High prevalence of Leishmania *spp. in from Central West Colombia*

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Summary
Leishmaniasis is a widespread disease caused by species of the genus *Leishmania*. In Colombia, this zoonosis is endemic in rural areas with a high prevalence in the departments of Antioquia, Santander, Meta, Tolima and Nariño. Dogs are the most important domestic reservoirs of the pathogen, given the epidemiological importance of dogs in the control of leishmaniasis is needed to determine the prevalence of *Leishmania* spp. in canines of the rural area of Ibagué and to identify potential risk factors related to the presence of this parasite. A cross-sectional study was carried out in 173 dogs from the rural area of Ibagué. *Leishmania* spp. was detected by amplifying the Internal Transcribed Spacer (ITS-1) and two regions of the *hsp70* gene through PCR. Factor associations were calculated through the Chi-square and odds ratio. Prevalence of *Leishmania* spp. infection in dogs was of 91.33% (158/173), where 36.71% (58/158) of the *Leishmania* spp. positive dogs showed one or more clinical signs of canine leishmaniasis and 63.29% (100/158) of the dogs were asymptomatic. Factors associated with the presence of the parasite did not show significant association. In addition, *hsp70D*-PCR was proved to be highly efficient for the detection of *Leishmania* spp.

Introduction

Leishmaniasis is a zoonotic disease caused by the invasion of protozoan parasites of the *Leishmania* genus, transmitted to mammals mainly by the hematophagous activity of sandflies belonging to the *Lutzomyia* and *Phlebotomus* genera (Ribeiro *et al*., 2018; Torres *et al*., 2017). This disease has a worldwide incidence of 0.7 million new cases per year and up to 65,000 deaths are reported annually (Alvar *et al*., 2012). There are around 53 species of *Leishmania*, of which parasitize mammals and 20 are pathogenic for humans (Akhoundi *et al*., 2016). These species are grouped by complexes according to the tropism to a specific tissue (Akhoundi *et al*., 2016) and they can infect macrophages in the bone marrow, lymph nodes, spleen, liver, kidneys, gastrointestinal tract, skin, and mucous membranes (Reis *et al*., 2009). This leads to the presentation of three clinical forms: cutaneous, mucocutaneous, and visceral leishmaniasis, characterized by presenting macroscopic lesions such as lymph node hypertrophy, ulcerative dermatitis (periorbital, nasal

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or disseminated), and edema in the limbs (Mümtaz, 2018). Leishmaniasis has a high prevalence in tropical developing countries and mainly affects the population in low-income countries (Oryan & Akbari, 2016). In Colombia, it is distributed in rural areas, with high prevalence in the departments of Antioquia, Santander, Meta, Tolima, and Nariño (Mendigaña, 2019), where nine species have been reported: *Leishmania panamensis, L. braziliensis*, *L. guyanensis*, *L. infantum*, *L. colombiensis*, *L. amazonensis*, *L. equatoriensis*, *L. lansoni* and *L. mexicana* (Salgado-Almario *et al*., 2019; Ramírez *et al*., 2016). According to the 2019 epidemiological bulletin were reported in the department of Tolima, 13 belonging to the municipality of Ibagué (incidence rate of 2.26) (Sistema de Vigilancia en Salud Pública, 2019). In 2020, 32 cases of CL have been notified with an incidence rate of 5.52% (Macedo & Urrego, 2020). Infection with *Leishmania* spp. in canines can be clinical or subclinical, becoming a public health concern due to the risk of transmission to humans in the presence of hematophagous vectors (Chinchilla & Vanessa, 2010; Flórez *et al*., 2006; Zoghlami *et al*., 2014). Several risk factors have been described related to *Leishmania* spp. infection including canine breed, infestation with ticks, proximity or contact with other animals (*e.g.* chickens, pigs, horses), improper garbage disposal, dwelling proximity to water sources, forest areas, and informal crops (Membrive *et al*., 2012; Oryan & Akbari, 2016; Silva *et al*., 2018). A higher prevalence of visceral leishmaniasis has been reported in shorthaired canines and purebreds such as Boxer, Pit Bull Terrier, Cocker Spaniel, and Great Danish (Almeida *et al*., 2010; Belo *et al*., 2013b; França-Silva *et al*., 2003). Active epidemiological surveillance based on the detection of infected animals is an initial step to establish appropriate control measures. Clinical examination allows the first approach to the diagnosis, however, in asymptomatic animals the use of diagnostic tests such as the Polymerase Chain Reaction (PCR) is required (Cavalcanti *et al*., 2012; Reis *et al*., 2013; Zoghlami *et al*., 2014). In the municipality of Ibagué, human leishmaniasis has been reported, nevertheless, the role of canines in the epidemiology of leishmaniasis is unknown. Thus, the present study aimed to determine the prevalence of *Leishmania* spp. in canines of the rural area of Ibagué and to identify potential risk factors related to the presence of this parasite.

Material and methods

Study location

Study was carried out in Ibagué, the capital city of the department of Tolima, Colombia, which has a population of 569,336 inhabitants (Centro de información municipal para planeación participativa, 2021) and a canine population of 40,016 animals (Ministerio de Salud y Protección Social de Colombia, 2018). Rural area is organized into 17 townships with approximately 144 villages.

Sample size

Sample size was estimated based on a population of 40,016 canines in the 17 townships of the rural area of Ibagué (Ministerio de Salud y Protección Social de Colombia, 2018) using the formula described by Thrusfield (2018), where the expected prevalence was 10%, the level of significance was 95% and the desired absolute precision was 5%.

Clinical examination, epidemiological survey and blood sampling

Study was carried out between July and November of 2019. Each canine`s owner signed a consent form before any procedure with the animals. Clinical examination was performed by a physical inspection to detect signs compatible with leishmaniasis including dermatological lesions (ulcers, alopecia, erythematous lesions), onychogryphosis, apathy, low body condition and abnormal peripheral lymph nodes. A survey was fulfilled in order to find possible risk factors related to the presence of *Leishmania* spp. in the canines, data included canine breed, dwelling features (a type of material of the house and the floor) and dwelling surround characteristics (disposal of garbage, presence of other animals, forest areas, agricultural activities and streams near the home). One hundred seventy-three samples of canine blood were collected by peripheral venipuncture in the saphenous or cephalic vein. Samples were placed in tubes with ethylenediaminetetraacetic acid and transported in an ice box until storage at -20 °C.

DNA extraction and endpoint PCR

Total genomic DNA extraction was performed from blood samples using phenol-chloroform-isoamyl alcohol (25:24:1).

DNA quality was verified by NanoDrop One spectrophotometer (ThermoFisher, USA) and stored at -20 °C until its use (Wang *et al.*, 2011). *Leishmania* spp. was detected by amplifying the Internal Transcribed Spacer 1 (ITS1) and regions C (1545-1778) and D (711-1089) of the hsp70 gene (Table 1).

Animals were considered positive to *Leishmania* spp. if one of the three tests was positive. PCR was performed in a T-100 thermal cycler (Bio-Rad,

Table I. *Sequences of primers for amplification of* ITS1 *and* hsp70 *genes.*

Gene	Amplicon size (bp)	Primer sequence (5´-3´)	Reference
ITS1	314	F: CTGGATCATTTTCCGATG	Schönian *et al.* (2003)
		R: TGATACCACTTATCGCACTT	
hsp70C	234	F: GGACGAGATCGAGCGCATGGT	Graça *et al.* (2012)
		R: TCCTTCGACGCCTCCTGGTTG	
hsp70D	379	F: CCGCCTCGTCACGTTCTTCAGC	Graça *et al.* (2012)
		R: GTTCAGCTCCTTGCCGCCGA	

Hercules, California, USA) using a final reaction volume of 25 µL, composed of 14.875 µL of distilled and deionized water (ddw), 5 µL of 5x Colorless GoTaq Flexi Buffer, 1 µL of dNTPs at 1.5 mM, 1 µL of each primer (forward and reverse) [10 pmol/µL], 1 µL MgCl2 at 25 mM, 0.125 µL of GoTaq Flexi DNA polymerase (Promega, Madison, Wisconsin, USA) and 1 µL of the gDNA sample as template. Amplification steps consisted of initial denaturation cycle at 95 °C for 3 minutes, followed by 35 denaturation cycles at 95 °C for 30 seconds, annealing at 55 °C for 30 seconds, extension at 72 °C for 30 seconds, and final extension at 72 °C for 5 minutes. Positive control of *L. infantum* (MCAN/CN/90/SC) and negative control (ddw) were used. PCR products were revealed by 2% agarose gel electrophoresis, stained with Hydragreen (ACTGene, Piscataway, New Jersey, USA), for 40 minutes at 100 V in the myGel Mini electrophoresis chamber (ACCURIS, Edison, New Jersey, USA) and visualized under ultraviolet light using an ENDURO GDS (LabNet Intl, Edison, New Jersey, USA).

DNA sequencing and phylogenetic analysis

hsp70C-PCR products of 234 pb were sequenced by the Sanger method (Macrogen, Seoul, Korea), sequencing was carried out by the same primer pair (*hsp70C*). Consensus sequences from forward and reverse reads were edited and analyzed using Geneious Prime software (Version 2021.0.3) (Biomatters, Auckland, New Zealand) (Kearse *et al.*, 2012), furthermore they were compared with the GenBank database (NCBI, USA) by using the BLASTN tool (Version 2.2.28+). Bioinformatic analysis included multiple alignments of our sequences and 18 *hsp70* sequences from 7 *Leishmania* species reported in GenBank database (NCBI, USA), and the construction of phylogenetic trees using Neighbor-Joining (NJ) method, with a bootstrap consensus inferred from 1000 replicates. It was used as outgroup the region C of *hsp70* gene sequence of *Trypanosoma cruzi* (GenBank accession No. MF144929.1). Sequences of the region *C* of *hsp70* gene of *Leishmania* spp. were submitted to GenBank in order to obtain accession numbers (MW509745, MW509746, MW509747, MW509748 and MW509749).

Statistical analysis

A database was created from the information collected in the clinical examinations and the epidemiological survey in the Epi Info software (CDC, Atlanta, Georgia, USA). Analysis of the independent variables in the epidemiological survey was performed using GraphPad Prism v.8 for macOS (La Jolla, CA, USA). Frequency tables were constructed and their relationship with the presence of *Leishmania* spp. was established through the Chi-square. Strength of this relationship was determined by calculating the odds ratio.

Results

Characteristics of the canine population

A total of 173 dogs were sampled. They were distribuited 65 from Totumo, 32 from Coello cocora, 14 from El Salado, 13 from Cay, 11 from San Bernardo, 11 from Calambeo, 9 from Gamboa and 9 from Carmen de Bulira, 9 from Buenos Aires. Of the 173 sampled dogs, 57.8% were males and 42.2% females. Crossbreed dogs were 79.19% and purebred 20.81%, they had short fur (65.32%) with an average age of 48.9 months. All animals were dogs with an owner, but 31.8% did not stay in the dwelling during the day and 22.5% did not stay overnight. Regarding the clinical sign compatible with leishmaniasis, 10.4% of the dogs had foci of alopecia, 12.72% skin lesions, 24.85% pruritus, and 2.89% onychogryphosis and lymphadenitis (Figure 1).

Figure 1. *Dogs with cutaneous lesions, mucocutaneous lesion, localized alopecia, cutaneous hypopigmentation and onychogryphosis hypopigmentation and onychogryphosis.*

Molecular diagnosis

Leishmania spp. were detected by amplification of 3 different targets by PCR assay, *hsp70C*, *hsp70D* and the ITS1 of the parasite. One hundred fifty eight of the 173 dogs were positive for *Leishmania* spp. in at least one of the three tests, which represents a prevalence of 91.33%.

On the other hand, 63.29% (100/158) of the PCR-positive dogs did not show any clinical sign compatible with the disease and only 36.71% (58/158) of the dogs presented one or more clinical signs of canine leishmaniasis (Table 2). In addition, 4.43% (7/158) of the positive dogs remain during the day in the houses, and 79.75% (126/158) stayed outside of the house at night, in the backyard or in a close neighborhood.

Efficiency of different PCR assays was compared, taking as a parameter the number of samples detected as positive for the presence of the parasite. One hundred thirty-eight DNA samples were positive for the *hsp70* gene of *Leishmania* spp. through the *hsp70C*-PCR, 140 by using the *hsp70D*-PCR, and 22 DNA samples were positive for the ITS-1 of *Leishmania* spp. (Table 2). According to which the hsp70D PCR was proven to be highly efficient for the detection of *Leishmania* spp. and ITS1-PCR had the fewest detections of the parasite in canine blood.

Risk factors

Regarding the housing conditions of the dogs, the wall and floor materials were mostly concrete (65.32% and 64.16%, respectively), the garbage disposal more frequent was in the municipal landfill (41.04%) and burning (35.26%), the 49.71% had agricultural production, specifically poultry. In the peridomiciliary zone, 56.65% of the ares had a water stream of 56.65%, wooded areas, and in 71.68% there was presence of mosquitoes. Of the dog's owners, 67.63% used protective measures for the mosquitoes, like bed nets to sleep (66.96%), and the remaining used repellents or fumigated their household (33.04%). According to the statistical analysis, characteristics of the canine, sociodemographic factors, home conditions and social interactions evaluated did not show associations with the presence of *Leishmania* spp. in the canines of Ibagué, and therefore, statistically significant risk factors were not determined.

Phylogenetic analyses

PCR products of *hsp70C* region (234 bp) from the LE_019, LE_021, LE_022, LE_024, LE_025, LE_029, LE_030, LE_053 and LE_064 samples were sequenced. Sequences LE_019 and LE_029 LE_025 have 100% identity with each other, the same as LE_021, LE_053 and LE_064, therefore, 5 partial sequences of the hsp70 gene of *Leishmania* spp. were submitted to GenBank.

Accession numbers of sequences were as follows: LE_019 (MW509745), LE_021 (MW509746), LE_022 (MW509747), LE_024 (MW509748) and LE_030 (MW509749).

Phylogenetic analysis bootstrapping provided strong support for all the nodes. *Leishmania* subgenres were separated for one cluster supported by 99.3%. *Viannia* subgenus, this was divided in L. braziliensis complex species and *L. equatorensis* with a support of 61.5%. Leishmania subgenus was divided in *L. mexicana* complex and *L. donovani* complex, where sequences of this study cluster with *L. donovani* complex (Figure 2).

Table II. *Distribution of* Leishmania*-positive canines by village.*

Village	Detection of *Leishmania* spp.							Clinical signs	
	ITS1		*hsp70D*		*hsp70C*		Positive canines (n)	+	-
	+	-	+	-	+	-			
Buenos Aires	0	9	9	0	9	0	9	6	3
Calambeo	0	11	11	0	11	0	11	4	7
Carmen de Bulira	4	5	6	3	6	3	9	4	5
Cay	0	13	13	0	13	0	13	9	4
Coello Cocora	5	27	26	6	26	6	28	9	19
El Salado	2	12	5	9	4	10	12	5	7
Gamboa	6	3	6	3	2	7	9	2	7
San Bernardo	0	11	11	0	11	0	11	0	11
Totumo	5	60	53	12	56	9	56	19	37
Total	**22**	**151**	**140**	**33**	**138**	**35**	**158**	**58**	**100**

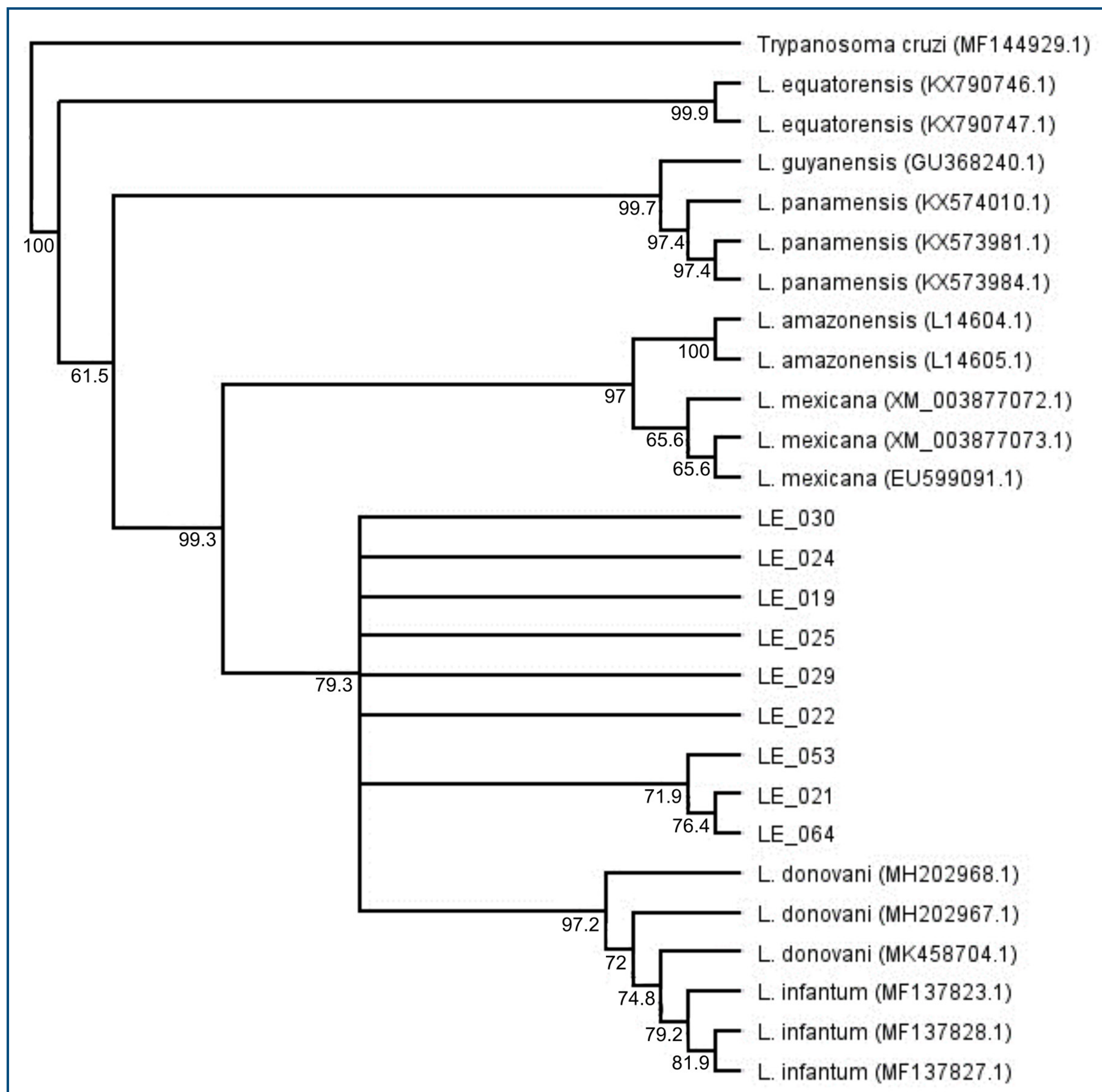

Figure 2. *Phylogenetic tree of Leishmania species and the studied samples, based in hsp70C region sequence. A bootstrapped NJ tree constructed using Geneious Prime software (Version 2021.0.3). Numbers at nodes represent the percentage of 1000 bootstrap iterations supporting the branch. T. cruzi was used as outgroup.*

Discussion

Molecular diagnosis

Prevalence of *Leishmania* spp. in canine population in Ibagué (91.33%) is highest compared with that reported by molecular methods in Chaparral-Tolima (7.3%) (Santaella *et al*., 2011), Sincelejo-Sucre (34.9%), Sampúes-Sucre (35.7%) and Ovejas-Sucre (11.1%) (Paternina-Gómez *et al*., 2013). In other countries, prevalence values were 24.8% by qPCR in Sichuan Province-China, 48.4% by IFAT in Italy, 26% by ELISA and 63% by PCR in Mallorca-Spain, and 66% by immunoblot and 80% by PCR in France (Berrahal *et al*., 1996; Paradies *et al*., 2006; Shang *et al*., 2011; Solano-Gallego *et al*., 2001). In Brazil, prevalence is reported between 3.2% to 50.3%, depending on the area and methods (Marcondes & Day, 2019), in this endemic country, serological tests were found to have low capacity for *Leishmania* spp. detection when it is compared with molecular tests in canines (Lopes *et al*., 2017).

The most widely methods used for the diagnosis of canine leishmaniasis in Latin America are the ELISA, IFAT and Western blot technique as well as PCR (Alves & Bevilacqua, 2004; Trevisan *et al*., 2015). PCR has high sensitivity and specificity, and its use reduces the costs of the *Leishmania* spp. detection (de Paiva *et al*., 2009; Sundar & Rai, 2002). On the other hand, PCR can detect the presence of *Leishmania* spp. in

different types of clinical samples such as blood, lymph nodes, skin, conjunctival smear and bone marrow aspirates (Lachaud *et al*., 2002; Lombardo *et al*., 2012; Manna *et al*., 2004; Reale *et al*., 1999; Reis *et al*., 2013).

Several studies reported that amplification of ITS1 through PCR is highly specific for detection of *Leishmania* spp. and highly sensitive as it is capable of detecting 0.2 parasites per sample (Schönian *et al*., 2003; Toz *et al*., 2013). Graça *et al*. (2012) reported a sensitivity of 81.4% of ITS1-PCR and 73.2% of *hsp70C*-PCR, using DNA extracted from skin biopsies. However, in this study, sensitivity of ITS1-PCR is lower than that obtained with *hsp70*-PCR, this may be due to the fact that the blood is less specific (80%) and sensitive (85%) than the aspirates of the lymph nodes (specificity, 100%; sensitivity, 100%) (Reale *et al*., 1999), given that blood tends to have a variable parasite load depending on the stage of infection (Manna *et al*., 2004). Nonetheless, the use of blood for the diagnosis of Leishmania spp. has shown to be effective in the detection of the parasite mainly in asymptomatic animals (Monteiro *et al.*, 2019)" for "… PCR is highly specific for detection of Leishmania spp. and highly sensitive as it may detect 0.2 parasites per sample … sensitivity of ITS1-PCR is lower than that obtained with hsp70-PCR, this may be due to the different matrix (Reale *et al.*, 1999; Manna *et al.*, 2004).Nonetheless, use of blood for the diagnosis of *Leishmania* spp. has shown to be effective in the detection of the parasite mainly in asymptomatic animals (Monteiro *et al*., 2019) as in our study and it is recommended as more suitable compared with aspiration-based methods (Albuquerque *et al*., 2017). Furthermore, blood sampling is a procedure,simple to perform, less stress, with low cost and its repeatability is easier compared to bone marrow sampling or biopsy of lymph nodes (Carvalho *et al*., 2009; Lachaud *et al*., 2002; Reale *et al*., 1999).

Amplification of the *hsp70* gene represents a valuable tool in the detection of *Leishmania* spp., since it contains between five and six *hsp70-I* copies followed by one *hsp70-II* copy (Ramirez *et al*., 2011) and its effectiveness in the detection of asymptomatic infections was evidenced in the present study. *hsp70* gene region is the most highly conserved in sequence and function in all organisms, with a lower rate of genetic diversity than other markers for instance *gp63* gene, rDNA genes, or ITS1 (Dabirzadeh *et al*., 2016), becoming a relevant target for the detection of *Leishmania* species.

Risk factors

Differences in clinical manifestations between dogs, such as the severity of clinical signs and the time of onset of the disease, may vary depending on the individual immune response of the infected animals as well as genetic factors, age, nutritional status, and the virulence of *Leishmania* strains (Alvar *et al*., 2004; Man *et al*., 2020; Moreno *et al*., 1999; Quinnell *et al*., 2003). In our study, 63.29% of the PCR-positive dogs did not present clinical signs of canine leishmaniasis or other symptoms. Similarly, it has reported that in endemic areas of visceral leishmaniasis 85% of dogs infected by *L. infantum* are asymptomatic (Dantas-Torres & Brandao-Filho., 2006). Nonetheless, it has been described that approximately 46% of infected dogs acquire the infection and develop the disease immediately, another 44% of dogs develop the disease later, and 10% of them can remain without clinical signs of visceral leishmaniasis throughout their lives (Moreno & Alvar, 2002).

Dogs have the ability to transmit *Leishmania* spp. to the vector and, consequently, to other canines or humans regardless of whether they present or no clinical signs. The transmission cycle of the parasite could be favored by the asymptomatic infection of *Leishmania* spp. in the canine (Dantas-Torres & Brandao-Filho, 2006; Moshfe *et al*., 2009; Soares *et al*., 2011). Presence of *Lutzomyia longiflocosa* has been reported in the rural zone of Ibagué, Tolima (Guzmán-Barragán *et al*., 2021). Furthermore, in several Tolima municipalities (Planadas, Rovira, Casablanca, Herveo, Ortega, San Antonio and Chaparral), it has been reported the presence of *L. columbiana*, *L. longiflocosa*, *L. micropyg*, *L. rangelina*, *L. suapensis*, *L. nuneztovari*, *L. atroclatava* (Bejarano *et al*., 2003; Bejarano *et al*., 2006; Cárdenas *et al*., 1999; Contreras *et al*., 2012; Morales *et al*., 1981; Pardo *et al*., 2006; Prado *et al*., 1999; Sierra *et al*., 2000).

Transmission potential of infected dogs can differ between symptomatic and asymptomatic animals. By xenodiagnosis methods it has been observed that asymptomatic dogs have a lower capacity to infect sandfly vectors (Travi *et al*., 2001; Verçosa *et al*., 2008), while other studies determined that symptomatic animals are more likely to spread the infection (Guarga *et al*., 2000; Molina *et al*., 1994).

Some studies have determined that the physical traits of dogs such as height, spaying, purebred, and age (older than 2 years) can have a positive correlation with the occurrence of leishmaniasis (Belo *et al*., 2013a).

Additionally, associated factors such as the presence of ectoparasites, contact with other animals, dog's permanence in the backyard, forest areas near dwelling and the presence of birds in the domestic environment, may play a role in attracting sandflies and predispose the infection of dogs (Belo *et al*., 2013b; Curi *et al*., 2014; Coura-Vital *et al*., 2011). However, significant associations were

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not found between the risk factors evaluated and the presence of *Leishmania* spp. in the dogs of the municipality. This can be attributed to the immune status of the individual dogs and because of both Leishmania-positive and Leishmania-negative dogs have homogeneous factors, therefore odds ratios did not show significance. Additionally, other studies have shown that there are not significant associations between age and the presence of *Leishmania* spp. in the canine population, age is not a strong predictor for *Leishmania* spp. infection and dogs of all ages may be reservoirs in the study areas. Moreover, according to Belo *et al*. (2013b), free-roaming dogs are more difficult targets to vector than dogs living at home; and it has been shown that the presence of chickens at home is not associated as an imminent risk factor since it does not correspond to a reservoir of the parasite.

Phylogenetic analyses

PCR-*hsp70C* amplifies a variable region of the hsp70 gene in eleven *Leishmania* species (Zampieri *et al*., 2016). However, it was not possible to differentiate the species using the sequence obtained with the amplification carried out. All sequences reported belong to the *Leishmania donovani* complex, according to the geographical distribution of *Leishmania species* in Colombia, the *Leishmania species* circulating in dogs from Ibagué-Tolima are *L. infantum* (Salgado-Almario *et al*. 2019).

This is the first molecular study of canine *Leishmania* infection in Ibagué, an endemic region in which a high prevalence of *L. donovani* complex was found. Additional investigations are required to assess the infective capacity of dogs towards sandfly vectors, looking for establishment of routine screening and preventive measures for canine and human leishmaniasis.

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Conflict of Interest Statement

The authors declare no conflict of interest.

Statement of animal rights

All the experimental procedures followed the Guidelines of the Bioethics Committee of the Central Research Office of the University of Tolima based on Law 84/1989 and Resolution 8430/1993 and complied with the guidelines for animal care and use in research and teaching. The Bioethic committee of the Central Research Office approved bioethics aspects in the agreement 2239 of June 25 at 2019, between the Municipal Health Secretariat and the University of Tolima.

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