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Prevalencia de Ehrlichia canis y Hepatozoon canis en perros de albergues en el sur del Valle de Aburrá, Colombia.

Prevalência de Ehrlichia canis e Hepatozoon canis em cães abrigados no sul do Vale do Aburrá, Colômbia.

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Abstract

Background: Pathogenic agents such as bacteria of *Anaplasmataceae* family and canine hemoparasitic protozoans transmitted by ticks are common in Colombia due to the circulation and biological adaptation of the vector *Rhipicephalus sanguineus* sensu lato (s.l.). **Objective:** To detect the circulation of *Ehrlichia canis* and *Hepatozoon canis* in sheltered dogs in three municipalities in southern Aburrá Valley, Antioquia. **Methods:** Primers were used to amplify the 16S rRNA associated with the *Anaplasmataceae* family, *dsb* for *Ehrlichia* sp. and 18S rRNA for *Hepatozoon* sp. **Results:** Of the 357 samples of venous blood obtained, representing all the sheltered dogs in the study zone, *Ehrlichia canis* DNA was detected

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in 2.2% individuals, showing identity of 100% with previous sequences from the GenBank. *Hepatozoon canis* showed a prevalence of infection of 8.7% (31/357), with 100% identity to genotypes from Japan, Brazil, and Spain. Only one sequence of *H. canis* exhibited a phylogenic divergence concerning *H. canis* previously reported in Brazil and the Old World. **Conclusions:** This study confirms the circulation of *E. canis* and *H. canis* in asymptomatic shelter dogs in the south-central zone of the Aburrá Valley, Antioquia Colombia. The present study is the first molecular detection of *H. canis* in the Province of Antioquia and the third report of canine hepatozoonosis from Colombia, highlighting the importance of considering this agent in the veterinary clinic.

Keywords: *Colombia; diagnosis; dog; Ehrlichia canis; Hepatozoon canis; infection; polymerase chain reaction; tick-borne pathogens.*

Resumen

Antecedentes: los agentes patógenos transmitidos por garrapatas como las bacterias de la familia Anaplasmataceae y los protozoos hemoparasitarios caninos, son comunes en Colombia debido a la circulación y la adaptación biológica del vector *Rhipicephalus sanguineus* sensu lato (s.l.). **Objetivo**: detectar la circulación de *Ehrlichia canis* y *Hepatozoon canis* en perros protegidos en tres municipios del sur del Valle de Aburrá, Antioquia - Colombia. **Métodos:** se usaron cebadores para amplificar el gen 16S rRNA asociado con la familia Anaplasmataceae y el gen *dsb* para *Ehrlichia* sp. y el 18S rRNA para *Hepatozoon* sp. **Resultados:** de las 357 muestras de sangre venosa obtenidas, que representan a todos los perros de albergues en la zona de estudio, 2,2% fueron positivas para *Ehrlichia canis*, con 100% de identidad con secuencias anteriores publicadas en todo el mundo. *Hepatozoon canis* mostró una prevalencia de infección del 8,7% (31/357), con una identidad del 100% con genotipos de Japón, Brasil y España. Solo una secuencia de *H. canis* exhibió una divergencia filogénica en relación con *H. canis* previamente reportada en Brasil y el Viejo Mundo. **Conclusiones:** este estudio confirma la circulación de *E. canis* y *H. canis* en perros asintomáticos de albergues en la zona centro-sur del Valle de Aburrá, Antioquia, Colombia. El presente estudio es la primera detección molecular en el Departamento de Antioquia y el tercer reporte de hepatozoonosis canina de Colombia destacando la importancia de considerar este agente en la clínica veterinaria.

Palabras clave: *Colombia; diagnóstico*; *perro*; *Ehrlichia canis; Hepatozoon canis; infección; reacción en cadena de la polimerasa; patógenos transmitidos por garrapatas.*

Resumo

Antecedentes: agentes patogênicos transmitidos por carrapatos, como bactérias da família Anaplasmataceae e protozoários hemoparasitários caninos, são comuns na Colômbia devido à circulação e adaptação biológica do vetor *Rhipicephalus sanguineus* sensu lato. **Objetivo:** nosso objetivo foi detectar *Ehrlichia canis* e *Hepatozoon canis* em cães abrigados em três municípios do sul do vale de Aburrá, Antioquia - Colômbia. **Métodos:** os primers foram utilizados para amplificar o rRNA 16S associado à família Anaplasmataceae, o *dsb* para *Ehrlichia* sp. e o rRNA 18S para *Hepatozoon* sp. **Resultados:** das 357 amostras de sangue venoso obtidas, representando todos os cães abrigados na zona de estudo, 2,2% foram positivas para *Ehrlichia canis*, com 100% de identidade com sequências anteriores publicadas em todo o mundo. *Hepatozoon canis* mostrou uma prevalência de infecção de 8,7% (31/357), com 100% de identidade com genótipos do Japão, Brasil e Espanha. Apenas uma sequência de *H. canis* apresentou divergência filogênica em relação a *H. canis* previamente relatados no Brasil e no Velho Mundo. **Conclusões:** este estudo confirma a circulação de *E. canis* e *H. canis* em cães de abrigo assintomáticos na zona centro-sul do vale de Aburrá, Antioquia Colômbia. O presente estudo é a primeira detecção molecular no Departamento de Antioquia e o terceiro relato de hepatozoonose canina na Colômbia, destacando a importância de considerar este agente na clínica veterinária.

Palavras-chave: *Colômbia; diagnóstico; cão; Ehrlichia canis; Hepatozoon canis; infecção; reação em cadeia da polimerase; patógenos transmitidos por carrapatos.*

Introduction

Urban development and environmental changes caused by humans have facilitated the appearance of emergent and re-emergent zoonoses, with the diseases transmitted by ticks being some of the most important (Dantas-Torres *et al.,* 2012). The epidemiological interest in ticks as ectoparasites is due to their vectorial capacity (mechanical and biological) to transmit multiple infectious agents such as viruses, protozoans, and bacteria to wild hosts (Soares *et al.,* 2017), and to domesticated mammals and humans (Little, 2010; Dantas-Torres and Otranto, 2011). The ability of ticks to survive in diverse ecosystems (Dantas-Torres and Otranto, 2011), and their capacity to feed on a wide range of hosts generate a worrisome scenary, where they could become reservoirs for the amplification or maintenance of several

pathogenic microorganisms (Viana *et al.,* 2014). Regarding the hosts, stray animals in shelters come from adverse conditions that directly compromise their immune response (Shaw *et al.,* 2017). Poor nutritional status, the absence of sanitary conditions, high ectoparasite loads, and other external stress factors may increase the susceptibility of these animals to developing infectious diseases (Rojas *et al.,* 2013).

Although *Ehrlichia canis* and *Hepatozoon canis* are frequently seen in the veterinary practice, they are poorly investigated in Colombia. Consequently, the lack of local reports about tick-borne pathogens (TBP) underestimate the importance of these pathogens in canines (*Canis lupus familiaris*) in our region, and the role that these domestic animals could be playing over the impact of those zoonoses on public health (Vargas-Hernández *et al.,* 2012).

Ehrlichia canis is the etiologic agent of canine monocytic ehrlichiosis (CME) and belongs to the Anaplasmataceae family. The members of this family are Gram-negative and obligate intracellular bacteria. CME is a multi-systemic disease that has acute, subclinical, or chronic presentations and causes a range of forms from asymptomatic to severe clinical signs characterized by depression, anorexia, lethargy, weight loss, and fever (Little, 2010). Canine hepatozoonosis is a disease caused by two parasites from the subphylum Apicomplexa: *Hepatozoon canis* and *H. americanum* (Baneth *et al.,* 2000). Although they are phylogenetically related, both species differ in several aspects, including clinical signs, life cycles, vectors, and host spectrum (O'Dwyer *et al.,* 2011).

The main objective of this study was to detect the circulation of *E. canis* and *H. canis* and measure the point prevalence in Shelter dog populations from south of the Aburrá Valley with only a cursory assessment of association. The study is highly relevant because targets to increase alertness about the presence of *E. canis* and *H. canis* in asymptomatic dogs, and improve their diagnosis and control, to avoid the spread among dogs and prevent their zoonotic transmission.

Materials and Methods

Ethical considerations

Field blood samples were obtained during the clinical follow-up of the patients, according to the norms stipulated by the Code of Ethics of the Professional Council of Veterinary Medicine and Zootechnics of Colombia (COMVEZCOL). The study was approved by the Ethical Committee for Animal Experimentation of the Corporación Universitaria Lasallista, statement of approval 06 of June 8, 2013.

Sampling and blood collection

We conducted a cross-sectional study involving the collection of blood samples and information about creole dogs in animal shelters from the municipalities of Caldas (Primavera and La Miel), La Estrella, and Sabaneta (Sabaneta and Pan de Azúcar; Figura 1), between July 2017 to February 2018. Shelter dog population was considered because it provided a defined and full access to the population of dogs from the south of the Aburrá Valley registered with the Health Secretaries. Accordingly, inclusion and exclusion criteria were not considered for this study.

Three hundred fifty-seven (357) blood samples were obtained from five municipal dog shelters located in the Aburrá Valley, Antioquia (Colombia). Samples were collected through cephalic vein puncture in tubes with EDTA and stored at -20 ^{o}C until processing.

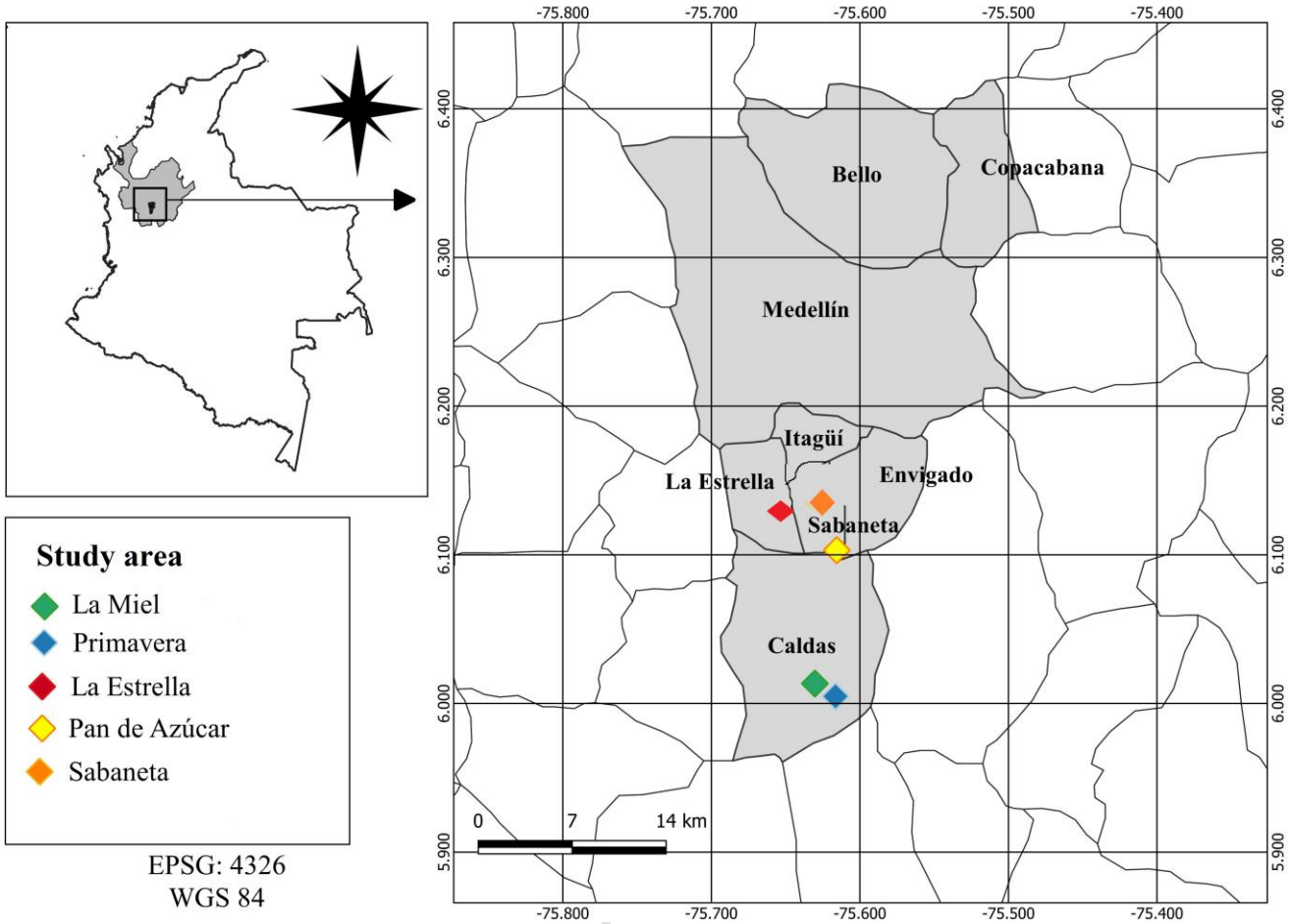

Figure 1. Geographical distribution of the study area (and the corresponding shelters in each municipality), for the molecular detection *E. canis* and *H. canis*. The following are the coordinates for each shelter: 1. La Miel, 6°06'11'N 75°36'56'W, located at 1,855 meters above the sea leve (m.a.s.l.); 2. Primavera, 6°00'17'N 75°36'59'W, located at 2,252 m.a.s.l.; 3. La Estrella, 6°08'57'N 75°38'14'W, located at 1,655 m.a.s.l.; 4. Pan de Azúcar, 6°08'00'N 75°37'49'W, located at 1776 m.a.s.l.; 5. Sabaneta, 6°08'39'N 75°36'45'W, located at 1,674 m.a.s.l

Nucleic acid extraction and polymerase chain reaction

The DNA was extracted from the blood samples using GeneJET Genomic DNA kits (Thermo Fisher Scientific Inc., USA), following the manufacturer's recommendations. The molecular detection of members of the Anaplasmataceae family was performed using a real-time PCR (qPCR), with the primers EC16SF and EC16SR (Table 1). Real-time detection was enabled using the SensiFAST™ SYBR Lo-Rox Kit (Bioline© London, UK). Each reaction was amplified using 10 µl of 1X of SensiFAST SYBR® (Meridian Life Science Inc. USA), primers (400 mM each), 6,4 µl of Nuclease-free Water DNA (New

England Biolabs Inc. UK) and 2 ul of DNA sample in a final volume of 20 µl. The β actin - *Canis lupus familiaris* (F 5'-GCGCAAGTACTCTGTGTGGAT-3' and R 5'-GTCGTACTCCTGCTTGCTGAT-3') was used as an internal control gene and *A. marginale* DNA was used as a positive control. Samples were considered positive by a melting curve between 80 to 85 °C. Positive samples were tested with a conventional PCR to amplify a fragment of the 16S rRNA (468 base pairs bp) and *dsb* (684 bp) genes. For the detection of *Hepatozoon* sp., we amplified a fragment that corresponded to the gene of the subunit 18SrRNA (Table 1). DNA from *H. canis* infected dog blood was used as a positive control. The ultrapure water was used as a negative control.

The conventional PCR reaction was amplified using 1U of TopTaq DNA Polymerase (Qiagen, Chatsworth, CA EEUU), 1x TopTaq PCR Buffer, 0.2 µM of each primer, 200 µM of each dNTP, 50~100 ng of DNA template, and ultrapure water, in a final volume of 50 µl. The PCR was performed using a cycling protocol of 94 °C for 3 min, and 35 cycles of 94 °C for 30 s, 60 °C (16S rRNA and *dsb*) and 58 °C for 30 s (18S rRNA), and 72°C for 1 min. The amplified products were separated on 2% agarose gel Tris Acetate-EDTA electrophoresis and visualized by staining with DNA EZ-Vision (Avantor®, EEUU).

Table 1. Primers used for detecting family Anaplasmataceae and *Hepatozoon* sp.

Target gene	Name	Sequence	Reference
16S rRNA	EC16S F	5'-TCGCTATTAGATGAGCCTACGT-3'	(Peleg *et al.*, 2010;
(120 bp)	EC16S R	5'-GAGTCTGGACCGTATCTCAGT-3'	Waner *et al.*, 2014)
16S rRNA	16sANA-F	5`-CAGAGTTTGATCCTGGCTCAGAACG-3`	(De la Fuente *et al.*,2006;
(468 bp)	16sANA-R	5`-AGTTTGCCGGGACTTCTTCTGTA-3`	Almazán *et al.*, 2016)
dsb	*dsb*F2	5`-CTTAGTAATACTAGTGGCAAGTTTTCCAC-3`	(Cruz *et al.*, 2012)
(684 bp)	*dsb*R2	5`- GTTGATATATCAGCTGCACCACCG-3`	
18S rRNA	PIRO A1	5'-AGGGAGCCTGAGAGACGGCTACC-3'	(Jefferies *et al.*, 2003)
(450 bp)	PIRO-B	5'-TTAAATACGAATGCCCCCAAC-3'	

bp: base pairs of nucleic acids.

Statistical analysis

The analysis was based on a level of confidence (Zα) of 95% for measuring the point prevalence and on a type I error level of 5% for detection of infection of *E. canis* and *H. canis* (dependent variable). Confidence intervals for the true prevalence were calculated using a standard formula (Evans and

O'Connor, 2007). Data were imported into IBM SPSS Statistics software (release 25.0, 2017–03, IBM Corp., New York, USA) and descriptive analyses was undertaken (frequencies for independent categorical data). Futhermore, we performed associations between infection of *E. canis* and *H. canis*, with age, sex and origin, using the Pearson's chi-squared test and odds ratio (OR of prevalence).

Sequence analysis and phylogenetic tree construction

All the positive products were sent to be sequenced at Macrogen Inc. in Seoul, Korea. Nucleotide sequences for the above-mentioned gene targets were edited, assembled and trimmed using the MEGA software v6.2013 (Pennsylvania, USA), and subsequently compared with existing sequences in the GenBank database using the BLAST algorithm on NCBI (www.ncbi.nlm.nih.gov/blast). Variance estimation for pairwise analysis for all gene sequences was carried out by the bootstrap method with 1000 replicates and uniform evolutionary rates among sites. The 16S rDNA, *dsb* and 18S rDNA, nucleotide sequences were furtherly tested by Maximum Likelihood (ML) phylogeny. For each of the phylogenetic tree buildings, we use a dataset for each of the genes. The corresponding dataset was tested in MEGA 6.0 to assess the model that was most suitable to generate the most reliable phylogenetic tree (Tamura *et al*., 2013).

Results

All positive dogs were clinically healthy. These dogs were located at 5 shelters in the 3 municipalities of our study and a summary of their demographics and history is presented in Table 2.

Table 2. Descriptive statistics for 357 dogs sampled at dog shelters in Southern Aburrá Valley during 2017 and 2018.

	N° of dogs per shelters						
Factor	*Shelter 1*	*Shelter 2*	*Shelter 3*	*Shelter 4*	*Shelter 5*	*Total*	*%*
Dog tested	**204**	**28**	**30**	**38**	**57**	**357**	
E. canis	4	2	0	1	1	8	2.2
H. canis	23	1	1	2	4	31	8.7
Age (years)							

1 - < 3	53	11	7	8	6	85	23.8
3 -< 5	62	8	11	12	12	105	29.4
5 -< 8	49	8	9	12	12	90	25.2
8 - 12	40	1	3	6	27	77	21.6
Sex							
Male	84	6	14	18	38	160	44.9
Female	119	23	16	20	19	197	55.1
Origin							
Urban	107	14	19	13	20	173	48.5
Rural	97	14	11	25	37	184	51.5

Shelters 1 and 2 Caldas, Shelter 3 La Estrella, Shelters 4 and 5 Sabaneta.

The odds ratios (OR) calculated according to the statistical analysis did not show 95% confidence intervals (CI) that excluded 1, thefore we could not show any associations between infection of *E. canis* and *H. canis* with age, sex and origin of the dogs sampled within the current study.

Prevalence of E. canis infection

Eight dogs were positive for *E. canis*, representing a prevalence of 2.2% (IC 95% 0.27-1.72). Partial DNA fragments from 16S rRNA and/or *dsb genes* were amplified and the sequences obtained for both genes exhibited 100 % identity with *E. canis* as shown on the distributed phylogenetic clusters (Figures 2 and 3). The sequences in this study were submitted from the GenBank and the sequence identification numbers are MT472811 to MT472820.

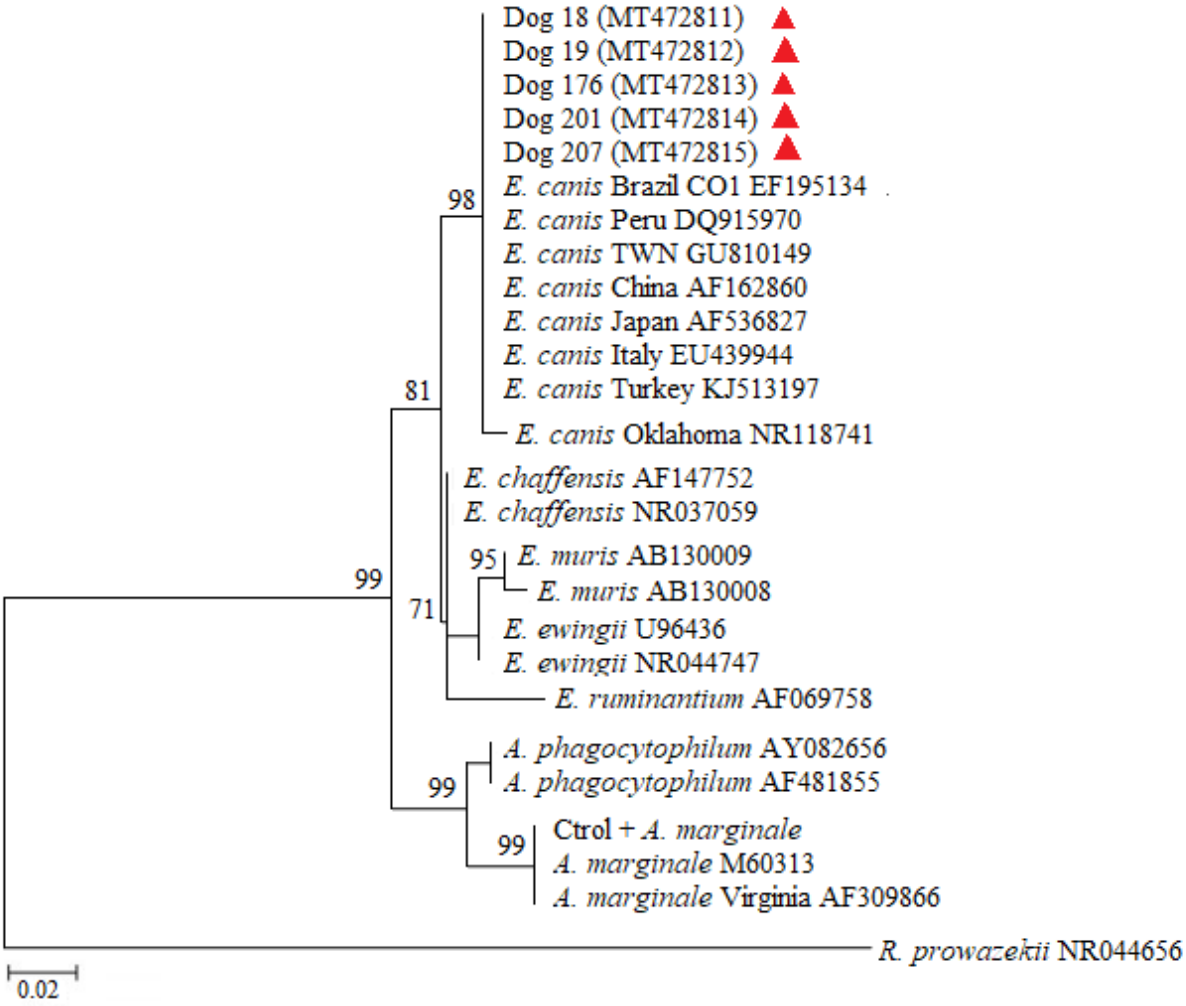

Figure 2. Tree of maximum likelihood and bootstrap method with 1000 replicates based on nucleotide sequences of DNA 16S rRNA gene. The red triangles identify the sequences obtained from the dogs that tested positive in the study. The evolutive distances were calculated using the *GTR + I + Γ* model. The size of the aligned sequences was about 460 nt, and the tree was rooted with partial sequence of strain Brein1 16S ribosomal RNA *Rickettsia prowazekii*.

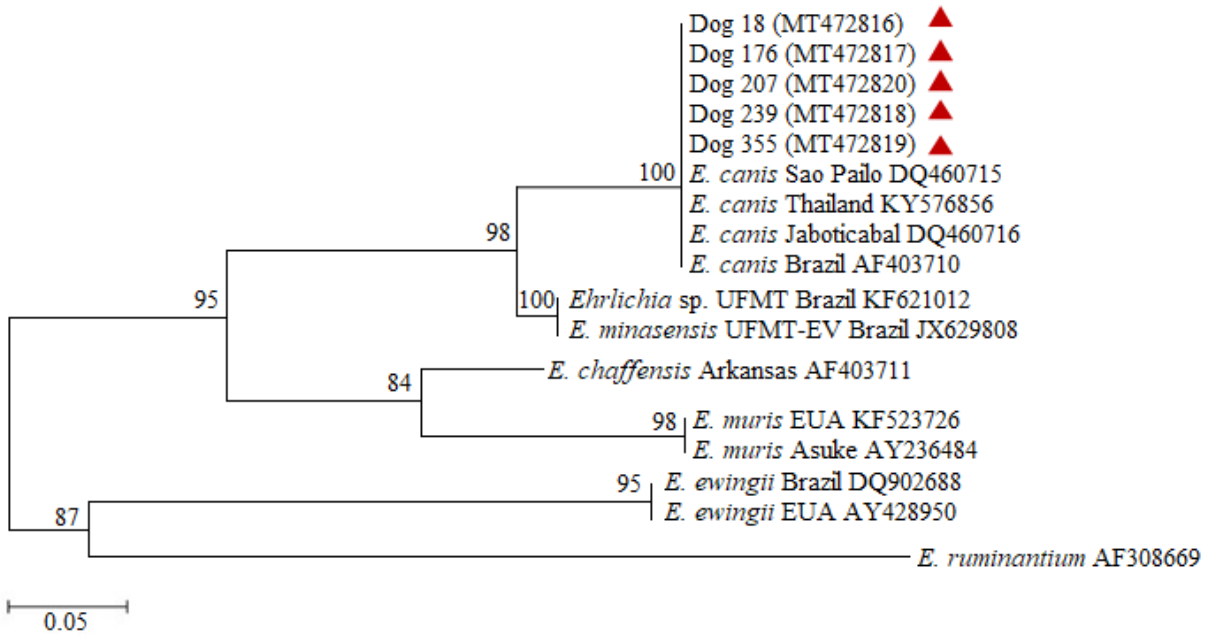

Figure 3. Tree of maximum likelihood and bootstrap method with 1000 replicates based on nucleotide sequences of DNA *dsb* gene. The evolutive distances were calculated using the *GTR + I + Γ* model. The size of the alignment was about 680 nt, and the tree was rooted with partial sequence of *Cowdria ruminantium* clone 18hw hypothetical outer membrane protein gene.

Prevalence of H. canis infection

Thirty-one of the samples were positive for *H. canis* (8.7% IC 95% 1.77-2.31), showing identity of 100% with sequences of genotypes from Japan (LC169075), Brazil (AY471615), and Spain (AY150067). However, the Dog 6 (MT678549) sequence was related with sequences of *H. canis* from Brazil (MF692036), Iran (KT736298), Italy (QU371447) and Hungary (KJ572976) (Figure 4). The sequences in this study were submitted to the GenBank and the sequence identification numbers are MT472821 to MT472834.

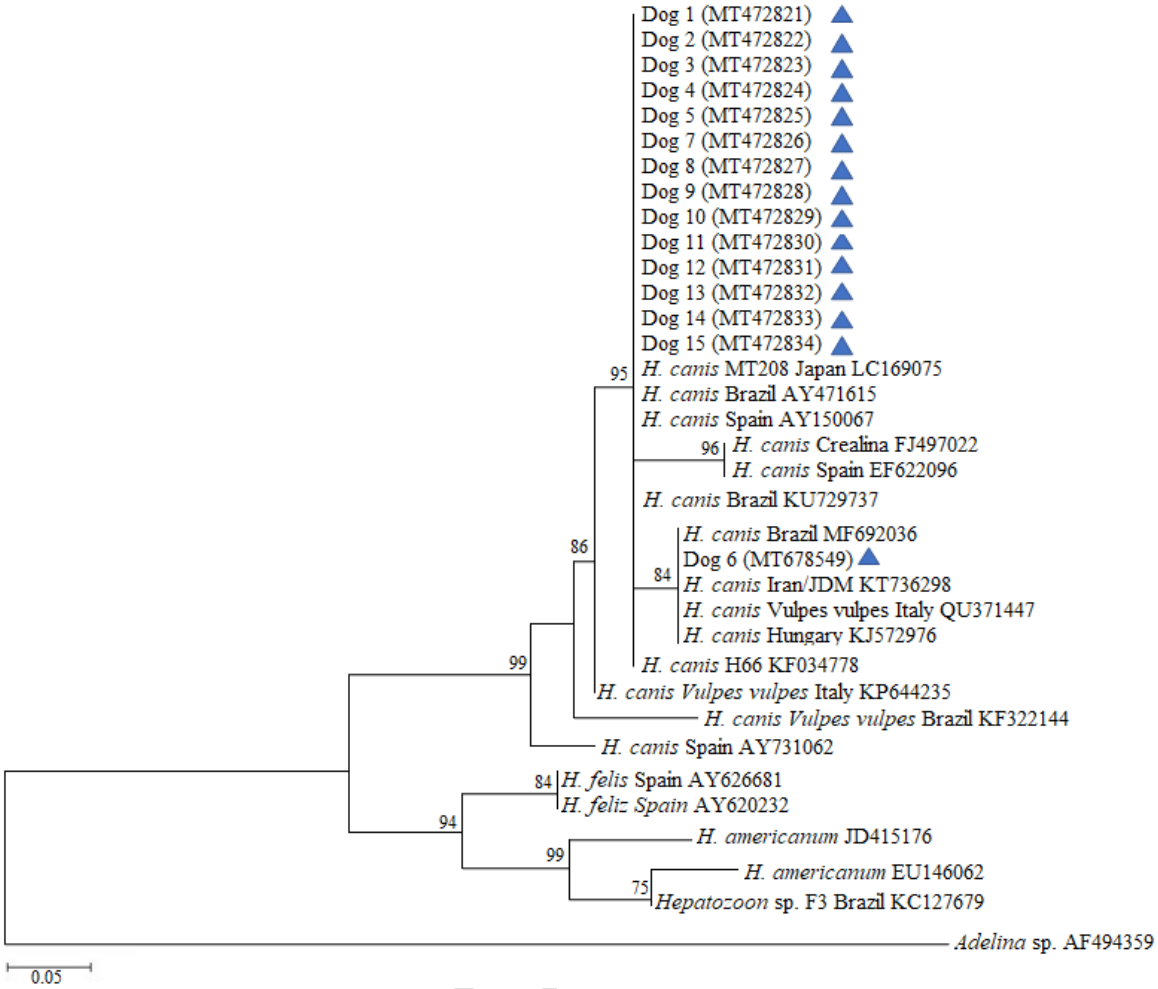

Figure 4. Tree of maximum likelihood and bootstrap method with 1000 replicates based on nucleotide sequences of DNA 18S rRNA that show the phylogenetic relationships between the apicomplexans. The blue triangles represent the sequences obtained from the positive dogs in the study. The evolutive distances were calculated using the *GTR + I + Γ* model. The size of the aligned sequences was about 450 nt, and the tree was rooted with *Adelina* sp. SH-2015 isolate HU4 18S ribosomal RNA gene.

Discussion

TBPs cause critical infections that are potentially fatal. The current study detected *E. canis* and *H. canis* DNA in asymptomatic stray dogs. This study did not show a risk association for age, sex, and origin; however, previous seroprevalence studies in countries such as Brazil and Costa Rica determined that age is a risk factor associated with *E. canis* seropositivity; stating that the older the dog, the greater the

probability of exposure to infection for TBPs (between 2-7 years (RR: 1.6, 95%CI: 1.2-2.2) and 8-15 years (RR: 1.8, 95%CI:1.2-3.0; Little, 2010; Barrantes-González, 2016).

The frequency of *E. canis* infection was 2.2% (8/357). This is lower than a previous study in Colombia (46 animals tested in 2016), which reported a prevalence of 10.9% (Posada-Zapata *et al.,* 2017). A further survey conducted in the southwest of the country found 54% (39/72) in stray domesticated canines with and without clinical signs (Rojas *et al.,* 2013). A 2012 study in the city of Medellin (22 km, about 14 miles, from the municipalities of the present study reported 33.3% of patients suspected to be infected with *E. canis* (Bonilla *et al.,* 2012) and a 2020 study of *E. canis* DNA (16S rRNA and/or *dsb*) was detected in 18% (53/300) of dogs with clinical signs of CME by PCR amplification (Arroyave *et al.,* 2020b). The partial sequences of 16S rRNA and *dsb genes* obtained in this study were consistent with previous phylogenetic analyses that show complete identity with *E. canis* (Waner *et al.,* 2014; Arroyave *et al.,* 2020b).

The present study is the first molecular detection of canine hepatozoonosis in the Department of Antioquia, and the third report from Colombia. The first molecular detection of *H. canis* reported a prevalence of 31.8% in 91 dogs sheltered of Bogotá, Bucaramanga, and Villavicencio cities (Vargas-Hernández *et al.,* 2012). After that, during the last year, we published the second molecular detection of *H. canis* in 350 samples of domestic dogs in the city of Cucuta, this study reported a frequency of infection of 8.6% (Chinchilla *et al.,* 2020). Additional reports of dogs infected with *H. canis* in Latin America, have been reported in countries such as Mexico (Jarquín-Díaz *et al.,* 2017), Brazil (Forlano *et al.,* 2007; Spolidorio *et al.,* 2009; O'Dwyer *et al.,* 2011; Vieira *et al.,* 2018), Argentina (Eiras *et al.,* 2007), and Venezuela (Criado-Fornelio *et al.,* 2007).

The primers PIRO A1 and PIRO-B have been commonly described to detect Babesia species (Jefferies *et al.,* 2003), however, the PCR assay using these primers in our study detected only *H. canis* in naturally infected dogs. Furthermore, the genetic sequence of each sample and their phylogenetic analysis confirmed this finding, suggesting that the same set of primers can be used to amplify species of the apicomplexan parasites. However, the evaluation and validity of this diagnostic test are beyond the scope of this study. Molecular studies utilizing the 18S rRNA gene have allowed the identification of the circulation of *Hepatozoon* spp. and the analysis of the phylogenetic divergence associated with its bio-geographic distribution (Vieira *et al.,* 2018).

E. canis is recognized as the most important tick-borne pathogen in Latin America (Dantas-Torres *et al*., 2012), however, other tick-borne pathogens could be in the shadow of canine ehrlichiosis. In addition, both pathogens are present in asymptomatic or severely ill dogs that show similar clinical signs such as lethargy, weight loss, cachexia, and anemia that might confuse the diagnosis (Little, 2010; Demoner *et al.*, 2013). All of the above, along with the low sensitivity of the buffy coat smear examination and the lack of a more accurate diagnostic tests, such as serological or molecular assays, may be underestimating the true importance of *H. canis.*

H. canis and *E. canis* are transmitted by the same vector, the brown dog tick, *R. sanguineus* s.l, which causes that the prevalence of *H. canis* overlaps in areas where canine ehrlichiosis is endemic (de Miranda *et al.*, 2014). Despite geographic and environmental conditions, none evidence of tick infestation was found on the dogs during the sample collection, however, a previous study reported that *R. sanguineus* s.l. was the main arthropod found in dogs with clinical signs compatible with canine ehrlichiosis from Medellín city (Arroyave *et al.*, 2020a).

In conclusion, this report confirms previous observations and epidemiological surveys from Colombia and other tick-endemic countries, which show evidence of infection with *E. canis* and *H. canis* in stray dogs (Chinchilla *et al.*, 2020). In addition, we observed that *H. canis* is the principal canine pathogen transmitted by ticks in the studied area, and its prevalence is in fact higher than expected, what suggests that *H. canis* may be commonly underreported (or underdiagnosed) in similar circumstances. Taken together, these results emphasize the need to test for multiple zoonotic TBPs in sheltered dogs and increase awareness about their likely threat to public health. Further investigation is needed to examine the genetic diversity of pathogenic microorganisms such as bacteria and eukaryotic agents in the blood of intermediate hosts, such as domesticated canines. Further research will also be required to infer the pathogenic relationships of the parasite with its natural host, and the risk of horizontal transmission where conditions for the presence of the vector are favorable. The analysis of these results is important for obtaining an epidemiological profile of the agents that silently circulate throughout the country where clinical symptoms are not obvious in dogs.

Declarations

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Conflicting interests

The authors declared no potential conflicts of interest concerning the research, authorship, and/or publication of this article.

Author contributions

Azucena Cabrera-Jaramillo: obtained samples and data collection, statistical analysis, conducted the experimental work, laboratory analysis, and writing of the manuscript. Esteban Arroyave: participated in the data collection analysis, Phylogenetic analysis and wrote, reviewed, or did a critical reading and editing of the paper. Santiago Monsalve: supervision of experimental protocol, experimental work, laboratory analysis and wrote the manuscript. Juan D. Rodas: wrote, reviewed, or did a critical reading and editing of the paper and the revised final version of the manuscript.

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