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Xenodiagnosis in four domestic cats naturally infected by *Leishmania infantum*

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Abstract

Leishmaniasis is a neglected tropical disease that continues to pose a serious public health problem. Albeit dogs have long been held as the major reservoirs of *Leishmania infantum*, the involvement of domestic cats in the zoonotic cycle of visceral leishmaniasis has gained prominence. Here, 240 cats were evaluated by clinical signs and haematological/biochemical changes compatible with leishmaniasis and were diagnosed by serological, molecular, and parasitological techniques. Thus, four cats naturally infected by *L. infantum* were submitted to xenodiagnosis. A total of 203 females of *Lutzomyia longipalpis* were subjected to feeding on four cats, with all females completing the blood meal. Parasitological and molecular assays were carried out to evaluate the presence of *L. infantum* in the sand flies' midgut. Promastigotes were observed in 10 females (6.5%) that fed on one cat, and *L. infantum* DNA was detected in 17 (8.4%) females that fed on two cats. Our results strengthen the evidence that naturally infected cats are capable of transmitting *L. infantum* to sand flies.

KEYWORDS
Feline leishmaniasis, *Leishmania infantum*, *Lutzomyia longipalpis*, visceral leishmaniasis

1 | INTRODUCTION

Visceral leishmaniasis (VL) is a neglected disease caused by intracellular protozoa of the *Leishmania donovani* complex that is transmitted by phlebotomine sand flies (Lana et al., 2018). In the Americas, Mediterranean Basin and Asia, *Leishmania infantum* is the causative agent of zoonotic VL, with dogs held as the main domestic reservoir (Miró & López-Vélez, 2018; Werneck, 2014).

The sand fly *Lutzomyia longipalpis*, the primary vector of *L. infantum* in Brazil, is adapted to different climatic conditions, possessing an opportunistic feeding behaviour (Lainson & Rangel, 2005). In the 1950s, this sand fly species predominantly inhabited rural areas (Alencar, 1959); nonetheless, it is now adapted to urban environments following human migration and changes to its natural ecosystem (Galvis-Ovallos et al., 2020; Oliveira et al., 2016). *Lutzomyia longipalpis* thrives in environments with the presence of domestic animals, fruit trees, and organic matter (Lana et al., 2018).

There has been an increase in geographical distribution of VL in Brazil possibly due to the spreading of the vector and deficiencies in control measures (Sevá et al., 2016, 2017). Of note, VL dynamic shows focal differences, requiring the evaluation of other parameters related to the vector dynamics, environmental variables, and the role of unknown urban reservoirs.

Feline leishmaniasis (FL) has already been described in the Americas, Europe, North Africa, and the Middle East (Pennisi & Persichetti, 2018; Vides et al., 2011). Similarly, there have been increasing reports

of domestic cats infected by *L. infantum* without clinical signs in endemic areas (Pennisi & Persichetti, 2018); notwithstanding, the role of these animals in *L. infantum* transmission dynamics have received little attention.

Xenodiagnosis is a technique that can be used to identify putative reservoir species of *L. infantum* since it makes possible to determine whether a particular host that is infected is able to transfer naturally the pathogen to a potential vector (Maia & Campino, 2011; Quinnell & Courtenay, 2009), and to evaluate the development of promastigotes in the sand flies' midgut.

In dogs, the *Leishmania* infection rate of phlebotomines seems to be related to the disease severity; however, studies evaluating this aspect in domestic cats are lacking, despite to have investigations with cats presenting clinical signs of leishmaniasis that developed *L. infantum* promastigotes in *Phlebotomus perniciosus* and *Lu. longipalpis* (Maroli et al., 2007; de Mendonça et al., 2020; da Silva et al., 2010).

Based on the foregoing observations, the present study aimed to evaluate the infectiveness of cats naturally infected by *L. infantum* for colonized *Lu. longipalpis* females and the development of promastigotes in their midgut, as well as to assess the clinical characteristics of infectious cats.

2 | MATERIALS AND METHODS

2.1 | Local

The cats included in the study were kept in two animal protection shelters, located at the municipality of Ilha Solteira (20°25′58″S 51°20′33″W), far west of São Paulo state, Brazil. Ilha Solteira is endemic for VL with five human cases notified from 2007 to 2019 (Brasil, 2019). On the last official census, there were 25,064 inhabitants in the municipality (Brasil, 2010). The prevalence of canine leishmaniasis in 2007 and 2008 is ranging from 3.0% to 14.5% among the five areas of Ilha Solteira (Paulan et al., 2012).

2.2 | Selection of animals

During 2015 and 2019, 240 cats passed through the two shelters and were screened for *L. infantum* infection. The seropositivity rate in domestic cats was found to be 25.4% in both shelters and in one of them *Lu. longipalpis* had already been captured (Leonel et al., 2020a). Clinical evaluation included the characterization of leishmaniasis signs and hematological and biochemical parameters. Additionally, parasitological, molecular, and serological diagnoses were performed. Lymph node and bone marrow aspirates and blood samples were collected and submitted to cytology and culture assays. Indirect Immunofluorescence Antibody Test (IFAT, cut off 1:40) and Indirect Immunoenzymatic Assay (ELISA, cut off ≥ 3) were carried out according to Vides et al. (2011) and Costa et al. (2010), respectively, to detect anti-*Leishmania* spp. antibodies. Blood samples were used for molecular analysis. The DNA was extracted with the DNeasy Blood & Tissue kit (QUIAGEN, USA), according to the manufacturer. The kDNA's mini-circle was amplified according to Rodgers et al. (1990), with 13A 5′-dGTG GGG GAG GGG CGT TCT-3′ and 13B 5′-dATT TTA CAC CAA CCC CCA GTT-3′ primers. It was possible to observe the amplification product through the 2% electrophoresis gel. A second PCR was performed only in samples positive for *Leishmania* spp., with amplification of the ribosomal DNA of the conserved region of the Internal Transcribed Spacer 1 (ITS1), according to El Tai et al. (2000), using the primers LITSR 5′-CTG GAT CAT TTT CCG ATG-3′ and L5-8S 5′-TGA TAC CAC TTA TCG CAC TT-3′. All PCR assays were performed using 5 μl of extracted DNA. Amplified ITS1 fragments were purified and sent to the DNA Sequencing Service of the Human Genome and Stem Cell Research Center-Biological Institute (IB) of the University of São Paulo (USP).

The nucleotide sequences were edited manually using the Sequence Scanner software 2.2 version and aligned on Clustal W software (available in BioEdit Sequence Alignment Editor, version 7.1.11; Ibis Biosciences, Carlsbad, CA, USA). *Leishmania* species identity was tentatively assessed by analyses of the obtained nucleotide sequences on the basis of the closest BLASTn match (identity ≥ 100% using MegaBLAST and considering a query cover no smaller than 98%) with homologous sequences deposited at GenBank.

Feline viruses were also screened (Feline Leukemia Virus Antigen-Feline/Immunodeficiency Virus Antibody Test, IDEXX Laboratories, One IDEXX Drive; REF 99-08354, lot MM813).

2.3 | Xenodiagnosis

Only the negative ones for feline immunodeficiency virus (FIV) and feline leukaemia virus (FeLV), with antibodies against *Leishmania*, harbouring *L. infantum* DNA in their blood were submitted to xenodiagnosis. The cats were exposed to sand flies in the Laboratory of Immunoparasitology of the São Paulo State University (UNESP), in Ilha Solteira, SP, Brazil. Considering potential differences in infectivity to the sand flies, the xenodiganosis was performed in two replicates for three cats (with 3 months of interval). In the second xenodiagnosis, there was an inclusion of a new cat. The two xenodiagnosis were equally performed (Figure 1).

2.3.1 | Sand fly specimens

Female phlebotomines of 3–5 days old, reared in a closed colony of *Lu. longipalpis* were used. The phlebotomines were maintained according to Killick-Kendrick & Killick-Kendrick (1991) and modified according to Galvis-Ovallos et al. (2017) at the Entomology Laboratory, Faculty of Public Health, of the University of São Paulo (USP).

2.3.2 | Exposition to sand flies

The cats were anaesthetized with an association of zolazepam hydrochloride, tiletamin hydrocloridate (11 mg/kg), and tramadol

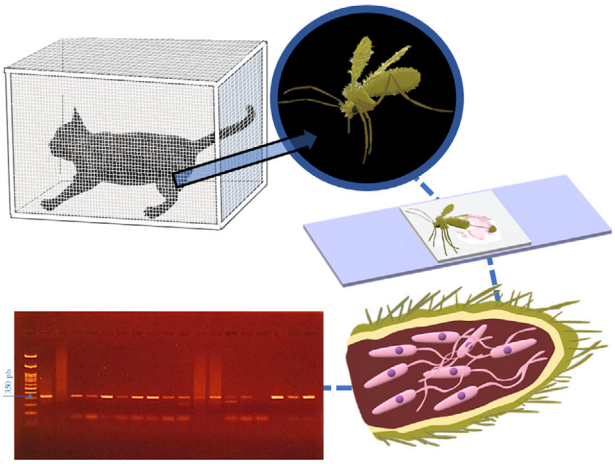

FIGURE 1 Schematic of xenodignosis performed in domestic cats naturally infected by *L. infantum*. First, anesthetized cats were exposed to *Lu. longipalpis* in entomological cages. Afterwards, the sandflies were retrieved for dissection followed by parasitological assay for observation of promastigotes in the midgut of sandflies. Finally, the sandflies' midgut were subjected to molecular assay to confirm *L. infantum* infection.

(2 mg/kg), and thereafter placed in individual 50 × 30 × 30 cm nylon cages. Following this, sand flies were released in the cages. Male phlebotomines were also released to stimulate the blood feeding of the females. The cats were kept completely exposed for 60 min (Figure 1).

2.3.3 | Parasitological assay

The dissection was performed in a stereomicroscope at a 60× amplification, shortly after the females died as described by Diniz et al. (2014). The surviving females up to 168 hours after the blood meal were euthanized by freezing at −12°C for 5 min. The midgut was analyzed with an optical microscope (400×) for the investigation of promastigotes. The intensity of promastigotes was expressed using a qualitative approach according to Travi et al. (2001), with (–) absence of promastigotes; (+) presence of 1–50 promastigotes (++), 51–100 (+++) 101–200; and (++++) > 201 promastigotes. After observation under a microscope, the coverslip was lifted with the aid of forceps, and washed with absolute alcohol; then, its contents were aspirated with the aid of a micropipette and transferred to a microtube. The same procedure was done in relation to the slide with the female gut, with all material transferred to the same microtube. The midgut samples were maintained at −20°C until DNA extraction.

2.3.4 | Molecular assay

Due to differences in sensitivity between dissection and molecular detection, the presence of *Leishmania* DNA was also evaluated in all females by PCR. The DNA extraction of sand flies was performed individually, according to Bruford et al. (1998) and modified by Galvis-Ovallos et al. (2017). The in-house protocol consisted on maceration of dissected sand fly into a cellular lyses detergent Digsol Buffer followed by addition of Proteinase K. Proteins denaturation occurred due the addition of ammonium acetate and DNA preciptation, due to addition of absulute alcohol followed by a 70% alcohol, resulting at 50 μl of re-suspended DNA in a median concentration (Galvis-Ovallos et al., 2017; Leonel et al., 2020b). The quality of the extraction was verified by amplifying the cacophonic gene IVS6 present in sand flies, with primers 5LIcac 5′-GTGGCCGAACATAATGTTAG-3′ and 3LIcac 5′-CCACGAACAAGTTCAACATC-3′, described by Lins et al. (2008) and PCR was performed as described by Pita-Pereira et al. (2005). To identify the presence of *Leishmania* spp. in samples of extracted DNA from female sand flies, the same molecular tests described above for detecting positive cats were performed.

3 | RESULTS

3.1 | Epidemiological characteristics of cats

Serological assays revealed that 151 (62.9%) of the 240 evaluated cats were seropositives. Regarding the seropositive cats, it was observed at least one clinical sign in 105 (69,6%) of them. Loss of body weight in 43 (40.9%), skin lesions in 39 (37.1%), including lesions on the ears, on the face and on the body; alopecia in 36 (34.3%) and lymph node enlargement 13 (12.3%) were the most commom clinical signs observed. Conversely, cachexia in six (5.7%), ocular lesions in three (2.8%), gingivitis in three (2.8%), and diarrhoea in four (2.8%) were also observed. Between haematological findings, leukocitosis and thrombocytopenia were the most observed, while increase of total proteins due to increase of globulins was the most observed change in biochemical analysis. Regarding the molecular assays, 11 (4.6%) cats had *Leishmania* spp.'s DNA amplified and ITS-1 sequences from 6 (54.5%) showed 100% identity with *L. infantum* (Table 1). Two cats died of unknown reasons before the first xenodiagnosis.

3.2 | Xenodiagnosis

Altogether, 203 female sand flies fed on four cats naturally infected by *L. infantum*, with all of them becoming completely engorged. The number of females varied from 39 to 63 for each cat in both experiments (Table 2). In the first experiment, three cats (cat no. 1–3) were exposed. A high mortality rate of females (50.5%) was observed in the first 48 h following feeding (Figure 2a). There was no identification of promastigotes in a total of 64 (66.0%) dissected sand flies; however, *L. infantum* DNA was detected in one female (Table 2) at 168 h after blood feeding (Figure 2a).

Three months after the former xenodiagnosis, another replicate was performed. This second xenodiagnosis included the three previously evaluated cats and one new cat (cat no. 4) was added, totalling four animals. The first three cats remained with the same clinical characteristics, antibody titres and PCR positivity, and were kept at the shelters,

TABLE 1 Clinical and diagnostic assessments of cats selected to undergo xenodiagnosis

Animal	Clinical signs	Blood count and biochemical profile	Culture (tissue)	Cytology (tissue)	Serology		Molecular		Sequencing
					ELISA/CO	IFAT/ Tit.	kDNA	ITS1	
1	Loss of body weight, enlarged popliteal lymph node, alopecia, skin peeling, microlesions on the tip of the ear	↑ Leukocytes (eosinophils); ↑ Urea, AST, AF and globulins; ↑ TP	+ Lymph node	+ Lymph node	+/6	+/1:40	+	+	99.7% *L. infantum*, access number MN648767.1
2	Loss of body weight, skin lesions throughout the body and alopecia	↑ Leukocytes (eosinophils); ↑ Globulins; ↑ TP	N	N	+/6	+/1:40	+	+	100% *L. infantum*, access number LR812960.1
3	Loss of body weight, ear and snout injuries	↑ Leukocytes (eosinophils); ↑ AF; ↑ TP.	N	N	+/5	+/1:40	+	+	*L. infantum*, access number LR812960.1
4	Loss of body weight	↑ Leukocytes and ACHC, ↓ ACV e platelets; ↓ GGT and albumin, ↑ TP and globulin; ↑ Lymphocytes	+Bone marrow +Blood	+Bone marrow +Lymph node	+/9	+/1:320	+	+	100% *L. infantum*, access number LR812960.1

Abbreviations: 13A/13B, kDNA of *Leishmania* spp.; ACHC, average corpuscular hemoglobin concentration; ACV, average corpuscular volume; AF, alkaline phosphatase; AST, aspartate aminotransferase; CO, cut-off ≥ 3; GGT, glutamyl transferase; N, negative; ITS1, rDNA–SSU-rDNA trypanosomatids internal trancribed spacer 1; Tit, titration – cut-off: 1:40; +, positive; TP, total protein.
↑: Levels above reference values; ↓: levels below the reference values, as proposed by Lacerda et al. (2014).

TABLE 2 Description of the results of xenodiagnostics 1, 2 and total

Feline	Xenodiagnosis 1					Xenodiagnosis 2					Total				
	N	Eng †	Dis‡	Paras §	PCR	N	Eng	Dis	Parasit	PCR	N	Eng	Dis	Parasit	PCR
1	38	38	15 39.5%	0	0	25	25	25 100.0%	0	0	63	63	40 63.5%	0	0
2	28	28	21 75.0%	0	0	23	23	19 82.6%	0	0	51	51	40 78.4%	0	0
3	31	31	28 90.3%	0	1 3.2%	19	19	18 94.7%	0	0	50	50	46 92.0%	0	1 2.0%
4	-	-	-	-	-	39	39	28 71.8%	10 35.7%	16 41.0%	39	39	28 71.8%	10 35.7%	16 41.0%
T	97	97	64 66.0%	0	1 1.0%	106	106	90 84.9%	10 11.1%	16 15.1%	203	203	154 75.9%	10 6.5%	17 8.4%

Abbreviation: N, number of sand fly females.
†Engorged females.
‡Number of females dissected and percentage (%).
§Parasitological result (compared to the percentage of dissected females); PCR: Molecular test for the 13A/13B kDNA and ITS1 targets (related to all females).

under the same conditions as before. In total, 67 female sand flies fed on the three cats evaluated in the former xenodiagnosis (ranging from 19 to 25 females for each animal). In the same way, 100% of females phlebotomines were engorged and 62 (92.5%) of them underwent dissection. A high mortality rate was observed during the first 24 h following feeding (Figure 2b). No promastigotes were observed in their midgut; likewise, there was no detection of *Leishmania* DNA (Table 2).

As aforementioned, a new cat was included in the second xenodiagnosis; 39 female sand flies fed on this animal and 28 (71.8%) of them were dissected. Contrastingly, a low proportion of these sand flies died during the first 72 h (26.0%). The highest proportion of deaths occurred 96 h after the blood meal, that is, 16 sand flies (41.0%) died (Figure 2c). Promastigotes were observed in the midgut of 10 (35.7%) out of 28 dissected females that fed on this cat, 96 h after blood meal (Table 2; Figure 2c). Regarding the intensity of promastigotes in the midgut, a low parasite load was observed in eight females (+); one female had moderate infection (++) and one a high parasite load (++++) (Figure 2d).

3.3 | PCR assays

A 220–250 bp fragment of the cacophonic IVS6 gene was amplified in all 203 sand fly's DNA samples. Additionally, all female sand flies were evaluated for the mini-circle kDNA PCR. First, 14 (6.9%), that is, 13 sand flies feed on cat no. 4 and one feed on cat no. 3, tested positive for kDNA PCR. Of these kDNA PCR positive sand flies, it was possible to observe promastigotes in the midgut of seven (50.0%) females. Moreover, three parasitological positive samples were negative by kDNA PCR, so they were included on the ITS-1 PCR. Thus, a total of 17 (8.4%) females were tested for ITS-1 PCR. From them, 16 (94.1%) had ITS-1 region amplified – only the positive kDNA PCR fed on cat no. 3 was negative. From 203 females, the molecular analyses (kDNA and ITS-1 PCRs) resulted in 17 positive females; of these, one female fed on cat no. 3 (Figure 2a), and 16 fed on cat no. 4 (Figure 2c; Table 2). The DNA amplified and sequenced from the midguts showed 100% identity with *L. infantum* (accession number LR812960.1).

4 | DISCUSSION

In the present experiment, all 203 *Lu. longipalpis* females exposed to the cats were engorged. Promastigotes were observed in 10 of the 154 dissected females (6.5%) while *L. infantum* DNA was detected in 17 of 203 (8.4%) evaluated females, which fed on two out of four cats submitted to the xenodiagnosis. The observation of parasitic forms in sand flies was only possible in the cat presenting the highest antibody levels determined by ELISA and IFAT and with positive parasitological tests (i.e., culture and citology) performed in different tissues (i.e., blood and bone marrow). Despite body weight loss was the only clinical sign compatible with leishmaniasis observed, this animal presented intense changes in laboratory parameters, including thrombocytopenia and hyperglobulinemia; this emphasizes the importance of performing haematological and biochemical tests for the diagnosis of the disease in endemic areas. Accordingly, alterations in biochemical parameters such as increased levels of serum urea and aspartate aminotransferase were observed by da Silva et al. (2010).

Xenodiagnosis is a highly specific technique, but with low sensitivity, since it requires a well-trained entomologist to perform the dissection of the vectors precisely (Singh et al., 2020). In this respect, when performed together, molecular assays have contributed to increase the sensitivity of xenodiagnosis (Bravo et al., 2012). To the better accuracy of xenodiagnosis, first the partial amplification of cacophony IVS6 region present in sand flies was performed to confirm the quality of the DNA extraction (Lins et al., 2002; Mazzoni et al., 2002). Then, the kDNA, which due to the thousands of copies in the parasite genome possesses a good sensitivity, was targeted (Fernandes et al., 2019; Liu

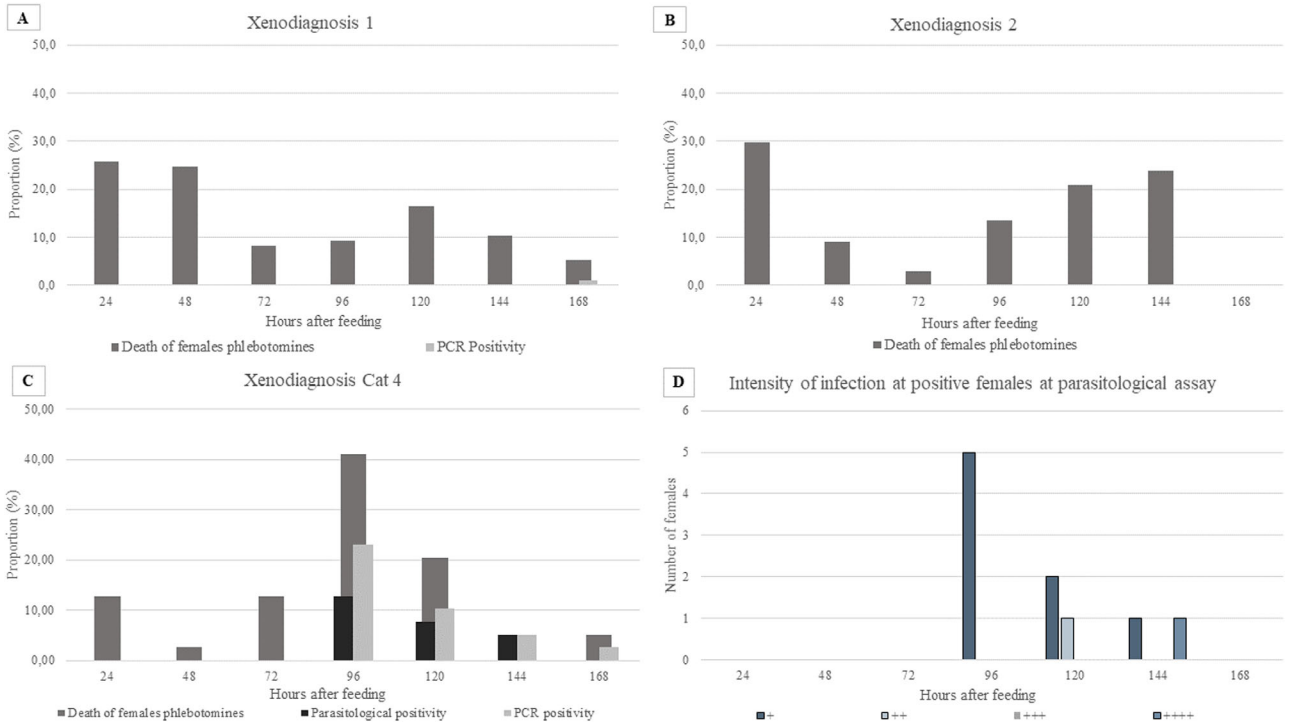

FIGURE 2 Death rates, PCR, and parasitological positivity of females sand flies in xenodiagnosis 1 and 2 during the course of 168 h after experiments, and intensity of infection of parasitological positive females. (a) Percentage of dead females phlebotomine and PCR positivity observed in xenodiagnosis 1; (b) proportion of death of females sand flies observed in xenodiagnosis 2; (c) proportion of death of females phlebotomine, PCR, and parasitological positivity arisen from the xenodiagnosis of cat number four; (d) parasitological classification, as proposed by Travi et al. (2001), of intensity of promastigotes females sand flies: (+) presence of 1–50 promastigotes (++), 51–100 (+++) 101–200; and (++++) > 201 promastigotes

et al., 2005). To complement the midgut dissection and kDNA-PCR results, the amplification of the ITS-1 was performed due to its usefulness for species identification and high specificity (Koltas et al., 2016). However, in our study three positive samples by both ITS-1 region and parasitological assays were not amplified by kDNA PCR.

Since the first report of FL (Sergent et al., 1912), new cases of the disease or infection have been described worldwide, including South America (Benassi et al., 2017; Oliveira et al., 2015; Savani et al., 2004; Vides et al., 2011), Europe (Maroli et al., 2007; Martín-Sánchez et al., 2007; Pennisi et al., 2013), and Asia (Hatam et al., 2010; Nasereddin et al., 2008). Hitherto, there have been reports of three xenodignostics confirming the transmissibility of *L. infantum* naturally infected cats to two proven vector species (Maroli et al., 2007; de Mendonça et al., 2020; da Silva et al., 2010). The first xenodiagnosis was carried out in Italy, using *P. perniciosus* females (Maroli et al., 2007), while the other two were performed in Brazil using *Lu. longipalpis* reared in colony (de Mendonça et al., 2020; da Silva et al., 2010). In the first study conducted in Brazil, only one ill cat was used to test its infectiousness to *Lu. longipalpis*, while the second (Mendonça et al., 2020) used 12 cats with clinical signs.

After dissection of the midgut, the presence of promastigotes from 96 to 144 h after the blood meal was observed, which indicates that the development of *L. infantum* after blood digestion is possible, showing that not only clinically symptomatic cats with parasitological positive tests, but also parasitological positive cats with laboratorial changes (i.e., increase of leukocytes, ACHC, TP, globulin and lymphocytes; decrease of ACV, platelets, GGT and albumin) may be infectious to the female sand flies.

Moreover, not all infected cats were infectious to the female sand flies. It is noteworthy that the cat infectious to sand flies had only signs of body weight loss, which is a nonspecific clinical sign; nonetheless, the cat was positive in the parasitological and serological tests as well as in the molecular analyses. In addition, the cat presented important hematological and biochemical changes. These results complement the study of Mendonça et al. (2020) who did not evaluate biochemical or hematological parameters. Furthermore, taking into consideration above studies and ours, most felines capable of transmitting *L. infantum* to the vector had at least three clinical and/or biochemical changes and all were positive by bone marrow cytology.

Our results showed a high mortality in non-infected females at the first 24 and 48 h after the blood meal in xenodiagnosis 1 and 2, respectively, while infected females started to die 96 h after repast – and no *L. infantum* DNA was found at 24, 48, and 72 h after feeding. Besides the lack of evidence if, in fact, the infection by *L. infantum* improves the survival of phlebotomines, it is feasible that the parasite develops surviving mechanisms in infected sand flies' midgut, extending the vector's life expectance (Kamhawi, 2006; Rogers & Bates, 2007). On the other hand, the small number of females, added to their exposition

to external conditions (i.e., the long distance between Ilha Solteira – where xenodiagnosis were performed – and São Paulo – where females were maintained and evaluated) could bias a statistical correlation.

Although human VL has been traditionally correlated with canine leishmaniasis (Belo et al., 2013; Camargo-Neves et al., 2001; Margonari et al., 2006), with a higher prevalence in dogs than in humans (Pennisi & Persichetti, 2018), inconsistent epidemiological data have already been reported in Brazil regarding the role of cats (Belo et al., 2013). The first autochthonous case of a cat infected by *L. infantum* in the Americas occurred in a municipality not endemic, at the time, of the state of São Paulo, Brazil, in which neither autochthonous human cases nor canine cases had been reported (Savani et al., 2004).

Also, the infectiouness of cats to sand flies and the development of the disease by cats may imply the use of measures to prevent felines of being infected by *L. infantum*. To the best of our knowledge, only collar impregnated with imidacloprid 10% and flumetrhin 4.5% was evaluated as preventive measure. It demonstrated to be safe and a potential tool to prevent FL, due to its efficacy of 75% against *L. infantum* infection (Brianti et al., 2017). However, it is also important to evaluate the efficacy of different types of application and active principles that have shown to be effective against phlebotomine bites in dogs and that is also available to use in cats, i.e., spot-on fluralaner, tested as systemic insecticide in dogs (Gomez, Chapman, et al., 2018; Gomez, Lucientes, et al. 2018).

5 | CONCLUSION

Our results show that cats with parasitological positive tests and few apparent clinical signs, but with haematological and biochemical alterations and high serological titres can be infectious to the vector *Lu. longipalpis*. Further epidemiological studies are warranted to shed light on the role of cats in VL transmission.

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ETHICAL APPROVAL

This study was approved by the Ethics Committee of the Faculty of Veterinary Medicine and Animal Science at São Paulo University, under protocol numbers: 7627010517 and 8541011019.

CONFLIT OF INTEREST

The authors declare that there is no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author Dr. Oliveira (tricia@usp.br) or the first author Mss. Vioti (geovanna.vioti@usp.br), upon reasonable request. Also, a short video is available on https://www.youtube.com/watch?v=1MuV_XSqu6k.

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