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Detection of *Leishmania* spp. using parasitological, serological and molecular assays in asymptomatic and sick cats from an endemic area of visceral leishmaniosis in Brazil

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ABSTRACT

Objective: To analyze the prevalence of feline leishmaniosis in 55 asymptomatic cats or assess clinical disease in a visceral leishmaniasis-endemic area using parasitological, serological and molecular techniques.
Methods: Fifty-five adult domestic and non-purebred cats held at an animal shelter in a rural area of the municipality of Ilha Solteira, São Paulo, Brazil, a region endemic for canine visceral leishmaniosis, were evaluated. A total of 5 mL of blood was collected from each animal, for blood culture, serological (indirect fluorescent antibody test, soluble extract ELISA, ELISA recombinant K39 antigen) and molecular tests (PCR). At the time of blood collection, the cats underwent clinical evaluation for the presence of clinical signs.
Results: Flagellate protozoa were found in nine blood cultures (16.4%). Seropositivity by indirect fluorescent antibody test was observed in 32 serum samples (62.7%). ELISA-SE showed seropositivity in 37 cats (72.5%), while ELISA-rK39 revealed positive results in 11 cats (21.6%). *Leishmania* spp. was detected by PCR in whole blood samples from five cats (9.1%). Based on clinical evaluation, it was possible to detect the presence of clinical signs (alopecia, emaciation, pinna lesions, nose lesions, skin lesions) in 30 animals (54.5%).
Conclusions: To the knowledge of the authors, this is the first report of *Leishmania* spp. in domestic cats in the epidemiological cycle of leishmaniosis in the studied area, requiring more attention in the establishment of preventive steps to control this disease.

1. Introduction

Visceral leishmaniosis (VL) is a zoonotic disease that affects humans and other species of wild and domestic mammals that are found chronically affected with several clinical signs[1]. Domestic and wild canids are reservoirs of great epidemiological importance[2]; however, in Brazil, other animal species can also be infected, including wild rodents, marsupials and cats[3-6].

Feline leishmaniosis (FL) cases have been reported in South America[5,7-11], and in Europe[12-18] and Asia[19-21]. More properly in many cats the *Leishmania* infection is associated with an immunosuppressive condition due to feline leukemia virus and feline immunodeficiency virus, and for this reason there can be an overlap of clinical symptoms or the immunosuppression can promote the development of visceral forms of leishmaniosis[22].

The lack of an early FL diagnosis in endemic areas may imply that the cats continue to represent a potential risk of *Leishmania*

transmission to vectors. Previous studies have shown evidence of transmission of *Leishmania* promastigotes from the cat to the vector[23,24] and that cats are more attractive to sandflies for blood meals when compared to other species[25], suggesting an important role for this species in the epidemiology of VL.

Research on *Leishmania infantum* (*L. infantum*) in domestic cats has revealed its sero-epidemiological characteristics by investigating the prevalence of infection indirectly through the detection of specific anti-*Leishmania* antibodies, where the variation of results of these serological research, with seroprevalence rates ranging from 0% to 68%, even in the cats from same areas, may be attributed to diferences in the serological tests and in their cut-off values or through the detection of the parasite DNA using molecular techniques (conventional PCR and real-time PCR) or direct parasitological techniques (histology, immunohistochemistry, culture)[16,26,27].

Due to the relevance of FL, the present study aimed to investigate the presence of *Leishmania* spp. in domestic cats (*Felis catus*) from an animal shelter in Ilha Solteira, São Paulo, Brazil, which is a VL-endemic area.

2. Materials and methods

2.1. Animals, sample collection and study site

Fifty-five adult domestic and non-purebred cats held at an animal shelter (20°24'40" S, 51°20'51" W), in a rural area of the municipality of Ilha Solteira, São Paulo, Brazil, a region endemic for canine VL, were evaluated. The animals were physically restrained and intramuscularly anaesthetised with Zoletil® (Tiletamine-Zolazepam; Virbac®, Brazil) (0.15 mg/kg) for blood samples collection via jugular venipuncture. A total of 5 mL of blood was collected from each animal. A fraction (3 mL) of the total blood sample was used for blood culture and molecular analysis as well, for the all 55 tested animals. The other fraction (2 mL) was used to obtain serum for serological tests in 51 cats considering that in four animals the amount of serum was insufficient. At the time of blood collection, the cats underwent clinical evaluation for the presence of clinical signs. The study was approved by the Animal Ethics Committee, School of Medicine, São Paulo State University (Universidade Estadual Paulista - UNESP), Botucatu campus (CEEA No. 862-2011).

2.2. Blood culture, direct parasitological diagnosis

The whole blood samples were incubated in liver infusion tryptose media and kept in an oven at 82.4˚F–86˚F. The blood cultures were observed biweekly under light microscopy (400×) over a period of 4 months according to Luz[28]. At the end of the culture period and/or after the visualization of the parasites, the cultures were centrifuged (1 400 r/min), washed in phosphate-buffered saline (pH 7.4) and prepared for DNA extraction according to Pinto[29].

2.3. Serological assays

2.3.1. Indirect fluorescent antibody test (IFAT)

Promastigotes of *L. infantum* (MHOM/BR/2002/LPC-RPV –

IOC/2906) were kept in tubes containing solid Novy-MacNeal-Nicolle medium plus liver infusion tryptose medium for the sensitization of the slides using 10 μL of the promastigote suspension. Species-specific conjugate (Anti-cat IgG FITC; Sigma®, USA) was used at a 1:100 dilution in 20% Evans blue solution, considering positivity as sera with a titre equal to 40. The IFAT technique was carried out according to Camargo[30].

2.3.2. Indirect ELISA

Indirect ELISA was performed according to the technique proposed by Lima *et al.*[31] and modified by Costa *et al.*[32]. The plates (Nunc® Maxisorp Thermo Scientific) were sensitized with 100 μL/well of antigen obtained from *L. infantum* (MHOM/BR/2002/LPC-RPV – IOC/2906) lysate at a concentration of 10 μg/mL (10 000 ng/mL) and of recombinant rK39 antigen at a concentration of 25 ng/mL at 50 μL/well. The animal sera were diluted in a buffered saline solution and 10% foetal bovine serum at 1:400 for soluble extract ELISA (ELISA-SE) and 1:50 for ELISA recombinant K39 antigen (ELISA-rK39). The peroxidase-conjugated total cat anti-IgG (A20-120P; Bethyl, Montgomery, USA) was used at a 1:40 000 dilution in phosphate-buffered saline-Tween 20 and tetramethylbenzidine dihydrochloride solution (BD Biosciences Pharmingen, San Diego, CA, USA). The reaction was stopped with 0.5 mol/L sulphuric acid, and the optical density was measured at 450 nm in an ELISA reader (Universal Microplate Reader - EL 800; Bio-Tek Instruments, Inc). After ELISA was performed, the cut-off point, ELISA levels (ELs) and sample values relative to the positive controls were calculated. The mean optical densities were grouped into ELs varying from 0 to 9 as described by Machado *et al.*[33].

2.4. Extraction and PCR for Leishmania spp.

DNA was extracted from the whole blood samples of cats using the Illustra™ Blood GenomicPrep Mini Spin commercial kit (GE Healthcare®, USA). The quality and quantity of DNA were evaluated by spectrophotometry (NanoDrop 2000c - Thermo Scientific). The 720-bp region containing the sequence-specific minicircle kDNA of *Leishmania* spp. was amplified with the primer pair LINR4 (5'-GGGGTTGGTGTAAAATAGGG-3') and LIN19 (5'-CAGAACGCCCCTACCCG-3') described by Ikonomopoulos *et al.*[34]. The PCR mix used Platinum *Taq* DNA Polymerase (Invitrogen®), and the amplification conditions followed the method of Aransay *et al.*[35]. The strain (MHOM/BR/2002/LPC-RPV − IOC/2906) of *L. infantum* was used as a positive control, and ultrapure water was used as a negative control. The amplified products were visualized by electrophoresis in 1.5% agarose gels and SYBR® safe (Invitrogen). A 100-bp DNA Ladder (Invitrogen) was used as the molecular weight standard.

2.5. Statistical analysis

The results of the various FL diagnostic methods were subjected to statistical analysis using the Fortran Power Station software (version 4; University of Economics and Business, Austria), evaluating the Kappa index (95% confidence interval) and the concordance index between the diagnostic methods. According to Landis and

Koch[36], κ < 0.4 is considered weak concordance; 0.41 ⩽ κ ⩽ 0.6 is accepted as moderate concordance; 0.61 ⩽ κ ⩽ 0.80 is regarded as good concordance; and κ > 0.8 is accepted as excellent concordance.

Tukey's test with a significance level of 5% was calculated by ANOVA using the SigmaStat 3.5 statistical software to evaluate the association of the seropositivity of cats for FL using each diagnostic method (ELISA-SE, ELISA-rK39, IFAT, PCR of blood and blood culture) with clinical signs (alopecia, emaciation, nose lesions, ear lesions, skin lesions) and with the absence of clinical signs.

3. Results

3.1. Parasitological, serological and molecular diagnosis

Flagellate protozoa were found in nine blood cultures (16.4%) of the 55 cats studied. The serological techniques were performed on 51 cats due to the insufficient amount of serum obtained from four cats. Seropositivity was found by IFAT in 32 serum samples (62.7%): five animals had titres of 40 (15.6%), 18 cats had titres of 80 (56.2%), eight cats had titres of 160 (25%), and only one cat had titre of 320 (3.1%). ELISA-SE revealed seropositivity in 37 cats (72.5%), distributed at ELs greater than or equal to the cut-off point (EL ⩾ 2), with the highest number of cats with positive reactivity observed for EL = 5 (8/37; 21.6%), while 27.4% (14 cats), were considered negative. ELISA-rK39 revealed positivity in 11 cats (21.6%), distributed at ELs greater than or equal to the cut-off point (EL ⩾ 3). PCR showed the detection of *Leishmania* spp. in blood samples from five cats (9.1%).

3.2. Clinical signs

The clinical evaluation of 55 cats allowed the detection of clinical signs such as alopecia, emaciation, pinna lesions, nose lesions and skin lesions in 30 animals (54.5%). There were no clinical signs in 25 animals (45.5%); however, both cats with clinical signs and normal cats, were positive for *Leishmania* spp. in at least one diagnostic test in 90% and 76% of the animals, respectively. The presence of alopecia was the most frequent clinical sign among the cats (27.3%), which showed the highest detection of infection by ELISA-SE (93.3%), PCR (20.0%) and blood cultures (40.0%). Among the clinical signs, such as weight loss and ear and nose lesions, there were no significant differences regarding to the methods applied. Among the cats with skin lesions, 76.9% were serum reactive by IFAT and ELISA-SE, and four animals had positive blood cultures (30.8%). Among the clinically normal cats, there was positivity using all techniques, emphasising that 32.0% of the animals were reactive by ELISA-rK39 (Table 1).

3.3. Comparative evaluation of the diagnostic methods

According to the results of the diagnostic tests, there was a moderate concordance between serological tests (IFAT × ELISA-

SE), with κ = 0.335 4, and between the parasitological and molecular exams (blood culture × PCR), with κ = 0.509 6, as observed in Table 2.

Table 1

Distribution of clinical signs based on diagnosis of *Leishmania* spp. by parasitological, serological and molecular assays in 55 cats [*n* (%)].

Clinical signs	Animals	Blood culture	IFAT	ELISA-SE	ELISA-rK39	PCR[b]
Alopecia	15 (27.3)	6 (40.0)	9 (60.0)	14 (93.3)	1 (66.7)	3 (20.0)
Emaciation	12 (21.8)	5 (41.7)	8 (66.7)	10 (83.3)	0 (0.0)	2 (16.7)
Nose lesions	2 (3.6)	1 (50.0)	1 (50.0)	1 (50.0)	0 (0.0)	0 (0.0)
Pinna lesions	10 (18.2)	2 (20.0)	7 (70.0)	7 (70.0)	2 (20.0)	0 (0.0)
Skin lesions	13 (23.6)	4 (30.8)	10 (76.9)	10 (76.9)	0 (0.0)	0 (0.0)
No clinical signs	25 (45.5)	1 (4.0)	12 (48.0)	17 (68.0)	8 (32.0)	2 (8.0)

[b]: PCR on whole blood.

Table 2

Comparative analysis of diagnostic methods for FL in cats [*n* (%)] classified as clinically normal and cats with clinical signs.

Techniques	N (*n* = 21)	CS (*n* = 30)	κ	Total (*n* = 51[*])
ELISA-SE × ELISA-rK39	11 (52.4)	12 (40.0)	0.126 1	23 (45.1)
	0.153 2	0.090 9	Weak	
ELISA-SE × IFAT	13 (61.9)	23 (76.7)	0.335 4	36 (70.6)
	0.176 5	0.461 5	Moderate	
ELISA-SE × PCR[b]	3 (14.3)	8 (26.7)	-0.151 2	11 (21.6)
	-0.203 8	-0.111 1	Weak	
ELISA-SE × BC	4 (19.0)	11 (36.7)	-0.092 9	15 (29.4)
	-0.098 5	-0.067 4	Weak	
ELISA-rK39 × IFAT	9 (42.8)	11 (36.7)	-0.061 8	20 (39.2)
	-0.105 3	0.000 0	Weak	
ELISA-rK39 × PCR[b]	13 (61.9)	24 (80.0)	-0.001 3	37 (72.5)
	0.056 2	-0.111 1	Weak	
ELISA-rK39 × BC	9 (42.8)	19 (63.3)	-0.116 8	28 (54.9)
	0.150 3	-0.170 2	Weak	
IFAT × PCR[b]	9 (42.8)	8 (26.7)	-0.074 0	17 (33.3)
	-0.024 4	-0.105 3	Weak	
IFAT × BC	10 (47.6)	8 (26.7)	0.091 1	18 (35.3)
	0.072 3	0.076 9	Weak	
PCR[b] × BC	19 (90.5)	25 (83.3)	0.509 6	44 (86.3)
	0.644 1	0.468 1	Moderate	
PCR[bc] × BC	20 (95.2)	22 (73.3)	0.000 0	42 (82.3)

[b]: PCR on whole blood; BC: Blood culture; κ: Kappa index, concordance between diagnostic methods; N: Cats clinically normal; CS: Cats with clinical signs; [*]: Insufficient sera in four animals.

4. Discussion

The present study was the first report of *Leishmania* infection in domestic cats from an area that is considered endemic for VL, with several reported cases of the disease in dogs and wild animals[37,38]. The animals of this study were from an animal rights association that shelters abandoned dogs and cats. Located in the countryside, the site is surrounded by woodlands, with accumulation of organic matter and the breeding of large and small domestic animals. According to Sherlock[39] and Camargo-Neves *et al.*[40], these factors provide a favourable environment for the *Leishmania* life cycle by facilitating the growth and spread of sandflies.

Domestic cats can become infected with various *Leishmania* species and may or may not become sick, act as VL reservoirs and contribute with the maintenance and spread of the disease in domestic environments[16,17,22-24]. However, little is known about the clinical manifestations and immune response to the

disease in these animals, which is well-established in dogs. Thus, various techniques have been used in the diagnosis of FL, such as serological, parasitological and molecular techniques[5,7,11,12,14,18, 32,41].

The present study found the presence of anti-*Leishmania* antibodies in most animals with greater rate of seropositivity by ELISA-SE (72.5%; 37/51), followed by IFAT (62.7%; 32/51); the lowest positivity was observed by ELISA-rK39 (21.6%; 11/51). There was moderate concordance (κ = 0.335 4) between serological tests (IFAT × ELISA-SE). Chatzis *et al.*[26], have shown that IFAT and ELISA are characterized by low sensitivity and high specificity for the detection of infected cats, and that the diagnostic performance is not improved by changing the cut-off values. The finding of anti-*Leishmania* antibodies using IFAT and/or ELISA-SE has also been reported in cats in other endemic regions but with a lower prevalence[8,11,42,43].

The utilization of total antigens makes the serological technique more sensitive. However, recombinant antigens can also be considered less specific possibly due to cross-reactivity with other trypanosomatids because the simpler and purer the antigen is, the greater the specificity of the technique, thus decreasing the probability of cross-reactivity with other antigens and false-positive results[44-46]. Thus, the present study shows the positivity of 21.6% by ELISA-rK39, which is in agreement with the results of de Silveira Neto *et al.*[8], who observed 15.9% of positivity in cats from Araçatuba, an endemic area, using ELISA-rK39. However, we observed that ELISA-rK39 was more effective in the detection of clinically normal cats, which contrasts in studies performed in dogs. Despite of the effectiveness in the detection of infection in dogs with clinical signs, there was poor efficacy in the use of recombinant protein in asymptomatic animals[38,46]. Thus, we observed the effectiveness of the use of recombinant proteins for the early diagnosis of infection in cats, as the clinical status of these animals is poorly known regarding *Leishmania* infection.

The parasitological diagnosis remains a simple way to determine the disease, with 100% specificity and a sensitivity ranging from 30% to 96%; however, factors such as the degree of parasitism and types and processing of biological materials and colouring can interfere with the sensitivity[26,47]. The culture from blood marrow was not possible to be performed; so, the present study used a parasitological blood culture technique, which is a good test for the isolation of the parasite but whose risk of contamination is high; additionally, furthermore, the species identification needs more diagnostic tests.

Promastigotes were found in cultures, obtaining positivity in 16.4% (9/55) of the examined animals. Our results agree examining those of Braga *et al.*[11], who reported 4% (2/50) of cats as positive using the blood culture technique, with animals from a non-endemic region, and so suggesting good applicability of the technique in endemic regions. Our parasitological result also agreed with other studies performed in domestic cats with a positive parasitological diagnosis, such as the lymph node imprint technique, with the finding of amastigotes in 0.7% (2/283) of animals, and bone marrow, spleen and liver aspirate smears,

showing positivity in 4% (8/200) of animals; both studies were conducted in cats from Araçatuba, a VL-endemic area[32,48].

Little is known concerning the parasite load in cats, reflecting the difficulty of finding promastigotes and amastigotes using a parasitological technique; *Leishmania* is an intracellular protozoan, so for this reason there is a need for its association with more specific and sensitive tests, such as molecular techniques from lymph node or bone marrow punctures, because they show higher positivity if compared with prevalence obtained from blood[49].

PCR is a technique that, when combined with other tests, becomes convenient for the detection of asymptomatic carriers of the parasite in endemic areas and for the evaluation of vectors and animal reservoirs involved in the cycle of leishmaniosis[50]. In the present study, the use of whole blood was shown to be more sensitive for the detection of *Leishmania* spp., with 9.1% (5/55) of positivity. Nevertheless, the choice of blood for PCR is commonly used, and our results agree with those found in other studies[4,20]. Because it is a specific and sensitive technique, with the use of a small amount of biological material, we believe in the efficacy of the combination of PCR with other tests for diagnostic confirmation, along with clinical confirmation and the animal origin of endemic areas.

Regarding clinical evaluation of the animals, cases of alopecia, weight loss, and pinna, nose and skin lesions were observed, which are clinical signs similar to those described in FL[5,16,51]. The association between skin lesions in cats from the endemic region and VL may exist because lesions can provide a gateway to sandflies, which can bite in areas with less hair; however, we cannot rule out the possibility that these animals have co-infections with feline immunodeficiency virus or feline leucemia virus, with clinical signs similar to those of VL[5,52,53].

We observed that among 54.5% (30/55) of cats that had clinical abnormalities, 90% (27/30) were positive for at least one type of diagnosis. However, 45.4% (25/55) of the animals showed no clinical signs, and 76% (19/25) were positive according to at least one technique, suggesting the importance of clinically normal cats as carriers of leishmaniosis, similar to what occurs in dogs. We also observed that animals with and without clinical signs had higher rates of reactivity by serological techniques, indicating that ELISA-SE and IFAT can be recommended for diagnostic screening. Similar results were not found by ELISA-rK39 in our study, which showed higher positivity in animals without clinical signs. In cats, rK39 antigen may not be also used for large-scale screening, contrary to what described in dogs[8].

Miró *et al.*[17], observed 5.7% (20/346) of cats with presence of clinical signs compatible with those found in cats with FL, and, of these, only one animal had positive serology. Chatzis *et al.*[16], found more positive results using the molecular technique in cats with clinical signs. Thus, regardless of the presence or absence of clinical manifestation in these animals, especially in endemic region for this zoonosis, the association of different diagnostic tests is necessary, since many times disease may be unnoticed due to lack of signs or confused with other infections[17].

Due to the high reactivity of the RIFI and ELISA tests in this

study, they should be considered as a population screening test in cats, mainly from regions endemic to VL; in this study, the isolation of promastigote forms, as well as the molecular detection of *Leishmania* spp. in blood samples, indicates the occurrence of infection by this agent in domestic cats from an endemic region for VL. This fact displays an alert to the authorities and veterinarians, as well as the need for more studies to clarify the epidemiological importance of domestic cats in the cycle of leishmaniosis and the educational measures for the better knowledge of this zoonosis for the community as well.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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