Original Article

Epidemiology of canine visceral leishmaniasis in a vulnerable region in Brazil

Soroepidemiologia da leishmaniose visceral canina em uma região vulnerável do Brasil

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Abstract

Visceral leishmaniasis (VL) is a neglected and endemic zoonosis that occurs throughout Brazil; nevertheless, few studies have focused on the early detection of the disease. The municipality of Ourinhos is a non-receptive, silent and vulnerable area for VL, where the seroprevalence of this disease has so far not been investigated. The present study aimed to determine the seroprevalence of canine VL in Ourinhos-SP, and to identify the presence of risk factors. Blood samples were obtained from 604 dogs during a rabies vaccination campaign together with application of a socioeconomic questionnaire, environmental and animal characteristics and tutor's knowledge about the disease. The samples were subjected to indirect ELISA and new samples were collected from reactive and suspect animals, including whole blood and lymph node aspiration evaluated by parasitological method, complete blood count and PCR. No animal was diagnosed as positive based on the combination of direct and indirect tests and the tutors' answers indicated little knowledge about leishmaniasis, being often confused with other diseases transmitted by arthropods; hence, according to the proposed methods, the presence of canine leishmaniasis in the city of Ourinhos was not confirmed and health education campaigns about the disease should be carried out.

Keywords: *Leishmania* spp., dog, indirect ELISA.

Resumo

A leishmaniose visceral (LV) é uma zoonose negligenciada e endêmica presente em todas as regiões do Brasil, mas mesmo assim poucos estudos têm objetivado a detecção inicial da doença. O município de Ourinhos - SP é uma área não receptiva, silenciosa e vulnerável à LV, não havendo até o momento estudos que tenham investigado a soroprevalência no município. Nesse sentido, o presente estudo objetivou determinar a soroprevalência da LV canina em Ourinhos-SP, bem como associar a presença de fatores de risco. Amostras sanguíneas de 604 cães foram obtidas juntamente com a aplicação de questionário socioeconômico, características ambientais e dos animais e conhecimento sobre a doença. As amostras foram submetidas à sorologia por ELISA e novas amostras coletadas de cães reagentes ou suspeitos foram analisadas por método parasitológico direto, hemograma e PCR. Nenhum animal foi considerado positivo na combinação de testes direto e indireto, e as respostas dos tutores indicaram pouco conhecimento sobre a leishmaniose, sendo muitas vezes confundida com outras doenças

transmitidas por artrópodes. Dessa forma, de acordo com os métodos propostos, a presença de leishmaniose canina, na cidade de Ourinhos, não foi confirmada. Por isso, campanhas de educação em saúde sobre a doença deveriam ser realizadas.

Palavras-chave: *Leishmania* spp., cães, ELISA indireto.

Introduction

Visceral Leishmaniasis (VL) is a zoonotic disease caused by protozoa of the genus *Leishmania* and transmitted by sandflies such as *Lutzomyia longipalpis*, which is considered the main vector in Brazil (Brasil, 2019). The disease is classified as a neglected tropical disease, and according to the World Health Organization (WHO), it is one of the six most important infectious diseases worldwide, ranking as the second parasitic disease that causes the most deaths in the world. About 350 million people live in endemic areas and are at risk of infection. In the Americas, VL is an important public health problem from Mexico to Argentina (WHO, 2016).

In the state of São Paulo, 8,553 cases of human VL were reported in 107 municipalities between 1999 and 2019 (Rangel et al., 2020). Previous studies have demonstrated that the presence of the vector and canine infection precede human cases, justifying the need for identification of the vector and infected dogs (Brasil, 2019; Rangel et al., 2020).

In domestic dogs – the main reservoir host of the parasite in the urban cycle, the disease is considered multisystemic, causing different clinical signs such as cutaneous lesions, keratoconjunctivitis, cachexia, onychogryphosis and enlarged lymph nodes. In the chronic phase, liver and splenomegaly and kidney disease are commonly observed (Marcondes & Rossi, 2014; Almeida et al., 2017a, b). The most common hematological abnormalities include normocytic and normochromic non-regenerative anemia, leukocytosis, neutropenia, lymphocytosis and thrombocytopenia. Signs of kidney injuries may be present in chronic stages due to the deposition of immune complexes (Ikeda-Garcia et al., 2003). Although a medication for the treatment of canine VL has recently been approved in Brazil, the federal agencies still recommend euthanasia as a way of controlling the disease (Brasil, 2019). Direct and indirect methods can be used for the diagnosis of canine infection; however, serology has been used for screening, while DNA detection and microscopy are commonly used to confirm the infection (Brasil, 2019), since serological tests may cross-react with other pathogens (Zanette et al., 2014; Lopes et al., 2017).

Ourinhos is a city located in the interior of the state of São Paulo, classified as silent, non-receptive and vulnerable according to the Paulista Epidemiologic Bulletin, due to the lack of autochthonous transmission, absence of sandflies and proximity to endemic cities such as Bauru and Marília, respectively (Rangel et al., 2020). Furthermore, to date, no studies have evaluated autochthonous transmission in both humans and dogs in the city. Due to the importance of public health and the rapid expansion in the country, serological surveys are essential to prevent the expansion and increase in the incidence of the disease. Thus, this study aimed to evaluate the epidemiology of canine visceral leishmaniasis in Ourinhos and to determine its association with risk factors.

Material and Methods

Ethics statement

All the procedures involving animals and their owners were approved by the Ethics Committee of the Centro Universitário das Faculdades Integradas de Ourinhos (no. 26/2017) and the Research Ethics Committee of the Faculdade de Medicina de Marília (no. 2.584.911). Dogs were included in the study only after obtaining their owners' consent.

Study area and sampling

The study was conducted in Ourinhos (22°58′59″S, 49°51′25″ W), a municipality of 295,818 km^2 in the state of São Paulo, with a population of 113,542.

The initial sample size of 530 animals was calculated using EpiInfo v.7 software, considering a population of 15,000 dogs according to the Municipal Health Department, an estimated prevalence of 50%, and a confidence level of 95%. Dogs were sampled in September and October 2017 during the municipal vaccination campaign against rabies at 25 vaccination stations distributed across the city. Blood collection from at least 10 dogs was carried out at each vaccination station, being randomly collected by sampling in 1 out of 10 dogs that were vaccinated

and had more than 6 months of age. The blood samples (2 mL) were obtained by cephalic puncture, placed in a tube containing clot activator (BD Vacutainer®, Becton-Dickson, New Jersey, USA) and centrifuged, and serum was stored at -20°C until the serological analysis. After blood sampling, a questionnaire about tutor's socioeconomic information, characteristics of the animal and its residence, and tutor's knowledge about VL was made available for completion by the tutor. Afterwards, each tutor received a brief explanation about VL and how to prevent it.

Approximately three months after the first blood collection (December 2017), 10 of these dogs that were suspected of having VL or were reactive by iELISA, underwent a physical examination and new blood samples and popliteal lymph node material was collected by aspiration for complete blood count, PCR and direct parasitological analysis. Other two dogs, one suspected (sample A-14) and other reactive (sample K-16) for canine VL, had no samples recolected because one of them died (K-16) and the other was missing at the time of resampling (A-16), so parasitological direct examination was not performed and PCR reactions of these samples were conducted from serum samples of the first sampling period.

Enzyme-linked immunosorbent assay (ELISA) and immunochromatographic test

iELISA was performed using the total antigen of *Leishmania infantum*, as previously described (Lima et al., 2003). Dogs were considered reactive when the optical density (OD) was greater than 0.270 and were considered suspect when the OD was between 0.198 and 0.269 as previously reported (Lima et al., 2003).

The serum samples from dogs reactive and suspected (n = 12) of CVL were also subjected to the Dual Path Platform (DPP) Canine Visceral Leishmaniasis serological test (Biomanguinhos/FioCruz, Rio de Janeiro, Brazil).

Complete blood count

Complete blood counts (CBC) and total plasma protein (TPP) concentration were performed in a veterinary hematology analyzer (HORIBA ABX Micros ESV 60, Paris, France) and using a portable clinical refractometer (ATAGO, Mod. Master-SUR-NM, Tokyo, Japan), respectively, following previous recommendations (Jain, 1986; Costa et al., 2020; Oliveira et al., 2020).

Direct parasitological examination

Part of the material obtained by aspiration biopsy of popliteal lymph node from reactive and suspected dogs (n = 10) was smeared onto microscope slides, and stained with commercial hematological dye (Panótico Rápido, Laborclin, Pinhais, PR, Brazil). The slides were examined under an optical microscope at 1000x magnification for at least 30 min, to detect amastigote forms of *Leishmania* spp.

Diagnosis of other infectious agents and PCR

A serological and molecular diagnosis of the main infectious diseases that could interfere in serological tests for canine leishmaniasis by iELISA (Zanette et al., 2014) was also performed. This included serological tests for canine monocytic ehrlichiosis (CME), anaplasmosis, borreliosis and heartworm disease (SNAP 4Dx Plus Test®, IDEXX Laboratories, USA) and molecular diagnosis of babesiosis, CME, neosporosis, toxoplasmosis, trypanosomiasis, and other common infection agents observed in our veterinary routine such as anaplamosis and hepatozoonosis according to PCR protocols described on Tables 1 and 2.

Samples destined for PCR reaction consisted of the remaining popliteal lymph node aspirates which was homogenized with 100 µL of sterile NaCl 0.9% solution and pooled with 100 µL of blood from the same dog from the dogs from which new samples (n = 10) were collected, and serum from the dogs from which new samples could not be collected (n = 2). The sample was then subjected to DNA extraction using a commercial kit (Norgen Biotek Corp, Canada), following the manufacturer's instructions. The DNA sample was eluted in 50 µL, and stored at -20°C until the moment of PCR processing.

Prior to the molecular detection, all the DNA samples were subjected to a PCR test targeting the housekeeping gene G3PDH, following a previously described method (Peters et al., 2007).

Nested PCR was used to detect *Leishmania* spp. DNA according to an earlier modified protocol (Albuquerque et al., 2017). To elicit a secondary *Leishmania* spp. reaction, the product of the first reaction was diluted 1:200. All the

Table 1. Reaction target, description and concentration of primers, product size and references used in polymerase chain reaction (PCR) for the detection of infectious agents and housekeeping genes.

Target	Primers	Primer concentration	Product size (pb)	Reference
G3PDH	FW: 5' TCAACGGATTTTGGCCCGTATTTGG 3' RV: 5' TGAAGGGGGTCATTGATGGCG 3'	0.2 µM	90	Peters et al. (2007)
Primary *Leishmania* spp.	FW: 5' GGTTCCTTTCCTGATTTACG 3' RV: 5' GGCCGGTAAACGTAAAGGCCGAATAG 3'	15 pM	603	Albuquerque et al. (2017)
Secondary *Leishmania* spp.	FW: 5' TCCATCGCAACCTCGGTT 3' RV: 5' AAAGCGGGCGCGGTGCTG 3'	15 pM	358	
Primary *Anaplasma platys*	FW: 5´ AGAGTTTGATCCTGGCTCAG 3' RV: 5´ TAGCACTCATCGTTTACAGC 3'	12.5 pM	760	Inokuma et al. (2001)
Secondary *Anaplasma platys*	FW: 5´ AAGTCGAACGGATTTTTGTC 3´ RV: 5´ CTTTAACTTACCGAACC 3'	12.5 pM	466	
Babesia canis	FW: 5' CCGTGCTAATTGTAGGGCTAATACA 3' RV: 5' GCTTGAAACACTCTARTTTCTCAAAG 3'	0.2 mM	551	Spolidorio et al. (2011)
Ehrlichia canis	FW: 5' CCTCTGGCTATAGGAAATTG 3' RV: 5' GGAGTGCTTAACGCGTTAG 3'	50 pM	761	Alves et al. (2006)
Hepatozoon canis	FW: 5´ CGCGAAATTACCCAATTCTA 3´ RV: 5´ TAAGGTGCTGAAGGAGTCGTTTAT 3'	0.2 µM	670	Spolidorio et al. (2009)
Neospora caninum	FW: 5´GGGTGTGCGTCCAATCCTGTAAC 3´ RV: 5´CTCGCCAGTCAAACCTACGTCTTCT 5´	10 pM	337	Liddell et al. (1999)
Toxoplasma gondii	FW: 5' GGAACTGCATCCGTTCATGAG 3' RV: 5' TCTTTAAAGCGTTCGTGGTC 3'	50 pM	194	Spalding et al. (2002)
Trypanossoma cruzi	FW: 5' AAATAATGTACGGGGGAGATGCATGA 3' RV: 5´ GGGTTCGATTGGGGTTGGTGT 3'	300 pM	330	Avila et al. (1990)
Trypanosoma evansi	FW: 5' AAACCCGTCCTCTTGGAGG 3' RV: 5' ATCCATCTAAGAGTTGT 3'	200 pM	315	Ventura et al. (2002)

Table 2. Polymerase chain reaction (PCR) conditions for the detection of infectious agents and housekeeping genes.

Target	Initial activation	Denaturation	Annealing	Extension	Cycles	Final extension
G3PDH	94ºC 5 min	95ºC 30 s	60ºC 30 s	72ºC 30 s	35	72ºC 7 min
Primary *Leishmania* spp.	94ºC 5 min	94ºC 30 s	60ºC 30 s	72ºC 30 s	35	72ºC 7 min
Secondary *Leishmania* spp.	94ºC 5 min	94ºC 30 s	65ºC 30 s	72ºC 30 s	35	72ºC 7 min
Primary *Anaplasma platys*	94ºC 5 min	94ºC 60 s	55ºC 60 s	72ºC 60 s	35	72ºC 5 min
Secondary *Anaplasma platys*	94ºC 5 min	94ºC 60 s	55ºC 60 s	72ºC 60 s	35	72ºC 5 min
Babesia canis	94ºC 5 min	95ºC 60 s	64ºC 30 s	72ºC 30 s	35	72ºC 7 min
Ehrlichia canis	94ºC 5 min	94ºC 60 s	54ºC 60 s	72ºC 60 s	40	72ºC 7 min
Hepatozoon canis	94ºC 5 min	95ºC 15 s	53ºC 40 s	72ºC 40 s	40	72ºC 5 min
Toxoplasma gondii	94ºC 5 min	94ºC 60 s	55ºC 45 s	72ºC 60 s	35	72ºC 7 min
Neospora caninum	94ºC 5 min	94ºC 40 s	63ºC 40 s	72ºC 60 s	40	72ºC 10 min
Trypanossoma cruzi	94ºC 5 min	94ºC 60 s	65ºC 60 s	72ºC 60 s	30	72ºC 10 min
Trypanosoma evansi	94ºC 5 min	95ºC 60 s	56ºC 120 s	72ºC 120 s	30	72ºC 5 min

reactions were performed using 1x MasterMix Red (Ampliqon, Denmark), 1 U Taq DNA polymerase, and primers in a final volume of 50 µL (Tables 1 and 2).

The PCR products were subjected to electrophoresis on 3% agarose gel (LE Agarose 1200, NeoTaq) with ethidium bromide 0.05% (Sigma Aldrich, USA), examined and photographed under UV light (MiniBis ProBio Imaging Systems). Molecular weight LowRanger 100pb DNA Ladder (Norgen Biotek Corp., Canada) was added to all the agarose gels, and ultrapure distilled water (RNA and DNAase free water, Gibco-Invitrogen, USA) was used as negative control in all the reactions, each of which had a positive control.

Results

Blood samples were collected from 604 dogs, but only 557 dog owners filled out the questionnaire completely, so that was the number of samples considered in the study. Of these, 54% came from males and 46% from females. There was a predominance of mixed breed dogs, i.e., 69%, followed by poodles (4.4%), labrador retrievers and pit bulls (4%), bassets (3%), lhasa apsos and rottweilers (2.25%) and doberman pinscher (2%), border collies and Brazilian terriers (1%), while other breeds corresponded to less than 1% of the dogs. As for their ages, 81 dogs were 6 months to 1 year old, 185 ranged from 1 to 3 years, 247 from 3 to 10 years, and 51 were older 10 years. The owners of 13 dogs did not know their age.

Table 3. Results of diagnostic methods for the detection of canine visceral leishmaniasis, including iELISA serology, Dual Path Platform (DPP) immunochromatographic serology, direct parasitological method from popliteal lymph node aspirate and polymerase chain reaction (PCR) to amplify the variable part of the small subunit rRNA gene of *Leishmania* spp., samples A-14 and K-16 had PCR reaction performed from serum of the first sampling.

Sample code	OD iELISA	DPP	Direct parasitological examination	PCR
A-14	0.259 / Suspect	Non-reactive	Not performed	Negative
J-07	**0.285 / Reactive**	**Reactive**	Negative	Negative
J-08	**0.411 / Reactive**	Non-reactive	Negative	Negative
J-31	**0.307 / Reactive**	**Reactive**	Negative	Negative
K-16	**0.298 / Reactive**	Non-reactive	Not performed	Negative
N-10	0.227 / Suspect	Non-reactive	Negative	Negative
O-05	**0.372 / Reactive**	**Reactive**	Negative	Negative
O-08	0.240 / Suspect	Non-reactive	Negative	Negative
O-19	0.257 / Suspect	Non-reactive	Negative	Negative
Q-24	0.198 / Suspect	Non-reactive	Negative	Negative
V-05	0.218 / Suspect	Non-reactive	Negative	Negative
V-06	**0.350 / Reactive**	Non-reactive	Negative	Negative

Dogs were considered reactive when the optical density (OD) was greater than 0.270 and were considered suspect when the OD was between 0.198 and 0.269.

Of the total of 577 samples analyzed by ELISA, 6 whose OD was higher than 0.270 were considered reactive, and 6 whose OD ranged from 0.198 and 0.259 were considered suspected. These samples were also subjected to the DPP serological test for Canine Visceral Leishmaniasis, and three were considered reactive (Table 3).

Blood samples could not be collected a second time from two of the dogs, because one of them died of a cause unknown to the owner (sample K-16) and the other was missing at the time of resampling (sample A-14). None of the reassessed dogs (n=10) presented amastigote forms of *Leishmania* spp. by direct parasitological examination of samples of blood smears and popliteal lymph node aspirates (Table 3), although all the lymph node samples contained sufficient material for cytological evaluation and PCR reaction.

All the samples (n=12) amplified G3PDH gene, which was used as DNA extraction control, including the serum sample from the two dogs (samples A-14 and K-16) from which blood samples could not be collected for a second time. All the dogs presented negative nested PCR to amplify the variable part of the small subunit rRNA gene of *Leishmania* spp. three months after the initial serological test (Table 3). When the presence of DNA from other infectious agents was identified by PCR analyses, one sample tested positive for *Anaplasma platys*, *Babesia canis vogeli*, *Ehrlichia canis* and *Hepatozoon canis*; two were positive for *A. platys*, *B. canis vogeli* and *H. canis*; two were positive for *B. canis vogeli*, *E. canis* and *H. canis*; one was positive for *B. canis vogeli* and *E. canis*; two for *E. canis* and *H. canis*; and two only for *H. canis.* None of the samples tested positive for *Neospora caninum*, *Toxoplasma gondii*,

Table 4. Results of polymerase chain reaction (PCR) and serology results on 4DX Plus for the detection of other infectious agents.

Sample code	PCR								4DX Plus			
	A. platys	*B. canis*	*E. canis*	*H. canis*	*N. caninum*	*T. gondii*	*T. cruzi*	*T. evansi*	*E. canis*	*A. platys*	*B. burgdorferi*	*D. immitis*
A-14	–	–	–	–	–	–	–	–	**Reactive**	Non-reactive	Non-reactive	Non-reactive
J-07	–	+	+	–	–	–	–	–	**Reactive**	Non-reactive	Non-reactive	Non-reactive
J-08	+	+	–	+	–	–	–	–	**Reactive**	**Reactive**	Non-reactive	Non-reactive
J-31	–	+	+	+	–	–	–	–	**Reactive**	Non-reactive	Non-reactive	Non-reactive
K-16	–	–	–	+	–	–	–	–	**Reactive**	Non-reactive	Non-reactive	Non-reactive
N-10	–	+	+	+	–	–	–	–	**Reactive**	Non-reactive	Non-reactive	Non-reactive
O-05	+	+	+	+	–	–	–	–	**Reactive**	**Reactive**	Non-reactive	Non-reactive
O-08	+	+	–	+	–	–	–	–	**Reactive**	**Reactive**	Non-reactive	Non-reactive
O-19	–	–	+	+	–	–	–	–	**Reactive**	Non-reactive	Non-reactive	Non-reactive
Q-24	–	–	+	+	–	–	–	–	**Reactive**	Non-reactive	Non-reactive	Non-reactive
V-05	–	–	–	+	–	–	–	–	**Reactive**	**Reactive**	Non-reactive	Non-reactive
V-06	–	–	–	+	–	–	–	–	**Reactive**	**Reactive**	Non-reactive	Non-reactive

+: pathogen DNA detection on PCR; –: absence of pathogen DNA on PCR.

Trypanosoma cruzi or *Trypanosoma evansi* (Table 4). The immunochromatographic serological tests for other infectious agents also detected that all the samples (n=12) contained anti-*E. canis* antibodies and five of them also presented anti-*A. platys* antibodies (Table 4).

The clinical reassessment of the dogs (n=10) revealed that 70% of them had ectoparasites, and all of them presented one or more clinical signs. The main signs were emaciation (60%), ulcerative skin lesions (60%), alopecia (40%), lymphadenomegaly (30%), mucopurulent eye discharge and hyperkeratosis (20%), and onychogryphosis (10%). The CBC indicated that these dogs suffered predominantly from hyperproteinemia with the presence of rouleaux formation (70%), thrombocytopenia (70%) and normochromic normocytic anemia (60%) (Table 5).

The questionnaire applied to dog owners revealed that 56% of them were aware of VL, 31% learned about it from TV, 22% from pamphlets, 19% from the Internet, and the rest cited two or more other sources. More than

Table 5. Physical findings and complete blood count of dogs reactive to and suspected of canine leishmaniasis, from which a second sample was collected (n=10).

Physical findings	
Presence of ectoparasites	70%
Emaciation	60%
Ulcerative skin lesions	60%
Alopecia	40%
Lymphadenomegaly	30%
Hyperkeratosis	20%
Eye discharge	20%
Onychogryphosis	10%

Complete blood count findings			
Parameter	Decreased (%)	Normal (%)	Increased (%)
Hematocrit	60	40	0
Platelet count	70	30	0
Total white blood cell count	30	60	10
Segmented neutrophil count	10	80	10
Lymphocite count	20	70	10
Monocyte count	0	100	0
Eosinophil count	10	80	10
Total plasma protein	0	30	70

80% stated that the source of transmission is a mosquito, 14% reported the tick as a vector, 3% stated bites, and 1% believed transmission was caused by blood from other animals, from rats, or did not know. The common names of sandflies such as "mosquito-palha" and "birigui" were recognized by 57% of the participants, 35% knew about them exclusively from TV, 20% from pamphlets, and 15% from the Internet. Almost 70% of respondents did not know about any preventive measures against the spread of VL. When questioned about disease control measures, 48% believed that euthanasia is a valid method, 29% cited castration, while 17% and 6%, respectively, cited screening windows and preventing standing water or keeping backyards clean.

Considering the main risk factors for VL, most of the interviewees lived in houses with backyard (61%), 38% had lawns or flower beds, and 1% lived in houses without a backyard. Grassy areas in the neighborhood were reported by 80%. Other green areas such as parks and gardens in the vicinity were reported by 81%. The presence of chicken coops in the neighborhood was reported by 15% of participants and 5% had their own chicken coop. The presence of other animals in the home environment was reported by 41% of dog owners, most of them being cats (62%) and some type of bird (34%). In addition, 24% of them lived in locations where there were farms animals in the surrounding area, and 34% of them were next-door neighbors of farms with farms animals.

As for preventive measures against VL, more than 90% of respondents stated that their dogs do not wear insect repellent collars and 60% of owners do not take their dogs to the veterinarian on a regular basis. Of the 577 animals, 20% were neutered and 38% were vaccinated only against rabies. With regard to how the owners acquired their dogs, 25% acquired their dog as an adult, 20% took them in off the street, and 40% adopted them from someone they know who lives in another location. Sixty percent of owners stated they regularly walk their dogs on different days and times. The majority (66%) of the animals have access to an open yard, but 43% sleep in an open yard without shelter. Short coats were predominant (75%), and 4% of dog owners took their dogs on trips.

Discussion

Of the 577 samples analyzed, six were reactive and six were considered suspect by iELISA for canine visceral leishmaniasis, as proposed by Lima et al. (2003). Of these samples, three were also serologically positive by DPP, a rapid screening test recommended by the Brazilian public health authorities for the diagnosis of canine leishmaniasis. However, three months after the initial serological test, none of the dogs presented amastigote forms of *Leishmania* spp. by the direct parasitological methods or *Leishmania* spp. DNA amplification by PCR, which are methods whose detection sensitivity ranges from 74 to 97% (Assis et al., 2010).

Our diagnostic investigation continued, particularly because the clinical signs presented by the dogs were also suggestive of canine leishmaniasis. Some of the dogs were emaciated and/or had ulcerative lesions, alopecia and lymphadenomegaly, which are clinical signs frequently observed in dogs with leishmaniasis (Marcondes & Rossi, 2014; Almeida et al., 2017a, b). Moreover, ectoparasites were detected on 70% of these animals. The abnormalities detected in the CBC included normocytic anemia, thrombocytopenia and hyperproteinemia, which also led us to consider the possibility of cross-reactivity in the serological test for canine leishmaniasis. Cesar (2008) evaluated the occurrence of ehrlichiosis in dogs with symptomatology compatible with the disease and confirmed the presence of *E. canis* DNA in 27 of 86 tested animals, all of which showed clinical signs such as lymphadenomegaly, splenomegaly and ocular lesions similar to those detected in the dogs of our study. Lymphadenomegaly, followed by anemia and thrombocytopenia, were the main clinical and laboratory findings in dogs with monocytic ehrlichiosis, corroborating the PCR results reported by Nakaghi et al. (2008). Borin et al. (2009) conducted a study involving 4407 dogs from a Veterinary Hospital, where *E. canis* morulae was detected in the blood smears of 251 dogs and 48 animals showed coinfection with babesiosis. Common clinical signs exhibited by these animals were apathy, anorexia, eye discharge, splenomegaly and anemia, most of which are clinical signs that can also occur in dogs with VL. The clinical signs of canine leishmaniasis may vary according to the time of infection and the host's immune response. That is why the signs are often nonspecific and can lead to mistaken diagnoses of other infectious and parasitic diseases, making it difficult to confirm a positive case based only on one diagnostic test (Marcondes & Rossi, 2014).

Zanette et al. (2014) evaluated serological cross-reactivity upon testing for canine leishmaniasis using the same methodology as that used in our study. They identified dogs with toxoplasmosis (n=10), ehrlichiosis (n=13), babesiosis (n=12), trypanosomiasis (by *T. cruzi*, n=14) and neosporosis (n=8), 42.1% of which tested positive by one of the three serological methods for the diagnosis of canine leishmaniasis: 10/57 (17.5%) in the iELISA with total crude antigen, 11/57 (19.3%) in the indirect immunofluorescence test and 3/57 (5.3%) in the immunochromatographic test (Kalazar Detect™). The above mentioned authors considered only dogs with trypanosomiasis and ehrlichiosis in the iELISA, with 64% and 7.6%, respectively, showing cross-reactivity. Given that canine monocytic ehrlichiosis is the most frequent infectious disease in our veterinary hospital care (unpublished data) and that 1.03% of all the collected samples were reactive in the iELISA for canine leishmaniasis, this percentage is still well below the possibility of cross-reactivity with ehrlichiosis reported by the authors. This is particularly relevant when considering that all dogs were reactive to serologic testing for ehrlichiosis by immunochromatographic test. Parasites of the genus *Leishmania* spp. belong to the family Trypanosomatidae, which explains the finding of high cross-reactivity with *Trypanosoma* spp. infection in several studies (Mendes et al., 2013; Matos et al., 2015). Costa & Vieira (2001) showed that 83.3% of the samples they subjected to serological tests showed cross-reactivity between *Leishmania* spp. and *T. cruzi*. However, none of the dogs tested in our study presented amplification of *T. cruzi* DNA in the pooled sample of blood and popliteal lymph node, leading us to assume that cross-reactivity occurs predominantly with ehrlichiosis.

In this context, several other studies have also demonstrated the cross-reactivity of other infectious diseases in serological tests for canine visceral leishmaniasis. Ferreira et al. (2007) applied indirect immunofluorescence and ELISA and observed cross-reactivity with canine leishmaniasis in dogs infected with *T. cruzi*, *L. braziliensis* and *E. canis*. A study involving 353 dogs carried out in the state of Bahia by Deiró et al. (2018) compared the occurrence of anti-*E. canis* antibodies, *Leishmania* spp. and *T. gondii*. These authors reported that coinfection of *Leishmania* spp. and *T. gondii* occurred in 7.1% of the cases, *Leishmania* spp. and *E. canis* in 5.7%, and that 4.5% of the dogs were coinfected with the three agents. These levels of infection are higher than those found in our study. Considering our results, we reiterate what has been discussed about the sensitivity of the different diagnostic methods for the diagnosis of canine VL (Lopes et al., 2017; Vaz et al., 2020). No test currently available has 100% specificity and sensitivity. This fact leads us to call into question the effectiveness of serological surveys used as screening for control of the disease by euthanizing serum reactive animals, a long-standing guideline of Brazilian government agencies (Alves & Bevilacqua, 2004; Gontijo & Melo, 2004; Assis et al., 2010). Some authors have suggested using a

combination of diagnostic tests, particularly of direct and indirect detection methods to confirm the infection, since results may differ as a function of the test, sample, disease evolution and immune response of the host (Gontijo & Melo, 2004; Queiroz et al., 2010; Sousa, 2012; Marcondes & Rossi, 2014).

In our evaluation of the population's understanding of VL in the municipality of Ourinhos, more than half of the interviewees stated they had no knowledge about the disease, and among those who stated they knew about leishmaniasis, many associated it with dengue fever. When asked where they saw or heard about the disease, the main means of information the research participants listed was TV, followed by pamphlets and the Internet. Most of them reported they had heard about it on TV newscasts, and many stated that the name "mosquito-palha" brought to mind the disease. In response to a questionnaire applied by Lobo et al. (2013) to 743 students in a public school to assess their knowledge about VL, 203 stated that the disease is transmitted by a vector and 197 said that the vector of transmission was the *Aedes aegypti* mosquito. In our study, *A. aegypti* was mostly also considered a vector of transmission, but as stated earlier herein, the disease was often mistaken for dengue, making it difficult to outline a real parameter about the population's understanding in this regard. Among the prevention methods, practically all the answers described the elimination of standing water, further reinforcing the mistaken idea of dengue fever.

We observed that most of the dogs in the municipality have free access to the street and do not wear any type of insect repellent collar, important risks factors for canine VL pointed by other studies. Studies evaluating the use of insect repellent collars to prevent VL in dogs have shown that this method is highly effective in controlling the disease. Moreover, dogs without such collars pose a risk factor for the occurrence of disease, since these collars act directly to reduce the number of vectors, of infected dogs, and thus also of human cases of the disease, including children, among whom lethality rates are higher (Mazloumi Gavgani et al., 2002; Sevá et al., 2016). However, the implementation of dog collar programs is still difficult due to the costs, loss of collars, adoption by the government, geographic diversity and adequate planning according to the reality of each region (Morais et al., 2015; Sevá et al., 2016; Bersusa et al., 2018). Leal et al. (2018) demonstrated that the incidence of leishmaniasis-positive dogs decreased by 61% in a period of 6 to 12 months, using deltamethrin-impregnated collars. With regard to the environment, more than 80% of the participants live in houses with backyard lawns or in neighborhoods with green areas. These are places where the odds of detecting ticks, mosquitoes and sandflies are higher, which could be considered risk factors for visceral leishmaniasis, according to Costa et al. (2015) and Menezes et al. (2016).

Most of the interviewees believe that neutering and euthanasia are ways to control the disease. This may be due to the fact that some of the interviewees relate the disease with how the canine population was historically controlled, which involved catching dogs on the street, or solving the problem of stray dogs by using these methods.

Taken together, the owners' responses to the questionnaires reveal that there are many risk factors for the occurrence of VL in the municipality of Ourinhos, SP, including dog handling practices, environmental conditions and proximity to endemic sites (Prado et al., 2011; Costa et al., 2015). A comparison of the results of studies conducted in both endemic locations and municipalities without confirmed cases revealed that Ourinhos presented factors in common with both of them. These factors include the paucity of dogs wearing collars, the fact that the majority of short-haired dogs spend most of their time outdoors in their owners' yards or in neighborhoods with green areas, the presence of production animals in the vicinity, and the population's lack of knowledge about the disease (Coiro et al., 2011; Menezes et al., 2016; Leal et al., 2018; Leite et al., 2018). This combination of factors underscores the municipality's vulnerability to the emergence of VL cases.

In this regard, alternatives to control the dissemination of the disease are extremely important, such as early diagnosis in dogs, which would help reduce human cases of VL if the disease began to be detected. Another important alternative is the population's health education and active surveillance of animals and the environment (Coura-Vital et al., 2014; Carvalho et al., 2018).

Although the municipality of Ourinhos is considered silent, non-receptive and vulnerable to canine leishmaniasis, we can state that there is evidence supporting the notion of the transmission of autochthonous cases should the disease and its vector arrive in the municipality, given the presence of numerous risk factors. It is also evident that the population lacks information about VL control, revealing that health education should be intensified.

Conclusions

The seroepidemiological diagnosis of canine visceral leishmaniasis in the municipality of Ourinhos enabled the detection of serum reactive dogs, but confirmation of the infection by other diagnostic methods revealed that

cross-reactivity may have resulted in false positive cases. Moreover, the population's knowledge about the disease and prevention methods must be improved.

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