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Epidemiological features of *Leishmania infantum* in dogs (*Canis lupus familiaris*) suggest a latent risk of visceral leishmaniasis in the metropolitan area of Bucaramanga, Santander, Eastern Colombia

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ABSTRACT

Visceral leishmaniasis (VL) is a disease caused by species of the *Leishmania donovani* complex that is mainly transmitted through the urban cycle involving dogs as the primary reservoir. In Colombia, the incidence of VL is increasing, along with the spread of potential vectors. This study aims to investigate the eco-epidemiological factors associated with *Leishmania* spp. infection in dogs from the Metropolitan Area of Bucaramanga (MAB), Santander, eastern Colombia, which is a region at risk for VL. We conducted molecular and serological surveillance of *Leishmania* spp. in 207 dogs from MAB to determine the epidemiological factors associated with infection. Subsequently, we carried out a molecular and serological analysis of phlebotomine and humans, respectively, in areas with a higher prevalence of infection, aiming to describe the main features associated with the transmission cycle. Out of the 207 dogs tested, 37 (17.8%, 95% CI = 12.6–23.1%) were positive for the presence of *Leishmania* antibodies by the IFAT test, and only 9 (4.3%, 95% CI = 1.55–7.15%) were positive for *L. infantum* by PCR. Multivariate analyses indicated that canine shelters and dogs with clinical signs commonly associated with canine VL had a higher prevalence of infection ($P < 0.05$). In the entomological survey, 69 blood-fed female phlebotomine of the genus *Lutzomyia* were captured in canine shelters, among them, 55% were identified as *Lutzomyia camposi*, 29% as *Lu. ovallesi*, 7% as *Lu. dubitans*, 6% as *Lu. torvida*, and 3% as *Lu. cayennensis*. The identified meal sources of the phlebotomine included human, pig, avian, cattle, and porcupine (*Coendou quichua*) blood. However, no phlebotomine positive for *Leishmania* spp. were detected by molecular analyses. Finally, 14 humans who had frequent contact with *L. infantum*-positive dogs were analyzed through rK39 test, but none tested was positive for IgG/IgM antibodies. The molecular and serological analyses indicate the circulation of *L. infantum* in dogs from MAB, with canine shelters having the highest prevalence of infection. The entomological survey of canine shelters showed a significant diversity of phlebotomine without potential vectors of *L. infantum*, suggesting the presence of infection in dogs from these areas could take place in other locations or through other transmission routes. The circulation of *L. infantum* in multiple dogs from MAB suggests a latent risk of zoonotic transmission of VL in these cities.

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1. Introduction

Leishmaniasis is a group of diseases caused by *Leishmania* parasites (order Kinetoplastida, family Trypanosomatidae), which mainly transmit to humans through the bite of female phlebotomine sand flies of the genus *Phlebotomus* or *Lutzomyia*. The disease can present in three clinical forms: cutaneous (CL), mucosal (mL) and visceral (VL) leishmaniasis (CFSPH, 2009). Visceral leishmaniasis, also known as kala-azar, is primarily caused by *Leishmania (L.) donovani* complex, which includes *L. donovani* and *L. infantum*. Although some strains of *L. amazonensis* have been also associated with this clinical form (de Souza et al., 2018). According to the World Health Organization (WHO), VL causes around 50,000–90,000 new cases annually worldwide, with Sudan, Bangladesh, Brazil, India, and Nepal being the most affected countries (WHO, 2020). In South America, VL poses a severe public health risk, and preventive measures include vector control, reducing human contact with phlebotomine, controlling reservoirs, and surveillance, among other strategies (Alves et al., 2016; Ávila et al., 2022).

In urban areas, dogs are the main domestic reservoirs of *L. infantum*, maintaining the parasitic life cycle and facilitating the transmission of parasites to humans and other mammals. Therefore, they have been considered a target for surveillance and control of VL (Dantas-Torres et al., 2019). The transmission of *L. infantum* to dogs is primarily through the bite of infected phlebotomine sand flies. Although less frequently, the parasite can also be transmitted vertically from an infected mother to her offspring, through venereal transmission during mating, through blood transfusions from infected donors, or directly from dog-to-dog through bites or wounds (Boggiatto et al., 2011; Naucke, Lorentz, 2012; Naucke et al., 2016). Infections in dogs typically result in cutaneous lesions, lymphadenomegaly, lethargy, weight loss, glomerulonephritis, keratoconjunctivitis, vasculitis, and eventually can lead to death in most cases (Miró and López-Vélez, 2018). The limited availability of licensed and effective vaccines for *L. infantum* in dogs (Velez and Gállego, 2020; Coelho and Christodoulides, 2023), along with environmental degradation of preserved areas, the phlebotomine plasticity, and the rise in stray dog populations in urban areas pose significant challenges to controlling VL and make eradicating this zoonosis nearly impossible (Morales-Yuste et al., 2022).

In Colombia, VL is endemic, with active transmission foci correspond to the distribution of *Lutzomyia evansi* in the subregion of Montes de Maria in Bolivar and Sucre, and *Lutzomyia longipalpis* in the Magdalena River Valley and its affluent in Tolima, Huila, Cundinamarca, Santander, and Norte de Santander (Castillo-Castañeda et al., 2021). From 2013–2018, 183 cases were reported, with 58% concentrated in the municipalities of Sucre, Bolivar, Huila, and Cordoba (INS, 2018). In Santander, the first case of VL was reported in a child from San Vicente de Chucurí municipality in 1943, considered the first demonstrated case in Colombia (Morales, Rodríguez, 1996). Since then, there have been sporadic outbreaks of VL near the Bucaramanga Metropolitan Area (MAB), the capital of Santander, including a significant outbreak in the municipality of Piedecuesta from 1998 to 2000 that affected eight children from peri-urban areas. The most recent outbreak occurred in the municipality of Giron in 2019 and affected an adult in an urban area (Flórez et al., 2006; Santander, 2019).

Concerning the dog reservoir studies, although serological surveillance in dogs from urban areas of Santander shows a low prevalence of *L. infantum*, a continuous risk of infection to people is present, as suggested by the occurrence of autochthonous cases of VL over the last years (Florez-Muñoz et al., 2022; Santander, 2019). Considering the paucity of molecular and ecological studies to understand the epidemiological cycle of VL in MAB, an area with a historical presence of phlebotomine species and frequent cases of canine leishmaniasis (Florez-Muñoz et al., 2022; Flórez et al., 2006), this study aims to explore eco-epidemiological traits associated with *Leishmania* spp. infection in dogs from this zone.

2. Methods

2.1. Ethics statement

Animal procedures were conducted in accordance with the Colombian code of practice for the care and use of animals for scientific purposes, as outlined in Law 84 of 1989 and Law 576 of 2000. Human procedures were performed according to resolution No. 08430 of 1993 of the Colombian Ministry of Health, and this study was classified as "Investigation with minimal risk". The Ethics Committee of the Cooperative University of Colombia approved the study, with concept 007–2021 in the Act No. 001 of 2021.

2.2. Study area and sampling

The MAB is the fifth most populous metropolitan area in Colombia, located in the department of Santander, in the Río de Oro valley, with an approximate human population of 1341,694 inhabitants. The central city is Bucaramanga, and its satellite municipalities are Floridablanca, Giron, and Piedecuesta (DANE, 2018).

The vegetation is a transition between the Tropical Dry Forest and Rainforest Premontane, with an altitude ranging from 706 to 959 m and an annual precipitation of 4426 mm. The climate are categorized as Tropical Rainforest (*Af* according to Köppen climate classification) (IDEAM, 2022). In the MAB, 69% of households have dogs, with a person-dog ratio of 4.7 persons per dog and an average of 0.67 dogs per household (Florez and Solano, 2019).

2.3. Canine evaluation

A cross-sectional study was carried out between June 2021 and August 2022, encompassing dogs from the MAB. The sample size was determined using Epi Info 7.0, based on a population of approximately 280,000 dogs in the MAB (Florez and Solano, 2019), and an expected prevalence of 4.8% from previous studies (Florez-Muñoz et al., 2022). The study aimed for a 99.9% confidence interval and a 5% statistical error, resulting in a calculated sample size of 198, which was increased by 5% to compensate for any sampling errors. Most of the dogs were randomly selected during anti-rabies vaccination campaigns in the MAB, while some were chosen through collaborations with veterinary hospitals and canine shelters. The latter represented peri-urban houses operated by anonymous organizations where stray, lost, or abandoned dogs were housed during the process of sterilization and adoption. Informed consent was performed from the owners during the sampling process. A pre-coded questionnaire, covering seven epidemiological variables (such as sex, age, breed size, municipality, socioeconomic level, habitat type, and garbage collection service), was performed. The age variable was divided into three categories: puppies (animals under 1 year of age), adults (animals between 1 and 12 years of age), and geriatrics (animals over 12 years of age) (Harvey, 2021). Socioeconomic status was determined based on the official stratum divisions as follows: level 1, lower-low; level 2, low; level 3, upper-low; level 4, medium; level 5, medium-high; and level 6, high (Farnsworth, 2017). The details categorization of other variables can be found in Table 1.

2.4. Collection and processing of samples

To collect samples, the dogs were first examined by a veterinarian and divided into symptomatic and healthy groups. The symptomatic group included dogs with clinical signs such as enlarged lymph nodes, poor nutritional status (body condition score less than 2/5), and skin lesions on the muzzle, ears, or skin, such as those commonly associated with canine VL in endemic areas of South America (Silva et al., 2017). The healthy group included visually healthy dogs based on clinical examination. The body condition of each dog was assessed using a five-scale scoring system ranging from emaciated (very thin = 1) to

Table 1
Bivariate analyses of infection by *Leishmania* spp., in canines from the Metropolitan Area of Bucaramanga, Santander.

Test	IFAT					IFAT + PCR			
Independent variables	Animals screened	Positive	Prevalence (%)	χ^2	*P*	Positive	Prevalence (%)	χ^2	*P*
Sex									
Female	125	18	14.4	2.548	0.110	1	0.8	8.729	0.004*
Male	82	19	23.1			8	9.7		
Age (yr)									
Puppy	81	11	1.3		0.363	0	0	6.782	0.034*
Adults	118	25	21.1	2.106		9	7.6		
Geriatric	8	1	12.5			0	0		
Breed size									
Small	45	4	8.8		0.555	0	0	1.790	0.409
Medium	130	26	20	1.248		7	5.3		
Large	32	7	21.8			2	6.2		
Clinical group									
Healthy	176	12	6.8	9.137	0.002 *	9	5.1	53.4	0.000*
Symptomatic	31	25	80.6			0	0		
Municipality									
Bucaramanga	116	18	15.5	4.543	0.198	5	4.3	2.437	0.487
Floridablanca	41	5	12.1			1	2.4		
Giron	36	10	27.7			3	8.3		
Piedecuesta	14	4	28.5			0	0		
Socioeconomic level									
One	112	26	23.2	4.778	0.092	8	7.1	5.221	0.074
Two	25	2	8			1	4		
Three	70	9	12.8			0	0		
Habitat type									
House	185	26	14.1	14.473	0.000*	2	1.1	40.893	0.000*
Canine shelters	22	11	50			7	31.8		
Garbage collection service									
Daily	76	11	14.4		0.001*	0	0	17.133	0.000*
Weekly	85	9	10.5	13.698		2	2.3		
Not service	46	17	39.9			7	15.2		

*= $P < 0.05$

obese (very fat = 5). For each animal in both groups, two blood samples of 5 mL were collected from the cephalic vein using EDTA.K3 and Serum tubes, respectively. In the symptomatic group, a fine-needle aspiration was also performed on the popliteal lymph node, as previously described (Silva et al., 2017). The aspirate was transferred into Serum vacutainer tubes with 1 mL of sterile PBS 1X. All samples were stored at 4 °C until processed.

Genomic DNA was extracted from 200 μL of blood or lymph node aspirate using the Genomic DNA Purification Kit (CorpoGen, Bogotá, Colombia®) according to the manufacturer's instructions. The DNA was then diluted with a 100-μL elution buffer and stored at −20 °C until molecular analysis. To determine the quantity and quality of DNA, a NanoDrop 2000 (Thermo Fisher Scientific, Massachusetts, USA) was used. For serum extraction, the samples were centrifuged at 5000 x g for 10 min, and the serum was stored at −20 °C until serological analysis.

2.5. Molecular detection tests

A PCR assay based on the *hsp70* gene was used to detect and identify *Leishmania* species in dog samples, using previously described primers HSP70F and HSP70R (Hernández et al., 2014). The reaction was performed in 25-μL volumes containing 1X reaction buffer (100 mM Tris-HCl, 50 mM KCl, pH 8.8), 0.2 mM dNTP, 1.5 mM MgCl2, 0.4 μM of each primer, 0.625 UI of Taq polymerase (CorpoGen, Bogotá, Colombia®), and 2 μL (20 ng) of DNA samples. PCR products were analyzed on a 2% agarose gel electrophoresis, stained with EZ-Vision (AMRESCO, Solon, OH), and visualized under ultraviolet light. Positive controls were derived from DNA samples of cultured *L. infantum*, *L. panamensis*, and *L. braziliensis*, as well as genomic DNA from two previously characterized *L. infantum*-positive dogs. Negative controls were derived from DNA samples of dogs previously characterized as negative for *Leishmania* spp.

PCR products that were positive were subjected to purification and sequencing in both directions using the Sanger method, which was performed by the Macrogen sequencing service located in Seoul, South Korea. The sequences were then edited using BioEdit v.7.1 software (Hall, 1999). The resulting products were compared with reference sequences that are available in the National Center for Biotechnology Information (NCBI). Table S1 provides information about the primers used in the study, including their sequences, annealing temperatures, and the sizes of the PCR products obtained.

2.6. Serological test in dogs

To assess IgG anti-*Leishmania* spp. antibodies, an in-house Indirect Immunofluorescence (IFAT) assay was employed, following OIE recommendations using the strain of *L. infantum (chagasi)* MHOM/BR/74/PP75. The assay employed a whole parasite antigen fixed on multi-spot slides (Huida Medical, Jiangsu, China) and fluorescently labeled with anti-canine gamma globulin as a conjugate (ThermoFisher Scientific, Massachusetts, USA). Animals were initially considered seropositive at a serum dilution of 1:40, following a previous studýs protocol (Herrera et al., 2019). Due to the known serological cross-reactivity between *Leishmania* spp. and *T. cruzi* (Zanette et al., 2014), animals positive for *Leishmania* spp. underwent a second IFAT to detect IgG anti-*T. cruzi*. This additional testing aimed to determine more accurate seropositivity, as reported in another study (Dario et al., 2016). The *T. cruzi* antigen used for this test was prepared from harvested cultures of epimastigotes of the Colombian strains Cas-15 and Gal-61. The cut-off for *T. cruzi* was determined as a 1:40 dilution of serum as reported elsewhere (Cantillo-Barraza et al., 2015). Animals positive for both tests were further evaluated by dilution of each test, starting at 1:80, until differences in positivity were observed. Animals with higher antibody titers for *Leishmania* spp. compared to *T. cruzi* were considered seropositive for *Leishmania* spp., while animals with an antibody titer higher for *T. cruzi* compared to *Leishmania* spp. were considered seronegative for *Leishmania* spp. Animals were considered to present both infections when titers were > 1:40 in each assay (Dario et al., 2016).

2.7. Phlebotomine sampling

Based on the serological analysis, three canine shelters were chosen for the capture of phlebotomine. Eight CDC (Centers for Disease Control and Prevention) light traps were placed overnight at each shelter: four traps were installed outdoors at a distance of 10 m from the kennels, and four were placed within the kennels with a maximum distance of 5 m. CDC traps were installed on different nights at each canine shelter, with a one-week interval between each installation. All traps were positioned 1.5 m above ground level and were activated from 18:00–6:00 h. The phlebotomine samples were collected between April and August 2022, within one month following the dog sampling.

2.8. Phlebotomine species identification

After immobilizing all insects at −4 °C for 12 h, phlebotomine specimens were separated from other insects based on their external morphological features using the keys previously reported (Young and Duncan, 1994). Only blood-fed females were stored in vials with 70% alcohol for molecular analyses. DNA was extracted using the DNeasy extraction kit (Qiagen, Hilden, Germany) following the manufacturer's guidelines. PCR was performed targeting the cytochrome c oxidase subunit I (COI) gene, using the primers LCO1490 and HCO2198, as previously described (Folmer et al., 1994). All positive PCR products were sequenced in both directions and their identities were confirmed by comparing the DNA sequence to NCBI databases.

2.9. Blood source analysis and detection of Leishmania DNA in phlebotomine

Blood source identification in phlebotomine was performed by sequencing the cytochrome b (*cyt b*) gene, using the primers CytBF and CytBR, which target vertebrates (Boakye et al., 1999). All positive PCR products were sequenced and identified by comparing the DNA sequence to NCBI databases using the Blastn tool. To detect the presence of *Leishmania* DNA in phlebotomine, the *hsp70* and ITS1 regions were amplified using PCR. The ITS1 region was amplified using the primers LITSR and L 5.8 S (el Tai et al., 2000), while the *hsp70* test was conducted as described previously for dogs.

2.10. Human infection evaluation

With the aim to evaluate the exposition of humans to *L. infantum*, a serological surveillance was carried out on owners or keepers of canine positive for *L. infantum* by PCR analysis. For each person, a blood sample of 5 mL was collected from the brachial vein using serum vacutainer, and sera was extracted as previously described. Serodiagnosis of VL was carried out using rK39-immunochromatographic test (Ad-bio *Leishmania* IgG/IgM Combo Rapid Test, Biotech, San Diego, USA) according to the manufacturer's instructions. This test has a sensitivity of 91.5% and a specificity of 93.2% for VL, as reported in a previous study (Herrera et al., 2019). All patients were informed about the study and provided their consent to participate, in accordance with international ethical regulations.

2.11. Data analyses

The prevalence of *Leishmania* spp. infection in each biological sample was determined with a 95% confidence interval (CI). To identify epidemiological variables associated with infection in dogs, serological results (positive or negative for *Leishmania* spp. in the IFAT test) were initially analyzed using bivariate analysis ($\chi 2$ test) (Table 1). Variables with a statistically significant association ($P < 0.05$) were then included in a multivariate model (Generalized Estimated Equations - GEE) using a binomial distribution, a log link function, exchangeable interclass correlation, and a stepwise method. Results were expressed as Exp (B) (an estimate of the prevalence ratio [PR]) and 95% CI. SPSS v.18.0 statistical software was used for data analyses. The same procedure was also used to analyze the results obtained through the PCR method to identify variables associated with active *Leishmania* spp. infection in dogs. Finally, the abundance of vectors and identification of blood sources were analyzed using RStudio version 2022.12.0 and the ggplot2 package.

3. Results

A total of 207 dogs were included in the study, with 56% (116/207) from Bucaramanga, 19.8% (41/207) from Floridablanca, 17.4% (36/207) from Giron, and 6.8% (14/207) from Piedecuesta. The age distribution of the dogs was 57% (118/207) adults, 39.1% (81/207) puppies, and 3.9% (8/207) geriatric. Regarding to clinical status, 85% (176/207) and 15% (31/207) correspond to healthy and symptomatic. In terms of living conditions, 89.4% (185/207) of the dogs lived in houses that provided access to parks and social areas, while 10.6% (22/207) were kept in canine shelters. The shelters were situated in peri-urban regions, comprising two in Bucaramanga and one in Giron, which keep between 70 and 120 dogs. In these places the feeding and care of the animals is financed through donations.

3.1. Surveillance of Leishmania spp. in dogs from the MAB using serological and molecular methods

Initially, 51 dogs were found to be positive for *Leishmania* spp. by the IFAT test, and of these, 16 showed reactivity to *T. cruzi*. Of the 16 dogs that were seropositive for both infections, only two had higher antibody titers to *Leishmania* spp. than to *T. cruzi*. No coinfected animals were identified. Finally, 37 dogs (17.8%, 95% CI = 12.6– 23.1%) were considered seropositive for *Leishmania* spp., with a higher prevalence in the symptomatic group (80.6%) compared to the healthy group (6.8%) ($P < 0.05$). In the bivariate analysis, socioeconomic level, habitat, and garbage collection were associated with seropositivity ($P < 0.05$), with a higher prevalence in low socioeconomic areas, canine shelters, and areas with inadequate garbage collection (Table 1). However, when these variables were analyzed in the multivariate analysis, only habitat was significantly associated with seropositivity, indicating that dogs living in canine shelters had a higher prevalence of infection (canine shelters vs. houses [PR = 3.625; 95% CI, 1.465 – 8.970]) ($P < 0.05$). There were no significant differences in seroprevalence between municipalities (Fig. 1, Table 1).

In the molecular analysis, 9 out of 207 dogs were found to be positive for *hsp70* amplification (4.3%, 95% CI = 1.55 – 7.15%), all of them positive in the IFAT test. These positive samples showed an identity greater than 99.6% with *L. infantum* (GenBank accession number OQ283567). All positive dogs identified by PCR were from the symptomatic group, and the majority of these (7 out of 9) were found in canine shelters. DNA of the parasite was detected in lymph node aspirates in six of the PCR-positive animals, while only two animals were positive in the blood sample. There were no positive animals in both tissues. Two of the dogs positive for *L. infantum* died due to clinical complications associated with leishmaniasis between one and two weeks after molecular diagnosis in veterinary centers, despite receiving anti-*Leishmania* treatment. In the bivariate analysis, all variables except breed size and municipality showed a significant association with the positivity of the PCR analyses ($P < 0.05$) (Table 1). However, in the multivariate analysis, only the clinical group shows a significant association with active infection by *L. infantum*, indicating that symptomatic dogs had a higher prevalence of infection compared to the healthy group (symptomatic vs. healthy [PR = 2.778; 95% CI, 1.326 – 5.817]) ($P < 0.05$). Further information on the positive animals is presented in Table S2.

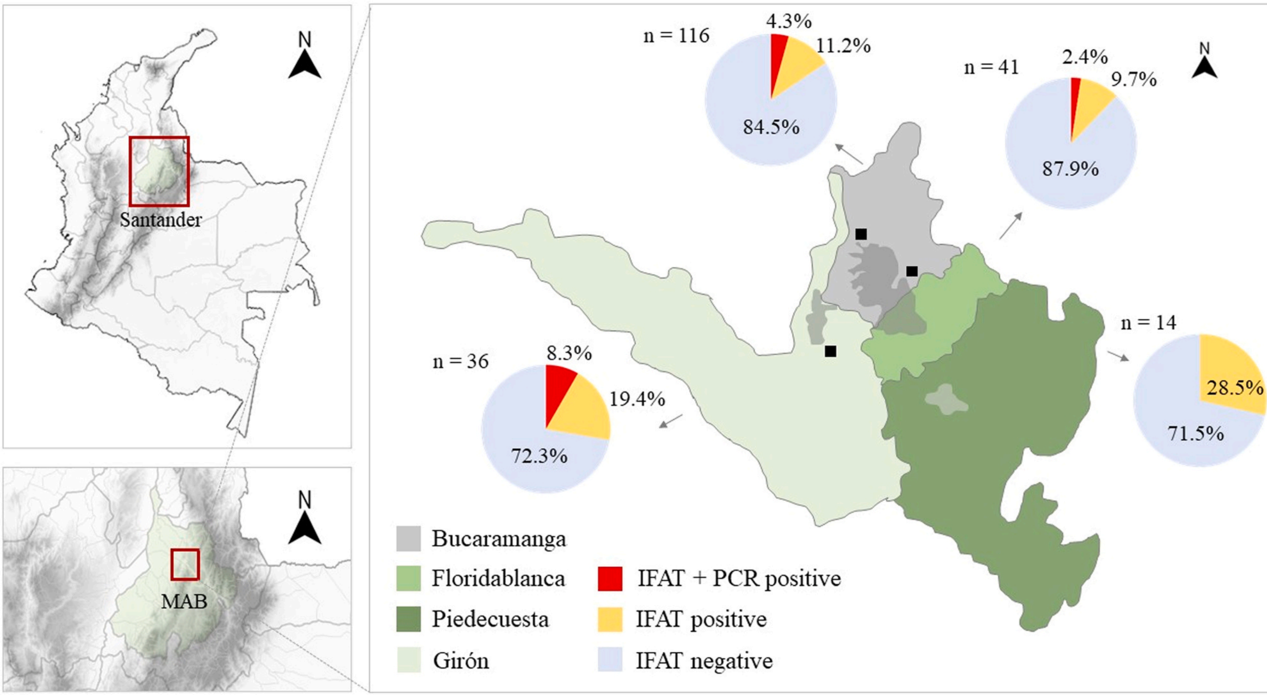

Fig. 1. Infection rate of *Leishmania* spp., detected by IFAT and PCR in canines from the Metropolitan Area of Bucaramanga, Santander. The three black dots correspond to canine shelters in Bucaramanga and Giron, where phlebotomine sampling was carried out.

3.2. Identification of blood sources and detection of Leishmania DNA in phlebotomine from canine shelters from MAB

Due to the high infection prevalence observed in canine shelters, entomological surveillance was conducted. A total of 128 *Lutzomyia* specimens were collected, with 90.6% (116/128) being female and 9.4% (12/128) being male. Of these, 13.2% (17/128) were collected in the kennels and the remaining specimens were captured in the peridomicile. Five species were identified, with *Lu. camposi*, *Lu. ovallesi*, *Lu. dubitans*, *Lu. torvida*, and *Lu. cayennensis* accounting for 55%, 29%, 7%, 6%, and 3% of the blood-fed females, respectively. The identity of the DNA sequences with the reference sequence reported in the NCBI databases is shown in Table S3. A total of fifteen DNA samples amplified successfully for the *Cyt b* gene of vertebrates: six of the samples contained human blood (four belong to *Lu. ovallesi*, and the others to *Lu. camposi*, and *Lu. dubitans*), five contained pig blood (*Sus scrofa domestica*), two avian blood (*Gallus gallus*), one cattle blood (*Bos primigenius taurus*), and one porcupine blood (*Coendou quichua*), the last sources were identified in *Lu. ovallesi* (Fig. 2). No *Leishmania* spp., infection was detected in the blood-fed females.

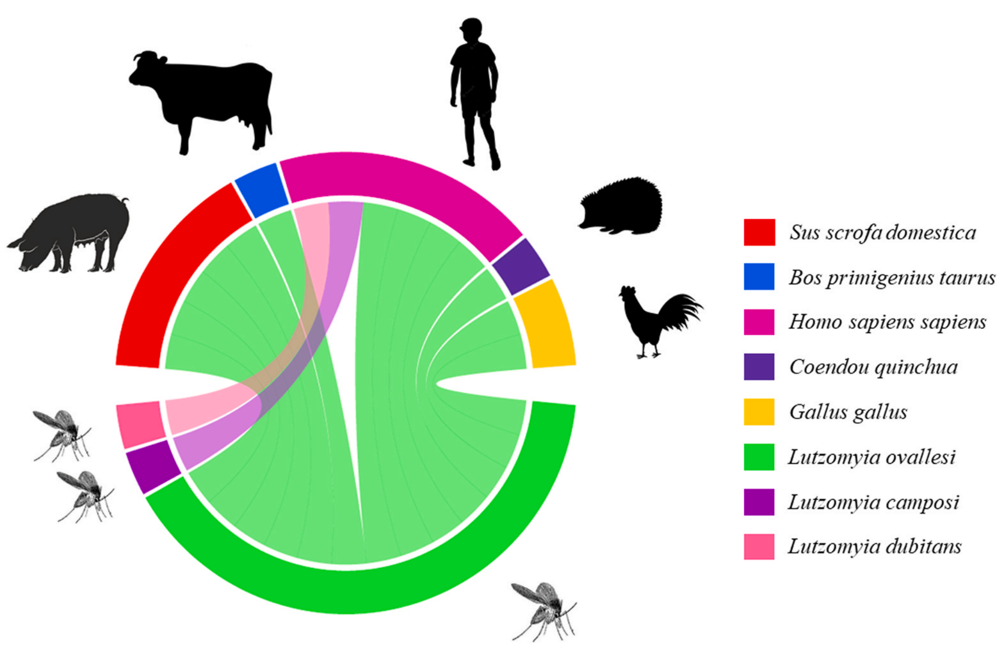

Fig. 2. (a) Chord plot representing the relative abundance of the feeding sources detected in each phlebotomine species. (b) The chord plot includes species in which food sources are detected.

3.3. Evaluation of anti-rK39 antibodies in owners or keepers of dogs infected with L. infantum

Fourteen asymptomatic humans (eight living in canine shelters, four veterinarians, and two owners) who had frequent contact with the *L. infantum* positive dogs identified through PCR analysis, were tested using the rK39-immunochromatographic test. However, none of them showed IgM/IgG antibodies against *L. infantum*.

4. Discussion

Visceral leishmaniasis is a significant public health issue in Colombia, with high morbidity and mortality rates, particularly in children (Morales, Rodríguez, 1996). However, information regarding its eco-epidemiological aspects in urban areas is limited. To address this, we conducted epidemiological surveillance of leishmaniasis in dogs in the MAB, one of the main metropolitan areas in Colombia. Our findings revealed a seroprevalence of 17.8% (95% CI = 12.6–23.1%) in dogs, with active infection by *L. infantum* primarily in symptomatic individuals. Statistical analyses indicated that canine shelters had the highest prevalence of infection. However, our entomological approach in these shelters did not identify *L. infantum* vectors, suggesting that dogs from these locations may become infected elsewhere or through alternative transmission routes. This information is crucial in understanding the eco-epidemiology of visceral leishmaniasis in urban areas, where there is a lack of the information on this serious public health issue in Colombia, particularly in Santander.

The canine seroprevalence observed in this study was lower than that found in hyperendemic areas for VL in Tolima, Huila, and Sucre departments (28–42%) using similar methods (Fernández et al., 2006; Romero et al., 2009; Rueda, 2015), but higher than that detected in Bogotá (Cundinamarca), MAB, and Barrancabermeja (Santander) (1.6–4.3%) (Florez-Muñoz et al., 2022; Rosypal et al., 2007). This suggests considerable variation in seroprevalence in the study area. It is noteworthy that the high seroprevalence values observed in this study compared to those in Santander during 2019 may be attributed to differences in the sensitivity of serological methods used (Florez-Muñoz et al., 2022). The previous study employed a commercial ELISA test with specific antigens of *L. infantum* (Ingezim LEISHMANIA, Madrid, Spain), while our study used the IFAT test, which has higher sensitivity due to the use of multiple antigens (Gutierrez et al., 2004). Some limitations of our study include the potential variability in the sensitivity and specificity of the serological test used to estimate the seroprevalence of *L. infantum* in dogs, as well as the significant margin of error considered for the sampling. To address this limitation, we recommend that future studies employ a second serological test to obtain a more accurate estimate of the true seroprevalence and reduce the significant margin of error during sampling. However, it is important to note that other factors, such as the sampling period and the characteristics of the canine population, may also contribute to the observed differences in seroprevalence.

Previous studies have estimated the seroprevalence of *Leishmania* spp. in canine shelters from Santander showing a seroprevalence of 4.1% in dogs from MAB (Florez-Muñoz et al., 2022); however, it is possible that the sampling restriction to canine shelters may have limited the authors' ability to identify epidemiological variables associated with this infection. Our statistical analyses reveal a higher prevalence of infection in dogs from shelters compared with domiciled dogs, which is consistent with observations in Asia, Europe, and South America (Estevam et al., 2022; Miró et al., 2012; Selim et al., 2021). This suggests that canine shelters may be a potential focus of infection in MAB. These findings are supported by molecular tests, which demonstrate a higher prevalence of *L. infantum* in dogs from shelters. Moreover, it is important to note that the origin of positive dogs in shelters could not be determined since most of them are stray dogs; hence, an increase in the prevalence of infection in shelters associated with the introduction of positive animals from other geographical areas cannot be ruled out. However, other factors such as economic status and the lack of garbage collection in domiciled dog habitats may also contribute to the infection.

On the other hand, the prevalence of *Leishmania* spp. infection in dogs from MAB, as estimated by molecular analyses, was lower than in hyperendemic areas of Sucre and Tolima departments using similar methods (7.3 – 34.9%) (Gómez et al., 2013; Santaella et al., 2011). This suggests a low frequency of active cases in MAB and a low probability of transmission to insect vectors. However, it is also possible that the lower detection limit of the markers used in these studies could explain these results (León et al., 2017). Alternatively, this discrepancy may be attributed to the high pathogenicity of the *L. infantum* strains in the MAB region, as suggested by the statistical analyses indicating a significant association between the PCR-detected infection and animals in the clinical group. Future clinical studies with different strains from Colombia are necessary to confirm this hypothesis. In terms of species diversity, our results differ from those observed in dogs from other rural areas of Colombia, where species such as *L. guyanensis*, *L. braziliensis* and *L. amazonensis* have been identified in these hosts (Castillo-Castañeda et al., 2022; Gómez et al., 2013; Herrera et al., 2018; Santaella et al., 2011; Vásquez-Trujillo et al., 2008), indicating a low exposure of canines from MAB to species of *Leishmania* associated with the sylvatic cycle.

Regarding sample types, our molecular analyses showed a significant variation in positivity frequency, with lymph node samples exhibiting a higher positivity than blood samples. This is consistent with findings in dogs from Europe using PCR, culture, and microscopic examination (Reale et al., 1999). Previous studies have demonstrated that skin or spleen biopsy has shown the most sensitivity for diagnosing these infections (Nunes et al., 2018; Santaella et al., 2011), we suggest lymph node aspiration as a suitable sample for molecular surveillance of *L. infantum* in dogs, as it is a less invasive procedure and easy to carry out in field conditions. However, we found some animals tested positive in blood and negative in lymph node, which suggests recent infections; therefore, both sample types should be considered to obtain a more accurate approximation of the real prevalence.

The entomological investigation in canine shelters revealed a dominance of three anthropophilic species, namely *Lu. camposi*, *Lu. ovallesi*, and *Lu. dubitans*, which were previously reported in Santander and the MAB with high frequency (Sandoval et al., 1998; Sandoval et al., 2006), and have been associated with transmission of CL but not VL (Niño and Pérez, 2021; Sandoval et al., 2006). These findings are supported by molecular analyses, which showed that these vectors lack *L. infantum* infection and have a low preference for feeding on dogs. These scenarios differ from what was observed during VL outbreaks in MAB, where *Lu. longipalpis* has been the major natural vector of *L. infantum* and found in 99.5% of the collected specimens (Cecílio et al., 2020; Flórez et al., 2006), which suggests that VL outbreaks in MAB may be associated with an increase in the population of potential vectors, as has been observed in other areas (Oliveira et al., 2018). The findings of the study, combined with the unclear origin of the infected dogs in the shelters, suggest that the transmission of *L. infantum* could occur in other areas where potential vectors are present. However, it is also possible that other routes of transmission, such as sexual or vertical transmission, or transmission through bites or wounds between dogs, could be contributing to the infection in the shelters (Boggiatto et al., 2011; da Silva et al., 2009; Gibson-Corley et al., 2008; Naucke et al., 2016). Therefore, further studies that investigate the temporal variation of phlebotomine populations in canine shelters and explore alternative modes of transmission are needed to confirm these hypotheses. Regarding *Lu. torvida* and *Lu. cayennensis*, two rare species with domiciliary reports in Cundinamarca and Cordoba (Niño and Pérez, 2021; Vivero et al., 2017), our results represent the first report of these species in the MAB, indicating the introduction of new species in the study area.

Finally, considering that many *Leishmania* spp. infections in humans in endemic areas remain asymptomatic (Faye et al., 2011), and that our study identified the presence of anthropophilic vectors sharing areas with dogs that are molecularly positive for *L. infantum*, we conducted a serological surveillance on people living with these dogs to assess their positivity for the parasite. Interestingly, no seropositivity was observed in this group, suggesting a low exposure to *L. infantum* infection, possibly due to the absence of potential vectors in the sampled canine shelters. However, it is important to note that the small sample size of human participants may also contribute to these results. Despite the lack of evidence of human exposure to *L. infantum* observed in this study, the presence of an autochthonous VL case reported in MAB during 2019 suggests that people living with *L. infantum*-positive dogs in the area may still face a considerable risk of infection (Santander, 2019). Further investigations, are necessary to understand the transmission dynamics of *L. infantum* in this area, including monitoring the temporal variation of phlebotomine populations in canine shelters, obtaining a larger number of human samples, and exploring alternative routes of transmission.

5. Conclusion

Our study indicates the circulation of *L. infantum* in dogs from MAB, with higher rates of infection in canine shelters suggesting a latent risk of zoonotic transmission of VL. However, our entomological investigation showed a lack of potential vectors of *L. infantum* in these areas, indicating that infection could occur elsewhere or through other routes of transmission. This may explain the absence of IgM/IgG antibodies against *L. infantum* in people living in canine shelters. Further research is needed to fully understand the transmission dynamics of *L. infantum* in dogs living in MAB shelters.

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Declaration of Competing Interest

The authors declare no competing interests.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.prevetmed.2023.106021.

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