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Research paper

Diagnosis and epidemiology of *Leishmania infantum* in domestic cats in an endemic area of the Amazon region, Brazil

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ABSTRACT

Visceral leishmaniasis is a zoonotic disease caused by *Leishmania infantum* for which dogs are the main reservoir. In South America, presence of this disease is expanding along with increasing dispersion of its principal vector, the sand-fly *Lutzomyia longipalpis*. Feline leishmaniasis is an emerging disease in domestic cats, but epidemiological studies in endemic areas of the Amazon region of Brazil are scarce and the role of cats as reservoirs of *L. infantum* has been debated. The aim of this study was to investigate *L. infantum* infection in cats living in the Amazon biome region, using serological and molecular methods. A total of 105 cats were subjected to clinical examination and blood samples were taken for immunofluorescent-antibody (IFAT) serological evaluation, to determine anti-*Leishmania* antibody titers. Conventional PCR and Sanger's sequencing targeting *L. infantum* chitinase and *Leishmania* species ribosomal internal transcribed spacer (ITS-1) encoding genes were performed on conjunctival swabs from these cats. Seropositivity was detected in 32 animals (30.48%), thus confirming that contact between these cats and the parasite was occurring. PCR followed by amplicon sequencing showed that three samples (2.86%) were positive for a chitinase gene and six (5.71%) were positive for the ITS-1 gene. Parasite-positive diagnoses presented a statistically significant association with free access to the streets ($p = 0.0111$), cohabitation with dogs affected previously by VL ($p = 0.0006$) and absence of backyard cleaning and garbage collection ($p = 0.00003$). These results emphasize that cats should be included in epidemiological surveys of leishmaniasis, especially in endemic areas, if not as the reservoir host (unproven), at least as a "sentinel host" that is useful for revealing situations of endemic circulation of *L. infantum*. Moreover, in these areas, feline leishmaniasis needs to be considered in the differential diagnosis among domestic cats presenting alopecia, rarefied hair, lacerations and ulcerative dermatitis.

1. Introduction

Visceral leishmaniasis (VL) is a neglected infectious disease with an estimated incidence of 300,000 new cases and 20,000 deaths annually, and over one billion people living in endemic areas are at risk of infection (WHO, 2017). Zoonotic VL is caused by the protozoan parasite *Leishmania infantum* (Espinosa et al., 2018; Marcili et al., 2014) and is transmitted by *Lutzomyia longipalpis* (Diptera: Psychodidae) (Freitas et al., 2012). Brazil accounts for the majority of VL cases in the Americas, where the numbers of cases are expanding (Georgiadou et al., 2015).

Domestic dogs participate in the spreading of VL caused by *L. infantum* and are the main reservoir for this parasite in Brazil. However, domestic cats can play a role as hosts for zoonotic VL (Maia and Cardoso, 2015; Marcili et al., 2014). Cats have become increasingly common pets in urban environments because of their behavioral traits, such as their cleaning habits, sociable nature and tolerance of remaining alone, in addition to their small size (Maia et al., 2008). In fact,

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feline leishmaniasis caused by *L. infantum* is considered to be an emerging feline disease (Pennisi and Persichetti, 2018).

The notion that cats (*Felis catus domesticus*) participate as alternative reservoir in the epidemiology of leishmaniasis is controversial (Maia and Campino, 2011) because only two reports worldwide (one from Italy and the other from Brazil) have shown that cats can be infectioustoy competent vectors (Maroli et al., 2007; da Silva et al., 2010).

Prevalence of *L. infantum* in cats ranges from 07 to 30% in studies conducted worldwide, including Brazil and Europeans countries (Asfaram et al., 2019; Pennisi et al., 2015), varying regionally in Italy (Iatta et al., 2019). Positive results have been obtained from using *Lu. longipalpis* for xenodiagnosis of *L. infantum* (da Silva et al., 2010). Several studies on cats in different parts of the world have revealed the presence of *L. infantum* through isolation (Mendonça et al., 2017), nucleic acid detection (Benassi et al., 2017; Coura et al., 2018; Mendonça et al., 2017) and serological tests (Akhtardanesh et al., 2017; Ayllon et al., 2008; Benassi et al., 2017; Bresciani et al., 2010; Coelho et al., 2010; Matos et al., 2018; Nasereddin et al., 2008; Oliveira et al., 2015a; Persichetti et al., 2017; Sarkari et al., 2009; Sousa et al., 2014a; Vita et al., 2005).

Distinct from canine leishmaniasis, feline leishmaniasis is manifested less characteristically and usually occurs in immunosuppressed animals (Pennisi et al., 2013). For diagnostic purposes, the parasite can be detected in peripheral blood, skin, lymphatic organs, liver, oral mucosa, nasal exudate, eyes, stomach, kidneys, etc. (Pennisi and Persichetti, 2018).

Occurrences of VL in Brazil are increasing in line with increasing dispersion of its principal vector, *Lu. longipalpis*. This sand-fly is well adapted to diverse climate conditions and habitats, with a capacity to feed on different animals. Its presence is related to urbanization, which is a risk factor for visceral leishmaniasis (Costa et al., 2013; Lainson and Rangel, 2005; Maia-Elkhoury et al., 2008).

On São Luis island, in the northeastern Brazilian state of Maranhão, dispersion of canine and human VL is occurring in association with environmental degradation of wild ecotypes due to urbanization, with increasing numbers of breeding sites, presence of garbage accumulation and presence of wild animal shelters within the urban perimeter. These factors favor proliferation of *Lu. longipalpis* (da Costa et al., 2015; Rebelo et al., 1999).

The present study had the aim of investigating occurrences of feline leishmaniasis and associated risk factors in an area endemic for *L. infantum* in the Amazon biome region of the state of Maranhão.

2. Material and methods

2.1. Legal aspects

This study was approved by the Ethics and Animal Experimentation Committee (protocol 032/2017) of the School of Veterinary Medicine, State University of Maranhão, Brazil, in accordance with the Ethical Principles for Animal Experimentation.

2.2. Study area

The study was carried out in the municipality of São Luís (02° 31′ 48″ S; 44° 18′ 10″ W), the capital of the state of Maranhão, in northeastern Brazil (Fig. 1), which is in the Amazon biome. It was conducted between July 2016 and June 2017.

The climate of the area is semi-humid tropical, with two well-defined seasons during the year (dry and rainy). The monthly maximum temperature ranges from 22 to 34 °C and the mean temperature is 28.5 °C. The mean relative humidity (RH) is 84.9% and the annual rainfall is 2290 mm (IBGE, 2015).

2.3. Animals and biological sample collection

The study population was composed of 105 domestic cats that were randomly chosen. They were of both genders and different ages (but preferably 6 months or older) and were living in their keepers' homes. This cross-sectional study was conducted using a convenience sample, and the sampling was carried out only after obtaining the pet owner's consent. All cats enrolled in the study were clinically evaluated for overall health conditions through physical examination regarding general appearance, body condition, mucous-membrane color and eyes.

All of these animals were subjected to clinical examination before blood sample collection, with the aim of ascertaining any presence of dermatoses and any clinical manifestations compatible with leishmaniasis (cutaneous and mucocutaneous lesions; nodular dermatitis on the head and legs; ulcerative-squamous lesions located on the head, muzzle, neck, elbows, ankles or ischial tuberosity; lymphadenomegaly; and the ocular condition of blepharitis, conjunctivitis, keratitis and unilateral uveitis).

An epidemiological questionnaire focusing on possible risk factors associated with *L. infantum* infection was applied to each keeper to obtain information about how they managed their cats and about their cats' characteristics (age, gender, free access to the streets, household status, cohabitation with chickens or other animals, presence of organic debris, backyard cleaning and garbage collection, including tree pruning, yard weeding and leaf/fruit and feces collection) that could possibly be linked to the VL cycle.

From each animal, a blood sample of 5 mL was collected from the jugular or cephalic vein and was transferred into sterile tubes (BD-Vacutainer). These samples were centrifuged at 1500 x g for 15 min, and the serum thus separated was transferred into plastic microtubes and stored at −20 °C until the time of serological analysis. Conjunctival epithelial cells were acquired from each cat's right and left eyes using a sterile cotton swab that was rubbed on the lower conjunctiva. These samples were placed in 1.5 mL nuclease-free microtubes and were kept at 4 °C until processing for nucleic acid extraction and the *Leishmania* sp. molecular diagnostic test.

2.4. Laboratory analyses

2.4.1. Serological diagnosis of L. infantum

The immunofluorescence antibody test (IFAT) was performed in accordance with Oliveira et al. (2008). Briefly, the procedure was as follows: *L. infantum* promastigotes (CBT 153 strain) were isolated from the popliteal lymph nodes of naturally infected dogs and were maintained in RPMI culture medium, which was used for antigen preparation. Serum dilutions were used in duplicate, starting at 1:40, and were placed over the antigen on slides at 37 °C for 30 min. The slides were then incubated with cat serum and afterwards with FITC rabbit anti-feline IgG conjugate (Sigma, USA), and were examined under a fluorescence-specific microscope. Samples that reacted at the screening dilution (1:40) were then titrated using serial two-fold dilutions to determine endpoint titers. Reference serum samples were included in the test as negative and positive controls.

2.4.2. DNA extraction and polymerase chain reaction (PCR)

DNA from epithelial cell samples from cat conjunctiva were extracted using the DNeasy® blood and tissue kit (QIAGEN®, Valencia, CA, USA) in accordance with the manufacturer's instructions. DNA quality was verified through visualization by means of electrophoresis on 1% agarose gel and the amount of DNA was measured by comparing band intensities with the low-mass ladder standard of Invitrogen/Thermo Fischer Scientific, Waltham, Massachusetts, USA. *Leishmania* DNA detection was performed using two PCR methods: one targeting a 999 bp fragment of *L. infantum* chitinase gene using the oligonucleotides LCQuitFow 5′ GCTGCCTGAGCGCTCTACT 3′ and LCQuitRev 5′ CTCCC TCGACCCATGTTG 3′ (Suzuki et al., 2015); and the other targeting the

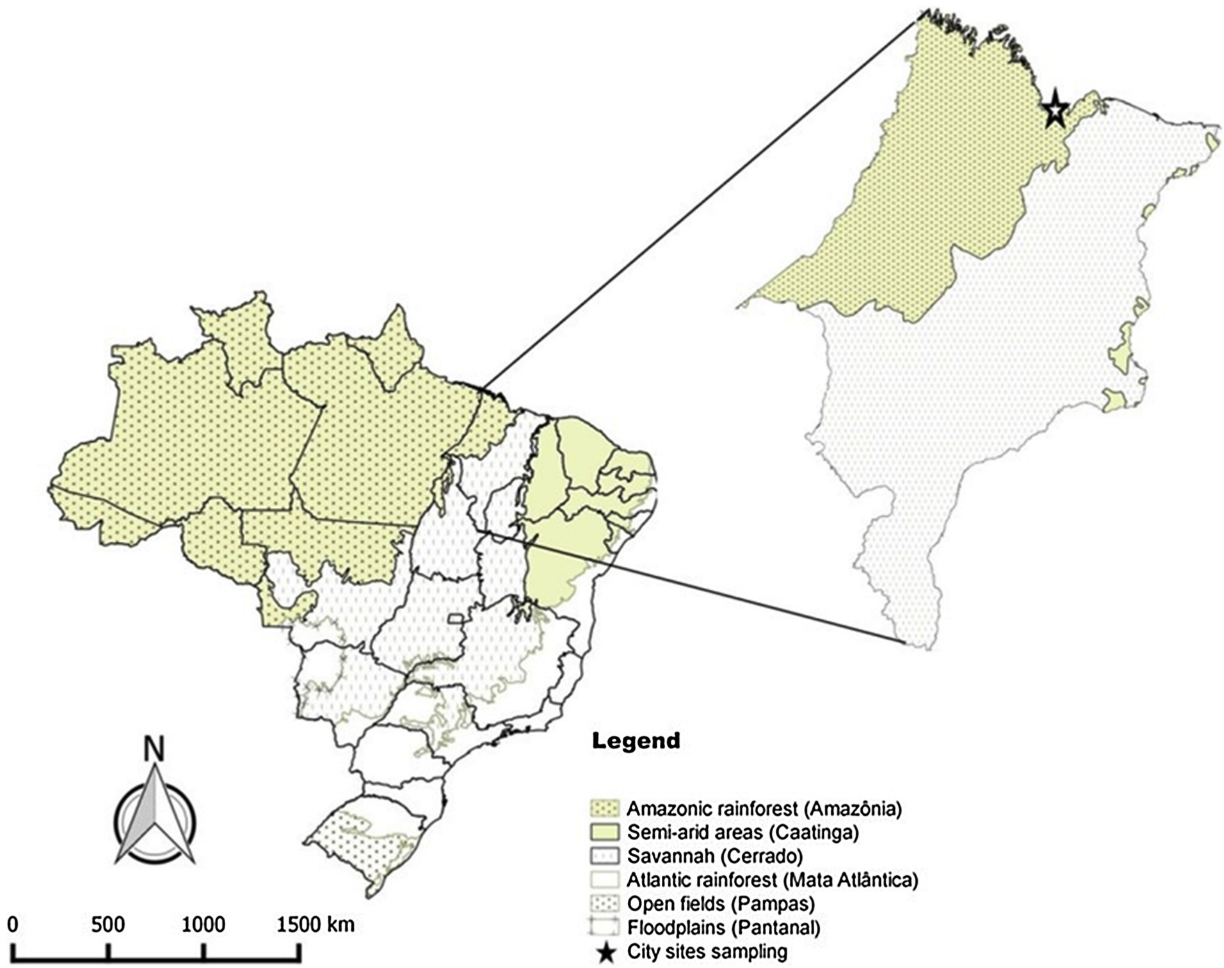

Fig. 1. Geographical map of Brazil with the state of Maranhão highlighted.

5.8S ribosomal internal transcribed spacer (ITS-1) of *Leishmania* species, which amplifies a 320 bp PCR fragment using the primers LITSR 5′-CTTGGATCATTTTCCGATG-3′ and L5.8S 5′-TGATACCACTTATCGC ATT-3′ (Schonian et al., 2003). The PCR products, which were stained with Gel Red™ (Biotium®, Hayward, CA, USA), were analyzed by means of electrophoresis on 1% agarose gel. Chitinase and ITS-1 amplicons were purified in GFX™ columns of PCR purification kits (GE Healthcare, Buckinghamshire, UK). They were then subjected to Sanger's sequencing reactions using the ABI PRISM® BigDye™ terminator kit (Perkin-Elmer® and Applied Biosystems®, Foster, CA, USA), in accordance with the manufacturer's specifications, with resolution in an automated sequencer (ABI PRISM® 310 genetic analyzer; Perkin-Elmer® and Applied Biosystems®, Foster, CA, USA).

3. Results

3.1. Cutaneous lesions were the main clinical findings relating to feline leishmaniasis

Out of the total of 105 cats evaluated, 34 (32.38%) were males and 71 (67.62%) were females; 103 (98%) were older than one year and 104 (99%) were crossbred. They had household habits 76 (72.38%) had not undergone any deworming and 44 (41.90%) had received an anti-rabies vaccination. On physical examination, 22 cats (20.95%) presented more than one clinical sign suggestive of feline leishmaniasis. The main findings were dermatoses characterized by alopecia, rarefied hair, lacerations and ulcerative dermatitis.

Regarding lifestyle, 59 cats (56.19%) had free access to the streets and 46 (43.81%) remained exclusively indoors; 95 cats (90.48%) cohabited with other animals of the same species or others (dogs and birds) and 10 (9.52%) did not.

3.2. Serological and molecular diagnoses revealed occurrences of L. infantum infection

Anti- *L. infantum* antibodies (IgG cutoff ≥ 1:40) were observed in 32 individuals (30.48%) of the feline population examined. The positive samples presented serological reactivity with the following titers: 1:40 (5/32); 1:80 (15/32); 1:160 (6/32); 1:320 (4/32); and 1:640 (2/32).

Out of 32 seropositive cats, only 10 presented clinical signs suggestive of feline leishmaniasis.

The molecular diagnostic test revealed that three samples (2.86%) were positive for a 999 bp chitinase gene specific for *L. infantum* and six (5.71%) were positive for a 320 bp fragment of *Leishmania* species ITS-1. After sequencing, all six amplicons of ITS-1 [GeneBank: MK510941-MK510946] showed a nucleotide similarity of 100% with *L. infantum* [GeneBank : MG778653.1]. All three samples positive for chitinase gene of *L. infantum* [GeneBank MK510947] were positive for ITS-1 and also confirmed its specificity by sequence [GeneBank XM_001464530.1]. All samples positive to PCR were also positive by IFAT.

3.3. Risk factors associated with feline leishmaniasis and keepers' knowledge of the disease

The results relating to the risk factors associated with positive

Table 1
Univariate analysis (Fisher exact test) on the association between independent variables and the serological and molecular diagnoses of presence of *L. infantum* in cats in São Luis, Maranhão, Brazil.

Variable	Category	Positive[#]/exposed	%	Negative[#]/exposed	%	p value
Sex	Male	6/34	17.65	28/34	82.35	0.0845
	female	26/71	36.62	45/71	63.38	
Access to the streets	Yes	24/59	40.67	35/59	59.33	0.0111*
	No	8/46	17.39	38/46	82.61	
Household status	Indoors	7/29	24.14	22/29	75.86	0.4799
	Outdoors	25/76	32.89	51/76	67.11	
Cohabitation with dogs with previous VL	Yes	16/28	57.14	12/28	42.86	0.0006*
	No	17/77	20.78	61/77	79.22	
Organic matter	Presence	15/57	29.82	42/57	70.18	1.0000
	Absence	17/48	31.25	31/48	68.75	
Backyard cleaning and garbage collection	Yes	6/52	11.54	46//52	88.46	0.00003*
	No	26/53	49.05	27/53	50.95	
Clinical symptoms	Yes	10/24	41.67	14/24	58.33	0.2096
	No	22/81	27.16	59/81	72.84	

* Statistically significant results.
[#] Relative to *L. infantum* diagnostic outcome.

serological and molecular diagnoses of feline leishmaniasis are presented in Table 1. The epidemiological characteristics that showed statistically significant associations with a positive diagnosis of *L. infantum* were the following: free access to the streets (p = 0.0111); cohabitation with dogs affected previously by VL (p = 0.0006); and absence of backyard cleaning and garbage collection (p = 0.00003). Human VL was reported in three homes where cat keepers said that they cohabited with dogs presenting VL. Out of the 105 cat keepers interviewed, 51 (48.57%) had some knowledge about leishmaniasis, since they had dogs, neighbors or relatives with the disease. Only two (2.1%) knew about the transmission forms. Concerning feline leishmaniasis, 83 keepers (79.05%) did not know that cats could be affected by this disease.

4. Discussion

Detection of *L. infantum* in cats in areas endemic for human and canine VL has been reported in different regions of the world (Nasereddin et al., 2008; Pocholle et al., 2012; Sarkari et al., 2009; Solano-Gallego et al., 2007). In Brazil, presence of feline leishmaniasis has been reported within different biomes. These have included the Cerrado in the states of São Paulo (Sobrinho et al., 2012; Sousa et al., 2014b), Mato Grosso do Sul (Braga et al., 2014; Metzdorf et al., 2017) and Piauí (De Mendonça et al., 2017); the Caatinga in Pernambuco (Silva Rde et al., 2014); the Atlantic rainforest in Rio de Janeiro (da Silva et al., 2008); and the Amazon in Pará (Oliveira et al., 2015a). Thus, to the best of the present authors' knowledge, our study is the second survey on feline *Leishmania* conducted in the Amazon biome.

In the present study, a species-specific IFAT serological test and PCR revealed frequencies of *L. infantum* of 33.4% and 5.7%, respectively, among domestic cats in a region of the Amazon biome in the state of Maranhão, Brazil, that is endemic for canine and human VL. Studies using serological methods have shown that the rates of *L. infantum* prevalence among cats vary widely, ranging from 2.4% to 23.1% (Bresciani et al., 2010; Fatollahzadeh et al., 2016; Figueiredo et al., 2009; Maia et al., 2010; Nasereddin et al., 2008; Oliveira et al., 2015b; Vides et al., 2011). These prevalence variations can be correlated with differences in the serological methodology used, including antigen concentration and quality; cutoff values; animal tissue source; circulation of different *Leishmania* species and other trypanosomatids; and epidemiological transmission risk factors relating to *L. infantum* (Dedola et al., 2018; Mancianti, 2004).

The titer of 1:80 was found most frequently among the animals and the highest titer observed was 1:640. In the present study, the titers detected were lower than those detected in similar studies in Spain, where the maximum titer found was 1:5120 (Martin-Sanchez et al., 2007), and in Brazil, where the titers ranged from 1:40 to 1:1280 (Sousa et al., 2014a). According to Quinnell et al. (2003), high antibody titers in cases of canine visceral leishmaniasis indicate situations of acute infection and therefore potential transmission to the vector (Quinnell et al., 2003). The same argument cannot be applied to cats infected by *Leishmania* (Oliveira et al., 2015a).

The source of the tissue that was used for making the molecular diagnosis of *L. infantum* was conjunctival swabs. These have proved to be efficient in epidemiological studies (Benassi et al., 2017; Oliveira et al., 2015b). The molecular diagnostic method used was based on PCR amplification of *Leishmania* sp. rRNA ITS-1. This identified six positive samples, all with 100% similarity to *L. infantum*, corresponding to 18.75% of the samples positive through IFAT. The specific *L. infantum*-encoding chitinase-based molecular diagnosis was shown to be less sensitive in conjunctival swabs than was ITS-1, and it confirmed the diagnosis in three PCR-positive samples.

Comparison between the serological and molecular tests showed that more animals were positive according to the serological test. This was already expected, since 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). Since all samples positive according to PCR were also positive according to IFAT, serological tests were used to identify cats that were positive for leishmaniasis and to investigate risk factors associated with *L. infantum* infection.

Presence of *L. infantum* was associated with dermatological alterations in 45% of the cats (Table 1). This may be suggestive of the symptoms of feline leishmaniasis, since infected cats mainly present cutaneous clinical manifestations (Bresciani et al., 2010; Pennisi et al., 2015; Pennisi and Persichetti, 2018). Although the clinical symptoms of feline leishmaniasis are non-specific, as also observed in dogs, presence of cutaneous lesions is strongly associated with co-infection with immunosuppressive viruses such as the feline immunodeficiency virus (FIV) and feline leukemia virus (FeLV) (Shaw et al., 2001; Sobrinho et al., 2012).

Although we found 10 animals with clinical signs suggestive of leishmaniasis and with positive serological tests, we were unable to confirm that these lesions were caused by *Leishmania* because the presence of co-infections was not investigated. The absence of clinical signs in positive animals, as observed here, can be explained by the hypothesis put forward by Solano-Gallego et al. (2007), who suggested that the immune response of cats is effective in controlling the infection when there are no immunosuppressive events (Solano-Gallego et al., 2007).

In the region investigated here, cohabitation of cats with dogs that were positive for VL, existence of free access for cats to the streets and absence of backyard cleaning and garbage collection presented significant associations with positive diagnoses of presence of *L. infantum* (Table 1). It is well known that these factors are associated with canine visceral leishmaniasis, thereby maintaining the environmental conditions for increased proliferation of phlebotomines whose oviposition and larval development occur in humid, shaded and organic matter-rich areas (Feliciangeli, 2004; Killick-Kendrick, 1990).

It is important to remember that in endemic areas, the role of cats may be important, considering that some studies have shown that cats can harbor the parasite without clinical manifestations (Chatzis et al., 2014; Sarkari et al., 2009) and that the vectors can become infected by feeding on infected cats, even in the absence of clinical signs (Afonso et al., 2012; da Silva et al., 2010; Maia et al., 2010; Maroli et al., 2007).

Some studies have shown that in São Luís, 28 species of phlebotomines have already been found associated with areas of secondary forest or peridomestic environments and eight species in primary forest areas (Rebelo et al., 1999; Vides et al., 2011). Nonetheless, more studies are required in order to ascertain the interaction between vectors and cats and thus to define the true role of cats in the epidemiology of VL. It is also important to state that the cat population is increasing in the area studied here. Therefore, epidemiological studies on human, feline and canine VL are essential, in order to aid public health staff in adopting efficient control measures.

Educational actions in relation to VL are essential for controlling this disease, principally among pet keepers (Alemu et al., 2013; Costa et al., 2014; Esch et al., 2012; Koirala et al., 1998). The results obtained from the population studied here demonstrate that the extent of such actions remains poor. This corroborates the results from studies carried out in the Brazilian states of Minas Gerais and Maranhão (Borges et al., 2008; França et al., 2013; Lobo et al., 2013), which pointed out that there was a lack of knowledge about the main aspects of leishmaniasis. Knowledge about leishmaniasis was generally restricted to keepers who had dogs that were positive for the disease or who had neighbors or relatives with dogs affected by VL. In this context, educational practices can be an effective way for engaging people in controlling the disease.

Declaration of Competing Interest

We declare that we have no conflict of interest.

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