

***Leishmania* infection in seropositive dogs in the Mucuri Valley Region, Minas Gerais, Brazil**

Infecção por *Leishmania* em cães seropositivos do Vale do Mucuri, Minas Gerais, Brasil

Infección por *Leishmania* en perros seropositivos en la region del Valle de Mucuri, Minas Gerais, Brasil

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Lidia Roedel Hinkelmann Berbert

ORCID: <https://orcid.org/0000-0002-0847-3545>
Federal University of the Jequitinhonha and Mucuri Valleys, Brazil
E-mail: liluedu@gmail.com

Camila Binder Soares de Souza

ORCID: <https://orcid.org/0000-0003-2883-021X>
Oswaldo Cruz Foundation, Brazil
E-mail: cbs@gmail.com

Gabriela de Paula Carli

ORCID: <https://orcid.org/0000-0003-3324-7686>
Federal University of Juiz de Fora, Brazil
E-mail: gabicarli15@gmail.com

Ygor Torres Pinto

ORCID: <https://orcid.org/0000-0001-6139-1672>
Federal University of Juiz de Fora, Brazil
E-mail: ygor.torresp@gmail.com

Fernando Leitão Rocha Júnior

ORCID: <https://orcid.org/0000-0002-7469-6472>
Federal University of the Jequitinhonha and Mucuri Valleys, Brazil
E-mail: fermn16@ufvjm.edu.br

Sandra Bertelli Ribeiro de Castro

ORCID: <https://orcid.org/0000-0002-5535-0919>
Federal University of the Jequitinhonha and Mucuri Valleys, Brazil
E-mail: sandrabertelliribeiro@gmail.com

Célia Maria Ferreira Gontijo

ORCID: <https://orcid.org/0000-0002-7034-535X>
Oswaldo Cruz Foundation, Brazil
E-mail: gontijo@cpqr.fiocruz.br

Alessandra de Paula Carli

ORCID: <https://orcid.org/0000-0002-7956-8947>
Federal University of the Jequitinhonha and Mucuri Valleys, Brazil
E-mail: alessandrapcarli@hotmail.com

Caio César de Souza Alves

ORCID: <https://orcid.org/0000-0001-9765-8527>
Federal University of the Jequitinhonha and Mucuri Valleys, Brazil
E-mail: caiosca@gmail.com

Abstract

Introduction: Canine Visceral Leishmaniasis (CVL) is a severe systemic zoonosis that has the dog as the main reservoir for human disease. As it is a neglected tropical disease, there are gaps in its aspects, especially in regional terms. In this sense, the aim was to evaluate the profile of infected animals and identify the prevalent species in canine infection in the macro-region of Mucuri Valley, Minas Gerais, Brazil, through the polymerase chain reaction (PCR-RFLP-ITS1). **Methodology:** Cross-sectional observational study carried out between August/2019 and February/2020 at the San Francisco Veterinary Clinic and the Municipal Dog Kennel. Positive animals to the DPP® were selected and biological samples were collected: venous blood (SNAP); ear skin fragment (imprint); and bone marrow aspirate to smear slides, isolation in culture environment, and molecular diagnosis. All animals underwent clinical examination and data collection: provenance, symptoms, fur, age, sex. **Results:** In 88 of the 132 samples, the presence of *Leishmania*'s DNA was detected and in 87.3% the species *L. infantum* was identified. Of these 88 animals, 69 had symptoms; 45 were males; 73 with short fur. The highest frequency of positive PCR occurred between 2-5 years (55 animals). 91.67% were SNAP test positive. **Conclusions:** The present study made it possible to understand aspects of

CVL in the evaluated samples and to identify the etiological agent most prevalent in the area, allowing the establishment of efficient strategies to control the disease in dogs and to prevent human infection in the region.

Keywords: Canine visceral leishmaniasis; Clinical features; *Leishmania* species identification.

Resumo

Introdução: A Leishmaniose Visceral Canina (LVC) é uma zoonose sistêmica grave que tem o cão como principal reservatório para a doença humana. Sendo uma doença tropical negligenciada, há lacunas sobre seus aspectos, sobretudo em caráter regional. Neste sentido, objetivou-se avaliar o perfil dos animais infectados e identificar as espécies prevalentes na infecção canina na macrorregião do Vale do Mucuri, Minas Gerais, Brasil, por meio da reação em cadeia de polimerase (PCR-RFLP ITS1). **Metodologia:** Estudo observacional transversal realizado entre agosto/2019 e fevereiro/2020 na Clínica Veterinária São Francisco e no Canil Municipal. Foram selecionados animais positivos ao Teste Rápido DPP®/Biomanguinhos, por meio da coleta de sangue venoso (SNAP); fragmento de pele de orelha (imprint por aposição); e aspirado de medula óssea para esfregaço em lâminas, isolamento em meio de cultura e diagnóstico molecular. Todos os animais passaram por exame clínico e coleta de dados: procedência, clínica, pelagem, idade, sexo. **Resultados:** Dentre 132 amostras, 88 (66,7%) identificaram a presença de *Leishmania* ao PCR-RFLP ITS1 e a identificada em 87,3% destes foi a *L. infantum*. Destes 88, 69 eram sintomáticos; 43 fêmeas, 45 machos; 73 com pelo curto. À idade, a maior frequência de PCR positivos ocorreu entre 2-5 anos (55 animais). 91,67% foram positivas ao teste sorológico SNAP, enquanto os parasitológicos foram majoritariamente negativos, apesar da presença de sintomas. **Conclusões:** O presente estudo possibilitou conhecer aspectos da LVC na amostra avaliada e identificar o agente etiológico prevalente na área de procedência, permitindo estabelecer estratégias eficientes no controle da enfermidade nos cães da região.

Palavras-chave: Leishmaniose visceral canina; Características clínicas; Identificação de espécies de *Leishmania*.

Resumen

Introducción: La Leishmaniasis Visceral Canina (LVV) es una grave zoonosis sistémica que tiene al perro como principal reservorio de la enfermedad humana. Al ser una enfermedad tropical desatendida, existen vacíos en cuanto a sus aspectos, especialmente en el carácter regional. En ese sentido, el objetivo fue evaluar el perfil de animales infectados e identificar las especies prevalentes en la infección canina en la macrorregión Vale do Mucuri, Minas Gerais, Brasil, por medio de la reacción en cadena de la polimerasa (PCR-RFLP ITS1). **Metodología:** Estudio observacional transversal realizado entre agosto/2019 y febrero/2020 en la Clínica Veterinária São Francisco y en el Canil Municipal. Los animales positivos al Test Rápido DPP®/Biomanguinhos fueron seleccionados por medio de extracción de sangre venosa (SNAP); fragmento de piel de oreja (impresión por aposición); y aspirado de médula ósea para frotis en lámina, aislamiento en medio de cultivo y diagnóstico molecular. Todos los animales fueron sometidos a examen clínico y recogida de datos: procedencia, pelaje, edad, sexo. **Resultados:** De 132 muestras, 88 (66,7%) identificaron la presencia de *Leishmania* por PCR-RFLP ITS1 y la identificada en el 87,3% de estas fue *L. infantum*. De estos 88, 69 eran sintomáticos; 43 mujeres, 45 hombres; 73 con pelo corto. Por edad, la mayor frecuencia de PCR se presentó entre los 2-5 años (55 animales). El 91,67% resultó positivo a la prueba serológica SNAP, mientras que las pruebas parasitológicas fueron en su mayoría negativas, a pesar de la presencia de síntomas. **Conclusiones:** El presente estudio permitió conocer aspectos de la LVC en la muestra evaluada e identificar la etiológica prevalente en la zona de origen, permitiendo establecer esquemas eficientes en el control del agente en perros.

Palabras clave: Leishmaniasis visceral canina; Características clínicas; Identificación de especies de *Leishmania*.

1. Introduction

Caused by many different species of *Leishmania*, leishmaniasis configures the second most common zoonosis in the world (BRASIL. Ministério Da Saúde. *Guia de Vigilância Epidemiológica*, 7. Ed., Cad. 11, Ministério Da Saúde, Brasília/DF, p. 1-64, 2009., n.d.) and is considered a Neglected Tropical Disease (Almeida et al., 2017), according to the World Health Organization (WHO). Its transmission among human beings and animals occurs by the bite of sandflies infected females (Brazil and Departamento de Vigilância Epidemiológica, 2003). Its wide range of etiological agents adds to the vast number of vector insects and potential reservoir animals explaining its difficulty in controlling the spread of infections (Teixeira-Neto et al., 2014).

Visceral Leishmaniasis (VL) also known as “Kala-azar” is a severe systemic disease that manifests among humans as well as in dogs (Travi et al., 2018). Besides being presented as an anthroponotic disease in countries where the current etiological agent is the *L. donovani*, in Brazil, VL is a zoonosis caused by *L. infantum*, in which the dog is the main reservoir of the parasite, especially considering the abundance of amastigotes forms in the skin of infected dogs (Alvar et al., 2004).

The disease in the animal is characterized by slow and insidious development and its presentation may be nonspecific and without pathognomonic signs. The clinical presentation depends on the immune response from the host and its primary manifestations are commonly associated with peeling and eczematous skin lesions. As the disease advances, it is common to verify onychogryphosis, splenomegaly, lymphadenopathy, alopecia, dermatitis, skin ulcers, keratoconjunctivitis, rhinorrhea, apathy, diarrhea, intestinal bleeding, paw edema, and vomit, besides hyperkeratosis. At the final stage of the disease, it's not unusual to see hind limb paresis, followed by cachexia, starvation, and death (Brazil and Departamento de Vigilância Epidemiológica, 2003). Nevertheless, about 60 to 80% of dogs that live in endemic areas may have contact with *L. infantum* and still be asymptomatic or oligosymptomatic for a long period of time, therefore taking an active role in the parasite transmission (Queiroz et al., 2010).

The diagnosis, of CVL, is still a challenge for public health due to the variety of different diagnoses, nonspecific histopathological alterations, and lack of 100% sensitive and specific diagnostic tests (Brazil and Departamento de Vigilância Epidemiológica, 2003). Because of this, there is a tendency towards underdiagnoses, which makes it difficult to control the disease (Berrahal et al., 1996). In this sense, as a highly specific method, etiological diagnosis by Polymerase Chain Reaction (PCR) has been used as a strategy for monitoring and defining prophylactic (Dantas-Torres, 2009; Lago et al., 2019; Solano-Gallego et al., 2011).

There are still gaps in many aspects of leishmaniasis that harden the disease management, among which can be cited the geographic distribution of parasites and vector insects; and the sources and risk factors associated with infection in dogs (Brazil and Departamento de Vigilância Epidemiológica, 2003). As each transmission area shows its own particularities (Teixeira-Neto et al., 2014) and, in Brazil, there are areas where the different species of *Leishmania*, that causes different clinical forms, are sympatric (Gontijo and Melo, 2004), the disease detailing - searching for the current species in each region - is needed in order to propose more specific and efficient local control measures.

Thereby, this study aims to create a profile of infected animals and to identify *Leishmania* species present in canine infection in the macro-region of Vale do Mucuri, Minas Gerais, Brazil, by using the PCR method, in order to establish efficient measures of epidemiological surveillance according to the etiologic agent.

2. Methodology

2.1 Study Design and sample handling

A cross-sectional observational study was accomplished between August 2019 and February 2020 at Veterinary Clinic São Francisco and at the Municipal Kennel, both located in the municipality of Teófilo Otoni, in the Mucuri Valley, State of Minas Gerais, Brazil. The methodological publications of the present study were developed in line with recent literature of specialized design (Severino, 2017).

At first, were selected the asymptomatic and symptomatic animals tested positive for the quick test DPP®/Biomanguinhos. From these animals were taken samples of venous blood to perform quick tests (DPP and SNAP); fragment of skin tissue taken from the animal's ear to imprint cytology; and bone marrow aspiration to perform smear on slide technique, isolation on culture medium, and molecular diagnosis.

Animal samples from the macro-region of Mucuri Valley, State of Minas Gerais, Brazil, were used in this study and all animals selected had been clinically examined and had their data been collected such as provenance, presence of clinical signs, pelage characteristics, age, and gender. The project was approved by the Animal Use Ethics Committee from the Federal University of Jequitinhonha and Mucuri Valleys under protocol 03/2019. The legally responsible for the dogs were informed about the research and signed the Informed Consent Form.

2.2 Serological diagnosis

The inclusion criteria in the study were positive in the DPP®/Biomanguinhos test (Figueiredo et al., 2018). DPP-positive dogs were therefore tested with Rapid Test SNAP/ELISA by IDEXX (SNAP® LEISHMANIA) to detect anti- *L. donovani* and *L. infantum* antibodies (Pedrassani et al., 2019).

2.3 Parasitological diagnosis

After trichotomy, skin disinfection, and anesthesia using 2% lidocaine, there were collected samples of skin from the left ear with 5 mm disposable PUNCH; bone marrow aspirate from the sternum bone using 40 mm x 12 mm fine-needle aspiration (FNA) technique. The skin slides were made by affixing (imprint), while the bone marrow slides by smear. Romanowski's coloring principle (Fast Panoptic) was selected for the direct parasitological examination (Barbosa et al., 2012; Dias et al., 2019). Samples that presented at least one amastigote form of the parasite were considered positive (Freitas et al., 2012).

2.4 Parasite Isolation

Samples from bone marrow aspirates were stored under refrigeration at 4°C for 24 hours in saline solution with antibiotics (streptomycin 100 µg/mL and penicillin 500 U/mL). After this period they were put into tubes containing NNN (Novy & Mc Nel, 1903; Nicolle, 1908) enriched with Liver Infusion Tryptose medium supplemented with 20% fetal bovine serum (FBS) and associated antibiotics (penicillin and streptomycin 100-200 µg/ml). The cultures were incubated at 25°C ± 1°C. The culture exam was performed weekly and considered positive if observed the presence of promastigote forms of *Leishmania*. Isolated samples were cryopreserved and deposited in the strain bank of the Leishmaniosis Study Group from the René Rachou Institute/Fiocruz Minas.

2.5 Molecular Diagnosis

The presence of the parasite was also investigated by amplifying the DNA directly isolated from the bone marrow aspirates and positive cultures. DNA extraction was performed by extraction kit "PureLink™ Genomic DNA Mini Kit" (Invitrogen®) and PCR/RFLP reactions (ITS-1/HAEIII) were used to detect and identify the presence of the parasite in the samples. The primers LITSR 5' CTGGATCATTTCGATG 3' and L5.8S 5' TGATACCACTTATCGCACTT 3' amplify 350 pb fragment through the following reaction: buffer solution 1x (200 mM Tris-HCl pH8,4, 500 mM KCl), 1,5 mM of MgCl₂, 0,2 of dNTP mix, 0,5 pmol of primer LITSR, 0,5 pmol of primer L5.8S, 1 U of Taq DNA polymerase platinum® (Invitrogen), 2 µl of template DNA within a final volume of 25 µl. Amplification was realized alternating 33 cycles of denaturation at 95°C for 30 seconds, then DNA annealing at 53°C for 1 minute and lastly extension at 72°C for 1 minute in automatic thermocycler equipment. The subsequent RFLP was realized in order to identify the species. It was prepared with a final volume of 15 µL which contains 1µL of HAEIII(10 U/µL), 1,5 µL of the enzyme buffer solution, and 10.0 µL of PCR products. The mixture was incubated at 37°C for 2 hours. The restriction profiles were analyzed in a solution containing 2% agarose gel stained with ethidium bromide and compared to the pattern obtained by the digestion of reference strains: *Leishmania amazonensis* (IFLA/BR/67/PH8), *L. braziliensis* (MHOM/BR/75/M2903), *L. infantum* (MHOM/BR/74/PP75) and *L. guyanensis* (MHOM/BR/75/M4147).

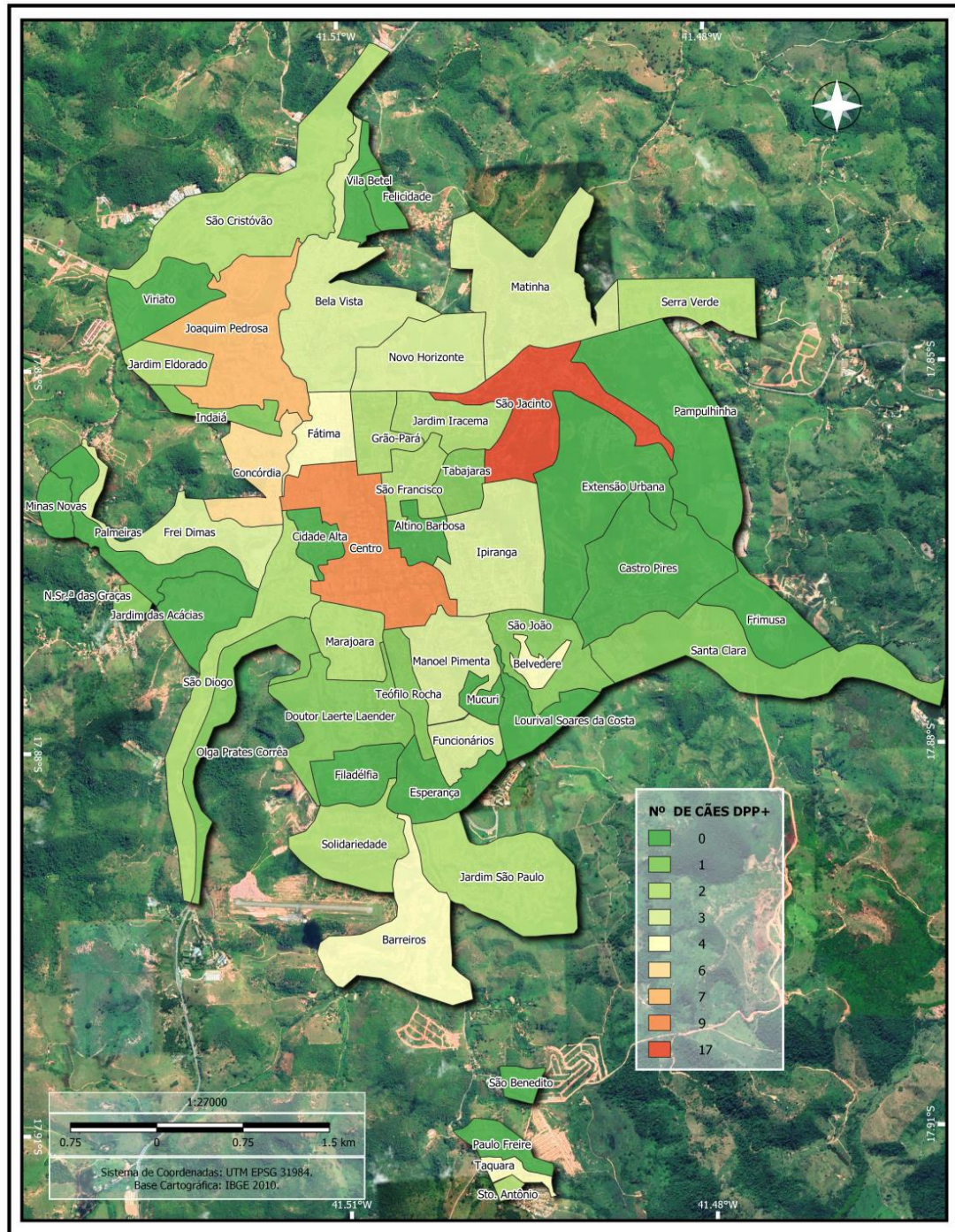
2.6 Statistical analysis

The software GraphPad Prism was chosen to analyze the data by using Fisher's exact test and chi-square test. Any p<0.05 variables were considered significantly associated.

3. Results

132 animals were selected from the positive DPP®/Biomanguinhos quick test. Concerning the animal origin, 58 dogs (43.94%) were from the São Francisco Veterinary Clinic, of which 37 (63.79%) had symptoms. In addition, 74 (56.06%) animals were from the Municipal Kennel and there were 65 (87.84%) symptomatic among those. According to Figure 1,

Figure 1: Distribution map of DPP®/Biomanguinhos seropositive dogs by neighborhood from the city of Teófilo Otoni, Minas Gerais State, Brazil.

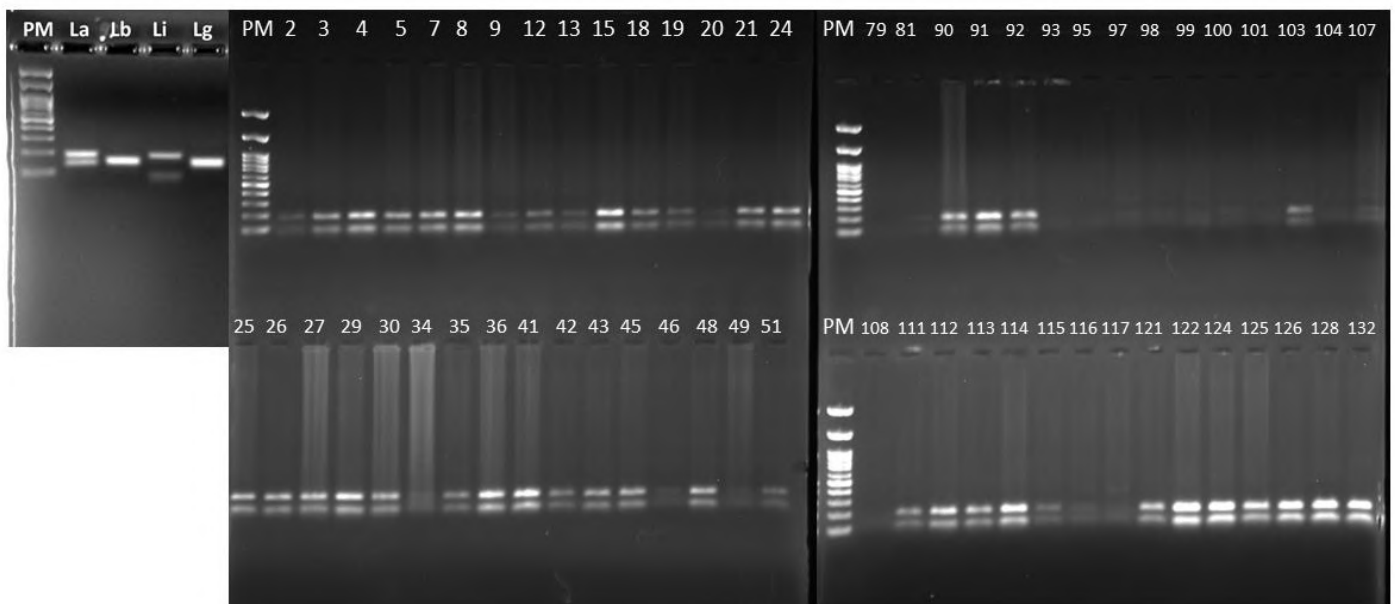


Source: Own authorship (2021).

The geographic distribution of positive dogs accounted for 127 dogs from the city of Teófilo Otoni of which only 22 animals came from the rural area. The remaining 105 animals came from the urban area. Merely 5 dogs were from other counties of the macro-region.

Bone marrow samples collected from 132 seropositive dogs were submitted to molecular diagnosis to confirm the infection and to identify the species. Isolated samples in the culture medium were also submitted to specific PCR-RFLP ITS1 identification. Figure 2 shows the restriction profiles of some samples identified as *L. infantum* in this study based on the comparison with the reference strain profiles.

Figure 2: Representative result of the digestion profile using the HaeIII enzyme fragments of the ITS1 intergenic region amplified from dogs' bone marrow samples. PM – 100 bp molecular weight marker; La – reference strain to *L. amazonensis* (IFLA/BR/75/PH8); Lb – reference strain to *L. braziliensis* (MHOM/BR/75/2903); Li – reference strain *L. infantum* (MHOM/BR/74/PP75); Lg – reference strain to *L. guyanensis* (MHOM/BR/75/M4147); 2 to 24 – samples from dogs.



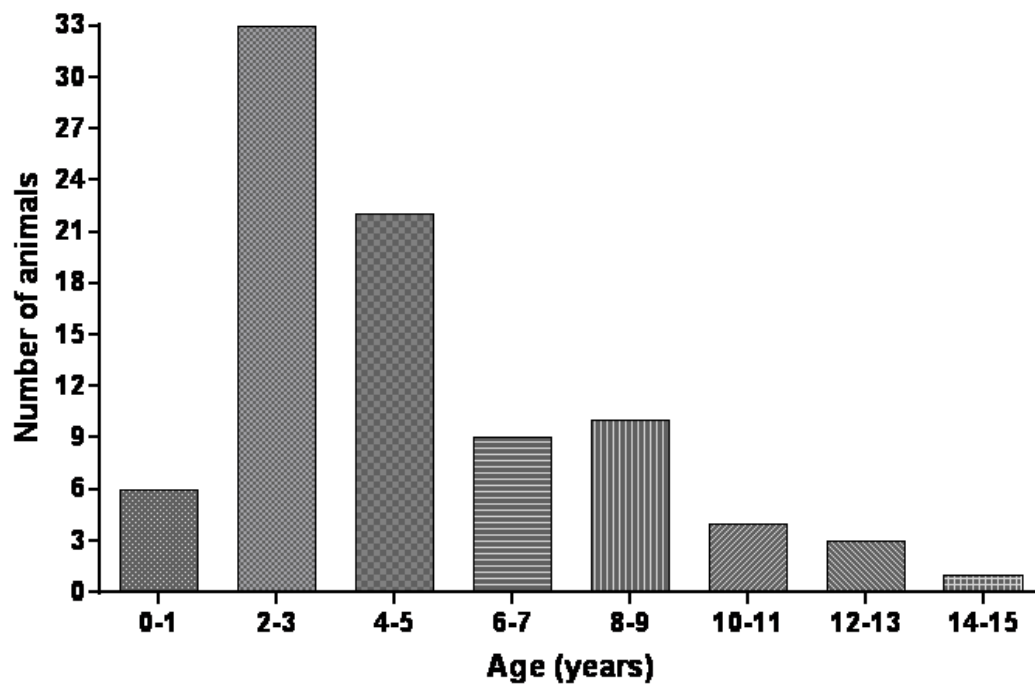
Source: Own authorship (2021).

Among the 132 samples, the presence of *Leishmania* was identified in 88 (66.7%), and 9 samples (10.2%) were only positive indirect examination of skin slide or bone marrow aspiration. From the samples submitted to PCR-RFLP ITS1, 87.3% of the species *L. infantum* was identified.

Of the 88 PCR-RFLP ITS1 positive animals studied, 69 (78.41%) had at least one clinical sign possibly related to CVL and 19 (21.59%) were asymptomatic. About the sex distribution, the sample consisted of 43 females (33 symptomatic) and 45 male dogs (36 symptomatic). Regarding the pelage type, 73 animals had short pelage and 15 had long pelage. Lastly, 46 came from the Municipal Kennel, while 42 came from the São Francisco Veterinary Clinic.

Concerning the age, the higher frequency of positive PCR occurred in the age group of 2 to 5 complete years, with 55 animals. The other distributions can be analyzed according to Figure 3.

Figure 3: Representative of the dogs studied according to age.



Source: Own authorship (2021).

Serological and parasitological diagnostic methods were correlated with the presence/absence of clinical signs in the 88 dogs PCR-RFLP ITS1 positive for *Leishmania*. The results are described in table 1. Due to the positivity of the DPP®/Biomanguinhosquick test being the inclusion criteria in the present study, it was not considered.

Table 1: Presence of clinical signs of CVL and qualitative results of serological and parasitological tests in dogs tested positive for *Leishmania* in the PCR-RFLP ITS1.

Diagnostic	Assays	Results	Clinical signs	
			Present	Absent
Serological	SNAP	(+) 91,67%	73,49%*	18,18%
		(-) 8,33%	3,79%	4,54%
Parasitological	Bone marrow smear	(+) 26,52%	21,18%	5,34%
		(-) 73,48%	55,98%	3,79%
	Ear skin	(+) 18,18%	3,79%	14,39%
		(-) 81,82%	62,88%	18,94%
	Culture	(+) 34,85%	29,49%	42,57%
		(-) 65,15%	42,57%	22,58%

*p = 0.017 when compared to absent clinical signs

Source: Own authorship (2021).

There was a predominant positivity to the SNAP serological test (91.67%), and of these, 73.49% were from symptomatic dogs. Regarding the parasitological tests, the results were mostly negative, both in the bone marrow smear

(73.48%), in the ear skin (81.82%), and in culture medium samples (65.15%), although there were symptoms in 55.98%; 62.88% and 42.57% respectively (Table 1).

4. Discussion (can be separated or together)

The occurrence of *Leishmania infantum* as the most prevalent species concerning cases of canine visceral leishmaniasis (CVL) in Brazil was confirmed in our study through the cultural isolation of this species and by the molecular identification from bone marrow aspirate of DPP+ dogs. It is important to highlight that this study is the first to identify the etiological agent species which cause CVL in the Mucuri Valley region.

Other species of *Leishmania* have already been reported in dogs (Alves Souza et al., 2019). In Brazil, the identification of *L. amazonensis* in animals with clinical manifestations of CVL was recorded in an endemic area for visceral leishmaniasis (Valdivia et al., 2017). However, *L. infantum* is still the predominant species in endemic areas to either canine or human leishmaniasis. In endemic areas, the dog is considered the main urban reservoir of *L. infantum*, and the identification of such species in suspected animals is very important for the Ministry of Health in Brazil, which establishes a series of measures from this identification and for the control of transmission for this species (Oliveira, n.d.).

In the last five years, 16 new cases of human visceral leishmaniasis were notified in the city of Teófilo Otoni (*Spatio-Temporal Modelling of Leishmania Infantum Infection among Domestic Dogs: A Simulation Study and Sensitivity Analysis Applied to Rural Brazil | Parasites & Vectors | Full Text*, n.d.), which is classified as a moderate transmission county according to the criteria of the Ministry of Health (Oliveira, n.d.). In such areas, the development of studies about CVL is relevant to promote better strategies to contain the expansion of both canine and human diseases.

The distribution of seroreactive dogs found in this study corroborates with the information provided by the Health Department of Teófilo Otoni about the regions with the highest incidence of canine and human visceral leishmaniasis in the period between 2015 and 2019. A higher incidence was found in the northern region and Center neighborhoods of the city. The São Jacinto Neighborhood, in the Northeast Region, stood out due to the high incidence in the present work. The higher prevalence in neighborhoods located in peripheral regions may be related to variables that facilitate the vector's biological cycle, such as a large number of trees and the accumulation of garbage and organic matter (Fernández et al., 2013), in addition to the advance of urbanization in areas previously represented by forest reserve (Bevilacqua et al., 2001).

The data on the prevalence of symptoms in seropositive animals, with mild to moderate dermatitis being the most frequent, is consistent with what is found in the literature, in which there was an absence of symptoms in 20 to 40% of seropositive dogs (Feitosa et al., 2000).

Regarding the distribution by sex, there was no significant heterogeneity of seropositivity and symptoms between males and females, a picture that corroborates previous study, which evaluated CVL in an endemic area of the city of Montes Claros, Minas Gerais, Brazil (França-Silva et al., 2003). However, in a research carried out in rural settlements in the hinterlands of Northeastern of Paraíba mesoregion, Brazil, found that females were more exposed to the risk of infection, possibly due to hormonal and immunological variations in the periods of estrus and gestation (Silva et al., 2017). Meanwhile, higher levels of infections in males, were shown in another study in a Brazilian municipality (Penaforte et al., 2013). Thus, there is no consensus on the disease frequency by sex (Gontijo and Melo, 2004).

As for the age group, the higher frequency of seropositive animals from 2 years onwards may be related to the long incubation periods of the disease and to the fact that adult animals are placed more often in peridomestic areas, so are also more exposed to the sandflies vector, as previously noted (Matos et al., 2006). However, a previous study observed the same probability of catching *L. infantum* infection at all ages, in order that other age groups are also important in the disease epidemiology (França-Silva et al., 2003).

Regarding the diagnosis of CVL, there are no 100% specificity and sensitivity tests. The presence of 11 negative SNAP IDEXX ELISA tests, even with positive DPP, corroborates the study that found that DPP Bio-Manguinhos (97.9%) was the most sensitive and SNAP IDEXX ELISA was the most specific (100%), which is a good combination of rapid serological tests for the previous diagnosis of CVL (Ribeiro et al., 2019). However, the disagreements between serological tests show the need for additional parasitological and/or molecular tests to confirm the diagnosis. In addition, the results of serological tests in asymptomatic animals should be carefully evaluated, since this usually presents low antibody titers. The low culture positivity in our study can be explained by the intrinsic limitations of the technique, related to the great variability of the parasite load observed in dogs and the heterogeneous distribution of the parasites in different tissues and organs of the same individual (Evans et al., 1989).

As described in a previous paper (Furtado et al., n.d.), in this study, imprint slides of ear fragments showed lower rates of amastigotes than those with bone marrow smears, a situation that can be explained by the presence of anemia often observed in dogs infected with *L. infantum*. Anemic dogs may present pale mucous membranes, one of the main clinical signs found in the study, and thus cause both lower circulation and parasite load in the ears (Feitosa et al., 2000; Torrecilha et al., 2016). In asymptomatic dogs, the sensitivity of direct examination for amastigotes was less than 30%, and it is probably due to the low parasite density, which can lead to false-negative results (Mendonça et al., 2017)

5. Conclusion

Finally, considering the lack of local studies on CVL, the need to implement measures that prevent the spread of the disease – above all, but not only, through the accurate diagnosis of suspicious dogs - is a prerogative for disease control and also a major challenge for public health.

The present study allowed the acknowledgment of some aspects of CVL in the sample evaluated, beyond identifying the prevalent etiological agent in the studied area. Our data can contribute to the establishment of strategies by surveillance agencies, allowing the implementation of public health measures for greater control of the disease in dogs and humans.

Finally, we suggest, for further studies, to expand the studied area to the Mucuri Valley countryside, in order to allow comparisons of the *L. infantum* prevalence between rural and urban regions, which has already been mostly studied in this work.

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