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Original Article
Small Animal Diseases

Detection of multiple circulating sandflies species and investigation of dogs and vectors naturally infected with *Leishmania* in the city of Ribeirão Vermelho, southeastern Brazil[1]

Ingrid M. Alvarenga[2], Joseane C. Castro[3], Leandro M.R. Melo[4], Mariana M. Oliveira[2], Pedro H. Castro[2], Tarcísio F. Milagres[5], José D.A. Filho[6], Camila B.S. Souza[6], Ricardo T. Fujiwara[7], Thales A. Barçante[3], Ana P. Peconick[2] and Joziana M.P. Barçante[3]*

ABSTRACT.- Alvarenga I.M., Castro J.C., Melo L.M.R., Oliveira M.M., Castro P.H., Milagres T.F., Filho J.D.A., Souza C.B.S., Fujiwara R.T., Barçante T.A., Peconick A.P. & Barçante J.M.P. 2024. **Detection of multiple circulating sandflies species and investigation of dogs and vectors naturally infected with *Leishmania* in the city of Ribeirão Vermelho, southeastern Brazil**. *Pesquisa Veterinária Brasileira 44:e07398, 2024*. Departamento de Medicina, Faculdade Ciências da Saúde, Universidade Federal de Lavras, Trevo Rotatório Professor Edmir Sá Santos s/n, Cx. Postal 37203-202, Lavras, MG, Brazil. E-mail: joziana@ufla.br

Leishmaniasis is a zoonotic disease caused by parasites of the *Leishmania* genus, resulting in various clinical forms, including a highly lethal visceral form. This study aimed to identify *Leishmania* spp. in sandflies and dogs in a small Brazilian town. DPP® Visceral Canine Leishmaniasis (CVL) was used for screening, and ELISA and conventional PCR were used for confirmatory testing, while sandflies were captured using CDC light traps and conventional PCR targeting ITS1. The Hill series was used to identify the diversity profile of species in the sampled area using R software. The study identified a *Leishmania* spp. prevalence of 4.02% in dogs, exceeding the 2% limit required by the Brazilian Ministry of Health. A total of 443 sandflies belonging to 14 different species were identified, with *Lutzomyia longipalpis* being the most abundant (73.81%). Negligence regarding leishmaniasis in small towns can lead to late diagnosis, hence the need to implement effective strategies, including early diagnosis and treatment of human and canine cases, vector control programs, and awareness campaigns to educate the public about risks and preventive measures. These measures can help prevent the spread of leishmaniasis and improve health outcomes for affected individuals and animals.

INDEX TERMS: Leishmaniasis, epidemiology, parasite, sandflies, dogs.

RESUMO.- [Detecção de múltiplas espécies de flebotomíneos circulantes e investigação de cães e vetores naturais infectados por Leishmania na cidade de Ribeirão Vermelho, sudeste do Brasil.] A leishmaniose é uma doença zoonótica causada por parasitas do gênero *Leishmania*, resultando em várias formas clínicas, incluindo uma forma visceral altamente letal. Este estudo teve como objetivo identificar *Leishmania* spp. DNA em flebotomíneos e cães em uma pequena

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[2] Departamento de Medicina Veterinária (DMV), Faculdade de Medicina e Zootecnia (FMVZ), Universidade Federal de Lavras (UFLA), Trevo Rotatório Professor Edmir Sá Santos s/n, Cx. Postal 37203-202, Lavras, MG, Brazil.

[3] Departamento de Medicina, Faculdade Ciências da Saúde, Universidade Federal de Lavras (UFLA), Trevo Rotatório Professor Edmir Sá Santos s/n, Cx. Postal 37203-202, Lavras, MG, Brazil. *Corresponding author: joziana@ufla.br

[4] Departamento de Biologia (DBI), Instituto de Ciências Naturais (ICN), Universidade Federal de Lavras (UFLA), Trevo Rotatório Professor Edmir Sá Santos s/n, Cx. Postal 37203-202, Lavras, MG, Brazil.

[5] Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul (UFRGS), Campus Saúde, Rua Ramiro Barcelos 2.600, Sala 625, Porto Alegre, RS 90035-003, Brazil.

[6] Instituto René Rachou (Fiocruz Minas), Av. Augusto de Lima 1.715, Barro Preto, Belo Horizonte, MG 30190-009, Brazil.

[7] Instituto de Ciências Biológicas (ICB), Universidade Federal de Minas Gerais (UFMG), Campus da UFMG, Av. Antônio Carlos 6627, Pampulha, Belo Horizonte, MG 31270-901, Brazil.

cidade brasileira. A investigação usou DPP® Visceral Canine Leishmaniasis (CVL) para triagem, e ELISA e PCR convencional para teste confirmatório, enquanto flebotomíneos foram capturados usando armadilhas de luz CDC e PCR convencional visando ITS1. A série Hill foi utilizada para identificar o perfil de diversidade das espécies na área amostrada, utilizando o software R. O estudo identificou uma prevalência de *Leishmania* spp. em 4,02% dos cães, ultrapassando o limite de 2% exigido pelo Ministério da Saúde do Brasil. Foi identificado um total de 443 flebotomíneos, pertencentes a 14 espécies diferentes, com *Lutzomyia longipalpis* sendo o mais abundante (73,81%). A negligência com a leishmaniose em cidades pequenas pode levar ao diagnóstico tardio, daí a necessidade de implementar estratégias eficazes, incluindo diagnóstico precoce e tratamento de casos humanos e caninos, programas de controle de vetores e campanhas de conscientização para educar o público sobre riscos e medidas preventivas. Essas medidas podem ajudar a prevenir a propagação da leishmaniose e melhorar os resultados de saúde para indivíduos e animais afetados.

TERMOS DE INDEXAÇÃO: Leishmaniose, epidemiologia, parasito, flebotomíneos, cães.

INTRODUCTION

Leishmaniasis is a complex of zoonotic infectious diseases caused by more than 20 species of parasites from the genus *Leishmania* Ross, 1903 (Kinetoplastida: Trypanosomatidae), whose biological cycle involves the participation of vertebrate (mammal) and invertebrate (insect) hosts (Pearson & Sousa 1996, Desjeux 2004, WHO 2023). It can present in different clinical forms, namely, tegumentary leishmaniasis (TL) and visceral leishmaniasis (VL), the latter being of particular importance due to its high lethality, especially in children, elderly individuals and immunocompromised individuals, if untreated (Pearson & Sousa 1996, Desjeux 2004, Gontijo & Melo 2004, Mourão et al. 2014, Zacarias et al. 2017).

In 2022, 1,684 cases of visceral leishmaniasis (VL) were reported in Brazil. The highest number of cases occurred in the Southeast Region (245 cases) and the Northeast Region (731 cases). In Minas Gerais, 151 cases were reported, representing 61.63% of the cases in the Southeast Region. The lethality of VL in Brazil in the same year was 11%. In the Southeast Region, the lethality was 14%. In Minas Gerais, lethality reached 15%, with 29 deaths reported (Brasil 2023). One of the main risk factors for VL is the presence of positive dogs for the disease. Although other animals have been described carrying the protozoan and are potentially capable of causing vector infection, under the current experimental conditions, dogs fulfilled the necessary conditions to be considered reservoirs because they are highly susceptible, have high cutaneous parasitism and have a close relationship with humans (WHO 2023).

Sandflies are of great importance to public health, as they transmit etiological agents of infectious diseases, mainly those of the genus *Leishmania* (Brasil 2014). *Lutzomyia longipalpis* (Neiva & Lutz, 1912) is Brazil's main species involved in transmitting *Leishmania infantum*. This species has a wide geographic distribution, in contrast to *Lutzomyia cruzi* (Mangabeira, 1938), another species involved in the epidemiology of VL in Brazil, which has its geographic distribution restricted to the Central-West Region (Andrade-Filho et al. 2017).

Given this serious public health problem, the aim of this study was to verify the e sandfly species in a small town and investigate the presence of *Leishmania* DNA in sandflies and dogs.

MATERIALS AND METHODS

Animal Ethics. The present study was carried out following all ethical precepts recommended in the Brazilian legislation on animals in scientific research. The study was approved by the Ethics Committee on the Use of Animals (CEUA) at the "Universidade Federal de Lavras" (UFLA) on 12/22/17, as stated in certificate no. 77/17.

Study area. This study was conducted in Ribeirão Vermelho (Fig.1), a municipality belonging to the mesoregion of Campo das Vertentes and the microregion of Lavras in southern Minas Gerais, which has an estimated population of 3,826 inhabitants and a territorial extension of 49,251km^2 (IBGE 2023). This region is mountainous, and the main hydrographic basin is Rio Grande. The city's population and economic growth are linked to the implementation of a railway in the region, which made it possible to build bridges, facilitated and increased trade, and provided employment opportunities. The main mineral product of this region is sand, and agriculture is based on coffee and corn. To date, there are no records of cases of human visceral leishmaniasis.

Serological tests. The protocols recommended by the "Ministério da Saúde" (Ministry of Health) were carried out for canine serological investigation. These consist of the rapid test DPP® Visceral Canine Leishmaniasis (CVL) – Bio-Manguinhos (Dual Path Platform), which is a qualitative immunochromatographic assay for the detection of anti-*Leishmania* antibodies and which uses the recombinant K28 protein (k26, k39 and k9 fragments) as an antigen, and the ELISA (Enzyme-Linked Immunosorbent Assay) test was performed as a confirmatory test. The field test was carried out by endemic disease control agents in the city of Ribeirão Vermelho. For the test, the animals were restrained using a muzzle. Then, a puncture with a sterile lancet was performed to collect 26 drops of total capillary blood. The test was carried out as recommended by the manufacturer.

Animals that were negative in the DPP® CVL – Bio-Manguinhos test were released soon after data collection from the owners. Five milliliters (5ml) of venous blood was further collected from animals

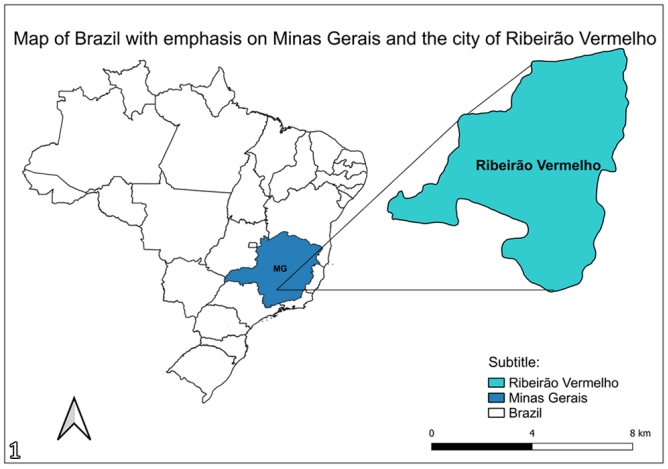

Fig.1. Map of Brazil emphasizing the state of Minas Gerais and the municipality of Ribeirão Vermelho.

whose tests showed a pattern of positivity. The collected material was used in the ELISA test.

PCR tests were performed on euthanized animals. Bone marrow was collected at necropsy. DNA extractions from biological samples were performed according to the procedures described in the manual of the Genomic DNA from Tissue kit (NucleoSpin® Tissue, Macherey-Nagel, Germany) with modifications only to the DNA elution phase, where 50µL of ultrapure water was added to the microtube, instead of 100µL of Buffer BE solution from the kit, and the incubation time at room temperature was changed to 10 minutes. The DNeasy Blood & Tissue® kit (QIAGEN, Germany) was used with three modifications to the procedure described in the manual for DNA extraction from the conjunctival swab. 1) The cotton from the swab was removed with a sterile scalpel blade and transferred to a 1.5mL DNAse-free microtube; 300µL ATL buffer was added and mixed with a vortex device in 5-second pulses. 2) Thirty-three microliters of proteinase K were added and mixed in pulses of 10 s; the microtubes were incubated at 56°C for 16 hours. 3) The cotton was removed, and the tubes were mixed in 15 s pulses; 334µL AL buffer was added and mixed by vortexing with 10 s pulses.

The other steps were followed according to the manufacturer's instructions, except for the incubation time of the microtubes at room temperature before the last centrifugation, which was changed to 10 minutes. After extracting the DNA from the bone marrow and swab samples, the DNA was measured in a spectrophotometer at 260/280nm and 260/230nm ratios.

Conventional PCR. Each sample had DNA adjusted to 20 to 100 nanograms/µL dosage. PCRs were conducted on a workstation after using ultraviolet light. The reactions were prepared according to the protocol adapted from (Lachaud et al. 2002), with 4µL 5X GoTaq, 0.4µL 10mM dNTPs, 0.25µL Taq (5U/µL), 1µL of each primer at 10µM and 11.75µL of a mixture of ultrapure water and sample DNA, which contained 20 to 100ng of DNA. Specific primers for the *Leishmania* subgenus were used for PCR that targeted minicircle kinetoplast DNA (kDNA).

Collection and identification of sandflies. HP models were used to collect sandflies CDC light traps (Pugedo et al. 2005). Collections were carried out monthly from February 2018 to May 2019. Fifteen initial collection points were defined, but not all points were collected over the period. The points were selected in the vicinity of homes that housed positive animals and in environments with green areas and regions with accumulated organic matter, chicken coops, kennels, and dog shelters in Ribeirão Vermelho/MG. The traps were installed and remained connected at the collection sites for three consecutive days, from 6:00 p.m. to 6:00 a.m. the following day. After the third day, the trap collection bags were removed and sent to the "Laboratório de Biologia Parasitária" (Parasitic Biology Laboratory - Biopar) at the UFLA, where they were placed in a refrigerator at 3°C for at least 72 hours.

After refrigeration, the collected insects were placed in a white tray to separate the specimens belonging to the Psychodidae family by macroscopic identification. The sandflies were placed in Petri dishes and separated into males and females with a stereoscopic microscope based on the external morphology in the final portion of the insect's body. Males were placed in polystyrene microtubes containing 70% alcohol and stored for further preparation, assembly and identification. Females were subjected to dissection and separation of the head and abdomen. The parts for morphological identification were stored in 70% alcohol, and the parts for research on *Leishmania* DNA were frozen. A taxonomic key proposed by Galati (2003) was used for morphological identification.

DNA extraction from female sandflies. One specimen at a time was macerated in a 1.5mL microtube, and 50µL NaCl (0.08M), sucrose (0.18M), EDTA (0.06), 0.5% SDS, and Tris-HCL (0.1), pH 8.6, was added. This homogeneous mixture was placed in a water bath at 65°C for 30 minutes, and then 7.1µL of potassium acetate (8M) was added before the mixture passed through the shaker. It incubated for another 30 minutes in a refrigerator or on ice at 4°C.

The tubes were centrifuged for 10 minutes at 13,000g, and the supernatant was transferred to a new tube. Then, 100µL of 95% alcohol was added, followed by another centrifugation for 10 minutes at 13,000g. After this step, the supernatant was discarded, and the dried tube was inverted against absorbent paper. Then, 100µL of 70% ethanol was added to the already dry tube and passed through a centrifuge at 13,000 for 10 minutes; the supernatant was discarded, leaving the tube dry.

Conventional PCR targeting ITS 1. One hundred fifty-seven female specimens of the 165 collected were tested. For PCR that targets the Internal Transcribed Spacer 1 (ITS1), the reaction was prepared for a final volume of 20µL containing 1µL (20ng) DNA from the sample to be tested, 4µL 5x GoTaq Green buffer solution, 2.0µL dNTP 2.0mM, 1.00µL of each of the primers (LITSR: 5'CTGGATCATTTTCCGATG3' and L5.8S: 5'TGATACCACTTATCGCACTT3') at 10µM, 0.25µL Taq DNA polymerase at 5U/µL, and 10.75µL of ultrapure H_2O (Andrade-Filho et al. 2017).

The amplification occurred in an automatic thermocycler Eppendorf® Mastercycler Gradient device using the following cycle: initial denaturation at 94°C for five minutes, followed by 30 repetitions of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 30 seconds. The final extension was at 72°C for seven minutes. The samples were then subjected to electrophoresis on a 2.0% agarose gel stained with ethidium bromide (10mg/mL), a molecular weight standard of 100 base pairs, 20µg DNA extracted from promastigote forms of *Leishmania infantum* (MHOM/BR/74/PP75) as a positive control and PCR reagents as a blank control.

Data analysis. The Hill series was used to identify the species diversity profile of the sampled area using R software.

RESULTS

Canine aspects

Three hundred forty-eight animals were tested: 186 (53.45%) females and 162 (46.55%) males. Of these, 14 animals (4.02%) showed positive serology in the two serological tests recommended by the "Ministério da Saúde" (Brasil 2011). Positive cases were found in almost all regions of the municipality, and in some homes, there was more than one positive dog.

Of the 14 dogs with two positive serologies, two were euthanized. They presented clinical signs of VL, such as lymphoid adenopathy, weight loss, alopecia, dull hair, ulcers, hyperkeratosis, keratoconjunctivitis, and onychogryphosis. They had never been vaccinated against canine visceral leishmaniasis nor used sandfly repellent collars (Fig.2-5). The owners of the remaining seropositive animals chose not to hand over the animal to the health service, or the animals died during the study for these reasons and were not clinically evaluated.

Entomological and environmental aspects

Of the 15 collection points initially defined, four residences (4, 5, 9 and 12) were discontinued at the beginning of the

study due to incompatibility of the owners' schedules. Thus, only the 11 households that had at least 11 months of follow-up since the beginning of the study were included in the present analyses: Households 7 and 10 had 15 months of data collection; Households 1, 6 and 14 had 14 months of data collection; Households 2, 3, 8 and 11 had 13 months of data collection; and Households 13 and 15 had 11 months of data collection, with a total of 5,256 hours of sampling effort.

A total of 443 specimens of sandflies were collected from February 2018 to May 2019, with 165 (37.25%) females and 278 (62.75%) males (Table 1). The specimens were identified as belonging to four subtribes: Brumptomyiina, Sergentomyiina, Lutzomyiina, and Psychodopygina; eight genera: *Brumptomyia, Micropygomyia, Lutzomyia, Migonemyia, Pintomyia, Expapillata, Evandromyia, Psathyromyia, and Nyssomyia*; and 14 species: *Brumptomyia brumpti* (3.39), *Brumptomyia nitzulescui* (0.22), *Brumptomyia* spp. (2.03), *Evandromyia cortelezzii* (4.84), *Expapillata firmatoi* (6.77), *Lutzomyia longipalpis* (73.81), *Micropygomyia* sp. (0.22), *Migonemyia migonei* (1.35), *Nyssomyia intermedia* (5.19), *Nyssomyia neivai* (0.90), *Nyssomyia whitmani* (0.90), *Pintomyia fischeri* (0.90), *Psathyromyia lanei* (0.22), and *Psathyromyia pascalei* (0.22). The most abundant species were *L. longipalpis* (73.81%), followed by *E. firmatoi* (6.77%) and *N. neivai* (5.19%).

L. longipalpis was found in nine out of 11 houses where the study was conducted, being the most abundant species in

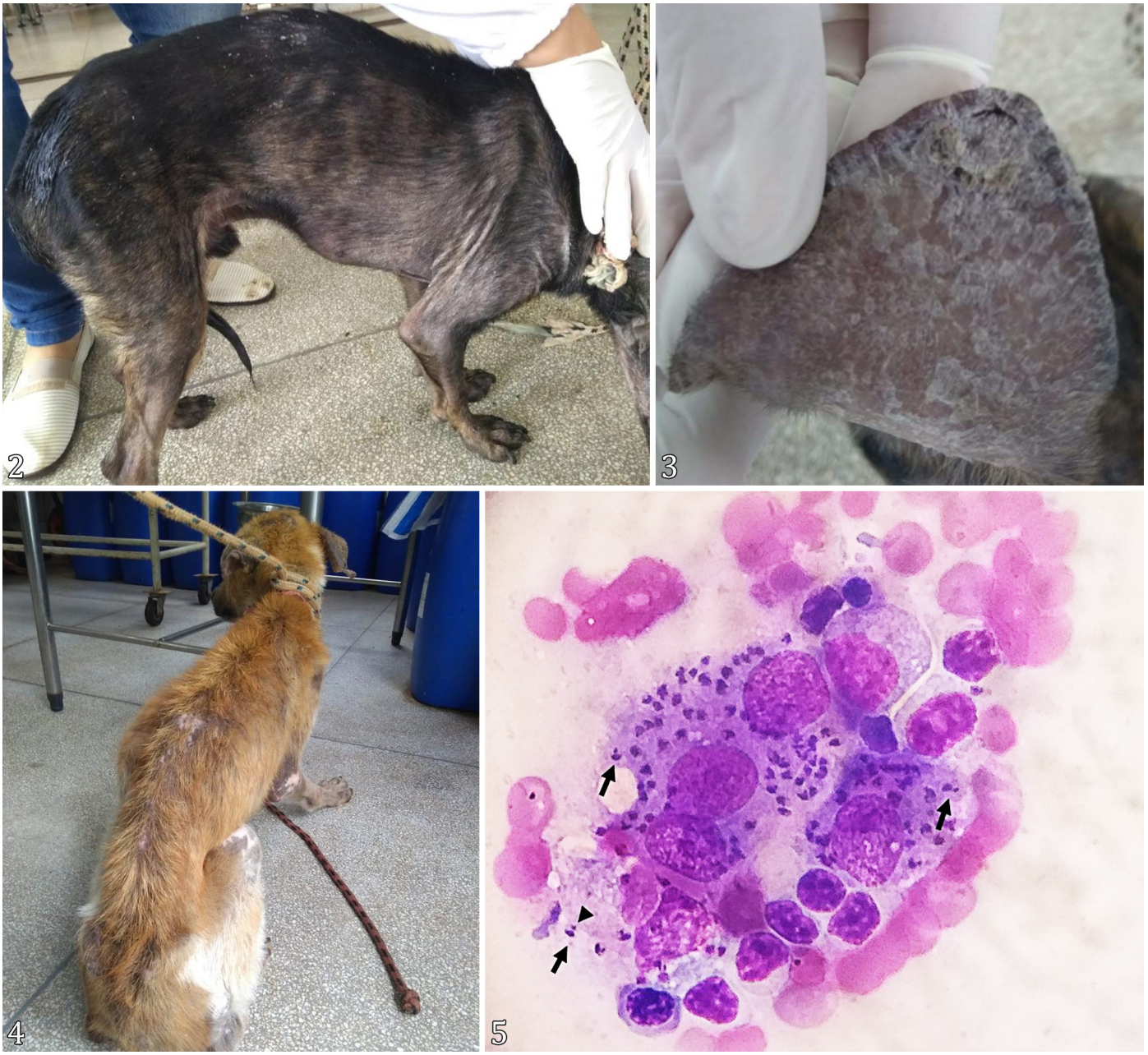

Fig.2-5. (**2**) Wasting and generalized alopecia; (**3**) hyperkeratosis; (**4**) emaciation, alopecia and ulcerated areas; (**5**) bone marrow smear from a dog with positive serology showing amastigote of *Leishmania* sp. on the black arrow; at the tip of the black arrow shows the kinetoplast. Giemsa, 100x magnification.

eight locations. Only in one locality, *N. whitmani*, an important vector of *Leishmania* (*Viannia*) *braziliensis* and *Leishmania* (*Viannia*) *shawi* (Costa et al. 2018), was more abundant, with three individuals collected. Using the Hill series to assess diversity profiles, the dominance of *L. longipalpis* in the area was observed, resulting in low diversity and high evenness in the sampled community where the increase in the value of (q) was followed by lower diversity. (Fig.6).

Molecular aspects

After molecular analysis, the presence of DNA from *Leishmania* spp. was detected in 21 of the 157 samples tested, which belong to the following genera and/or species: *L. longipalpis, Brumptomyia* spp., *M. migonei, E. firmatoi, E. cortelezzii* and *P. fischeri* (Fig.7).

DISCUSSION

The longitudinal structure of the city's urban areas allows extensive contact with rural areas and preserved environments. Initially of rural character, visceral leishmaniasis is currently urbanized and found in large population centers (Barçante et al. 2015).

The change to urban areas in the epidemiological profile of leishmaniasis is visible mainly in the peripheries of cities, where human and dog population density is high. According to data from the Health Surveillance of Ribeirão Vermelho, the dog population is estimated at approximately 1,200 animals, based on the anti-rabies vaccination campaigns, while the human population is 3,826 inhabitants. This proximity to rural areas and high dog/human ratio (1:3) merit attention about VL.

The clinical signs observed in euthanized dogs corroborate with the dermatological lesions that have been reported in approximately 90% of symptomatic dogs (Rossi et al. 2016). Hyperkeratosis is caused by increased keratin production, and alopecia is related to the action of the parasite in the hair follicle. This sign may also be associated with immune complexes, frequent in symptomatic dogs, in which keratin is deposited in the basement membrane of the skin and induces

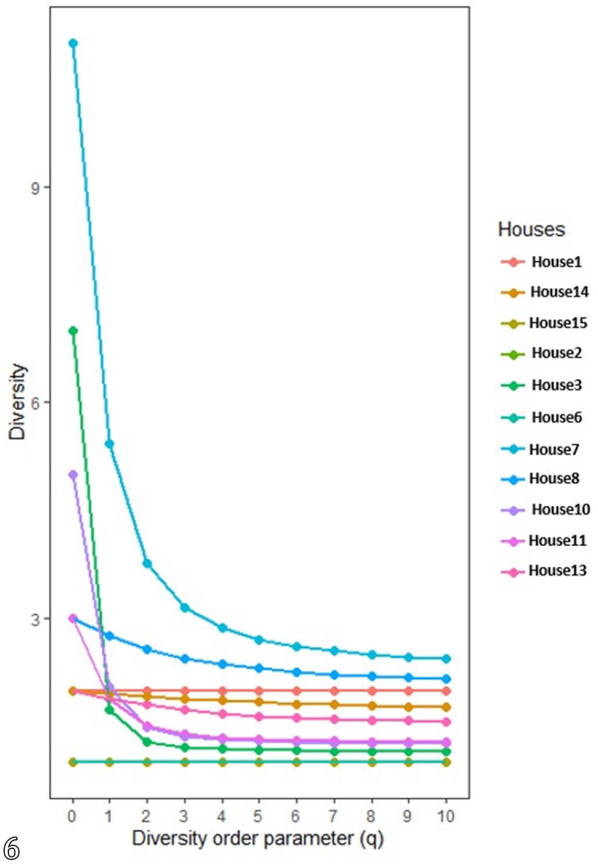

Fig.6. Graph showing the diversity of sandfly species using the Hill series generated from the R software.

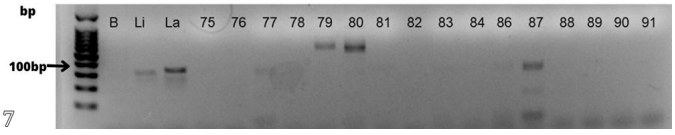

Fig.7. B = white, Li = *Leishmania infantum*, La = *Leishmania amazonensis*, 75-85, 86-91 = samples of female sandfly specimens, 77,79,80 and 87 = PRC reactive base pair bands.

Table 1. Species of sandflies collected in the municipality of Ribeirão Vermelho/MG from February 2018 to May 2019

Genus/Species	Absolute number (%)		
	Female	Male	Total
Brumptomyia brumpti	0 (0100)	15 (5.39)	15 (3.39)
Brumptomyia nitzulescui	0 (0.00)	1 (0.36)	1 (0.22)
Brumptomyia spp.	8 (4.85)	1 (0.36)	9 (2.03)
Evandromyia cortelezzii	15 (9.09)	2 (0.72)	17 (4.84)
Expapillata firmatoi	30 (18.18)	0 (0.00)	30 (6.77)
Lutzomyia longipalpis	75 (45.45)	252 (94.65)	327 (73.81)
Micropygomyia sp.	1 (0.61)	0 (0.00)	1 (0.22)
Migonemyia migonei	6 (3.63)	0 (0.00)	6 (1.35)
Nyssomyia intermedia	23 (13.94)	0 (0.00)	23 (5.19)
Nyssomyia neivai	1 (0.61)	3 (1.08)	4 (0.90)
Nyssomyia whitmani	2 (1.21)	2 (0.72)	4 (0.90)
Pintomyia fischeri	2 (1.21)	2 (0.72	4 (0.90)
Psathyromyia lanei	1 (0.61)	0 (0.00)	1 (0.22)
Psathyromyia pascalei	1 (0.61)	0 (0.00)	1 (0.22)
TOTAL	165	278	443

an autoimmune reaction, or even disorders in the metabolism of pantothenic acid (vitamin B5) due to liver damage caused by the infection (Hommel 1978). Alopecia exposes large areas of the animal's skin, facilitating transmission (Zacarias et al. 2017). The lymphadenopathy observed is due to the proliferation of the parasite and destruction of cells in the mononuclear phagocytic system and is one of the most frequent and early clinical signs of CVL (Ribeiro et al. 2018). Another author demonstrated the strong presence of lymphadenopathy in dogs with VL in a study of 173 dogs from an endemic area in the provinces of Sicily (Lombardo et al. 2012).

Only the species *Leishmania infantum* was verified in the tested sample due to the band pattern. Although parasitological confirmation is indisputable, it does not allow for species identification. Therefore, as this was the first recorded instance of CVL in the municipality of RV, performing PCR-RFLP was essential to exclude the possibility of parasitism by other species of the *Leishmania* subgenus since some studies report the possible visceralization of *Leishmania amazonensis* (Tolezano et al. 2007).

This study identified 4.02% of dogs with double-positive serology, which is noteworthy as it is higher than the 2% limit required for the "Ministério da Saúde" to consider an area free of VL. The VL Surveillance and Control Manual recommends a series of actions in municipalities that have a prevalence greater than 2%, including the active search for dogs with clinical suspicion, the development of health education activities, the training of professionals for early diagnosis and treatment, the euthanasia of seroreactive dogs and surveillance and monitoring (Brasil 2014). Therefore, the results of the present study will be fundamental in guiding health monitoring in the city of Ribeirão Vermelho since, in addition to reporting the first case of CVL, this study also points to an initial prevalence of 4.02% in the studied sample.

It is still important to emphasize that the screening tests recommended by the "Ministério da Saúde" provide heterogeneous data and may present false negatives, even though these tests have high sensitivity. Gondim et al. (2022) found low sensitivity in immunochromatographic tests used for screening. False-negative results are high when a screening test presents a relatively low sensitivity value. In this same study, carried out in Lavras, a municipality adjacent to Ribeirão Vermelho, Gondim et al. (2022) found that more than half of the infected dogs did not display clinical signs characteristic of VL and were no longer detected by the screening tests. This finding is particularly important since dogs without clinical signs can present high transmissibility and potential for maintenance and dissemination of the parasite (Tolezano et al. 2007).

In studies carried out in Belo Horizonte, the state's capital, *L. infantum* was confirmed to be in 24.7% of the dogs sampled using PCR. However, only 15.9% were positive in the serology test, demonstrating the importance of conducting more specific tests, particularly in the case of a zoonosis whose two possible outcomes are canine euthanasia (Brasil 2014, Coura-Vital et al. 2014) or treatment, which is a control measure. A study in Ipatinga, another small town in MG, found a prevalence of 14.8% of positive dogs in at least two serological tests, the same ones used in the present study. A total of 9,139 dogs were sampled, 4,183 from active search and the other 4,953 from passive search. In our study, there was a prevalence of 4.02% sampled by passive search, in which the owner took the dog to perform the test. In the Ipatinga study, which took place in an endemic area for VL, there was a greater sampling effort in neighborhoods with positive cases for VL and TL (Lana et al. 2018).

Tests that are more specific than serological tests are important for confirming the first cases in previously VL-free regions (Paltrinieri et al. 2016). In the municipality of Iguatama/MG, the first canine case was confirmed after serological tests using parasitological and molecular tests that could detect *L. infantum* (Faria et al. 2017). In the municipality of Lavras/MG, serological, parasitological and molecular tests were performed to confirm the first canine case of VL in 2013. Since then, the municipality has no longer been considered VL-free; instead, it is considered an area of transmission, already recording eight cases of human VL and two deaths, which corresponds to a 25% fatality rate and a prevalence of 6.53% of canine cases.

Although cases of human VL have not yet been reported in the area of the current study, the results presented here point to the need to make efforts to carry out surveillance and control activities, as it is known that canine enzooty precedes the occurrence of human cases. According to the Visceral Leishmaniasis Surveillance and Control Manual (VLSCM), it is estimated that the first human cases will appear when the dog prevalence is equal to or above 2% (Brasil 2014).

Recent studies in small cities in the state of Minas Gerais have revealed concerning results, indicating the presence of the main vector of *L. infantum*, *Lutzomyia longipalpis*, in urban areas (Barçante et al. 2015, Faria et al. 2017). These findings point to a potential expansion of leishmaniasis in these regions, especially due to the characteristics of the vector, which has been shown to be abundant and dominant in areas where the study of this disease is still incipient and neglected. This is particularly relevant for small cities that, until recently, did not consider leishmaniasis to be a serious public health problem. Therefore, advancing studies in these municipalities is essential to understanding this disease's progress in Brazil's interior.

The identification of the species that act as vectors of *L. infantum* is of utmost importance for the implementation of effective control measures to prevent the spread of leishmaniasis – a disease that affects both humans and animals. In this study, *L. longipalpis* was found to be the most abundant species, highlighting its critical role as the primary vector of *L. infantum* in the region.

The low diversity of sandflies observed in the collection sites where *L. longipalpis* was predominant raises concerns about the potential impact of this species on the ecosystem's stability. Different regions of Brazil, where visceral leishmaniasis is endemic, have shown the predominance of *L. longipalpis* over other species (Pinto et al. 2010, Silva et al. 2019). The Hill series analysis performed in this study showed that, as the number of specimens collected increased, the evenness of species diversity increased as well. This finding suggests that the high abundance of *L. longipalpis* in the region may contribute to reducing sandfly species richness and ultimately affect the ecosystem's equilibrium.

The ability of *L. longipalpis* to adapt to anthropized environments compared to other species is also a matter of concern. This adaptation may increase the risk of leishmaniasis

transmission in urban areas (Soares Santana et al. 2021), where human populations are at greater risk of contracting the disease. The fact that *L. longipalpis* accounts for most sandfly specimens found in homes with dogs infected with *L. infantum* highlights the need for effective control measures to reduce the transmission of this disease.

The presence of multiple sandfly species in the study region, including those previously reported in Lavras and Ribeirão Vermelho, underscores the need for continued monitoring of sandfly populations to track changes in species diversity and abundance. Such monitoring will aid in identifying potential shifts in the vector populations and the implementation of timely control measures to mitigate the spread of leishmaniasis.

Lutzomyia longipalpis is an insect that can occupy several ecological niches, including those resulting from anthropic action (Margonari et al. 2006, Barçante et al. 2018). It has a wide geographic distribution, being present from southern Mexico to northern Argentina and Paraguay, with the occurrence of VL coinciding in these regions. Although it is considered the main transmitting species of *L. infantum* in the Americas, in some regions, positive human and dog cases have been verified in areas without *L. longipalpis*, as is the case in Jaciara, Mato Grosso, where the species *L. cruzi* seems to be mainly responsible for cycle transmission (Missawa et al. 2011), and in Corumbá, Mato Grosso do Sul, where *L. cruzi* was identified as a vector of *L. infantum* (Oliveira et al. 2016). In addition, there is strong evidence that other species, such as *Migonemyia migonei* (França, 1920), *Pintomyia fischeri* (Pinto, 1926), *Lutzomyia gaminarai* (Cordero et al. 1928) and *Nyssomyia intermedia* (Lutz & Neiva, 1912), play a role in the transmission of *L. infantum* in some areas (Carvalho et al. 2010, Saraiva et al. 2010, Brasil 2014, Moya et al. 2015, Galvis-Ovallos et al. 2017, Castro et al. 2019, Rêgo et al. 2019, 2020, Galvis-Ovallos et al. 2021, Milagres et al. 2022).

Neglect of leishmaniasis in small towns is a significant issue that needs to be addressed. Leishmaniasis is often considered a neglected disease, and small towns are particularly vulnerable to its effects due to inadequate resources, poor infrastructure, and insufficient funding for health care services. Neglect of leishmaniasis in small towns can lead to delayed diagnoses, inadequate treatment, and poor management of the disease. As a result, the disease can spread rapidly in these areas, affecting both humans and animals.

Efforts should be made to raise awareness about leishmaniasis in small towns, and resources should be allocated to improve surveillance and control measures. Effective measures can include early diagnosis and treatment of human and canine cases, implementation of vector control programs, and awareness campaigns to educate the public on the risks and prevention measures. By addressing the issue of neglect in small towns, we can help prevent the spread of leishmaniasis and improve the health outcomes of affected individuals and animals.

CONCLUSIONS

The findings of this study reveal a concerning public health situation in the municipality of Ribeirão Vermelho/MG, where autochthonous cases of canine visceral leishmaniasis have been identified. The presence of at least 13 species of sandflies, especially in residential areas with dense vegetation and domestic animals, suggests a conducive environment for disease transmission. The predominance of the species *Lutzomyia longipalpis* and the detection of *Leishmania* DNA in the guts of female sandflies underscore the importance of this region as a recent transmission focus.

Therefore, it is imperative to urgently implement the surveillance and control measures recommended by the "Ministério da Saúde" to prevent the spread of canine visceral leishmaniasis and, consequently, human cases of the disease. Additionally, continuing epidemiological research is essential to identify the determinants driving transmission, contributing to a more effective approach to combating this illness.

Authors' contributions.- Alvarenga M.I.: Wrote and revised the manuscript and participated in the entire methodological part.

Castro J.C. and Fujiwara. T.R.: Wrote and revised the manuscript and participated in the molecular diagnosis.

Melo L.M.R.: Wrote and revised the manuscript, participated in the entire methodological part, and carried out the statistics of the present study.

Oliveira M.M.: Wrote and revised the manuscript and assisted in the serology of the dogs, collection of the animal's bone marrow, and identification of *Leishmania* sp. in the smears.

Castro P.H.: Wrote and revised the manuscript, following the magazine's standards.

Miracles T.F., Filho J.D.A., and Binder C.: Wrote and revised the manuscript and participated in identifying sandfly species.

Barçante T.A. and Barçante J.M.P.: Provided guidance, participated in the delimitation of the study and reviewed the manuscript.

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