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Use of the intradermal leishmanin test (Montenegro skin test) for feline visceral leishmaniosis: Detection of cellular immunity

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ABSTRACT

This study evaluated the humoral and cellular response in 100 cats living in an endemic area of visceral leishmaniosis (VL) using the Montenegro Skin Test (MST) and serological diagnosis and compared the MST with other diagnostic techniques. Sixty 60%, (60/100) cats were positive for MST and the diameter of positive skin reactions ranged from 5 to 9 mm. By serological methods, 74% (74/100) and 34% (34/100) had antibodies against *Leishmania* spp. by Immunofluorescence Antibody Test (IFAT) and Indirect Enzyme-Linked Immunosorbent Assay (ELISA), respectively. Comparing tests, the observed profiles were (1) IFAT (+)/MST (−) = 27 cats, (2) IFAT (−)/MST(+) = 13 cats, (3) IFAT(+)/MST(+) = 47 cats, (4) ELISA(+)/MST(−) = 12 cats, (5) ELISA(−)/MST(+) = 38 cats and (6) ELISA(+)/MST(+) = 22 cats. Through the combination of serological diagnosis and MST, a positivity frequency of 87% (87/100) by IFAT + MST and 72% (72/100) by ELISA + MST was identified in this cat population. Five cats (5%) were positive for *Leishmania donovani* complex DNA by molecular analysis, and two cats (2%) had *Leishmania* spp. amastigotes in lymph node smears. Therefore, the agreement between tests was classified as poor for all tests by Kappa index. The IFAT (+)/MST (+) response was the most frequent considering all cats (47%; 47/100); nonetheless, the most frequent immune expression in Polymerase Chain Reaction (PCR)-positive cats was the IFAT (+)/MST (−) profile (80%; 4/5). Five sick and PCR-positive cats, negative for Feline Immunodeficiency Virus (FIV) and Feline Leukemia Virus (FeLV), that PCR sequencing matched 100% with *L. donovani* complex, all but one were MST negative. These results suggest that cats develop a significant cellular response against infection by parasites of the *L. donovani* complex, and most PCR and parasitological positive cats may be unable to develop a significant cellular response.

1. Introduction

The delayed-type hypersensitivity (DTH) is characterized by inflammatory reactions initiated by lymphocytes, responding specifically to allergens by the release of lymphokines and the development of specific cytotoxicity, without the participation of free antibodies (Godfrey and Gell, 1978). Locally, it is manifested by lymphocytic and mononuclear cell infiltration at the site where the antigen is injected (Weck, 1998). The Montenegro Skin Test (MST) is a skin hypersensitivity test that measures DTH reactions (De Luca et al., 2001) to an intradermal injection of a suspension of killed *Leishmania* spp. promastigotes (Manzur and Bari, 2006). Skin hypersensitivity tests are also used for the diagnosis of tuberculosis (Zijlstra and EL-Hassan, 1993), and cutaneous leishmaniosis (CL) (Antonio et al., 2014).

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In humans, MST has been used since the 1920s (Montenegro, 1926), with a sensitivity rate of 94% and 98% for cutaneous lesions of 4- and 6-weeks duration, respectively, in patients with CL (Manzur and Bari, 2006). However, in a study of diffuse CL, patients from the Dominican Republic showed a monocyte suppression of antigen-specific lymphocyte, been negative in MST (Petersen et al., 1984).

Primarily indicated to aid in CL's diagnosis, some studies have proven MST effectiveness in identifying humans and dogs with visceral leishmaniosis (VL) (Crescente et al., 2009; Solano-gallego et al., 2001).

In studies of VL, MST can be a useful tool for diagnosis, where the test is negative during the acute stage but becomes positive following the resolution of clinical symptoms (Pearson and Sousa, 1996). The peripheral blood mononuclear cells of human patients with acute untreated VL, when stimulated with *L. infantum* extract, present a lower proliferative response than those obtained from individuals in a control group or cured of VL (Carvalho et al., 1981); thus, VL is associated with impaired cell-mediated immunity (Alexander and Phillips, 1980; Bryceson, 1970). In that regard, MST may be a useful tool for assessing the prevalence of inapparent infection with *Leishmania,* to quantify the transmission rate over time (Souza et al., 1992) and follow-up in vaccination programs (Mayrink et al., 1979). According to Carstens-Kass et al. (2021), the DTH observed by the use of leishmanin skin test can be a valuable tool for surveillance, epidemiological studies and vaccine clinical trials in VL elimination programs.

In dogs, MST has proven to be an interesting tool to detect animals with a strong cellular immune response that protects the animals against parasite proliferation, so that, most of them would be resistant to infection and/or disease progression.

Symptomatic VL has been associated with several mechanisms involving T cells, such as decreased T cell numbers and the low expression of gamma interferon (IFN-γ) and interleukin-2 in peripheral blood mononuclear cells, and the absence of delayed-type hypersensitivity (Cardoso et al., 1998; Pinelli et al., 1994; Silveira et al., 2012). There is still no knowledge of official diagnostic techniques for feline leishmaniosis (FeL), thus, VL diagnostic tools that are available for dogs are also used in cats, being them serological, cytological, histological, culture and molecular (Pennisi et al., 2015). Among the serological methods, Immunofluorescence Antibody Test (IFAT) and Indirect Enzyme-Linked Immunosorbent Assay (ELISA) are the most used in epidemiological studies (Persichetti et al., 2017).

Employing cytological diagnosis, more precisely through direct parasitological examination of the popliteal lymph node aspirate from an infected cat, amastigote forms were visualized under a microscope (Coelho et al., 2010). Using histological technique, Navarro et al. (2010) described changes in microscopic findings, including a diffuse granulomatous inflammation with macrophages in tissue biopsies from cats with FeL. These findings emphasize the value of histopathological and immunohistochemical techniques tests in the diagnosis of FeL.

Regarding molecular techniques, DNA of *Leishmania* spp. was detected in blood samples and conjunctival swabs from cats (Oliveira et al., 2015; Alves-Martin et al., 2017; Benassi et al., 2017). Considering that there are no studies with cats concerning MST, this work aimed to evaluate the humoral and cellular response based on serological diagnosis and MST in cats from a VL endemic area and compare the MST with other conventional diagnostic techniques. The data presented in this study add information to our current understanding of the repertoire of immune responses responsible for FeL development.

2. Materials and methods

2.1. Ethics statement

This study was approved by the Ethics Committee on Animal Experimentation and Animal Welfare of the School of Veterinary Medicine and Animal Science, University of São Paulo (FMVZ-USP), São Paulo, Brazil, under protocol number 3737090317.

2.2. Animals, locations and clinical exams

The study included all 100 cats living in two animal shelters located in the northwestern region of the state of São Paulo, Brazil at the time of sampling. The city of Ilha Solteira (20° 25′ 58″ S e 51° 20' 33" W) is considered an endemic area for VL (CVE, 2019). All cats were anesthetized (11 mg/kg ketamine + 1.1 mg/kg xylazine) before collection of biological samples and MST.

Peripheral blood was obtained through the jugular vein, where 5 mL of blood were collected in vacuum tubes without anticoagulant to obtain serum for ELISA and IFAT, and in tubes with anticoagulant (EDTA) for PCR and parasitological diagnostic. Obtained samples were stored at −20 °C until the tests were performed.

Fine needle aspiration biopsy was performed from lymph nodes or bone marrow aspirates from the sternum of cats. The obtained aspirates were deposited on a glass slide for smearing and staining.

During the sampling, cats were submitted to veterinary medical examination. Thus, each animal was carefully examined by several parameters [mucosal color, nutrition status, abdominal and lymph node alterations (lymphadenopathy), ocular and dermatological alterations (alopecia, dermatitis, onychogryphosis, and conjunctivitis)] (Pennisi et al., 2015).

2.3. Montenegro skin test

The method for MST in cats was based on the method described by Silveira et al. (2012) with some modifications. Thus, cultured promastigote forms in RPMI 1640 medium (Sigma-Aldrich, St Louis, MO, USA) of *L. infantum* (MHOM/BR/2002/LPC-RPV – IOC/2906), kindly provided by Fundação Oswaldo Cruz (FIOCRUZ), Rio de Janeiro, were used. These cultures were washed three times in phosphate-buffered saline (PBS) and fixed in chlorhexidine digluconate saline solution (1:10,000) with a final concentration of approximately 4×10^7 promastigotes/mL. These cultures were rapidly frozen (−70 °C), thawed (37 °C) and vortexing (1 min), seven times and stored at −20 °C. Then, 0.1 mL of antigen solution was inoculated intradermally in the cats' right ear, and as a control for the *Leishmania* antigen, 0.1 mL of chlorhexidine digluconate saline solution (1:10,000) was administered intradermally in the opposite ear of each individual. The skin reactions were measured after 24, 48, and 72 h. Indurations with diameters ≥5 mm at any time were considered positive (Sokal, 1975).

2.4. Parasitological diagnostic

Parasitological (PA) diagnosis was performed with smears on slides from blood, lymph nodes, and bone marrow aspirates. The smears were stained by the Rapid Panotic® kit (Laborclin®) according to the manufacturer's recommendations. The slides were examined by bright-field microscopy (400–1000 x magnification). The smears stained that presented basophilic structures suggestive of *Leishmania* spp. were considered positive. If the parasite was not visualized in at least 100 well stained and free of stain precipitate fields, it was considered negative.

2.5. Serology diagnostic

A fraction (3 mL) of the total blood sample was used to obtain serum for serological tests. The serological evaluation of antibodies against *Leishmania* spp. was performed by ELISA, according to De Lima et al. (2005) and adapted by Costa et al. (2010). The optical density determination (OD) for the cut-off point and ELISA level (EL) classification (EL ≥ 3) was performed according to (Oliveira et al., 2008).

The IFAT was performed according to a previous study (Oliveira et al., 2008), with a 1:40 dilution as a cut-off point.

2.6. Molecular diagnostic

2.6.1. DNA extraction

DNA extraction from blood samples was performed with the DNeasy® Blood & Tissue kit (Ref: 69506, QIAGEN, GERMANY) according to the manufacturer's recommendations. The extracted DNA was stored at −20 °C until conventional PCR (PCR).

2.6.2. PCR for Leishmania spp. kDNA and Trypanosomatidae rDNA

DNA extracted from blood was submitted to PCR amplification for the conserved region of *Leishmania* spp. kinetoplast minicircle DNA (kDNA), using primers 13A (5′-dGTG GGG GAG GGG CGT TCT-30) and 13B (5′- dATT TTA CAC CAA CCC CCA GTT-30), as described by Rodgers et al. (1990). The samples were also tested using primers [(LITSR (5′-CTGGATCATTTTCCGATG-3′) and L58S (5′TGATACCACTTATCG CACTT-3′)] targeting the internal transcribed spacer region 1 (ITS-1) of the ribosomal DNA (rDNA) for Trypanosomatidae (El Tai et al., 2000). All PCR reactions were performed in a Veriti® thermocycler (Applied Biosystems). *L. infantum* (MCAN/BR/1984/CCC-17.481) DNA was used as a positive control. Sterilized ultrapure water was used as a negative control.

2.6.3. DNA sequencing

The ITS-1 PCR-positive amplified products were removed from the gel and purified using the GE Healthcare kit (Illustra®, GFX PCR DNA and GEL Band Purification Kit, catalog number 28-9034-70) according to the manufacturer's instructions. The sequences were analyzed at the DNA Sequencing Service of the Human Genome and Stem Cell Research Center, Biologic Institute (IB), USP. Chromatograms obtained with the forward and reverse primers were assessed with the Sequence Scanner Software 2 v2.2. The sequences were manipulated with Clustal W available in the BioEdit Sequence Alignment Editor version 7.1.11 to align and generate consensus sequences. The contig sequences were submitted to GenBank in order to obtain accession numbers. These records were compared with deposited accession numbers in GeneBank to find the similarity and mismatching between ITS1 sequences of *Leishmania* spp. isolates and NCBI-Genbank *Leishmania* spp. strains using the Basic Local Alignment Search Tool (BLAST) (Jian et al., 2006).

2.7. Feline Immunodeficiency Virus (FIV) and Feline Leukemia Virus (FeLV) detection

The serum was used to detect FIV and FeLV using the IDEXX SNAP FIV/FeLV Combo Test diagnostic kit (IDEXX Laboratories, Markham, Ontario) according to the manufacturer's recommendations.

2.8. Statistical analysis

A nonparametric statistical analysis was used to evaluate the diagnostic method performance in cats. The agreement index between the diagnostic methods was assessed by the Kappa index (κ) and interpreted according to Landis and Koch (1977). $\kappa \leq 0.4$ is considered to be poor agreement; $0.41 \leq \kappa \leq 0.6$ is accepted as moderate agreement; $0.61 \leq \kappa \leq 0.80$ is considered good agreement; and $\kappa > 0.8$ is accepted as excellent agreement.

3. Results

Of the 100 cats, the presence of clinical signs compatible with FeL was observed in 74% (74/100). The most frequent clinical signs were dermal lesions, thinness, lymph node enlargement, alopecia, eye lesions, gingivitis, diarrhea, and dehydration. Based on the number signs observed, 38% (38/100) had one, 22% (22/100) two and 14% (14/100) had three or more signs during the physical clinical examination at the time of the study.

Sixty 60% (60/100) of the cats were positive for MST (Fig. 1). The diameter of the positive skin reactions ranged from 5 to 9 mm and none of the cats showed non-specific reactions to the diluent. But of the 40% (40/100) MST negative cats, 29 animals showed clinical signs, and of these, four sick cats were blood PCR positive whose amplicon sequencing matched 100% with *Leishmania donovani* complex and were negative for FIV and FeLV. Obtained nucleotides sequences were submitted to the GenBank-database and recorded on the website with 5 accession numbers OM731928- OM731932 (Table 1). Thus, of the five

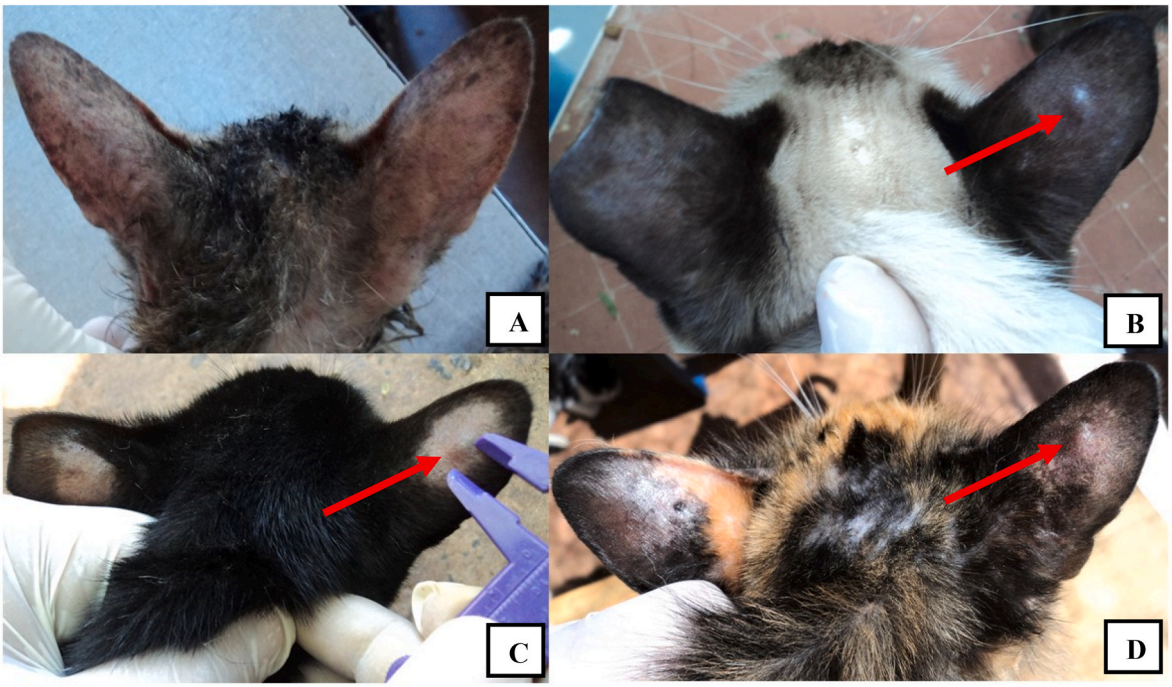

Fig. 1. Montenegro Skin Test for *Leishmania infantum* in cats from a Visceral Leishmaniosis endemic area. A: Negative cat; B, C, and D: nodule reactions in Montenegro Skin Test-positive cats (red arrow). Ilha Solteira, SP, Brazil.

Table 1
NCBI-BLAST homology sequence identity between the *Leishmania donovani* complex ITS1 blood PCR obtained sequences and NCBI-Genbank isolates species.

Animals	Genbank submission accession number	NCBI-BLAST Homology Sequence identity				
		NCBI BLAST identity isolate	Accession number	E-value	Identity (%)	Query cover
Seq1_Cat10	OM731928	*L. infantum*	NW_004057905.1	3e-152	100%	100%
Seq2_Cat13	OM731929	*L. infantum*	NW_004057905.1	1e-94	100%	100%
Seq3_Cat17	OM731930	*L. infantum*	NW_004057905.1	2e-123	100%	100%
Seq4_Cat20	OM731931	*L. infantum*	NW_004057905.1	8e-117	100%	100%
Seq5-Cat75	OM731932	*L. infantum*	NW_004057905.1	2e-127	100%	100%

L. donovani complex PCR positive cats, only one cat was positive for MST. Two of the 4 PCR positive and MST negative cats were positive for parasitological tests, and all 5 PCR positive cats were positive for IFAT and ELISA with titers ranging from 1:40 to 1:320 in IFAT (Table 2).

According to the other tests, the positive rates were 74% (74/100), 34% (34/100), 5% (5/100), and 2% (2/100) by IFAT, ELISA, PCRs (rDNA/kDNA) and parasitological tests, respectively. The same five cats that were positive by PCR rDNA were also positive by kDNA- PCR. Regarding the positive cats by other diagnosis and their MST diagnosis: of the positive cats for IFAT, 47 were MST positive, and 27 were MST negative, while of the positive cats by ELISA, 22 were positive, and 12 were negative by MST. Concerning molecular diagnosis, only one cat was MST positive, and the remaining cats were MST negative. By parasitological diagnosis, all the positive cats were MST negative (Fig. 2). Regarding ELISA seropositive cats (EL $\geq$ 3), the ELs found were: EL = 3 (10/34); EL = 4 (9/34); EL = 5 (5/34); EL = 9 (4/34); EL = 6 (3/34); EL = 8 (2/34) and EL = 7 (1/34). The antibody titers found using IFAT were 1:40 (49/74), 1:80 (22/74), 1:160 (2/74) and 1:320 (1/74). A total of 32/100 (32%) cats were IFAT and ELISA positive, and of these, 21/32 (65.6%) cats were MST positive. Concerning PCR positive cats (5/100), three had antibody titers of 1:40 and the other two cats had antibody titers of 1:80 and 1:320 (Fig. 3).

Regarding immunological expression, serological diagnosis and MST reactivity of cats revealed the following profiles: IFAT(+)/MST(−) = 27 cats; IFAT(−)/MST(+) = 13 cats; IFAT(+)/MST(+) = 47 cats; ELISA (+)/MST(−) = 12 cats; ELISA(−)/MST(+) = 38 cats and ELISA(+)/MST (+) = 22 cats. Through the combination of serological diagnosis and MST, a frequency of 87% (87/100) by IFAT + MST and 72% (72/100) by ELISA + MST was identified in this cat population (Table 3). As for agreement between MST and other diagnostic methods, they were classified as poor for all tests, with Kappa indexes of PCR ($\kappa = -0.0678$), parasitological ($\kappa = -0.0403$), ELISA ($\kappa = 0.0602$) and IFAT ($\kappa = 0.1150$).

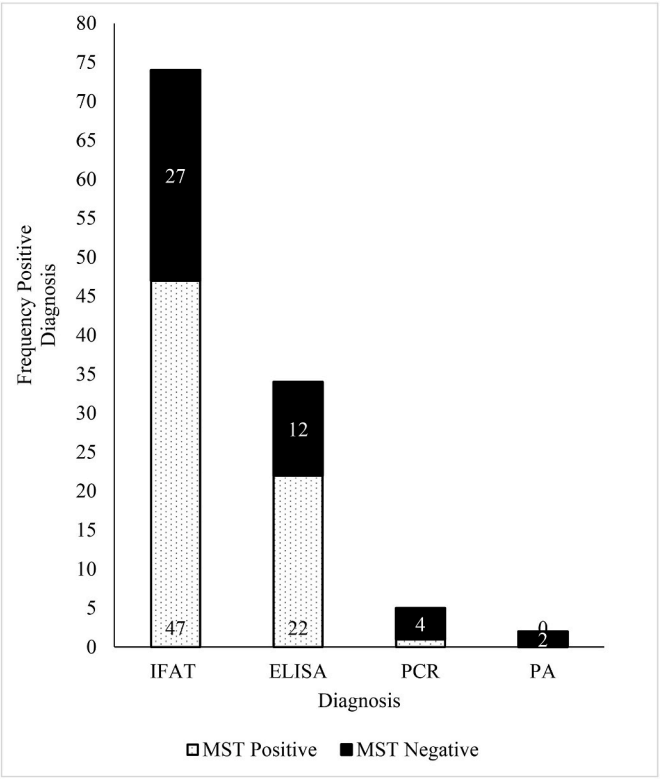

Fig. 2. Number of cats positives for *Leishmania donovani* complex by serological, molecular, and parasitological tests, and their Montenegro Skin Test diagnosis. Ilha Solteira, SP, Brazil.
Legend: Enzyme-linked immunosorbent assay (ELISA); Indirect immunofluorescence test (IFAT); Montenegro skin test (MST); Parasitological direct exam (PA); Polymerase Chain Reaction (PCR).

4. Discussion

The present study represents the first study carried out in an endemic area of VL that examined 100 cats to determine the response to MST

Table 2
Results of five *Leishmania donovani* complex PCR positive cats by parasitological, serological and Montenegro skin test, Ilha Solteira, SP, Brazil.

Animals	Clinical Signs	FIV/ FeLV	Serological diagnosis		Molecular diagnosis			Parasitological	Montenegro skin test
			ELISA/ EL	IFAT/ Tit.	Trypanosomatidae rDNA (ITS-1)	*Leishmania* spp. KDNA (13A/13B)	Sequencing		
Cat 10	Skin lesion, alopecia, cachexia	N	+/6	+/1:40	+	+	*L. infantum*	N	N
Cat 13	Skin lesion, alopecia, cachexia, lymph node enlargement, pinna lesion	N	+/6	+/1:80	+	+	*L. infantum*	+	N
Cat 17	Skin lesion, alopecia, pinna lesion	N	+/5	+/1:40	+	+	*L. infantum*	N	N
Cat 20	Lymph node enlargement, pinna lesion, weight loss	N	+/4	+/1:40	+	+	*L. infantum*	N	+
Cat 75	Weight loss	N	+/9	+/1:320	+	+	*L. infantum*	+	N

Legend: positive (+); negative (N); Feline Immunodeficiency Virus (FIV); Feline Leukemia Virus (FeLV); Enzyme-linked immunosorbent assay (ELISA) with respective ELISA levels (EL); Indirect immunofluorescence antibody test (IFAT) with respective antibody titers.

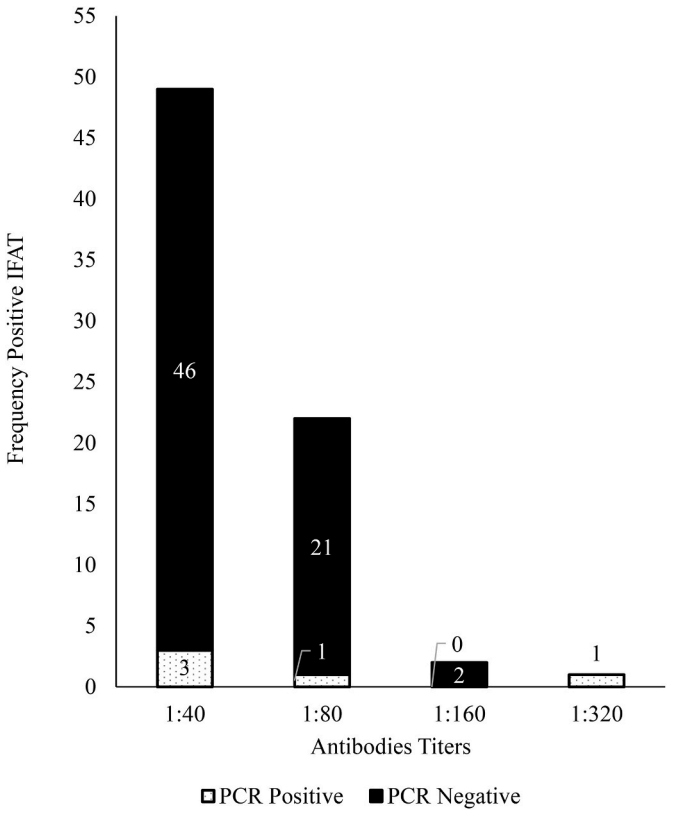

Fig. 3. PCR positivity according to *Leishmania* spp. antibody titers by Immunofluorescence Antibody Test in cats from Ilha Solteira, SP, Brazil.
Legend: Indirect immunofluorescence test (IFAT); Polymerase Chain Reaction (PCR).

against *L. infantum*. To that end, an MST was standardized for the detection of the *Leishmania* cellular response in cats. It is important to highlight that the cats studied here belong to animal shelters, where the occurrence of sand fly vector of *L. infantum* (*Lutzomyia longipalpis*), canine visceral leishmaniosis (CanL), and FeL has already been reported in previous studies (Alves-Martin et al., 2017; Fernandes et al., 2019; Franco Leonel et al., 2020; Spada et al., 2014).

Until recently, case reports of cats infected by *Leishmania* spp. were rare, and felines were considered resistant to the infection (Pennisi et al., 2015). Perhaps the feline immune system is less susceptible to the range of immunomodulatory salivary proteins contained within arthropod saliva and the cat is more competent at generating protective or sterilizing immune responses to arthropod-borne pathogens (Day, 2016, 2011). Cats are as exposed as dogs and humans to the risk of infection; nonetheless, the cellular response of cats against *L. infantum* infection may be more expressive than in dogs (Day, 2016) and may differ from that of dogs (Pennisi et al., 2015).

In the county of study, using the same molecular and parasitological techniques herein described the frequency of CanL was estimated to range from 13.1% by molecular tests (Pereira et al., 2016) to 40.3% in parasitological exams (Silva et al., 2014), in comparison with the 5% of PCR-positive cats and 2% by parasitological exams in the present study. The difference in the immune response of these two species may explain the higher prevalence of CanL than FeL, which has also been reported in other studies (Otranto et al., 2017; Poli et al., 2002). In a study with cats from CanL endemic areas, 18% produced IFN-γ after stimulation with *Leishmania infantum* soluble antigen and the authors concluded that cats could activate a cell-mediated response against the parasite that is variably associated with blood PCR or positive antibodies, since most IFN-γ producers cats were negative to antibody and DNA detection in blood (Priolo et al., 2019).

Herein more than half of the studied cat population (60%) was MST positive to *Leishmania* antigen. Similar studies carried in other species, observed 15% MST positive dogs from in an endemic area of Bahia, Brazil (Baleeiro et al., 2006) as well 9.7% (31/320) and 11.2% (106/946) MST positive dogs and humans, respectively, both in an endemic area of VL in the Amazonian, Brazil (Crescente et al., 2009; Silveira et al., 2012). In other studies, in Brazilian endemic areas at Maranhão, the prevalence rate of infection measured by MST, in children, were 61.7% (Nascimento et al., 2005) and 26.6% (Caldas et al., 2001). Also in dogs, Ibizan hounds has been reported to be more resistant to *Leishmania* infection than do dogs of other breeds; and in a study conducted in a CanL endemic region of Spain, 81% of Ibizan Hounds were MST positive, while only 48% of the other dogs were MST positive (Solano-gallego et al., 2000). Regarding the humoral response, antibody titers found in these cats by IFAT were mainly 1:40, as in other studies with cats (Franco Leonel et al., 2020; Vides et al., 2011). The low antibody rate may also be related to the fact that infected or exposed cats do not suffer from an impaired ability to mount a strong and effective cell-mediated response (Otranto et al., 2017). This may be due to the efficient and predominant Th1 immune response in cats when compared to dog (Day, 2016).

An IFAT cut-off of 1:80 has been recommended for serodiagnosis (Pennisi et al., 2015; Persichetti et al., 2017). However, in this study, a cut-off of 1:40 in IFAT was a better screening test. Herein we observed a 1:40 antibody titer in most sick and PCR positive cats (3/5). This lack of standardization of serological techniques may underestimate the real prevalence of *Leishmania* spp. infection in cats living in endemic areas and contribute to the spread of the disease (Portús et al., 2002; Maia et al., 2010). With a cut-off point of 1:80, the prevalence of exposure herein observed by IFAT would be 22% (22/100) instead of 74% (74/100).

The immune expression IFAT (+)/MST (+) was the most prevalent type of feline response in the present study, and it suggests that most naturally infected cats in the endemic area develop cellular and humoral responses simultaneously. Alexander and Phillips (1980) showed that cellular cooperation effectively occurs with the synergy between cellular and humoral immune responses. In a study with 188 dogs from Portugal, it was found that the combination of serological diagnosis and MST is a good tool to assess the immune response of dogs (Cardoso et al., 2007) and in the study of Priolo et al. (2019), the association of a cell-mediated immune response test, specific for *L. infantum,* with serological and molecular tests provided a better estimate of cat exposure to the parasite.

On the other hand, the IFAT(+)/MST(−) profile was the most frequent (80%) immune expression in PCR and parasitological positive

Table 3
Frequency of immunodiagnostic response to *Leishmania infantum* in cats from Ilha Solteira, SP, Brazil a visceral leishmaniosis endemic area.

Number of cats surveyed	Immunodiagnostic response *n* (%)					
	IFAT x MST (F = 87%)			ELISA x MST (F = 72%)		
	$IFAT^{(+)}/MST^{(-)}$	$IFAT^{(-)}/MST^{(+)}$	$IFAT^{(+)}/MST^{(+)}$	$ELISA^{(+)}/MST^{(-)}$	$ELISA^{(-)}/MST^{(+)}$	$ELISA^{(+)}/MST^{(+)}$
100	27 (27%)	13 (13%)	47 (47%)	12 (12%)	38 (38%)	22 (22%)

Legend: Frequency (F); positive (+); negative (−); enzyme-linked immunosorbent assay (ELISA); indirect immunofluorescence antibody test (IFAT); Montenegro skin test (MST).

sick cats observed here. In an endemic area, the IFAT(+)/MST(−) profile was the most prevalent on canine immune expression (Silveira et al., 2012). In VL, sick individuals, during the active phase of the disease showed depression of mediated immune response by cells and high levels of anti-*Leishmania* antibody (Badaró et al., 1986; Carvalho et al., 1981; Carvalho and Badaró, 1985), which is consistent with the observation that most PCR-positive cats of the study do not respond to the intradermal injection of *Leishmania* antigen.

The combined analysis of MST and IFAT provided an overall *Leishmania* spp. exposure frequency of 87%, contrasting with human (34.1%) and canine (43%) frequency in other VL endemic areas by the combined analysis (Crescente et al., 2009; Silveira et al., 2012). As in the Ibizan Hound study (77%), the cats in this work showed a high-frequency rate combining ELISA and MST (72%). If we consider that animals presenting a specific cellular or humoral response have been infected, the infection rate is very high (Solano-gallego et al., 2000).

Reports of FeL are increasing (Benassi et al., 2017; Franco Leonel et al., 2020), including those reporting infection of vectors by cats (Maroli et al., 2007; Da Silva et al., 2010; De Mendonça et al., 2020; Vioti et al., 2021). Four of the five PCR positive cats in this study underwent xenodiagnosis, and one of them (MST negative) was able to transmit *Leishmania* to colonized *Lutzomyia longipalpis* (Vioti et al., 2021).

The frequency of cat exposure increases using MST to detect *Leishmania*-specific cellular immunity in comparison with the frequency obtained with other techniques (Cardoso et al., 1998), which suggests that the frequency of feline exposure to *Leishmania* is underestimated, and that this species probably develops an efficient cellular immune response that decreases the number of FeL cases when compared to CanL.

5. Conclusion

Results show that most cats in this study tested positive for MST but were negative by parasitological and blood PCR. This suggests that cats in an endemic area, once exposed, probably develop a significant cellular response against the infection of parasites of the *L. donovani* complex. The depression of cell-mediated immune response is marked in VL and therefore most sick cats, PCR and parasitological positive, as observed, did not respond to MST. Considering the results obtained, further studies are needed to better understand the cellular immune response in *L. donovani* complex infected cats, and the use of MST as a tool to identify cats exposed to *Leishmania* spp. parasites in epidemiological studies.

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Maria Luana Alves: Conceptualization, Methodology, Investigation, Writing - original draft. Diogo Tiago da Silva: Conceptualization, Formal analysis, Investigation, Writing - Review & Editing. Julio César Pereira Spada: Conceptualization, Investigation, Writing - Review & Editing. João Augusto Franco Leonel: Conceptualization, Investigation. Julia Cristina Benassi: Investigation, Supervision. Nuno Wolfgang Balbini Pereira: Investigation. Geovanna Vioti: Investigation. Maria Fernanda Alves-Martin: Resources, Writing - Review & Editing. Nathália Frigo de Almeida Paula: Investigation. Wilma Aparecida Starke-Buzetti: Conceptualization, Methodology, Resources, Supervision. Trícia Maria Ferreira de Sousa Oliveira: Conceptualization, Methodology, Resources, Writing - Review & Editing, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that there are no potential conflicts of interest concerning the research, authorship, and/or publication of this article.

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