control, and it enables optimization of human resources, since knowledge of CVL cases can serve as an indicator for human cases. In addition, investigations on factors relating to patterns of occurrence and dissemination of communicable diseases will define priority areas for implementation of surveillance and control measures (Azevedo et al., 2019; da Silva Zuque et al., 2022).

4. Conclusions

The tests used, regardless of the percentages of positivity, made it possible to report on the occurrence of L. *infantum* among dogs in the municipality of Belágua for the first time. This expands the area of occurrence of this parasite in the state of Maranhão. The results indicated that canine visceral leishmaniasis is well dispersed in this municipality, thus putting at risk the human population. There is a need to alert epidemiological surveillance agencies, in order to establish measures for preventing and controlling this zoonosis, including educational and sanitary actions that encompass the concept of one health (man, animal and environment). From this research, it was possible to develop an educational booklet for the population and healthcare professionals of Belágua, Maranhão.

Declaration of Competing Interest

None.

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