

Leishmania infantum in domestic cats from the municipality of Teresina, state of Piauí, Brazil

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SUMMARY

Feline leishmaniasis has been reported in various parts of the world in recent years, occurring mainly in areas where zoonotic visceral leishmaniasis (VL) is endemic. The purpose of this study was to assess the occurrence of natural infection by *Leishmania* spp. in domestic cats (*Felis catus*) in the municipality of Teresina, Piauí state, Brazil, an endemic area of VL in Brazil. The prevalence of infection by *Leishmania* spp. in the population under study was 4% (3/83) in the enzyme-linked immunosorbent assay (ELISA) and 4% (3/83) by smear observation and isolation in a culture medium, using popliteal lymphnode sample. Only one of the three infected cats was positive for ELISA, also being positive for feline immunodeficiency virus. In the haematologic parameters, normocytic normochromic anaemia was the most common change, being present in the three infected animals. In the biochemical measurements also were observed alterations in infected animals. The *Leishmania* spp. strains isolated from the culture medium were characterized as *L. infantum*. The presence of *L. infantum* infection in cats in the city of Teresina, an area endemic for VL, suggests the possible involvement of these animals in the epidemiological chain of *L. infantum* in high-transmission areas.

Key words: Feline leishmaniasis, ELISA, PCR–RFLP, *Leishmania infantum*.

INTRODUCTION

Cats are known to be susceptible to leishmaniasis, but their participation in the cycle of the disease is still unclear. Since the description of the first case of leishmaniasis in a domestic cat in 1912 (Sergeant *et al.* 1912), several cases of leishmaniasis have been reported in cats (Schubach *et al.* 2004; Simões-Mattos *et al.* 2004; Da Silva *et al.* 2008; Vides *et al.* 2011), occurring mainly in visceral leishmaniasis (VL) endemic areas (Saló *et al.* 2007; Maia *et al.* 2008; Costa *et al.* 2010).

Although dogs are the main urban reservoir hosts of zoonotic VL (Souza *et al.* 2008), it has been observed by xenodiagnosis that cats infected by VL are able to infect *Lutzomyia longipalpis* (Maroli *et al.* 2007; Silva *et al.* 2010), and are thus potential reservoirs of this zoonotic disease. Studies about feline LV are limited and the disease in cats is still considered neglected (Pennisi *et al.* 2015).

VL is a zoonotic disease that occurs in the Old and New World, and Brazil is one of the countries with the highest incidence of the disease (Alvar *et al.* 2012). The first case of natural *Leishmania* infection in a feline in Brazil was recorded in 1939 in a cat with

ulcers on the ears and nose, in the state of Pará, but the species causing the infection was not identified (Mello, 1940).

Other studies conducted in Brazil reported *Leishmania* spp. infection in domestic cats in Aurá, state of Pará (Mello, 1940), *Leishmania* subgenus *Viannia* in Belo Horizonte, state of Minas Gerais (Passos *et al.* 1996), *L. infantum* in Cotia, state of São Paulo (Savani *et al.* 2004), Araçatuba, state of São Paulo (Vides *et al.* 2011) and Andradina, state of São Paulo (Coelho *et al.* 2011), and *L. amazonensis* in Campo Grande, state of Mato Grosso do Sul (Souza *et al.* 2005) and Ribas do Rio Pardo, state of Mato Grosso do Sul (Souza *et al.* 2009). The presence of anti-*Leishmania* spp. antibodies in cats has also been reported in other regions in Brazil, such as Barra Mansa, state of Rio de Janeiro (Figueiredo *et al.* 2009) and Recife and Petrolina, state of Pernambuco (Silva *et al.* 2014).

Several clinical signs have been observed in infected cats, such as the presence of ulcers in the ears and nose (Mello, 1940), interdigital ulcers (Passos *et al.* 1996), nodes in the nose, weight loss, dehydration, swollen lymph nodes (Savani *et al.* 2004), skin alterations (Bresciani *et al.* 2010) and facial dermatitis with scabbing and sores and in palmar and plantar areas (Coelho *et al.* 2010). However, the clinical diagnosis is complex and difficult because other skin diseases are common among these animals.

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The investigation of the history and the relation of epidemiological aspects are important points to consider for obtaining a correct clinical diagnosis because, in practice, skin diseases, which account for the majority of cases in the clinical routine of these animals, can be confused with some of the signs found in leishmaniasis.

Given that cats can be infected by several species of *Leishmania*, and that these animals do not always present a detectable humoral immune response by the techniques used in canine serological diagnostics (Poli *et al.* 2002; Solano-Gallego *et al.* 2007), the species of parasite involved in the infection should be identified and characterized (Vides *et al.* 2011).

The diagnostic confirmation and correct identification of *Leishmania* spp. is important for proper species-specific therapy as well as for epidemiologic studies. The method, internal transcribed spacers 1 (ITS1), polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), using the restriction enzyme approach was developed as an alternative to existing diagnostic procedures such as direct detection of parasites by microscopic examination of clinical specimens or by cultivation (Schonian *et al.* 2003).

Due to the increase in reported cases of *Leishmania* spp. and especially of *L. infantum* in domestic cats, the aim of this study was to use a diagnostic method for leishmaniasis, which combines high sensitivity with the differentiation of species, to determine the occurrence of *L. infantum* infection in domestic cats and to investigate the importance of this species in the epidemiological chain in an area where VL is highly endemic, the municipality of Teresina.

MATERIAL AND METHODS

Animals and samples

The study involved 83 domestic cats of both sexes and a variety of breeds, ages, sizes and weights. The inclusion criteria were animals from the Collects were conducted in the period of February to June of 2013. Inclusion criteria were 10% of attended cats in University Veterinary Hospital from Federal University of Piauí (HVU/UFPI) ($n = 22$) and 10% of the total population of cats of two neighbourhoods (Planalto Uruguai and Parque Itararé) of the municipality of Teresina, with high LV prevalence, according to information of Zoonosis Management of Teresina, using random houses.

The animals were subjected to a clinical examination, weighed and their nutritional status was assessed. To collect biological material, the animals were given an intramuscular anaesthetic of acepromazine (0.1 mg kg^{-1}) and ketamine (5 mg kg^{-1}). Five millilitre of blood was then drawn into EDTA vacutainer tubes for complete blood count

(CBC) and 5 mL in vacutainer tubes without anti-coagulant for biochemical tests, serology for retroviruses and *Leishmania*, as well as popliteal lymph node aspirates to examine for *Leishmania*.

Parasitological tests

Samples of popliteal lymph node aspirate were placed in test tubes containing NNN (Novy, MacNeal, Nicolle) biphasic culture medium with 1 mL of Schneider's supplemented with penicillin (100 IU mL^{-1}), streptomycin (100 ug mL^{-1}), and 10% fetal bovine serum, which were then incubated in a Biochemical Oxygen Demand (B.O.D.) chamber at 25°C . The culture was examined every 5 days up to day 20 in an optical microscope under $40\times$ magnification to observe the promastigote forms of the parasite. Popliteal lymph node sample was also performed smeared on a slide and stained by the Giemsa method to visualize *Leishmania* amastigotes.

Haematological and biochemical tests

The CBC was performed in a Mindray BC-2800 Vet automatic haematology analyser and the differential leucocyte count in Giemsa stain blood smears.

Serum biochemical quantifications of urea, creatinine, aspartate aminotransferase (AST), albumin and total proteins were performed in a BIOCLIN BA-88 semi-auto chemical analyser using BIOCLIN kits, as recommended by the manufacturer. The globulin concentration was calculated by subtracting the albumin from the total protein.

Detection of feline leukaemia virus (FeLV) antigen and antibodies to feline immunodeficiency virus (FIV)

To evaluate the impact of immunosuppressive retroviruses, 83 of them were tested for FeLV antigen and for FIV antibody. The detection of FeLV antigen (p27) and FIV antibody was performed using a commercial assay kit (SNAP[®] FIV Antibody/FeLV Antigen Combo Test; IDEXX Laboratories, Westbrook, ME), according to the manufacturer's recommendations.

Leishmaniasis detection enzyme-linked immunosorbent assay (ELISA)

An ELISA protocol was followed as recommended by the manufacturer, using FIOCRUZ Bio-Manguinhos kits (EIE – Canine Visceral Leishmaniasis – Bio-Manguinhos) (Ashford *et al.* 1993; Alves & Bevilacqua, 2004), with minor modifications. The sera were diluted 1:200 and anti-mouse IgG conjugated cat. A20-120P at 1:80 000. Testing was performed in duplicate, and the procedure was repeated on different days using different slides to confirm the results. Optical density (OD) was

measured at 450 nm in a URIT-660 Microplate Reader (URIT Medical Electronic Co., Ltd, Guangxi, China). Sera of the 10 control cats were placed on the same plate to calculate the limits of the cutoff. These animals became from neighbourhoods from Teresina where LV (canine and human) presents low prevalence and negative bone marrow PCR. The cutoff was calculated multiplying the mean of the negative controls by two (approximately two standard deviations). In addition, the manufacturer of kit suggests adding 20% to the cut-off value to increase the specificity of the test. Therefore, animals that presented optical density higher than the cutoff plus 20% were considered positive.

DNA Extraction

The DNA of the promastigote forms was extracted using the Genomic DNA from tissue kit (NucleoSpin® Tissue, Macherey-Nagel, Dürren, Germany), following the protocol described by the manufacturer.

Molecular analysis and identification of isolates

Leishmania-specific ribosomal ITS1 gene was amplified using the primers LITSR (5-CTG GAT CAT TTT CCG ATG-3) and L5-8S (5-TGA TAC CAC TTA TCG CAC TT-3). The PCR reaction mixture contained 1.0 µL of DNA solution, 0.2 mM of each dNTP, 0.1 nM of each primer, 2.5 U of AmpliTaq Gold® (Applied Biosystems), 2.5 µL of 10× buffer [Tris-HCl 50 mM, (pH 8.3), KCl 50 mM] and 2.0 mM MgCl₂ in a final volume of 15 µL. Positive controls with *L. infantum* (MHOM/1973/BH46), *L. amazonensis* (IFLA/BR/1968/PH8) and *L. braziliensis* (MHOM/1975/M2903) were used. Genomic DNA was used at 1.0 ng µL⁻¹. All the tests included a negative control without DNA. The amplifying conditions were: initial denaturation at 95 °C for 2 min, 32 cycles at 95 °C for 20 s, 53 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 6 min. The amplicons of about 300–350 base pairs (bp) fragments were analysed at 100 V in buffer (89 mM tris (pH 7.3), 89 mM boric acid and 2 mM EDTA) in 5% acrylamide/bis-acrylamide 39:1 gels stained with silver nitrate.

PCR products (5–10 µL) were digested with endonuclease HaeIII enzyme, according to the manufacturer's instructions. Restriction fragments were analysed at 100 V in buffer [89 mM tris (pH 7.3), 89 mM boric acid and 2 mM EDTA] in 5% acrylamide/bis-acrylamide and compared with the *Leishmania* species used as control.

Analysis of data

The *Kappa* agreement index was determined using version 8.2 of the SAS System for Windows

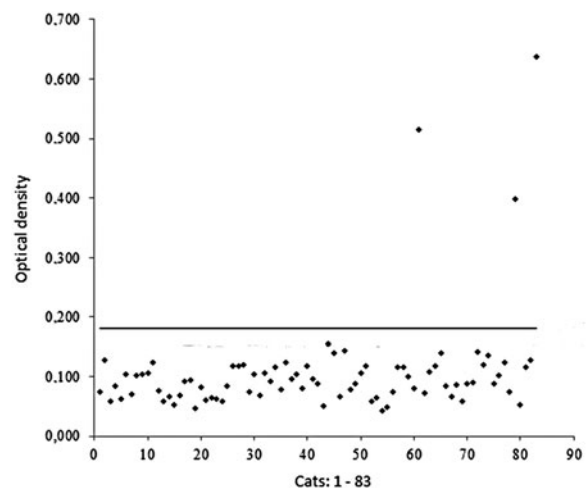


Fig. 1. ELISA anti-*Leishmania* antibody assay of 83 domestic cats in Teresina, PI, Brazil. Dots (optical density of each animal). The horizontal line indicates the cutoff (mean of the negative controls $\times 2 \times 1.2$). The three animals were considered positive with optical density value above of the cutoff.

(Statistical Analysis System) (SAS Institute Inc., Cary, NC, USA).

RESULTS

The prevalence of VL was 4% (3/83) by ELISA (Fig. 1), and 4% (3/83) animals presented *L. infantum* in popliteal lymph node aspirate, in both Giemsa-stained smear and culture medium.

The agreement between tests (parasitological and ELISA) was good (0.31, $P = 0.005$), based on the classification of the agreement index *Kappa* described by Landis & Koch (1997).

Among the 83 animals studied, only one of the cats with positive parasitological tested positive by ELISA, was also positive for FIV, presenting cachexia as a clinical sign suggestive of leishmaniasis. This was the only one of the 83 cats that presented a change in the clinical evaluation. The three cats with positive parasitological were females and adults (>6 months).

In the three animals with positive parasitological examination, a more detailed evaluation of possible biochemical changes was performed. In the haematological tests, animal I presented normocytic normochromic anaemia, with white blood cell count (WBC) within the normal range (Tables 1 and 2). Serum biochemistry showed urea levels above the physiological parameters, hyperproteinaemia, hypergammaglobulinaemia, hypoalbuminaemia and low albumin/globulin (A/G) ratio. The ELISA was positive for *Leishmania*, FIV test positive and FeLV negative (Table 3).

Animal II presented normocytic normochromic anaemia, and WBC showed eosinophilia (Tables 1 and 2). Serum biochemistry showed urea levels

Table 1. Haematological parameters of the cats under study

Cat	No. of cats	Infected	Percentage (%)
Anaemia			
No	77	0	0
Yes	6	3	50
Thrombocytopenia			
No	61	3	5
Yes	22	0	0
Leukocytosis			
No	60	2	3
Yes	23	1	4
Neutrophilia			
No	63	2	3
Yes	20	1	5
Eosinophilia			
No	60	2	3
Yes	23	1	4
Eosinopenia			
No	81	3	4
Yes	2	0	0
Lymphocytosis			
No	69	3	4
Yes	14	0	0
Lymphopenia			
No	80	3	4
Yes	3	0	0
Polycythemia			
No	81	3	4
Yes	2	0	0

above the physiological parameters. ELISA (VL), FIV and FeLV tested negative (Table 3).

Animal III presented normocytic normochromic anaemia, with WBC showing neutrophilic leucocytosis (Tables 1 and 2). Serum biochemistry showed urea levels above the physiological parameters, hyperproteinaemia, hypergammaglobulinaemia, hypoalbuminaemia, and therefore, low A/G ratio. ELISA (VL), FIV and FeLV were negative (Table 3).

The molecular biology revealed the presence of amplified fragments of 300–350 pb in the strains isolated from the three infected cats. The causative parasites were identified as *L. infantum* using ITS1 PCR and subsequent RFLP analysis (Fig. 2).

DISCUSSION

Teresina, the state capital of Piauí, is located in northeastern Brazil, 72 m above sea level, at 05°05' 21" latitude, 42°48'07" longitude. This region is considered endemic for human and canine VL. Transmission of the disease in the city started in 1981, aided by the precarious living conditions of the population and the environmental changes caused by the intense urbanization process, which presumably facilitated the vector's adaptation to the urban environment (Costa *et al.* 1990).

The provenance and domicile of cats in regions endemic for VL, living close to infected humans, dogs and rats, may trigger the transmission of *Leishmania* spp., causing them to participate as accidental hosts. The three infected cats of this study came from the same neighbourhood on the outskirts of Teresina, most of which lack basic sanitation and have tree vegetation in the proximities. The brick houses were unfinished.

The presence of phlebotomine sand flies in the city is widespread, and according to the reports of the Teresina Zoonoses Management, *L. longipalpis* can be captured throughout the year, with an incidence of 44.4% during the rainy season (December, January and February) (Gezoon, 2010).

The non-selective and remarkably zoophilic feeding behaviour of some sandfly species such as *L. longipalpis*, which is present in and around human dwellings, reinforces the hypothesis that felines can act as a food source for the vector, favouring *Leishmania* spp. infection (Dantas-Torres *et al.* 2006).

Table 2. CBC of cats with positive parasitological examination for leishmaniasis

CBC	Positive cats			Reference values ^a
	I	II	III	
Haemocyte ($\times 10^6 \mu\text{L}^{-1}$)	3.91	4.93	4.98	5–10
Haemoglobin (g dL ⁻¹)	9.3	11.2	11.4	8–15
Haematocrit (%)	17.5	22.8	24.0	24–45
MCV ^b (fl)	44.8	46.4	48.3	39–55
MCHC ^c (%)	53.1	49.1	47.5	30–36
Blood platelets ($\times 10^3 \text{ mm}^3$)	517	635	551	200–800
Total leucocytes (10^3)	9.6	13.6	24.0	5.5–19.0
Segmented	7296	5984	19 680	2500–12 500
Lymphocytes	2208	5168	3840	1500–7000
Eosinophils	0	2312	240	0–1500

^a (Jain, 1993).

^b Mean corpuscular volume.

^c Mean corpuscular haemoglobin concentration.

Table 3. Biochemical and serological tests for the identification of retrovirus in cats with positive parasitological examination for leishmaniasis

Parameters	Positive cats			Reference values ^a
	I	II	III	
Urea (mg dL ⁻¹)	43.1	69.1	59.7	20 a 30
Creatinine (mg dL ⁻¹)	1.0	1.0	1.6	0.8 a 1.8
AST (U/L)	38.2	18.6	24.0	26 a 43
Total proteins (g dL ⁻¹)	10.1	7.6	9.0	5.4 a 7.8
Albumin (g dL ⁻¹)	1.89	3.11	1.81	2.1 a 3.3
Globulin (g dL ⁻¹)	8.21	4.26	7.17	2.6 a 5.1
A/G ratio (g dL ⁻¹)	0.23	0.73	0.25	0.45 a 1.19
ELISA (VL)	+	–	–	
FIV	+	–	–	
FeLV	–	–	–	

^a (Kaneko *et al.* 1997).

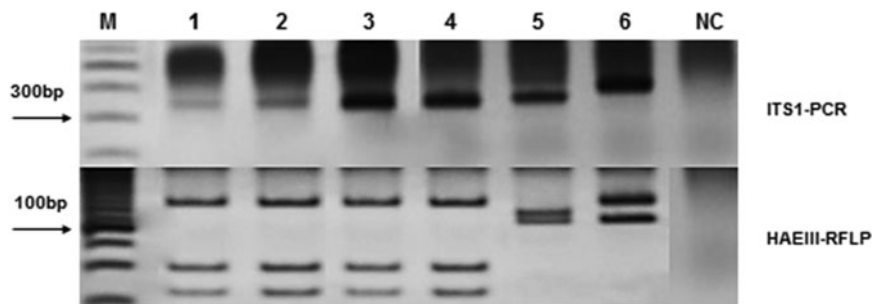


Fig. 2. ITS1 PCR gel electrophoresis of DNA of *Leishmania infantum* isolates from three cats (lines 1, 2 and 3 represent cats 1, 2 and 3, respectively). Top panel 300–350 bp, *Leishmania* ITS1 PCR product. Bottom panel shows the HaeIII restriction fragments of the ITS1 PCR product. Note the band pattern, identical to the pattern of *L. infantum* control (line 4). Line 5, *L. braziliensis*; line 6, *L. amazonensis*; NC, negative control reaction; M, molecular marker.

The low prevalence of *Leishmania* spp. in cats may be attributed to ineffective diagnosis and the natural resistance of the cat to the parasite, due to genetic factors (Maia *et al.* 2008). This possible resistance was observed in an experimental infection performed by Kirkpatrick *et al.* (1994), who detected the presence of *L. infantum* in the post-mortem inspection of all the animals in the 4th week of infection, but detected no parasites in the 24th week.

The ELISA-based serological test, the prevalence was 4% (3/83), but only one of these tested positive for *Leishmania* spp. by parasitological method. Silveira-Neto *et al.* (2011), who evaluated serum samples from cats in Araçatuba, state of São Paulo, an area endemic for human and canine VL, using ELISA with crude antigen, fucose–mannose ligand-ELISA and ELISA-rK39, reported that 23, 13.3 and 15.9% of the analysed samples, respectively, were positive.

Vides *et al.* (2011), who studied *Leishmania* in cats with skin lesions, demonstrated that the congruence between serological and parasitological tests was very low. In fact, when they evaluated the results of the positive animals, they found that most of the cats with positive serology showed negative

parasitology, and those with positive parasitology showed negative serology. This incongruence may be related to the natural resistance to infection by *L. infantum* of cats, which usually do not develop the severe visceral form but a cutaneous form with low production of specific antibodies (Solano-Gallego *et al.* 2007).

Only one animal in this study presented clinical signs suggestive of VL, a fact that is corroborated by other reports which state that a large proportion of these animals are subclinically infected (Vita *et al.* 2005; Solano-Gallego *et al.* 2007; Nasereddin *et al.* 2008; Sobrinho *et al.* 2012); hence, the actual number of infected cats may be underestimated (Miró *et al.* 2014). However, in other studies, clinical signs observed in cats with VL were crusty dermatitis, ulcerative lesions, subcutaneous nodules, weight loss, dehydration and enlarged lymph nodes (Savani *et al.* 2004; Coelho *et al.* 2010).

Leishmaniasis in cats coinfecting with FIV and FeLV has been reported in the literature (Grevot *et al.* 2005; Duarte *et al.* 2010). However, immunosuppression caused by infection by these viruses and the relationship with feline leishmaniasis should be further investigated (Poli *et al.* 2002; Grevot *et al.*

2005). In this work, one of the animals with VL tested positive for FIV, and it is suspected that retroviral coinfections increase the susceptibility of cats to leishmaniasis, being the only one to present clinic manifestation (weight loss). The lymph nodes of this animal presented an average of three amastigotes for field, possibly due to immunosuppression caused by viral infection associated with VL.

Unlike dogs affected by VL, which present immune response, particularly humoral, cats seem to have a predominantly cellular immune response to *Leishmania* infection, a fact that decreases the value of serology for diagnosis (Solano-Gallego *et al.* 2007).

The usefulness of haematological and biochemical laboratory data for the diagnosis of VL in cats is limited, although these parameters are important for the evaluation of the animals' clinical status and prognosis of the disease (Hagiwara, 2003). According to Solano-Gallego *et al.* (2009), some laboratory findings may be related to canine VL, such as renal azotemia, non-regenerative anaemia, leukocytosis or leukopenia, hyperglobulinaemia, hypoalbuminaemia, low A/G ratio, and changes in liver enzyme activities.

In this study, the cats infected by *L. infantum* showed normocytic and normochromic anaemia. Anaemia in canine VL may occur by different mechanisms: diminished erythropoiesis, chronicity of the disease, blood loss, lysis of red blood cells and erythrocyte depletion due to the production of autoantibodies, which lead to splenic sequestration (Ikeda *et al.* 2003).

Two animals (I and III) presented hyperproteinaemia with hyperglobulinaemia and hypoalbuminaemia. In canine VL, hyperglobulinaemia may be due to the humoral immune response of polyclonal B lymphocytes, which is evidenced by increased gamma globulin, decreased albumin, and inverted A/G ratio (Abreu-Silva *et al.* 2008; Trópia de Abreu *et al.* 2011; Nicolato *et al.* 2013). Hypergammaglobulinaemia may cause the formation of immune complexes, which are determinants in the pathogenesis of the infection, especially in kidney lesions. In this study, the cats I, II and III presented urea elevation (suggestive of kidney failure). However, the levels of creatinine of these animals were within normal parameters. It is therefore likely that the increase of urea in these animals is due to extra- or pre-renal factors.

It was also observed a change in AST in two cats with positive parasitological examination. Both presented AST below of the normal parameters, but this alteration not shows relation with the LV. One of the causes that favours the reduction of this enzyme in the blood are hormonal problems, however the low concentration of AST presents little clinical importance.

Molecular techniques have proved to be sensitive and powerful tools for detecting *Leishmania* directly in clinical samples as well as for parasite characterization by PCR. Several scientific papers based on ITS1 analysis have been published on the diagnosis of leishmaniasis and the identification of *Leishmania* species (Schonian *et al.* 2003). Using molecular characterization, this study identified *L. infantum* in cats in the municipality of Teresina, an area highly endemic for VL (Brasil, 2012).

Finally, the presence of cats infected with *L. infantum* in the city of Teresina, an area endemic for canine and human VL, indicates a possible participation of these animals in the epidemiological chain of *L. infantum* in intra- and peridomestic environments. Natural infection of this species in endemic areas for VL requires more studies in order to shed light on the possible role of these animals in the transmission of VL cycle.

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CONFLICT OF INTEREST

None.

ETHICAL STANDARDS

All procedures contributing to this work comply with the ethical standards on the care and use of animals in experiment. The study was approved by the Ethics Committee for Animal Experimentation – CEEA/UFPI, under Protocol No. 012/12.

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