


Detection of tick-borne rickettsial pathogens in naturally infected dogs and dog-associated ticks in Medellín, Colombia

Detecção de patógenos rickettsiais transmitidos por carrapatos em cães naturalmente infectados e carrapatos associados a cães em Medellín, Colômbia

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Abstract

Tick-borne rickettsial pathogens (TBRP) are important causes of infections in both dogs and humans. Dogs play an important role as a biological host for several tick species and can serve as sentinels for rickettsial infections. Our aim was to determine the presence of TBRP in dogs and in dog-associated ticks and their potential risk to human diseases in Medellín, Colombia. DNA for *E. canis* (16S rRNA and *dsb*) and *A. platys* (*groEl*) was detected in 17.6% (53/300) and 2.6% (8/300) of dogs, respectively. Antibodies against *Ehrlichia* spp. 82 (27.3%) and *Anaplasma* spp. 8 (2.6%) were detected in dogs. Antibody reactivity against both agents were found in 16 dogs (5.3%). Eight dogs showed antibody for *Rickettsia* spp. with titers that suggest 3 of them had a probable exposure to *R. parkeri*. *Rhipicephalus sanguineus* s.l. (178/193) was the main tick in dogs, followed by *R. microplus* (15/193). The minimum infection rates (MIR) in *R. sanguineus* were 11.8% for *E. canis* and 3.4% for *A. platys*. *E. canis* and *A. platys* are the main TBRP infecting dogs and ticks and *R. sanguineus* s.l. is likely involved in the transmission of both agents. Interestingly, we found serological evidence of exposure in dogs for spotted fever group rickettsiae.

Keywords: Tick-borne diseases, Rickettsiales, *Rhipicephalus sanguineus*, dogs, Colombia.

Resumo

As riquetsias transmitidas por carrapatos (RTC) são causas importantes de infecção em cães e humanos. Os cães exercem um papel essencial como hospedeiros biológicos para diversas espécies de carrapatos, assim como podem ser úteis como sentinelas de infecções por riquetsias. O intuito deste estudo foi determinar a presença de RTC em cães, assim como em seus carrapatos, para determinar o risco potencial de doença humana em Medellín, Colômbia. DNA de *Ehrlichia canis* (16S rRNA e *dsb*) e *Anaplasma platys* (*groEl*) foi detectado em 17,6% (53/300) e 2,6% (8/300) dos cães, respectivamente. Anticorpos contra *Ehrlichia* spp. (82; 27,3%) e *Anaplasma* spp. (8; 2,6%) foram detectados nos cães. Reatividade de anticorpos contra ambos patógenos (*Ehrlichia* e *Anaplasma*) foi detectada em 16 cães (5,3%). Oito animais apresentaram anticorpos contra *Rickettsia* spp., e 3 deles sugerem uma provável exposição a *Rickettsia parkeri*. *Rhipicephalus sanguineus* s.l. (178/193) foi a principal espécie de carrapatos, seguida de *R. microplus* (15/193). A taxa de infecção mínima em *R. sanguineus* foi 11,8% para *E. canis* e 3,4% para *A. platys*. *E. canis* e *A. platys* são as principais RTC que infectam cães e *R. sanguineus* s.l. provavelmente está envolvido na transmissão de ambos os agentes. É evidente, porém, a exposição sorológica dos cães a riquetsias do grupo da febre maculosa.

Palavras-chave: Doenças transmitidas por carrapatos, Rickettsiales, *Rhipicephalus sanguineus*, cães, Colômbia.

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Introduction

Obligate intracellular bacteria of the order Rickettsiales cause several tick-borne diseases of human and veterinary medical importance. This order encompasses two families: Anaplasmataceae that includes several pathogens of humans and animals within the genera *Ehrlichia* and *Anaplasma*, which are transmitted by species of ixodid ticks to mammalian hosts (Rar & Golovljova, 2011) and Rickettsiaceae containing pathogenic *Rickettsia* species that are found throughout the world and continue to emerge and reemerge as important causes of febrile illnesses in humans and numerous domestic and wild animals (Fang et al., 2017). Dogs are considered important sentinel animals for human rickettsial infection since they may suffer a clinical illness similar to humans or may be asymptomatic and chronically infected, serving as reservoir host. Even if dogs are not the main reservoirs, or amplifying hosts for rickettsial pathogens, they may serve as definitive feeding hosts for ticks or carry ticks infected by these pathogens to human dwellings (Sabatini et al., 2010; Nieri-Bastos et al., 2013; Szabó et al., 2013).

Ehrlichia canis and *Anaplasma platys* are the etiological agents of Canine Monocytic Ehrlichiosis (CME) and Canine Infectious Cyclic Thrombocytopenia (CICT), respectively. These agents are the most common tick-borne pathogens detected in dogs in places around the world where *Rhipicephalus sanguineus* sensu lato (s.l.) is present (Sainz et al., 2015; Cárdenas et al., 2007). *Ehrlichia canis* is also considered the agent of an emerging zoonosis in Latin America (Carvalho et al., 2017; Bouza-Mora et al., 2017). *Rickettsia rickettsii* is the etiologic agent of Rocky Mountain spotted fever (RMSF), the severest tick-borne human disease in the Americas. Dogs can serve as sentinels in endemic regions for RMSF because, like humans, they are susceptible to infection with *R. rickettsii* with potentially fatal outcomes, and they have relatively high rates of exposure to infected ticks (Breitschwerdt et al., 1985; Demma et al., 2005; Piranda et al., 2008; Labruna et al., 2009; Levin et al., 2014). Recently, other species of the spotted fever group rickettsiae (SFG) such as *R. parkeri* and *R. massiliae* have been associated with infection in humans and dogs from South America (Spolidorio et al., 2010; Cicuttin et al., 2004; Londoño et al., 2014).

Cases of canine infectious cyclic thrombocytopenia by *A. platys* and RMSF by *R. rickettsii* in dogs could easily be misdiagnosed and confused with CME by *E. canis* since these three pathogens share clinical signs and also could be transmitted by the same vector, the brown dog tick, *Rhipicephalus sanguineus* (s.l.) (Grindem et al., 1999; Piranda et al., 2008; Labruna et al., 2009). Clinical disease in dogs experimentally and naturally infected with *E. canis*, *A. platys* and *R. rickettsii* results in variable and nonspecific clinical signs, such as fever, lethargy, anorexia, weight loss, pale mucous membranes, petechiae, nasal discharge, and lymphadenopathy (Harvey, 2006). Although CICT tends to be less severe than CME or RMSF, co-infection by *A. platys* with other tick-borne pathogens could exacerbate the clinical manifestations (Sainz et al., 2015; Gaunt et al., 2010).

R. sanguineus s.l. is considered the most widespread ectoparasite in dogs in the world and also a well-recognized vector of numerous pathogens for dogs and humans (Dantas-Torres, 2008). This tick is the main vector of *E. canis* and *A. platys*, although its vector competence for *A. platys* has not been firmly established (Simpson et al., 1991; Aktas & Özübek, 2017; Ipek et al., 2018). Even though *R. sanguineus* s.l. rarely feeds on humans, it has been involved in the transmission of *R. rickettsii* in two recent RMSF outbreaks in the United States and Mexico, where stray and free-roaming dogs appeared to play an important role in the propagation and the dispersal of infected ticks (Demma et al., 2006; Álvarez-Hernández et al., 2017).

The first study about tick-borne rickettsial diseases on humans in Colombia occurred during an outbreak between 1934 and 1936 in Tobia, Department of Cundinamarca, and was named "Tobia spotted fever", which was caused by *R. rickettsii* (Patiño et al., 1937; Patiño, 1941). Seventy years later, two new fatal cases of RMSF were confirmed in the same region (Hidalgo et al., 2007). A year later, a serological survey was conducted in domestic animals, finding that 18% and 31.8% of dogs had antibodies to spotted fever group (SFG) rickettsiae and *Ehrlichia* spp., respectively (Hidalgo et al., 2009). In northwest Colombia, the second known endemic area of RMSF, the *R. parkeri* strain Atlantic rainforest was isolated from *Amblyomma ovale* ticks collected from a dog, and a human case of mild rickettsiosis was reported (Londoño et al., 2014; Acevedo-Gutiérrez et al., 2019). Nevertheless, no clinical cases due to any SFG rickettsiae have been documented in dogs in Colombia. Conversely, seroepidemiological data suggest that the frequency of antibodies to *Ehrlichia* spp. and *Anaplasma* spp. in dogs vary between 23 and 80%, and 11 and 53%, respectively (Hidalgo et al., 2009; McCown et al., 2014a,b). The aim of the present work was to investigate serological and molecular evidence of tick-borne rickettsial pathogens (TBRP) in dogs and in dog-associated ticks and their potential risk to human diseases in Medellín city, Colombia.

Materials and Methods

Sample collection

The study was performed between July 2013 and January 2015. Blood samples were collected from 300 dogs that were referred to two veterinary teaching hospitals in the metropolitan area of Medellín city, Colombia. We considered as inclusion criteria, dogs that presented with non-specific clinical signs associated with CME such as fever, lethargy, anorexia, and weight loss. In addition, we included apparently healthy dogs in which the owner reported tick infestation one year before the consultation.

Blood was obtained from the cephalic vein, collected into sterile tubes with anticoagulant (EDTA), and kept at 4 °C until arrival to the laboratory. Subsequently, whole blood (1 mL) was aliquoted for DNA extraction, and the remaining sample was centrifuged at 700 x g for 10 min to obtain plasma. All dogs included underwent a hematological test through Abacus Junior Vet®, Diatron normalized hematologic analysis device, the hematology panel included RBCs (no./ μ L), mean (red) cell volume (MCV, fL), Hb (g/dL), red cell distribution width (RDW, %), platelets (no./ μ L), WBCs (no./ μ L), neutrophils (NE, no./ μ L), lymphocytes (no./ μ L), monocytes (no./ μ L), basophils (no./ μ L), and eosinophils (no./ μ L). Hematological values of dogs with detected rickettsial pathogens were compared with defined reference values of healthy dogs (Bossa-Miranda et al., 2012).

The whole blood and plasma were stored at -80 °C. Ticks retrieved from the sampled dogs were immediately transported to the laboratory and identified using the taxonomic key of Onofrio et al. (2006). The ticks were separated into pools of no more than 4 specimens each, by species, developmental stage and sex, and stored at -80°C until DNA extraction.

Serologic testing

Canine plasma was tested by immunofluorescence assay (IFA) using DH82 cells infected with *E. canis* strain Jake (USA) and Vero cells infected with *R. rickettsii* strain Sheila Smith as previously described (McBride et al., 2001; Horta et al., 2004). Samples showing reactivity at a dilution of 1:100 for *E. canis* and 1:64 for *R. rickettsii* were further titrated using serial 2-fold dilutions to determine the endpoint IgG titer. Additionally, samples positive at a titer \geq 1:64 in the screening test with *R. rickettsii* antigens were also tested with four antigens derived of rickettsial isolates from Brazil including *R. bellii* strain Mogi, *R. amblyommatidis* strain Ac37, *R. felis* strain Pedreira and *R. parkeri* strain At24. Plasma showing a titer at least fourfold higher than that observed for any other *Rickettsia* species was considered homologous to the highest titer *Rickettsia* species or to a very closely related strain (Saito et al., 2008). Sera from dogs experimentally infected with *E. canis* and *R. rickettsii* and negative dog plasma were used as positive and negative controls, respectively. The slides were incubated with fluorescein isothiocyanate-labelled rabbit anti-dog IgG (Sigma, St Louis, MO, USA) as the secondary antibody reagent. In addition, the commercial enzyme-linked immune-sorbent assay (ELISA) SNAP 4Dx® Plus (IDEXX Laboratories, Inc., Westbrook, Maine) was used for the detection of antibodies against *Anaplasma* spp., (Stillman et al., 2014) according to manufacturer's recommendations.

PCR amplification and sequencing

Genomic DNA was extracted from 200 μ L of whole blood samples using a DNeasy® Blood & Tissue kit according to the manufacturer's protocol (Qiagen, Chatsworth, CA, USA). Nucleic acid was eluted into 100 μ L of elution buffer and stored at -80 °C for molecular detection. PCR amplification was performed by targeting the following genes (Table 1).

Each reaction was amplified using 1U of platinum Taq DNA Polymerase (Invitrogen, Brazil), PCR buffer (20 mM Tris-HCl), 1.5 mM MgCl₂, dNTP mixture (0.2 mM each) (Invitrogen, USA), 100-200 ng of DNA template, and ultrapure water from Milli-Q with DEPC (AMRESCO) in a final volume of 25 μ L. DNA from *E. canis*, *A. platys* and *R. rickettsii* were used as positive controls, and DNA from an uninfected dog blood was used as a negative control. The PCR was performed in a thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA), using a cycling protocol of 94°C for 5 min and 35 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 1 min, for 16S rRNA and *groEl* genes, and the annealing temperature for *gltA* and *dsb* were 54 °C and 48 °C for 30 s, respectively. The amplified products were separated on a 2% agarose gel Tris Acetate-EDTA electrophoresis, and visualized by staining with DNA GelRed (Biotium®, Fremont, CA, USA). To confirm the absence of PCR inhibitors in DNA extractions, a fragment of β -actin (259bp) or 12S rDNA (400 bp) gene in canine or tick DNA templates, respectively, was amplified as previously described (Agudelo-Ruiz et al.,

Table 1. Primers used for the amplification of some tick-borne pathogens.

Pathogens	Gen (bp)	Primer	Sequences	Reference
Anaplasmataceae	16S rRNA (345)	EHR16SD	5'-GGTACCYACAGAAGAAGTCC-3'	Inokuma et al. (2000)
		EHR16SR	5'-TAGCACTCATCGTTTACAGC-3'	
<i>Ehrlichia</i> spp.	dsb (409)	DSB330	5'-GATGATGTCTGAAGATATGAAACAAAT-3'	Doyle et al. (2005)
		DSB728	5'-CTGCTCGTCTATTTTACTTCTTAAAGT-3'	
<i>Anaplasma platys</i>	groEl (750)	HS475F	5'-AAGGCGAAAGAAGCAGTCTTA-3'	Inokuma et al. (2002)
		HS1198R	5'-CATAGTCTGAAGTGGAGGAC-3'	
<i>Rickettsia</i> spp.	gltA (401)	Cs-78	5'-GCAAGTATCGGTGAGGATGTAAT-3'	Oteo et al. (2014)
		Cs-323	5'-GCTTCCTAAAATTCAATAAATCAGGAT-3'	

2017; Murrell et al., 1999). The PCR on the ticks collected was performed in pools and the result was expressed as the minimum infection rate (MIR) representing the minimum number of ticks with detectable rickettsial pathogen, expressed as: $MIR = (\text{number of infected pools} / \text{total number of tested ticks}) \times 100$ (Anderson et al., 1993).

DNA sequencing and phylogenetic analysis

The PCR products were sequenced by Macrogen (Seoul, Korea), and the sequences (forward and reverse) were aligned using GeneStudio (2020) to obtain the consensus sequences. The alignments were made with MUSCLE (Multiple Sequence Comparison by Log-Expectation) and the phylogenetic analyses were performed with the MEGA 7.0.26 program. Best-fitting substitution models were determined through the ML model test implemented in MEGA 7.0.26. The phylogenetic trees were constructed based in Maximum-Likelihood (ML) algorithms using Tamura 3-parameter model (T92 +G+I). Support for the topologies was tested by bootstrapping over 1000 replications. The sequences of *A. platys* obtained in this study (GenBank accession numbers MT135102 to MT135108) were aligned with 18 sequences from the *groEl* gene retrieved from GenBank including sequences of *A. platys* from South America (Venezuela: AF399916, Argentina: KR826285, KR929453 and Uruguay: KX792012), Africa (Republic of Congo: AF478129 and Zambia: LC373039) and Asia (Japan: AY077621, Thailand: KU765205 and Philippines: JN121382); to *A. ovis* (AF441131), *A. marginale* (AF414864), *A. centrale* (AF414866), *A. phagocytophilum* (EU552920) and *A. bovis* (JX092093); for *Ehrlichia* spp. we used the sequences of *E. ewingii* (AF195273), *E. muris* (KF312362) and *E. canis* (U96731), and as a root we used *Neorickettsia risticii* (U96732). All positions with less than 90% site coverage were eliminated.

Statistical analysis

The hematological data were first tested to determine the normality of the distribution. The hematological mean values were compared among the positive dogs by PCR to *Anaplasma* spp. and *Ehrlichia* spp. Student's t-test and Mann-Whitney test were performed for the parametric and nonparametric values, respectively. The tests were implemented with SPSS Statistics for Windows Version 22.0 (SPSS, Inc., Chicago, IL, U.S.A.), and a p-value of <0.05 was considered to indicate statistical difference.

Results

Of the 300 canine plasma samples tested, 82 (27.3%) contained antibodies reactive to *E. canis* with endpoint titers varying from 100 to 12,800, whereas 24 (8%) plasma samples were seroreactive to *Anaplasma* spp. through the ELISA (SNAP 4DX plus) and 16 of these samples reacted to both genera (*Ehrlichia* and *Anaplasma*). Overall, 8 (2.6%) dogs were seroreactive to the screening dilution 1:64 to *R. rickettsii* antigen by IFA. These eight canine samples were also reactive at the 1:64 dilution to some of the other *Rickettsia* species (*R. bellii*, *R. felis*, *R. amblyommatis*, or *R. parkeri*). Among these, three canine plasma showed endpoint titers to *R. parkeri* at least 4-fold higher than those to any of the other five antigens, suggesting that *R. parkeri* or a very closely related species stimulated the antibody response in these three dogs (Table 2).

Table 2. Indirect immunofluorescence assay (IFA) antibody titers for five *Rickettsia* species in canine plasma.

Dog plasma	IFA serological endpoint titers according to <i>Rickettsia</i> species					Probable antigen-stimulating antibody response
	<i>R. parkeri</i>	<i>R. rickettsii</i>	<i>R. bellii</i>	<i>R. felis</i>	<i>R. amblyommatis</i>	
32	1024	256	-	-	-	<i>R. parkeri</i>
39	1024	256	-	64	-	<i>R. parkeri</i>
81	256	128	-	-	-	undetermined
89	-	128	-	-	-	undetermined
114	-	64	64	64	-	undetermined
132	1024	512	-	64	64	undetermined
182	64	64	-	-	-	undetermined
357	1024	64	-	-	-	<i>R. parkeri</i>

-, <1:64.

Ehrlichial DNA was detected in 17.6% (53/300) of canine blood samples through the amplification of the partial fragment of the genes *16S rRNA* and *dsb*, showing 100% of identity with *E. canis* by phylogenetic analysis in both genes (data not shown). On the other hand, we partially sequenced the *groEL* gene of *Anaplasma* spp. in 2.6% (8/300) canine samples. The sequences obtained by *groEL* exhibited 100% identity with *A. platys*. None of the canine or tick samples revealed *Rickettsia* DNA.

Only two tick species were collected from the dogs: 178 *R. sanguineus* s.l. collected from 298 dogs from urban and peri-urban, and 15 *Rhipicephalus microplus* ticks collected from two dogs that lived in a peri-urban area. Ticks were separated into pools of 1 to 5 individuals resulting in 69 pools of *R. sanguineus* s.l. distributed in 2 pools of larvae, 17 pools of nymphs, 20 pools of male adults and 30 pools of female adults. For *R. microplus*, we made 6 pools including one of nymphs, one of adult males and four of adult females. We detected ehrlichial DNA (*dsb*) in 21/69 (30.4%) pools of *R. sanguineus* s.l. (7 nymph pools, 6 male pools and 8 female adult tick pools), and *A. platys* DNA (*groEL*) in 6/69 (8%) pools (3 males and 3 females adult tick pools), two of which also contained ehrlichial DNA. Interestingly, these 6 pools of ticks were retrieved from 4 dogs that were negative by PCR to *A. platys*, and only one of them had detectable antibodies to *Anaplasma* spp. The minimum infection rate (MIR) for *Ehrlichia* spp. and *A. platys* in *R. sanguineus* s.l. was 11.8% and 3.4%, respectively (Table 3). All the DNA pooled samples of *R. microplus* were negative by PCR for *Ehrlichia* and *Anaplasma* spp. and no *Rickettsia* spp. DNA was detected in ticks.

Table 3. Number of *Rhipicephalus sanguineus* s.l. ticks classified by developmental stage, and MIR (Minimum Infection Rate) of *Ehrlichia canis* and *Anaplasma platys*.

Developmental stages	Number of ticks	Number of pools	<i>E. canis</i>		<i>A. platys</i>	
			Positive pools	% MIR	Positive pools	% MIR
Larva	4	2	0	0 (0/4)	0	0 (0/4)
Nymph	74	17	7	9.4 (7/74)	0	0 (0/17)
Adult (male)	51	20	6	11.8 (6/51)	3	5.9 (3/51)
Adult (female)	49	30	8	16.3 (8/49)	3	6.1 (3/49)
Total	178	69	21	11.8 (21/178)	6	3.4 (6/178)

All the *dsb* sequences obtained from either dogs or ticks, shared an identity of 100% with *E. canis*. Specific PCR assays for *A. platys* revealed that 14 samples contained DNA of the *groEL* gene (8 dog samples and 6 tick pools). However, only 5 dog samples and 2 pools of ticks were sequenced; the remaining samples showed a weak band in the gel and poor quality sequences. Phylogenetic analysis of partial sequences from the *groEL* operon (655 bp) showed that the sequences from dogs and ticks shared an identity of 100% with sequences of *A. platys* from different regions, including South America (Venezuela, Argentina and Uruguay), Africa (Republic of Congo and Zambia) and Asia (Japan, Thailand and Philippines) (Figure 1).

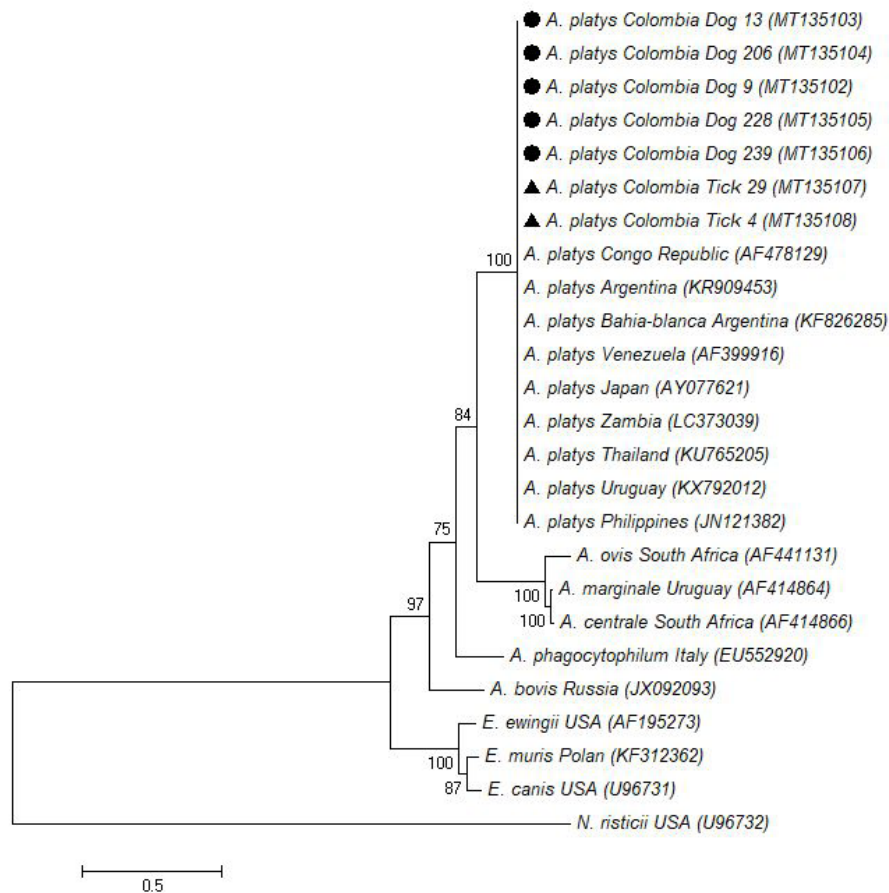


Figure 1. Maximum likelihood phylogenetic tree of *groEL* operon of *Anaplasma platys* (partial sequences, 655 pb) identified in this study and other strains of the Anaplasmataceae family. The numbers at the nodes represent the percentage of 1000 bootstrap resampling. ● dog samples, ▲ tick samples. We used the orthologous sequence *groEL* of *Neorickettsia risticii* as an outgroup.

The most common abnormal hematological findings in dogs with positive PCR for *A. platys* or *E. canis* were thrombocytopenia (*A. platys* median: 86×10^3 platelets count, *E. canis* median: 98×10^3 platelets count, reference value 290.8 ± 100), and low hematocrit (*A. platys* mean: $38.89\% \pm 9.46$; *E. canis* mean: $36.55\% \pm 10.72$; reference value 52.8 ± 6.5), however, there were no statistically significant differences between the hematologic parameters of canines with *A. platys* and those infected with *E. canis* (t-test or nonparametric Mann-Whitney test, $p > 0.05$).

Discussion

Overall, 92.2% (178/193) of the ticks collected from the dogs were *R. sanguineus* s.l. This result is consistent with other authors that described that this tick inhabits both rural and urban environments but is particularly abundant in dogs from urban and peri-urban areas (Jones et al., 2017; Cicuttin et al., 2015). The *R. sanguineus* complex is, probably, the most widely distributed group of ticks in the world and should be considered as one of the most important ectoparasites of dogs throughout Latin America (Dantas-Torres, 2008; Venzal et al., 2007). The role of *R. sanguineus* in the transmission of tick-borne pathogens in humans is often not considered important due to the low affinity of *R. sanguineus* to feed on this host; however, there are some reports confirming frequent human exposure to this ticks in different countries (Harrison et al., 1997; Estrada-Peña & Jongejan, 1999; Dantas-Torres et al., 2006; Guglielmone et al., 2006; Dantas-Torres, 2008). In addition, there is evidence that during periods of warmer temperatures *R. sanguineus* increases its aggressiveness and propensity to bite hosts other than dogs, including humans (Parola et al., 2008).

Different reports have shown that in the New World, the taxon *R. sanguineus* encompasses at least two different species, the formerly temperate lineage or *R. sanguineus sensu stricto* (s.s.), and a yet to be defined species, the so-called tropical lineage of *R. sanguineus* s.l. (Moraes-Filho et al., 2011,2015; Nava et al., 2018). Among these two

species, only *R. sanguineus* s.l. tropical lineage has been reported in Colombia and other tropical regions of South America (Moraes-Filho et al., 2011; Rivera-Páez et al., 2018).

A previous study has experimentally demonstrated that *E. canis* was successfully transmitted by the *R. sanguineus* s.l. tropical lineage, but not by *R. sanguineus* s.s. (temperate lineage) (Moraes-Filho et al., 2015). Recent reports of cases of CME in dogs from *R. sanguineus* s.s.-prevailing areas of Argentina suggest that this temperate lineage may have some degree of vector capacity for *E. canis* or that *R. sanguineus* s.l. tropical lineage infected with *E. canis* is migrating to these areas, probably when climatic conditions are favorable to the temporary establishment and infection of susceptible hosts (Cicuttin et al., 2017; Tarragona et al., 2019).

R. sanguineus s.l. tropical lineage is distributed from southern Brazil to northern Mexico and the United States and is the only lineage that has been reported in Colombia (Moraes-Filho et al., 2011; Dantas-Torres et al., 2013). Although in our study, the taxon of *R. sanguineus* was not identified, we expect that it belongs to the tropical lineage considering the geographic location and that *E. canis* was the most prevalent tick-borne pathogen in both dogs (IFA: 82% PCR: 18%) and ticks (PCR: 30.4%). In addition, our previous results showed the presence of three genotypes of *E. canis* (strain United States, Brazil, and Costa Rica) infecting dogs in Colombia, in which one of these strains has been associated with human infection in Costa Rica (Arroyave et al., 2020; Bouza-Mora et al., 2017).

Despite the small number of studies regarding *A. platys* published in Colombia, all of them are consistent in showing positive seroprevalence in canines, even with frequencies above 40% (McCown et al., 2014a,b). We found anti-*Anaplasma* spp. antibodies in 8% (24/300) of plasma samples by ELISA; this frequency is comparable to a previous results in the same area that reported 11% of dogs having antibodies against *A. platys* (McCown et al., 2014a). Notably, 16 of 24 samples were also reactive to *Ehrlichia* spp., demonstrating the frequency of co-infection.

In the present work, the distribution of *E. canis* and *A. platys* overlapped where *R. sanguineus* s.l. was the only tick found on the dogs that came from the urban area, suggesting that both agents may share the same vector. The high percentage of nymphs and adults of this tick infected with *A. platys* reinforces the hypothesis that *R. sanguineus* s.l. ticks have vector competence to transmit *A. platys* (Ramos et al., 2014; Carvalho et al., 2017; Cicuttin et al., 2015; De Almeida et al., 2012; da Silva et al., 2016). However, the first experimental study attempting to confirm *R. sanguineus* s.l. as a vector of *A. platys* was unsuccessful (Simpson et al., 1991). The attempt to prove the vectorial competence of this tick species likely failed due to the low sensitivity of the diagnostic method or the tick lineage of *R. sanguineus* (Dantas-Torres et al., 2013; Ramos et al., 2014; Moraes-Filho et al., 2015). A more recent study reveals the trans-stadial transmission of *A. platys* in *R. sanguineus* s.l. from larvae to nymph and nymph to adults, although the lineage involved as a vector was not confirmed (Aktas & Özübek, 2017). Our results reinforce the hypothesis that *R. sanguineus* s.l. tropical lineage is involved in the transmission of *A. platys*, since *A. platys* DNA was found in 6 of 69 pools (MIR: 3.4%) of *R. sanguineus* s.l. removed from dogs that did not test positive for *Anaplasma* spp. by serologic or molecular means, indicating that these ticks likely acquired this agent in the previous life stage.

Natural and experimental evidence of co-infection of dogs with *E. canis* and *A. platys* has been reported (Sainz et al., 2015; Santamaria et al., 2014; Gaunt et al., 2010). In the current study, we show evidence of co-infection in 16 dogs with serological response against both agents, although, co-infection through detection of DNA of *E. canis* and *A. platys* was only found in two adult tick pools and in one dog. Co-infection with *E. canis* and *A. platys* contributes to atypical manifestations of disease including increased severity of clinical signs and exaggerated hematological abnormalities (Kordick et al., 1999). However, we did not find hematological differences among the dogs infected with *E. canis* or *A. platys*, and the dog infected with both agents.

Our results showed serological evidence of exposure to SFG rickettsiae in eight dogs. Of these, three showed endpoint titers to *R. parkeri* with at least 4-fold higher than those to any of the other four antigens suggesting that this rickettsia was involved in the infection. The remaining five samples with low titers to SFG rickettsiae were considered undetermined, suggesting that other *Rickettsia* species not present in our antigens stimulated the antibody response. Previous studies in dogs experimentally infected with *R. rickettsii* (pathogenic) and *R. montanensis* (non-pathogenic) have shown that the non-pathogenic species stimulate low antibody responses (~1:64) compared with the pathogenic species (Breitschwerdt et al., 1988). In the present study, some of the indeterminate samples showed low titers (1:64 to 1:128) suggesting that dogs may have been infected with a non-pathogenic rickettsia.

R. parkeri strain Atlantic rainforest emerged in 2010 in Brazil causing febrile illnesses in humans (Spolidorio et al., 2010; Silva et al., 2011). One year later *R. parkeri* was isolated from *Amblyomma ovale* ticks collected on a free-roaming domestic dogs in a rural area from Colombia (Londoño et al., 2014). Experimental studies have shown that *A. ovale* is a reservoir and competent vector of *R. parkeri* strain Atlantic rainforest (Krawczak et al., 2016; Brustolin et al., 2018). Endemic regions of *R. parkeri* strain Atlantic rainforest are associated with rural areas, especially areas with

vegetation (forest, pastures) where the adult stage of *A. ovale* attaches to and feeds on dogs, which typically become infested in the forest (Szabó et al., 2013). Medellín is primarily an urban area and the second most important city in Colombia, but border areas are essentially composed of forest where wildlife abounds, which could provide optimal conditions for the establishment of *A. ovale*. Common activities in these areas can make dogs have access to forest fragments and become infested and carry infected ticks to homes, becoming a risk to humans.

R. rickettsii, the etiologic agent of RMSF, is the most severe tick-borne disease in the New World. Dogs and both *R. sanguineus* s.s and the tropical lineage, have been involved in two recent outbreaks of RMSF in Arizona (USA) and Sonora and Baja California (Mexico), respectively (Demma et al., 2006; Álvarez-Hernández et al., 2017). However, in South America, the role of *R. sanguineus* in the transmission of RMSF remains a source of speculation (Labruna et al., 2008; Piranda et al., 2011). Our current results detected no evidence of rickettsial DNA in *R. sanguineus* s.l. ticks, and serologically we have not demonstrated *R. rickettsii* infection in our tested dogs. In contrast; in a previous study, we showed 4-fold higher antibody titers (by IFA) against *R. rickettsii* compared to other Rickettsia species on two dogs from a rural area close to Medellín, and related with a cluster of lethal SFG human cases (Londoño et al. 2019).

Conclusions

Ours serologic and molecular findings confirm to *E. canis* and *A. platys* as a main TBRP infecting dogs in this study and *R. sanguineus* s.l. is likely involved in the transmission of both agents. More studies are needed to elucidate the role of *R. sanguineus* as a vector of *A. platys* in America. We also show serological evidence of exposure of dogs to SFG rickettsiae, which supports the likely role of dogs as sentinels for human infection by TBRP.

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