SHORT COMMUNICATION

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Importance of *Canis familiaris* in the maintenance of visceral leishmaniasis in the endemic area of Diamantina municipality (Minas Gerais State, Brazil)

Importância de Canis familiaris na manutenção de leishmaniose visceral na área endêmica de Diamantina (Estado de Minas Gerais, Brasil)

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ABSTRACT: The present study aimed to evaluate the role of dogs in the epidemiology of visceral leishmaniasis (VL) in the municipality of Diamantina. Phenotypic information of seroreactive dogs were analysed and the identification of *Leishmania* was performed by Ln-PCR and sequencing. The predominant profiles observed were dogs residing in the rural area, males, mongrel and short-haired dogs. The most clinical signs were onychogryphosis and cachexia. The presence of *Leishmania infantum* circulating in the dogs of the municipality is worrying and indicates that urgent measures for surveillance and control of VL should be adopted in this endemic area.

KEYWORDS: Epidemiology; canine visceral leishmaniasis; dogs; *Leishmania infantum*.

RESUMO: O presente estudo teve como objetivo avaliar o papel dos cães na epidemiologia da leishmaniose visceral (LV) no município de Diamantina. Informações fenotípicas de cães sororreativos foram analisadas e a identificação de *Leishmania* foi realizada por Ln-PCR e sequenciamento. Os perfis predominantes observados foram cães residentes na zona rural, machos, sem raça definida e cães de pelagem curta. Os sinais clínicos mais frequentes foram onicogrifose e caquexia. A presença de *Leishmania infantum* circulando nos cães do município é preocupante e indica que medidas urgentes de vigilância e controle da LV devem ser adotadas nesta área endêmica.

PALAVRAS-CHAVE: Epidemiologia; leishmaniose visceral canina; cães; *Leishmania infantum*.

INTRODUCTION

Visceral leishmaniasis (VL) represents a serious problem of public health in Brazil. Dogs (*Canis familiaris*) play an important role in the maintenance of these disease in the human environment serving as reservoirs for *Leishmania*, for having great ability to transmit this parasite to the vector. In infected dogs, the disease manifests itself in a chronic and progressive way and, classically, the signs of canine visceral leishmaniasis (CVL) are skin lesions, onychogryphosis, splenomegaly, local or generalized lymphadenopathy, conjunctivitis, apathy, epistaxis, diarrhea, anorexia, severe dehydration, paw edema, and hyperkeratosis (Brasil, 2014).

In Brazil, the infection in dogs has preceded the occurrence of the disease in humans, mainly in new outbreaks of transmission. Studies on risk factors for the occurrence of CVL indicate that the sex, breed, age and fur type of dogs can positively influence the infection of animals. This risk increases proportionally the greater the number of dogs present in the residence, also increasing the possibility of VL occurrence in humans (França-Silva *et al.*, 2003; Coura-Vital *et al.*, 2011).

The municipality of Diamantina (Minas Gerais State) is considered an endemic area for VL, with daily reports of positive animals and records of human disease, including some of these resulting in death (Ursine *et al.*, 2016; Batista-Santos *et al.*,

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2021). Thus, considering the role of dogs as reservoirs of VL, this study aimed to evaluate the role of dogs in the epidemiology of VL, identifying the presence of *Leishmania* in dogs by serological and molecular tests, as well as to identify factors possible associated with canine infection in this VL endemic area.

MATERIAL AND METHODS

Study area

The study was conducted between the months of January and December 2019 in Diamantina municipality (18º 14' 58" S - 43º 36' 01" W), Minas Gerais State, which is considered an endemic area for human VL. It has a population of 47,702 inhabitants distributed in an area of approximately 3,900 km² and a canine population estimated at 5,000 dogs. Diamantina is an urban city small, but is characterized by being pole of the region, with Human Development Index=0.716. A large part of the population lives in modest homes and in inadequate sanitary conditions.

Its geographical location directly influences its biogeography, since the city is located in the region known as Serra do Espinhaço. Serra do Espinhaço is a mountain range that extends across several Brazilian states, and is considered one of the main transition biomes between the Atlantic Forest and the Cerrado. The climatic regime of the region is predominantly humid temperate (Cwb - Köppen classification), characterized by mild, humid summers from October to April, followed by colder, drier winters from June to August. Annual average precipitation ranges from 1250 to 1550 mm. The city is situated at an altitude of 1,280 meters. The average annual temperature falls within the range of 18° to 19°C, remaining predominantly mild throughout the year. Relative air humidity is consistently high, with annual averages of 75.6%. Further details of the study area can be seen in Batista-Santos *et al.* (2021).

Canine population survey

Blood collections were performed by spontaneous demand (when the owner asked the health team to provide care for the animal that showed any sign of disease). Venous blood samples from dogs were collected for serological diagnosis, as recommended by the Brazilian Ministry of Health: first, using the Dual Path Platform TR-DPP® as a screening immunochromatographic test and, then, seroreactive dogs at this stage were submitted to the confirmatory enzyme linked immunosorbent assay ELISA®.

Dog sampling

An individual form for each examined dog was filled out, which contained general characteristics, such as the owner's name, residence area, sex, breed and fur type. After clinical examination, they were grouped into asymptomatic or symptomatic, according to the absence or presence of at least one signs of VL infection (i.e., cutaneous lesions, onycogryphosis, splenomegaly, local or generalized lymphadenopathy, conjunctivitis, apathy, epistaxis, diarrhea, anorexia, severe dehydration, paw edema, and hyperkeratosis), respectively (based on Mancianti *et al.*, 1988, with modifications).

Detection of Leishmania by Ln-PCR

Blood samples from ten CVL-positive dogs (randomly selected) were subjected to DNA extraction using the GE Healthcare kit. After DNA assessment, the presence of *Leishmania* DNA was tested by *Leishmania* nested PCR (LnPCR) targeting the small subunit ribosomal RNA (*SSU-rRNA*) gene (Van Eys *et al.*, 1992; Cruz *et al.*, 2002). A positive control (DNA from a reference strain of *L. (V.) braziliensis* (MHOM/BR/75/M2903) and negative control (no DNA) were included in every set of reactions. The amplified products were analyzed by electrophoresis on a 2% agarose gel stained with ethidium bromide and examined under ultraviolet light.

Identification of Leishmania spp. by sequencing

DNA sequencing was performed using the BigDye® Terminator v3.1 Cycle kit and an ABI 3730 automated DNA sequencing platform (Applied Biosytems) as described in Lopes *et al.* (2019). The nucleotide sequence for each sample was aligned and compared to *Leishmania braziliensis* (M80292.1), *Leishmania amazonensis* (M80293.1), and *Leishmania infantum* (M81430.1); the sequences were deposited in the GenBank® database. BioEdit (www.mbio.ncsu.edu/bioedit.bioedit.html), BLAST (www.ncbi.nlm.nih.gov/BLAST), and MacVector® (www.macvector.com, MacVector Inc.) tools were employed for multiple sequence alignments.

Statistical analysis

All statistical analyzes were performed by using Software R version 4.2.2 (R Core Team, 2022). Bivariate analysis between the variables: sex (female or male), breed (American Foxhound, Labrador, German Shepherd, Pinscher, Pit Bull, Poodle, Mongrel), fur type (short or long) and residence area (rural or urban) being performed by using the Fisher's exact test for count data or Pearson's chi-squared test with Yates's continuity correction. The significance of the results is represented by the *p*-value, and the association was considered statistically significant when the *p*-value was less than 0.05.

Ethics statement

The activity of blood collection that involves the handling of animals is part of the routine of endemic agents of the local Municipal Health Department, and therefore, it is exempt from approval

by the Council of Ethics in the Use of Animals. Positive dogs in both tests were considered seroreactive. As recommended by the Brazilian Ministry of Health, these animals were submitted to euthanasia, in accordance with Resolution No. 1000, May 11, 2012, of the Federal Council of Veterinary Medicine (http://www3.cfmv.gov.br/portal/public/lei/index/id/326).

RESULTS AND DISCUSSION

In 2019, 534 tests were performed on dogs from different urban and rural districts of the municipality of Diamantina, of which 121 were seroreactive for CVL. The predominant profiles observed were dogs residing in the rural area (69.4%), males (66.1%) and mongrel (70.2%). The large number of American Foxhound animals among the seroreactives also called our attention (Table 1). Considering the variables under study (sex, breed, fur type and residence area) there was a significant association only between breed and residence area (p=0.0033 - Fisher's exact test). Similar results were observed by França-Silva *et al.* (2003) in Montes Claros/MG, although they also studied other variables not included in the present study.

Figure 1a shows the distribution of seroreactive dogs for VL according to symptomatology and fur type in the municipality of Diamantina in the year 2019. Eighty-three percent of cases were considered symptomatic. Short-haired dogs were the most frequent (90.9%) among the seroreactive animals analyzed. The most frequent clinical signs were onycohgryphosis (27.8%) and caquexia (24.5%), as evidenced in Figure 1b.

Figure 2 shows dog DNA amplification products obtained with SSU-rRNA gene primers visualized after agarose gel electrophoresis. It can be observed that two samples (2 and 8) were positive to the test, confirming the circulation of *Leishmania* in dogs domiciled in the municipality (Figure 2). After performing the alignment, the DNA sequencing showed (data not shown), with 99% of identity, that *Leishmania infantum* was the species found circulating in the dogs of the municipality.

Between 2016 and 2018, Batista-Santos *et al.* (2021) carried out a study in this same municipality and found a high density of *Lutzomyia longipalpis*, high canine prevalence and a record of 8 human cases of VL, with 3 deaths. On that occasion, the results revealed that all elements of the transmission chain were present in the municipality of Diamantina.

The most seroreactive animals were classified as symptomatic dogs, as they had at least one clinical sign for VL. In the present study, we observed that male, mongrel and short-haired dogs were predominant among seroreactive animals, as evidenced by França-Silva *et al.* (2003). The main clinical signs observed in animals with CVL were onychogryphosis and cachexia. These data are consistent with observations made by other authors in another endemic area of the world (Koutinas *et al.*, 1999).

Studies have also shown that short-haired animals are more likely to become infected, since the short fur facilitates the access of the sand fly to the bite site (Sideris *et al.*, 1996; Moreira-Jr *et al.*, 2003). However, there is no evidence of predisposition to CVL in relation to these and other variables in Brazil (Brasil, 2014, França-Siva *et al.*, 2003). In fact, several host-related factors, such as sex, age, genetics, nutritional status or concomitant infections, can play a key role in the dog's response to infection, which also depend on other specific variables from each endemic area (Sideris *et al.*, 1996, Solano-Gallego *et al.*, 2011).

Finally, the presence of *Leishmania infantum* circulating in the dogs of the municipality proved the role of the dog (*Canis familiaris*) as a reservoir of the parasite. This finding is worrying, given the high prevalence of CVL and the high density of *Lutzomyia longipalpis* seen in other studies (Borges *et al.*, 2009; Michalsky *et al.*, 2011; Barata *et al.*, 2011; Dias *et al.*, 2011; Lana *et al.*, 2018). This scenario indicates that urgent VL surveillance and control measures must be adopted in this endemic area.

Table 1. Profile of VL seroreactive dogs by fur-type, breed and sex from different areas of the municipality of Diamantina (Minas Gerais State, Brazil) in 2019.

Fur Type	Breed	Area				Frequency	
		Rural		Urban		n	%
		F	M	F	M		
Short-Hair	Mongrel	17	36	10	16	79	65.2
	American Foxhound	8	13	1	1	23	19.0
	Pinscher	0	2	1	3	6	5.0
	Pit bull	0	0	0	1	1	0.8
	Labrador	0	0	0	1	1	0.8
Long-Hair	Mongrel	2	3	0	1	6	5.0
	Poodle	1	0	1	1	3	2.5
	German Shepherd	0	2	0	0	2	1.7
Total (%)		28(23.1)	56(46.3)	13(10.8)	24(19.8)	121	100
		69.4%		30.6%			

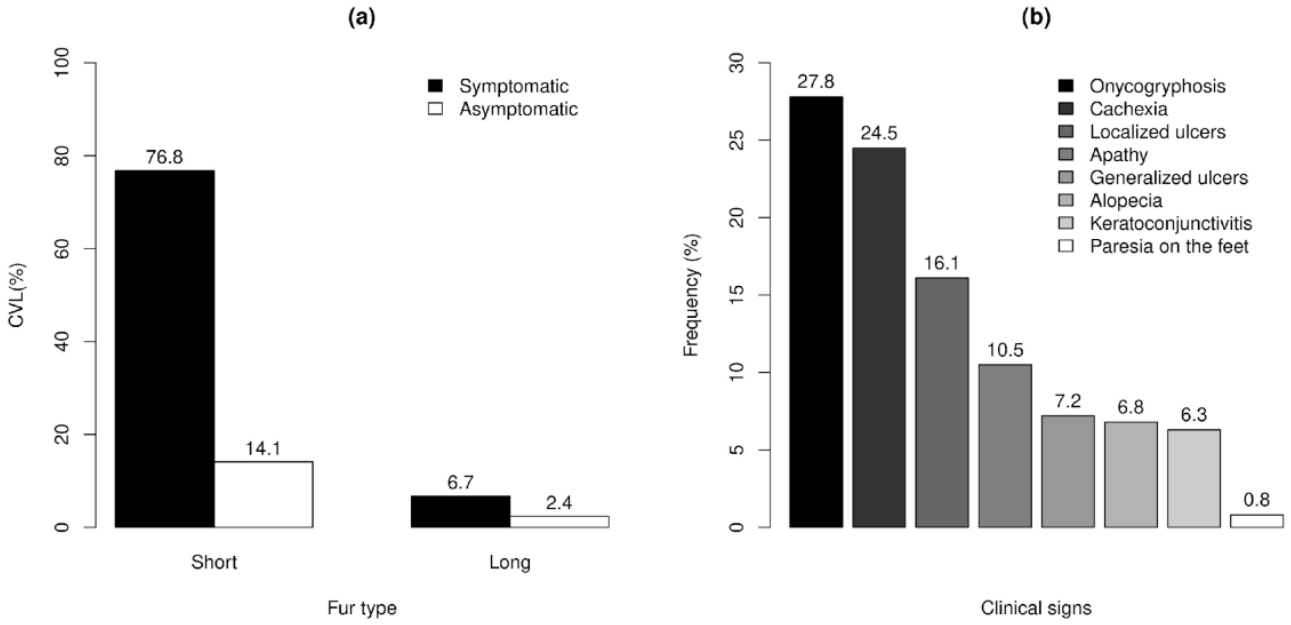

Figure 1. Profile of VL seroreactive dogs by fur type and clinical status (a) and the recorded clinical signs of CVL (b) of the municipality of Diamantina (Minas Gerais State, Brazil) in 2019.

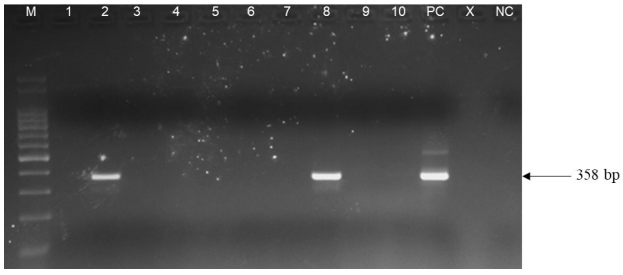

Figure 2. Electrophoresis on 2% agarose gel of the products obtained after amplification of dog blood samples total DNA by LnPCR. The 338 bp amplicon is *Leishmania*-specific (Cruz *et al.*, 2002). M- 100 bp DNA ladder; Channels: samples 1 a 10: samples positives 2 e 8; PC- positive control (*L. infantum* DNA); NC- negative control (no DNA).

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CONCLUSIONS

It was found that *Leishmania infantum* was the circulating species in seroreactive dogs in the municipality of Diamantina, proving the role of the dog (*Canis familiaris*) as a reservoir of the parasite. The results showed that the municipality of Diamantina has a high prevalence of CVL, and that prevention, control and surveillance actions are necessary and urgent, especially in locations where transmission occurs actively.

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