Tick-borne rickettsial pathogens in naturally infected dogs and dog-associated ticks and their role as sentinels of zoonotic rickettsial diseases in Medellin, Colombia

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CONTENTS

SUMMARY

Introduction

Tick-borne rickettsial agents are obligately intracellular bacteria in the order *Rickettsiales* that cause several diseases of human and veterinary medical importance (Parola et al., 2005). Microorganisms within the genera *Rickettsia*, *Ehrlichia* and *Anaplasma* are found throughout the world and continue to emerge and reemerge as important causes of febrile illness in humans (Walker et al., 2008). Dogs are considered important sentinel animals for tick-borne pathogens, since humans and canines are particularly intertwined in their respective roles in the life cycle of the rickettsial pathogens, where humans are susceptible hosts and dogs can serve as reservoirs for these pathogens, as definitive feeding hosts for vector ticks and as mechanical tick-transporters into human habitats (Eremeeva and Dasch, 2015a).

At least five well-established human rickettsial pathogens circulate in dogs and ticks in different and often overlapping parts in the Americas, *Rickettsia rickettsii, R. parkeri, Ehrlichia chaffeensis, E ewingii,* and *Anaplasma phagocytophilum,* There are other rickettsial species in which their pathogenicity in humans has not yet been clearly demonstrated, such as *R. amblyommatis, R. montanensis, R. rhipicephali, R. bellii, E. canis and A. platys*.

The first rickettsial human diseases investigations in Colombia were recorded during an outbreak that occurred between 1934 and 1936 in Tobia, Department of Cundinamarca, for which the diseases was named “Tobia spotted fever”, corresponding to Rocky Mountain spotted fever (RMSF) caused by *R. rickettsii* (Patiño et al., 1937; Patiño, 1941). This disease affected 20% of the population and had a 95% lethality (62

out of 65 patients died in this outbreak). Seventy years later, two new fatal cases of RMSF were confirmed in Villeta, a town close to the first outbreak, involving *R. rickettsii* (Hidalgo et al., 2007). The second endemic area of RMSF was recognized after two important outbreaks in 2006 (Acosta et al., 2006), and 2008 (Pacheco et al., 2008) occurred in the Uraba region in northwestern Colombia, an area near Medellin where the current study was conducted. Recently, a serological survey showed seroprevalences of 24% to 41% in humans and 42% to 58% for domestic animals such as canines and horses, respectively (Quintero et al., 2017). Evidence of a mild rickettsiosis related to the *R. parkeri* strain Atlantic rainforest was recently described in Colombia and this strain was isolated from an *A. ovale* tick retrieved from a dog in the area of the cases (Londoño et al., 2014; Acevedo-Gutiérrez et al., 2019). However, despite the continuous findings of human cases, RMSF rickettsiosis in Colombia, is not a reportable disease.

Ehrlichia canis and *A. platys* are the most prevalent canine pathogens of *Anaplasmataceae* family. These agents are frequently detected in domestic dogs of Latin America, but little evidence has been published with regards to human infections. On the other hand, *E. chaffeensis* is the major etiologic agent of human monocytotropic ehrlichiosis (HME), whereas *A. phagocytophilum* is the major cause of human granulocytic anaplasmosis (HGA). These zoonotic diseases occur predominantly in the United States, but they are not exclusive from North America becuase DNA of both agents has also been detected in South America (Sacchi et al., 2012; Guillemi et al., 2019). Although cases of HME and HGA have been serologically diagnosed in Colombia and Brazil, the epidemiology of these diseases is not well understood (Hidrón Botero et al., 2014; da Costa et al., 2006; Calic et al., 2004).

Tick-borne pathogens have complex cycles that involve microorganisms, vectors, humans and animal hosts. Thus, is important to elucidate the relationships among them, to understand the potential impact of these pathogens on public health. In countries such as Colombia, where there is a lack of rigorous epidemiological surveillance programs on human tick-borne agents (TBA), follow up of infected vectors and sentinel animals could shed some light on human and animal risks due to this disease exposure.

Based in the above information, our main hypothesis as that domestic canines serve as sentinels of tick-borne pathogens and create a potential zoonotic risk for disease transmission to humans in an urban area of Colombia. To test this hypothesis, this investigation is focused on develop serological and molecular evidence of tick-borne rickettsial pathogens (TBRP) in dogs with clinical signs and their ticks and determine the potential risk for transmission to humans in Medellin, Colombia".

To achieve the previous goal, our specific aims were:

1. Detect IgG antibodies against TBRP in canine samples.
2. Detect DNA from *Ehrlichia*, *Anaplasma* and *Rickettsia* spp. in canine using molecular based testing.
3. To determine the proportion of infection of TBRP in ticks collected from the same canines

This thesis includes the following four chapters:

Chapter I: General introduction. This chapter is a review that focuses on the TBRP most commonly recognized in humans and dogs in the New World including spotted fever group (SFG) *Rickettsia*, *Anaplasma* and *Ehrlichia* spp.

Chapter II: Detection of TBRP in naturally infected dogs and dog-associated ticks from Colombia. This chapter is an original article that describes serological and molecular approaches to determine the exposure and prevalence of rickettsial agents in dog and their tick infections.

Chapter III: *E. canis* TRP36 diversity in naturally infected-dogs from an urban area of Colombia. This chapter is an original article, which was accepted for publication “Ticks and Tick-borne Diseases". This paper describes the phylogenetic and serological diversity of *E. canis* in dogs demonstrating the presence of 3 genotypes in naturally infected dogs, including a genotype (Costa Rica) associated with human infections.

Chapter IV: This chapter provides a general discussion, conclusions and perspectives based on the results of the investigation.

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Chapter I review: Tick-borne rickettsial pathogens in dogs and potential zoonotic risk in the New World.

Overview

Obligately intracellular bacteria in the order *Rickettsiales* cause several tick-borne diseases of human and veterinary medical importance. This order encompasses two families, the family *Anaplasmataceae* that includes several pathogens of humans and animals within the genera *Ehrlichia* and *Anaplasma*, where all bacteria of this family are transmitted by ixodid ticks to mammalian hosts, and the family *Rickettsiaceae,* that contains pathogenic *Rickettsia* species that are found throughout the world and continue to emerge and reemerge as important causes of febrile illness in humans but also cause infections in numerous domestic and wild animal species. Pathogenic *Rickettsia* species are transmitted among a variety of hematophagous arthropod vectors, which includes ticks, lice, mites, and fleas, however, the tick-borne rickettsiae belonging to the spotted fever group are naturally transmitted by ixodes ticks. The tick-borne organisms are maintained in complex cycles in nature involving different domestic or wild animal hosts and arthropod vectors. Dogs and humans share the susceptibility to several diseases transmitted by ticks, the risk of exposure to any of these pathogens depends mainly on their geographical location, environment, and outdoor activities. Both are particularly intertwined in their respective roles and risks for diseases transmitted by ticks, where dogs can serve as reservoirs for these pathogens, as definitive feeding hosts for vector ticks and as mechanical tick transporters to human habitats. They, indeed, serve as sentinel indicators of risk for human rickettsial diseases.

1.1 General ecology of tick-borne pathogens

Tick-borne rickettsial pathogens (TBRP) have evolved complex life cycles that develop in two very different biological hosts, the infection within the invertebrate vector and in the terrestrial vertebrate host. Ticks are arthropods belonging to the Class Arachnida, where approximately 10% of 900 currently known tick species belong to two main large families, Ixodidae (hard ticks) and Argasidae (soft ticks), which have medical importance or veterinary significance (Estrada-Peña, 2016). In the New World, only about a dozen species of Ixodid ticks parasitize humans and dogs with some frequency and are known to transmit rickettsial pathogens. Usually, only one or two species of tick have the ability to acquire, maintain, and transmit a given pathogen. (Fritz, 2009). The ixodid life cycle includes the larva (which hatches from the egg), the nymph (the intermedium phase), and the adult (male and female). Ixodid ticks feed once in each active stage and ingest massive amounts of blood necessary to molt and develop to the subsequent stage. The immature stages (larvae and nymphs) usually feed upon small hosts, such as rodents and/or birds, while adults commonly feed upon large animals, including carnivores and ungulates (Estrada-Peña, 2016). Ticks acquire the bacteria while feeding on a rickettsemic animal known as an amplifier vertebrate host and transmit it to a new host through feeding of an infected stage on a new host, which could be a clinically susceptible host or serve as a reservoir or amplifier vertebrate host (Fritz, 2009; Parola et al., 2005). Pathogens belonging to the *Rickettsiaceae* family are maintained in the tick vector from adult female to her eggs (transovarial transmission) and/or during transfer from one life stage to the next (transstadial transmission) (Parola et al., 2005). In *Anaplasma* and *Ehrlichia*, the bacteria are preserved mainly though transstadial transmission, because

transovarial transmission has not been demonstrated (Woldehiwet, 2010).

1.2 General characterization of *Rickettsiaceae* and *Anaplasmataceae* families

Rickettsiaceae and *Anaplasmataceae* families are genetically related microorganisms, belonging to the class *Alphaproteobacteria* in the order *Rickettsiales*, that share the characteristic of being small bacilli and coccobacilli (0.3–0.5 x 0.8–2.0 mm) gram-negative obligately intracellular bacteria and additionally reside in an arthropod during a part of their life cycle (Fang et al., 2017). Currently, there are 7 genera within the order Rickettsiales: *Rickettsia and Orientia* in the *Rickettsiaceae* family, and *Ehrlichia, Anaplasma, Neorickettsia, Neoehrlichia* and *Wolbachia in Anaplasmataceae* family. Except for *Wolbachia* (endosymbiont organism), most of the described members above are considered as emerging and re-emerging pathogens in many places of the world (Walker et al., 2008). This review focuses on the TBRP most commonly recognized in people and dogs in the New World caused by spotted fever group (SFG) *Rickettsia*, *Anaplasma* and *Ehrlichia* species. These agents are briefly described in the annex 1.

1.3 Tick-borne *Rickettsiae*

The genus *Rickettsia* consists of 27 species grouped into 4 categories: the SFG, typhus group (TG), ancestral group (AG), and transitional group (TRG) (Fang et al., 2017; Gillespie et al., 2008). Pathogenic species (17 currently described) are only recognized in TG, (transmitted by lice and fleas), SFG, (transmitted by hard ticks - Ixodidae) and TGR, (transmitted by mites and ticks) (Fang et al., 2017). Once in the bloodstream of a vertebrate host, rickettsiae invade endothelial cells of the vasculature ultimately leading to detachment and death of the infected cells. Rickettsiae can move from cell to cell by

actin mobilization and invade new endothelial cells causing a vascular injury, which is responsible for the clinical and hematological abnormalities that occur in tick-borne rickettsioses (Walker et al., 2003). Rickettsiae can be ingested by a hematophagous arthropod during a blood meal. Inside of the arthropod, they infect and replicate within the epithelial cells lining the midgut, circulate in the hemolymph and may invade the ovaries, salivary glands and other tissues (Yoshimizu and Billeter, 2018). Below we describe the tick-borne rickettsiae-associated with human diseases involving the canines.

1.3.1 *Rickettsia rickettsii*

R. rickettsii is the etiological agent of the Rocky Mountain spotted fever (RMSF), the most severe tick-borne disease in the new world, with high fatality rate ranging from 23 – 80% in untreated human cases (Oteo et al., 2014). In 2017, in the USA more than 6,248 cases of spotted fever rickettsioses (SFR) were reported (CDC, 2019). In North America (Canada and the United States), the distribution of cases of RMSF is related to the distribution and abundance of the major vectors of *R. rickettsii*, which infect *Dermacentor variabilis* (the American dog tick) in the central and eastern states, *D. andersoni* (the Rocky Mountain wood tick) in the western states, and *Rhipicephalus sanguineus* (the brown dog tick) in the southwestern US and northern Mexico (Alhassan et al., 2018). Although occasionally other hard ticks, including *Amblyomma americanum* (the lone star tick) may be involved in the transmission of this pathogen to humans and animals (Levin et al., 2017). In Latin America (from Mexico to Argentina), ticks of the *A. cajennense* complex, that is formed by six species, namely *A. cajennense* sensu stricto, *A. interandinum, A. mixtum, A. patinoi, A.sculptum* and *A. tonelliae,* are the principal vectors

of *R. rickettsii* (Guedes et al., 2005*;* Tarragona et al., 2015*;* Labruna, 2009). Similarly, other ticks of the genus *Amblyomma*, different from the *A. cajennense* complex, have also been implicated in the natural maintenance of *R. rickettsii*, including *A. aureolatum, A. dubitatum,* and *A. tenellum* (published as *A. imitator*) (Guedes et al., 2005*;* Pinter and Labruna, 2006*;* Oliveira et al., 2010*;* Labruna, 2009).

Humans and domestic dogs are both similarly susceptible to infection with *R. rickettsii* and clinical cases of canine RMSF with fatal outcomes have been described (Breitschwerdt et al., 1985; Demma et al., 2005; Piranda et al., 2008; Labruna et al., 2009; Levin et al., 2014). Dogs serve as sentinels for the risk of RMSF in people due to their susceptibility to *R. rickettsii*, and the relatively high rates of exposure to ticks. The clearest example occurred in endemic areas of Arizona (southwestern US) and in Sonora and Baja California in the north of Mexico, where the brown dog tick *R. sanguineus* sensu lato (s.l.), the most common tick on dogs, was involved in outbreaks of RMSF in humans 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). In the case of Arizona, eight years before the human outbreak of RMSF, the seroprevalence of anti- spotted fever group *Rickettsia* (SFGR) antibodies in dogs was 6%, and during the outbreak, 70% of the dogs showed serologic evidence of SFGR. In the northern regions of Mexico, in the affected communities, the local dogs were consistently infested with *R. sanguineus* s.l., and these ticks were frequently observed on walls and earth floors of the adobe houses (sun-dried brick) and mattresses of patients (Álvarez-Hernández et al., 2017). Despite the coincidence in time and the proximity of the two regions where these outbreaks occurred, apparently, there is no evidence of a relationship between them, since recent reports

suggest that *R. sanguineus* ticks in Arizona and Mexicali belonged to different lineages (template and tropical, respectively) and also the *R. rickettsii* organisms are genetically distinct (Eremeeva et al., 2011; Traeger et al., 2015).

In Brazil, *R. rickettsii* has been detected and isolated in cell culture from *R. sanguineus* s.l. collected from dogs, suggesting that this tick could be involved as a vector in some RMSF-endemic areas (Cunha et al., 2009; Gehrke et al., 2009; Rozental et al., 2009; Silva et al., 2017; de Almeida et al., 2013; Pacheco et al., 2011). Similarly, in Villeta, an endemic area for *R. rickettsii* in Colombia, the continuous circulation of SFG *Rickettsia* spp. (seroprevalence 18% by IFA) has been documented in dogs. Interestingly, ticks parasitizing the dogs from this region, belong to the *A. cajennense* complex, the primary vectors of *R. rickettsii* in South America, and *R. sanguineus s*.l. (Hidalgo et al., 2009; Faccini-Martínez et al., 2017). The most recent outbreak occurred in Uramita, a municipality in northwest Colombia, close to an area where previous RMSF outbreaks have been reported. In this outbreak, there were four human cases, two of them fatal. Two dogs belonging to these cases showed IFA titers greater than 16,384, and *R. sanguineus* ticks were present on them, but did not contain *Rickettsia* spp. when examined by PCR (Londoño et al., 2019).

Although, there is evidence of association among dogs, ticks and the human cases of RMSF, the knowledge regarding the role that dogs play in the transmission of these diseases to humans is incomplete. Studies with dogs experimentally infected with *R. rickettsii* (intraperitoneally inoculated and through *A. aureolatum* feeding), showed that dogs develop a rickettsemic period between 3 - 13 days, during which 15.2% of larvae, 37.9% of nymphs, and 35.8% of adults *R. sanguineus* ticks, acquired the bacteria from

an infected dog. Consequently, these ticks transmitted also this agent to another susceptible host (Piranda et al., 2011). This result indicates that domestic dogs can act as a competent amplifier host of *R. rickettsii* and *R. sanguineus* is a potential vector of this pathogen. In addition, *R. sanguineus* can maintain *R. rickettsii* by transstadial and transovarial transmission without lethal effect, while *A. cajennense* is highly susceptible to lethal infection with *R. rickettsii* (Labruna et al., 2008). Despite these results, natural human or canine cases of RMSF involving *R. sanguineus* in transmission have not been documented in Latin America, except for the cases described in the northern Mexico.

In Brazil, tick species of the *A. cajennense* complex and *A. aureolatum*, are considered the main vectors of *R. rickettsii* (Szabó et al., 2013). *Amblyomma aureolatum* is a Neotropical three-host tick, found in undisturbed Atlantic rainforest areas at eastern South America (Guglielmone et al., 2003). The immature stages of *A. aureolatum* feed on passerine birds and a few rodent species while the adult stage, uses carnivore species as a primary host (Labruna, 2009). Thus, human Brazilian spotted fever (BSF) cases associated to *A. aureolatum* seem to occur when domestic dogs living close to forested areas are infected during time spent in the rainforest and carry infected *A. aureolatum* ticks to human dwellings where ticks attach to people, or dogs may become infected and transmit the infection to *R. sanguineus* ticks (Pinter et al., 2004; M. Szabó et al., 2013).

1.3.2 *Rickettsia parkeri*

Rickettsia parkeri was first shown to cause spotted fever in humans in the United States in 2004, after almost 6 decades that this bacterium was isolated from *A. maculatum* tick (Paddock et al., 2004). Also in 2004, this agent was found to have infected *A. triste*

tick in Uruguay (Venzal et al., 2004), and during the following years, was also reported in *A. triste* from Brazil and Argentina (Silveira et al., 2007; Nava et al., 2008), in *A. maculatum* sensu stricto (s.s.) from Perú (Flores-Mendoza et al., 2013), and in *A. tigrinum* from Uruguay, Argentina, Bolivia and Brazil (Lado et al., 2014; Tomassone et al., 2010; Weck et al., 2016). These three tick species (*A triste, A maculatum* s.s. and *A. tigrinum*) are morphologically and genetically closely related, forming the *A. maculatum* complex (Estrada-Peña et al., 2005), and according to previous reports, this tick species complex are the potential vectors of *R. parkeri* sensu stricto (s.s.) in the New World (Nieri-Bastos et al., 2013; Romer et al., 2014; Nieri-Bastos et al., 2018).

Human cases of *R. parkeri* rickettsiosis seem to be a milder compared to *R. rickettsii* (Conti-Diaz et al., 2009; Romer et al., 2011). In South America, although infected ticks belonging to the *A. maculatum* complex are apparently widely distributed, human clinical cases of spotted fever rickettsioses confirmed to be caused by *R. parkeri* s.s. have only been reported in Uruguay and Argentina, where the adult stage of *A. triste and A tigrinum* feed on dogs as the main host (Venzal et al., 2004; Lado et al., 2015). Although some evidence suggests the association between human cases, seropositive domestic dogs and their ticks (Tomassone et al., 2010; Grasperge et al., 2012; Venzal et al., 2008), further knowledge regarding the susceptibility of dogs to *R. parkeri* s.s. and their potential role in the eco-epidemiology of the disease is needed.

A novel SFG strain of *R. parkeri* emerged in 2010 in Brazil causing febrile illness in humans that is milder than BSF (Spolidorio et al., 2010; Silva et al., 2011; Krawczak et al., 2016). This novel agent was isolated from adult *A. ovale* ticks collected from dogs in the same area where the patient acquired the infection and was designated *R. parkeri*

strain Atlantic rainforest. Subsequent studies showed that 88,6% of the sampled dogs from the endemic area of *R. parkeri* strain Atlantic rainforest carried also antibodies against SFG with highest endpoint titers to *R. parkeri* (Szabó et al., 2013a*;* Szabó et al., 2013b). Infection with *R. parkeri* strain Atlantic rainforest was also reported in *A. ovale* and *A. aureolatum* ticks*,* both of the *A. ovale* complex, and in *R. sanguineus* ticks collected from dogs and collected from the environment (Sabatini et al., 2010; Barbieri et al., 2014; Medeiros et al., 2011). In addition, experimental studies showed that *A. ovale* is a reservoir and a competent vector of *R. parkeri* strain Atlantic rainforest (Krawczak et al., 2016*;* Brustolin et al., 2018) while vector competence of *A. aureolatum* and *R. sanguineus* for this agent has not been established.

Rickettsia parkeri strain Atlantic rainforest had also been reported infecting *A. ovale* ticks in Belize (Lopes et al., 2016) Nicaragua (Vogel et al., 2018), Argentina (Lamattina et al., 2018) and Colombia (Londoño et al., 2014). Considering that *A. ovale* is one of the most important human-biting ticks and has a broad distribution in the neotropical region, with established populations from Mexico to Argentina (Guglielmone et al., 2003), it is possible that this pathogen has a wide distribution in Latin America. In the Urabá region, in northwest Colombia, where a previous outbreak of *R. rickettsii* infections was reported, *R. parkeri* Atlantic rainforest-like strain was isolated from *A. ovale* ticks collected from a dog (Londoño et al., 2014), and evidence of a mild human case of rickettsiosis has been observed (Acevedo-Gutiérrez et al., 2019). The epidemiological nexus in this region of Colombia is comparable to the ecological characteristics described in endemic areas of Brazil, where *R. parkeri* strain Atlantic rainforest is endemic and unrestrained dogs are infested with adult *A. ovale* ticks when accessing Atlantic forest patches bringing the

infected ticks to the human dwellings (Sabatini et al., 2010; Nieri-Bastos et al., 2013; Silveira et al., 2007; M. Szabó et al., 2013).

1.3.3 *Rickettsia amblyommatis*

Rickettsia amblyommatis (formerly *Candidatus* Rickettsia amblyommii) was isolated in 1973 from an adult *A. americanum* tick from Tennessee USA (Karpathy et al., 2016). The first studies of *R. amblyommatis* concluded that this organism was probably not pathogenic for humans due to its inability to cause disease in guinea pigs (Burgdorfer et al., 1981). However, subsequent reports have associated these bacteria with human SFR cases (Billeter et al., 2007; Apperson et al., 2008; Moncayo et al., 2010). Based on circumstantial evidence of both asymptomatic seroconversion and conversion after self-limited illness in soldier from Arkansas, heavily exposed to lone star ticks highly positive to *R. amblyommatis*, the pathogenic potential of this pathogen remains unclear. Exposure of dogs to *R. amblyommatis* is not surprising since questing *A. americanum* ticks have rates of infection that often exceed 40% (Karpathy et al., 2016), and these ticks are promiscuous feeders that will readily attached to people, domestic animals (including dogs), and a variety of other medium-to-large mammals as hosts (Kollars et al., 2000*;* Estrada-Peña and Jongejan, 1999). Repeated exposure to infected ticks can induce very high serological titers in dogs, and there is recent molecular evidence that this organism can infect them, but no clinical signs in dogs have been documented. Conversely, infection of dogs with *R. amblyommatis* may protect them against a more pathogenic rickettsial species such as *R. rickettsii* because *R. amblyommatis* infection may provide a cross-protective immune response against subsequent infection with *R. rickettsii*

(Blanton et al., 2014; Rivas et al., 2015). *Rickettsia amblyommatis* may also play a role in the ecology and epidemiology of SFGR, since the primary infection in a tick with this rickettsia could block or decrease transovarial or trans-stadial transmission of a second SFGR species, including *R. rickettsii* or *R. parkeri,* through a refractory phenomenon that avoids the maintenance of a secondary infection (Macaluso et al., 2002; Sakai et al., 2014; Levin et al., 2018, Scott et al., 2016).

In Latin America, *R. amblyommatis* is detected in *Amblyomma* spp. ticks, especially in *A. cajennense* complex species from Mexico, Panama, Costa Rica, Colombia, Argentina and Brazil (Moreira-Soto et al., 2016; Sánchez-Montes et al., 2016; Sosa-Gutierrez et al., 2016; Hun et al., 2011; Faccini-Martínez et al., 2016; Labruna et al., 2004). However, *R. amblyommatis* is also found in other tick species such as *A. varium* in Colombia (Quintero et al., 2017), *A. auriculatum* in Brazil (Saraiva et al., 2013), *A. neumanni* in Argentina (Labruna et al., 2007) and *A. ovale, D. nitens,* and *R. sanguineus* in Central America (Bermudez and Troyo, 2018). Thus, *R. amblyommatis is* probably the most prevalent and widely distributed SFG rickettsia in ticks in the Americas (Karpathy et al., 2016). Canine infection with titers to *R. amblyommatis* four-fold higher than those to any of the other rickettsial antigens has been described in Panama (Bermúdez et al., 2009, 2011), Brazil (Costa et al., 2017) and in Costa Rica, where seroreactivity was detected in dogs from sites associated with human cases of SFGR (Moreira-Soto et al., 2016). In Colombia, there are evidence of *R. amblyommatis* infection in one dog and some horses in a endemic area of SFG rickettsial infection (Quintero et al., 2017).

1.3.4 *Rickettsia massiliae*

Rickettsia massiliae belongs to the spotted fever group, and for many years its pathogenicity was unknown. In 2005, the first human case worldwide was confirmed in Italy through a retrospective study conducted on archived samples. The sample was obtained in 1980s from a patient that was hospitalized with fever, maculopapular rash on the palms and soles, and an eschar (Vitale et al., 2006). The same year the only human case of *R. massiliae* infection so far described in the New World, originated in Buenos Aires, Argentina (García-García et al., 2010). To date, few human cases of *R. massiliae* infection have been confirmed by molecular methods, mainly from North Africa and southern European countries bordering the Mediterranean Sea (Cascio et al., 2013). *Rhipicephalus sanguineus* ticks has been suggested as the main vector of *R. massiliae*. In the New World, this pathogen has been isolated from *R sanguineus* ticks in the endemic area of RMSF in Arizona, (Eremeeva et al., 2006) and this tick species infected with *R. massiliae* was identified in California (Beeler et al., 2011) and in North Carolina (Fornadel et al., 2013). Similarly, the prevalence of *R. massiliae* ranged from 3.4% to 20% in *R. sanguineus* collected from dogs in Buenos Aires (Cicuttin et al., 2004; Cicuttin et al., 2014). No human cases have been reported from North America, though evidence of mild to moderate canine rickettsioses with specific antibodies response against *R. massiliae* was reported in four dogs where *R. massiliae*-infected ticks were collected (Beeler et al., 2011). In Argentina, sereoprevalence of 80% (121/152) in dogs showed antibodies against *Rickettsia* spp. but not clinical signs were reported, suggesting high exposition by any SFGR species (Cicuttin et al., 2004).

Recent studies determined the existence of different geno-species under the taxon

R. sanguineus. In the New World, at least two lineages (tropical and temperate) with a different geographic distribution and vectorial competence have been reported (Moraes-Filho et al., 2011; Moraes-Filho et al., 2015; Nava et al., 2015). The fact that the geographical distribution of the *R. sanguineus* temperate lineage occurs in regions of the United States and the southern cone of South America where *R. massiliae* has been detected suggests that the temperate lineage has vectorial competence for this pathogen and is the main vector in the American continents (Jones et al., 2017; Monje et al., 2016; Nava et al., 2018).

1.4 *Anaplasmataceae* family

Among emerging tick-borne pathogens, agents belonging to family *Anaplasmataceae* stand out due to their worldwide distribution and zoonotic potential. In the New world, *Anaplasma* and *Ehrlichia* species are the most important tick-borne pathogen infecting animals and humans, both transmitted by hard ticks. The absence of transovarial transmission in ticks for both species, determines the essential role of the vertebrate reservoir hosts for the maintenance of these pathogens in the environment (Woldehiwet, 2010). When ticks feed on infected hosts, *Ehrlichia* and *Anaplasma* first enter the tick midgut epithelium, where their primary replication takes place (Rar and Golovljova, 2011a). Then, pathogens move to the hemolymph and to the tick salivary glands invading the epithelial cells where a second replication cycle occurs. From epithelial cells, microorganisms are released to salivary gland secretions and pass on to the next vertebrate host when ticks feed on them (Kazimírová and Štibrániová, 2013). Transmission times have not been established for the majority of tick-borne pathogens,

but in general, for the *Anaplasmataceae* family, transmission is thought to occur within the first 4–24 hours of tick attachment (Fourie et al., 2013). The type of cells infected in the vertebrate host are directly related to the *Ehrlichia* or *Anaplasma* spp. involved in the infection (erythrocytes, bone marrow-derived phagocytic cells, endothelial cells and platelets). Inside of the infected cell, bacteria are present as macro colonies (morulae) within intracytoplasmic vacuoles (Dumler et al., 2001). The cytoplasmic morulae have variable sizes (between 1.5 µm and 6 µm in diameter) and shapes (usually coccoid to ellipsoidal). Some of the bacteria appear as small dense structures (dense-cored or DC) while others are larger and less dense (reticulate cell or RC). RC and DC represent replicating and infectious forms, respectively (McBride and Walker, 2011).

1.4.1 *Ehrlichia* genus

To date, the genus *Ehrlichia* includes six species: *E. muris* (murine pathogen), *E. ruminantium* and *E. mineirensis*, (pathogens in domestic ruminants) and three species - *E. chaffeensis, E. ewingii* and *E. canis*— causing infection in dogs and humans. In this review we will focus on the last three species, which are relevant in canine and human infections.

1.4.1.1 Ehrlichia canis

Ehrlichia canis is the primary etiologic agent of canine monocytic ehrlichiosis (CME). This disease is widely distributed in dogs around the world, but is more prevalent in tropical and subtropical regions (Rar and Golovljova, 2011a). In the New World, the main vector of *E. canis* is the tropical lineage of the tick *R. sanguineus* (Dantas-Torres et

al., 2013). This tick is distributed from southern Brazil to the northern Mexico and United States, where inhabits both rural and urban environments, although is particularly abundant in urban and peri-urban areas (Jones et al., 2017; Cicuttin et al., 2015). In North America, *Dermacentor variabilis* (the American dog tick) has also been shown to have vector competence in experimentally infected ticks (Johnson et al., 1998). CME is currently the most prevalent canine tick-borne disease in Latin America; epidemiological studies based on seroprevalence and occasionally on DNA-based analysis conducted in several countries of Central and South America, showed that between 15 and 60% of dogs carried antibodies against *E. canis* (Martínez-Vega et al., 2016; Barrantes-Gonzalez et al., 2016; Araes-santos et al., 2015). In Colombia, prevalence of antibodies to *E. canis* as high as 50% in dogs has been reported in urban areas (McCown et al., 2014).

Ehrlichia canis infections are rare in humans. Evidence of *E. canis* DNA has been detected in symptomatic and asymptomatic human samples from Venezuela, Mexico and Panama (Perez et al., 1996; Daza et al., 2018; Silva et al., 2014) and in human blood donors in Costa Rica (Bouza-Mora et al., 2016). The few reported cases of human monocytic ehrlichiosis (HME) caused by *E. canis* show moderate to severe clinical signs with fever over 39°C, rash, headache, myalgia, thrombocytopenia (Perez et al., 2006), and in more severe cases with respiratory distress (Daza et al., 2018). The genetic and antigenic comparison between *E. canis* isolates from humans and dogs obtained from the same area where human cases occurred, suggests transmission from dog-to-human (Unver et al., 2001). The absence of human cases and the low human seroprevalence (3.3% - 5%) in areas with high prevalence of *E. canis* in dogs and ticks in South America can be explained by the low affinity of *R. sanguineus* to feed on humans (Ríos et al.,

2008; Barrios et al., 2013). Nevertheless, there is evidence that *R. sanguineus* has increased aggressiveness and propensity to bite other hosts than dogs during periods of warmer temperatures (Parola et al., 2008).

1.4.1.2 Ehrlichia chaffeensis

Ehrlichia chaffeensis is the etiological agent of human monocytic ehrlichiosis (HME). This disease occurs predominantly in United States, although is not exclusive from North America since *E. chaffeensis* DNA has also been detected in South America (Guillemi et al., 2019). *Ehrlichia chaffeensis* is transmitted primarily by the lone star tick (*A. americanum*), and the white-tailed deer (*Odocoileus virginianus*) is recognized as the main reservoir, but there is evidence of natural infection in other mammals including goats, calves, domestic dogs, red foxes and coyotes (Paddock and Childs, 2003; Lockhart et al., 1997). Human infection by *E. chaffeensis* is the most frequently diagnosed tick-borne disease in the southern United States (Heitman et al., 2016). A total of 4,613 cases of HME with a date of illness onset between 2008 and 2012 have been reported, but this incidence is based on passive surveillance and it is a likely a 10-100 fold underestimation of the actual disease incidence (Heitman et al., 2016) as determined by active prospective surveillance. The symptoms of ehrlichiosis in humans are nonspecific and typically begin within 7–14 days of exposure. Acute infections result in a moderate to severe (sometimes even fatal) illness, characterized by fever, headache, lethargy and myalgia. In addition, a maculopapular rash is present in a minority of cases in early stages and can be petechial in late stages (Dumler et al., 2007).

Dogs are susceptible to disease caused by *E. chaffeensis* (Breitschwerdt et al.,

1998), and DNA of *E. chaffeensis* has also been found in ticks collected from dogs. *Amblyomma americanum* is commonly found on dogs and persons in the southeastern and south-central United States, and dogs can serve as host for all stages of the life cycle of this tick. Thus, dogs provide a convenient vehicle for transport of infected *A. americanum* ticks from various habitats into the peri-domestic environment (Paddock and Childs, 2003). Other species of ticks such as *R. sanguineus* have been infected naturally and experimentally with *E. chaffeensis*, but their role as a natural vector of HME has not been definitively established (Stoffel et al., 2014). Serological surveys conducted in dogs in areas endemic for HME in the USA have shown antibodies reactive to *E. chaffeensis* in 38% (28/74) from southeast Virginia and 10.8% (7/65) from Oklahoma. In addition, *E. chaffeensis* DNA was detected in whole-blood samples from 8/74 and 4/65 dogs, respectively (Dawson et al., 1990; Murphy et al., 1998).

The epidemiology of HME in South America is not well elucidated, although in Colombia and Brazil cases of ehrlichiosis in humans have been diagnosed serologically; the *Ehrlichia* species associated with the infection has not been determined (Hidrón Botero et al., 2014; da Costa et al., 2006; Calic et al., 2004). In fact, little is known about the native mammalian hosts and vectors involved in its transmission cycle in South America. Some studies in Argentina and Brazil have shown DNA evidence of an organism related to *E. chaffeensis* in free-ranging marsh deer (*Blastocerus dichotomus*), suggesting this mammal as a potential vertebrate host to *E. chaffeensis* (Sacchi et al., 2012; Guillemi et al., 2019). In previous studies, molecular evidence has suggested *A. tigrinum* and *A. parvum* ticks as potential vectors in the natural cycle of *E. chaffeensis* in South America, where the *E. chaffeensis* DNA sequences were molecularly closely

related to the strain detected in marsh deer (Cicuttin et al., 2017; Tomassone et al., 2008). Humans, dogs and other domestic animals such as horses and cattle are fed upon by both tick species, increasing the risk of inter-species transmission of potentially zoonotic agents including *E. chaffeensis* and a novel *Ehrlichia* spp. (Lado et al., 2016). Further research should focus on clarifying the possible roles of different mammals as reservoir hosts and the vectors involved in the transmission.

1.4.1.3 Ehrlichia ewingii

Ehrlichia ewingii is a granulocytotropic member of the genus *Ehrlichia* and is the etiological agent of human ewingii ehrlichiosis (HEE). *Ehrlichia ewingii* was first described in 1992 when it was attributed to being the etiologic agent of canine granulocytic ehrlichiosis (Anderson et al., 1992). Subsequent work has demonstrated that humans also develop this disease (Buller et al., 1999; Masters et al., 2009; Allen et al., 2014). However, infection with *E. ewingii* is a less common human disease compared to *E. chaffeensis*, but some proportion of cases currently reported as *E. chaffeensis* infection may actually be by *E. ewingii* due to serologic cross-reactions between them. Also some cases might be missed because of limited sensitivity of molecular tests (Harris et al., 2016). This disease became reportable in the USA in 2008 with 185 cases of HEE reported between 2008 and 2017(CDC, 2019).

E. ewingii probably occupies a similar zoonotic cycle as *E. chaffeensis,* due to both agents being transmitted by the lone star ticks and white-tailed deer being a key host to all stages of the lone-star tick as the principal reservoir for *E. chaffeensis* (Yabsley et al., 2002). Domestic dogs have an important role in the transmission cycle of *E. ewingii*

because canines may be asymptomatic and chronically infected, serving as a reservoir for this organism (Liddell et al., 2003). *Ehrlichia ewingii* Infection in dogs and humans manifest as a milder disease compared to *E. canis* or *E. chaffeensis*. As described for other tick-borne infections, it is likely that dogs and humans share similar exposures to infecting ticks.

HEE has been identified only in the US where *A. americanum* is considered the main vector (Rar and Golovljova, 2011a), but natural infection in other tick species such as *R. sanguineus* s.l. and *D. variabilis*, has also been reported in the US (Hudman and Sargentini, 2018; Murphy et al., 1998) and in Cameroon where *E. ewingii* was detected in dogs and in *R. sanguineus* s.l. (Ndip et al., 2007). In Korea, *E. ewingii* DNA was detected in *H. longicornis* ticks and *Apodemus agrarius* voles (Kim et al., 2006) and in *R. microplus* in Colombia (Miranda and Mattar, 2015). However, experimental infection of *R. sanguineus* and *D. variabilis* tick was unsuccessful (Yabsley et al., 2011). Further studies are needed to determine the vectorial competence of alternative tick species.

1.4.2 *Anaplasma* genus

Anaplasma species transmitted by ticks show morphology, genomic organization and life cycle similar to the *Ehrlichia* species. The genus *Anaplasma* includes *A. phagocytophilum*, which infects several species of mammals including humans and canines. Ruminant pathogens include *A. marginale*, *A. centrale*, *A. ovis*, and *A. bovis*. We will focus on *A. phagocytophilum* and *A. platys* that have been documented to infect humans and dogs.

1.4.2.1 Anaplasma phagocytophilum

Anaplasma phagocytophilum, formerly known as *E. phagocytophila* or *E. equi* (Dumler et al., 2001), is the etiologic agent of human granulocytic anaplasmosis (HGA). This disease was first described in 1994 in six patients from the US (Chen et al., 1994), but before the human reports, this agent was considered an important veterinary pathogen, responsible for equine and canine granulocytic anaplasmosis (CGA) in the USA and Europe, and for tick-borne fever in goats, cattle and sheep in Europe (Woldehiwet, 2010). *Anaplasma phagocytophilum* is transmitted by ticks of the *Ixodes ricinus* including *I. scapularis* in New England and North Central United States, *I. pacificus* and *I. spinipalpis* in the Western United States, *I. ricinus* in Europe, and *I. persulcatus* in Asia (Rar and Golovljova, 2011a). Under natural conditions, *A. phagocytophilum* is naturally maintained in a cycle involving ticks and rodents although many ground-dwelling vertebrate species and some birds are infected, and some of them may have a role as competent reservoirs (Woldehiwet, 2010). In the North Central United States, the main reservoirs of HGA include the white-footed mouse (*Peromyscus leucopus*), grey squirrel (*Sciurus carolinensis*) and raccoon (*Procyon lotor*), while in the western US, there are multiple hosts that maintain *A. phagocytophilum*, including the dusky-footed woodrat (*Neotoma fuscipes*), gray squirrel (*Sciurus griseus*) and chipmunk (Tamias) (Rar and Golovljova, 2011; Hongtao et al., 2012; Keesing et al., 2012).

Several genetic variants of *A. phagocytophilum* have been described with distinct biological and genetic properties that seem to be associated with vector competence, host tropism and geographic distribution (Stuen et al., 2013; Massung et al., 2003; Dugat et al., 2015). The variant (Ap-ha) seems to be associated with disease in humans and

dogs in the US, the country where most of the cases have been described (Nicholson et al., 2010; Bakken and Dumler, 2015). Between 2000 and 2017 the incidence of HGA in the US increased from 1.4 cases to 17.9 cases per million people (CDC, 2017). Patients with HGA ranged from asymptomatic infection to fatal disease presenting nonspecific febrile symptoms, such as acute onset of fever, headache, malaise, and myalgias (Keesing et al., 2012; Bakken and Dumler, 2015).

The first case of canine granulocytic anaplasmosis (CGA) caused by *A. phagocytophilum* in dogs was identified in California in 1982 (Madewell and Gribble, 1982). Since this report, evidence of dog exposure to *A. phagocytophilum* continues to be detected in upper Midwestern, Northeastern, and Western states of the US (Bowman et al., 2009). Dogs are considered as accidental hosts of *A. phagocytophilum* since apparently they do not have an important role in transmission to other host species (Carrade et al., 2009). Dogs naturally infected with *A. phagocytophilum* exhibit a mild to severe acute illness, often accompanied by anorexia, lethargy, fever, lameness, and thrombocytopenia (Kohn et al., 2008); however, most dogs remain apparently healthy as indicated by the presence of *A. phagocytophilum* DNA and high seroprevalence in healthy dogs in endemic areas (Beall et al., 2012; Bowman et al., 2009; Carrade et al., 2009).

Until now, human cases associated with HGA have not been documented in South America despite the serological evidence of infection in humans. In Colombia, there are two surveillance studies that reported IFA detection of IgG antibodies against *A. phagocytophilum* with a seroprevalence of 20% in an asymptomatic population from a rural area (Máttar and Parra, 2006a) and 6.7% in patients with undifferentiated acute febrile syndrome (Faccini-Martínez et al., 2017). However, the possible antibody

response against *A. phagocytophilum* may have been caused by exposure to *A. platys*, an agent apparently common in dogs with some cases in humans in South America, due to cross-reactivity among species within the same genogroup (Suksawat et al., 2000).

Molecular evidence of the presence of *A. phagocytophilum* in South America is limited, DNA from genotypes similar to *A. phagocytophilum* have been reported in wild animals, including carnivores (André et al. 2012), birds (Machado et al. 2012), gray brockets (*Mazama gouazoubira*) (Silveira et al. 2012) and Andean tapir (Pesquera et al., 2015). Therefore, the vector of this agent in nature has not been identified (André, 2018). On the other hand, *A. phagocytophilum* DNA has been detected in domestic dogs where one case was associated with an organism closely related to *A. phagocytophilum* in a sick dog from Colombia (Vargas-Hernandez et al., 2016), and two studies in Brazil showed that 6 – 7 % of domestic dogs without clinical symptoms were infected with the same *A. phagocytophilum* strain that infect dogs and humans in the US (Santos et al., 2011; Santos et al., 2013). Another study reported a co-infection with *E. canis* and *A. phagocytophilum* in a dog from a urban area of Brazil (Silveira et al., 2015). Ticks infected with *A. phagocytophilum* collected from dogs in Brazil (6/235), included one *Am. cajennense* sl and five *R. sanguineus* s.l (Santos et al., 2013); however, this finding does not confirm the role of these tick species as vectors. Further studies are needed to elucidate the enzootic cycle and the tick species that play a role as a vector in the transmission of *A. phagocytophilum* in South America.

1.4.2.2 Anaplasma platys

Anaplasma platys together with *E. canis* are the main canine pathogens of the

Anaplasmataceae family. These agents are frequently detected in domestic dogs in Latin America, but have also been reported in the US, Europe and Asia (Rar and Golovljova, 2011; Carvalho et al., 2016; Sainz et al., 2015). *A. platys* is the etiologic agent of canine infectious cyclic thrombocytopenia (CICT) (Sainz et al., 2015) and is thought to be a re-emerging zoonosis (Bouza-Mora et al., 2016; Breitschwerdt et al., 2014; Cárdenas et al., 2007; Maggi et al., 2013; Sainz et al., 2015). Dogs experimentally and naturally infected with *A. platys* showed similar clinical signs to the ones showed by *E. canis*, which include fever, lethargy, anorexia, weight loss, pale mucous membranes, petechiae, nasal discharge, and lymphadenopathy (Harvey, 2006). *A. platys* unlike *E. canis* can also cause asymptomatic infection and tends to be less severe than CME (Sainz et al., 2015), although co-infection with other tick-borne pathogens such as *A. phagocytophilum*, *E. chaffeensis, E. canis, E. ewingii* and *Borrelia burgdorferi* could enhance the clinical manifestations of CICT (Gaunt et al., 2010).

Anaplasma platys (formerly known as *E. platys*) is the only rickettsial species to infect platelets (Dumler et al., 2001). The dog is the primary reservoir host for this agent, presumably transmitted by *R. sanguineus* s.l. ticks (Inokuma et al., 2000); however, the only experimental study attempting to confirm *R. sanguineus* s.l. as vector of *A. platys* was unsuccessful (Simpson et al., 1991). According to previous attempts to confirm the hypothesis regarding the vector competence of *R. sanguineus* s.l. in *A. platys* likely failed due to the low sensitivity of the diagnostic method, the tick strain, or lineage of the *R. sanguineus* complex used in the vector competence experiments (Dantas-Torres et al., 2013; Ramos et al., 2014; Moraes-Filho et al., 2015). The high percentage of nymphs and adult ticks infected with *A. platys* in both lineages of *R. sanguineus* (tropical and

temperate) in the American continent, reinforces the hypothesis that these ticks have vector competence to transmit *A. platys* (Ramos et al., 2014; Carvalho et al., 2016; Cicuttin et al., 2015; De Almeida et al., 2012; Lopes et al., 2016). Further studies are recommended to elucidate the role of *R. sanguineus* as a vector of *A. platys.*

Little evidence has been published about human infections by *A. platys*. Two humans with A*. platys* infection were documented in Venezuela. Both patients experienced chronic, nonspecific clinical signs including headaches and muscle pains and reported the presence of dogs infested with ticks. Intra-platelet inclusion bodies resembling *A. platys* were observed in buffy coat smears, and *A. platys* DNA was amplified and sequenced in both patients. Two dogs belonging to one patient also had petechial lesions on the abdomen, thorax, and legs, thrombocytopenia, and antibodies against *Anaplasma* spp. (Arraga-Alvarado et al., 2014)

In Colombia, only a few studies of *Anaplasma* infections in dogs and ticks have been reported. The seroepidemiological data suggest that the frequencies in dogs from urban areas vary between 11% and 53% for *Anaplasma* spp. (Hidalgo et al., 2009 McCown, 2013; McCown et al., 2014). Despite the high seroprevalence in dogs, the only two studies that search for *A. platys* DNA in Colombia showed low frequencies of infection in dogs (1% to 6%) (Posada-Zapata et al., 2017; Vargas-Hernandez et al., 2016), which is consistent with the prevalence in other Latin American countries such as Uruguay (4.2%; Carvalho et al., 2016), Mexico (3%; Almazán et al., 2016) and Costa Rica (1%; Bonilla et al., 2017). However, higher prevalence ranging between 16% and 48% have been demonstrated in urban areas of Brazil, Chile, Venezuela, Argentina and Cuba (Lopes et al., 2016; Huang et al., 2005; Abarca et al., 2007; Bahía et al., 2014; Ramos et al., 2010).

1.5 Clinical symptoms of tick-borne rickettsial diseases in humans

Clinical symptoms of tick-borne SFG rickettsioses in humans generally begin 4 to 10 days after a tick bite and typically include fever accompanied by headache, muscle pain, rash, local lymphadenopathy, and, for most of these diseases, a characteristic inoculation eschar at the site of the bite. However, these signs vary depending on the rickettsial species involved, and typical signs may be absent or unnoticed by an unexperienced clinical examination (Parola et al., 2013). In cases of human anaplasmosis or ehrlichiosis (HGA, HME and HEE), the disease presents as undifferentiated febrile illnesses that closely resembles the disease in dogs. After infection (1-2 weeks) following an exposure to infected ticks, patients experience a prodromic phase characterized by malaise, low-back pain, fever, and/or gastrointestinal disorders. Other less frequent symptoms include arthralgia, lymphadenomegaly, conjunctivitis, dysuria, and peripheral edema (Paddock and Childs, 2003; Bakken and Dumler, 2015). A skin rash, similar to SFG rickettsioses is observed in patients with HME but is very infrequent, present in less than 10% of adults (Ismail and McBride, 2017; Thomas et al., 2009).

1.6 Clinical signs of tick-borne rickettsial diseases in dogs

Clinical signs in dogs infected with RMSF, ehrlichiosis or anaplasmosis are nonspecific and indistinguishable from each other, may include general signs such as fever, weakness, lethargy, anorexia, lymphadenopathy, splenomegaly, hepatomegaly, and weight loss; however, the clinical signs may be variable, depending on the bacteria strain, the dog breed, the dog's immune response, and the presence of co-infection with other tick- or flea-borne pathogens (Sainz et al., 2015). Here, we will describe the clinic-

pathological features of each tick-borne rickettsial infection in dogs.

The most severe tick-borne diseases in dogs is RMSF, caused by *R. rickettsii*. This disease has a more rapid and severe course of clinical illness than ehrlichiosis or anaplasmosis, but in the acute phase it is difficult to differentiate among them (Greene et al., 1985). Cases of RMSF in dogs have been described in North America and South America. Evidence of more virulent *R. rickettsii* strains have been reported in Brazil (human case-fatality rates 10-40%) compared to those RMSF in the United States (human case-fatality rate 5-10%) (Labruna et al., 2014). In contrast to the human cases, the most severe clinical signs were observed in dogs experimentally infected with *R. rickettsii* strains from North America compared to a relatively mild illness without hemorrhagic or neurological abnormalities in dogs infested with ticks carrying a Brazilian *R. rickettsii* strain Taiaçu (Piranda et al., 2008; Levin et al., 2014). The most prominent signs of rickettsial infection in dogs start with fever over 39.5˚C that appears 3–7 days after tick bite. The fever is followed by depression, anorexia, and widespread cutaneous and mucosal hemorrhages on day 5 post infection. The abrupt decline in hematologic values including thrombocytopenia become evident at 6–8 DPI, when neurological signs, including tremors, begin. Dogs experimentally infected with a North American strain of *R. rickettsii* commonly develop petechiae on both the ocular and oral mucosa within 6–11 days after infection. Conversely, Brazilian spotted fever in dogs seems very rarely to cause ocular lesions (Levin et al., 2014). In the New World, mild to moderate canine rickettsioses involving to *R. massiliae* have been described in four dogs from California and in dogs from Argentina (Beeler et al., 2011; Cicuttin et al., 2014; Monje et al., 2016). On the other hand, dogs do not appear to exhibit clinical symptoms when they are infected

with *R. parkeri* or *R. amblyommatis*; and it is unknown if they become rickettsemic (Yoshimizu and Billeter, 2018; Grasperge et al., 2012; Barrett et al., 2014).

CME is the second most severe tick-borne disease in dogs. Experimental and naturally occurring *E. canis* infection in dogs courses through three phases: acute, subclinical, and chronic, although the distinction among these phases is not straightforward in dogs with naturally-occurring disease (Waner et al., 1997). In the acute phase (2 to 4 weeks), clinical signs may vary but commonly include fever, lethargy, lameness, oculo-nasal discharge, thrombocytopenia, non-regenerative anemia, leukopenia, hyperglobulinemia, and proteinuria, but these clinical signs may disappear spontaneously, even without treatment. In the subclinical phase (from months to years), the dogs do not present clinical signs; however, hematologic abnormalities such as subnormal platelet concentrations may be detected (Mylonakis et al., 2019). Some, but not all infected dogs can progress to a chronic phase. In this phase, dogs show many of the same clinical signs as in the acute phase; however, dogs in the chronic phase have bone marrow hypoplasia, severe pancytopenia, uveitis, weight loss and hemorrhages (Sainz et al., 2015; Gaunt et al., 2010). Clinical laboratory findings in dogs infected by *E. chaffeensis* or *E. ewingii* are similar to those caused by CME. In general, infections by any *Ehrlichia* spp. are difficult to distinguish by clinical or hematological observations; however, infection by *E. ewingii* in dogs is more likely to be associated with polyarthritis than other *Ehrlichia* spp., but a differential diagnosis should be made with infections caused by *A. phagocytophilum* (Qurollo et al., 2019; Modarelli et al., 2019).

After tick transmission of *A. phagocytophilum* in dogs, the clinical illness usually appears within 1–2 weeks, but most of the exposed dogs will not develop overt clinical

disease (Eberts et al., 2011; Eberts et al., 2011). The most common clinical signs caused by *A. phagocytophilum* are non-specific; they include lethargy, inappetence/anorexia, and fever, although other findings may occur, such as pale mucous membranes, tense abdomen, vomiting and diarrhea (Sainz et al., 2015). Musculoskeletal signs, such as reluctance to move and lameness, have also been reported (Carrade et al., 2009). On the other hand, the clinical signs of *A. platys* infection in dogs are similar to CME and anaplasmosis caused by *A. phagocytophilum*, with variable and nonspecific clinical signs (Harvey JW., 2006). *A. platys* infection often causes asymptomatic infection, and when the clinical signs occur, they tend to be less severe than in CME (Sainz et al., 2015). After infection periods of thrombocytopenia and fever appear and disappear cyclically every 1-2 weeks.

Coinfection by various rickettsial pathogens contributes to the presentation of atypical manifestations, influences the severity of the clinical disease, and potentiates hematological anomalies (Gaunt et al., 2010). Simultaneous infection with tick-borne organisms can occur as a result of the transmission of multiple organisms by the same tick or as a result of the independent transmission of infections by different ticks at different times (Kordick et al., 1999; Gutiérrez et al., 2008). The most frequently co-infection in dogs involve *E. canis* and *A. platys* (Sainz et al., 2015; Santamaria et al., 2014). In an experimental infection, dogs infected with both pathogens developed a more prolonged infection in conjunction with more severe thrombocytopenia and anemia (Gaunt et al., 2010). The lack of evidence of coinfection among any rickettsia pathogen with other tick-borne pathogens may be due to limitations in the diagnosis of *Rickettsia* spp infection, which will be discussed later.

Hematological abnormalities in dogs infected of some TBD do not serve as indicators to differentiate among any specific pathogen due to similar drop in the packed cell volume (PCV). Cyclical thrombocytopenia, previously reported with *A. platys* infection, has also been observed with *E. canis* and *A. phagocytophilum* but as persistent thrombocytopenia, although the cell tropism of these pathogens are monocytes and granulocytes, respectively (Nair et al., 2016). In dogs with RMSF, leukocytosis and thrombocytopenia are more frequent, while ehrlichiosis and anaplasmosis more frequently by manifest with anemia, leukopenia and thrombocytopenia (Greene et al., 1985).

1.7 Diagnostic

The diagnosis of tick-borne rickettsial diseases is one of the most difficult because multiple rickettsial agents share the same geographical distribution and the vector competence of some ticks for several pathogens. The limitations in the diagnosis are related to the similarity in the clinical presentation, cross-reactions in some serological tests, the low sensitivity and specificity by reference methods during the acute phase and, the unavailability of timely laboratory confirmation. We will describe the most common methods used for the diagnosis of rickettsial diseases in dogs.

1.7.1 Microscopic evaluation of a blood smear:

The members of the genera *Ehrlichia* and *Anaplasma* are observed in the form of a morula through the staining with the Wright, Diff-Quik, or Giemsa stains of a peripheral blood smear. The type of cell where the morula is detected is useful for diagnosis,

because although the morulae are indistinguishable, the type of infected cell (granulocyte, monocyte or platelet) can suggest which agent is involved in the infection. For example, the morulae of *E. ewingii* and *A. phagocytophilum* are detected in granulocytes, compared with the morulae of *E. canis* and *E. chaffeensis* that infect mononuclear cells. In contrast, *A. platys* is observed especially in platelets (Sainz et al., 2015). In dogs experimentally infected with *A. phagocytophilum*, the morulae appear as early as 4 days after inoculation and persist for 4-8 days (Egenvall et al., 2000). This method has a low sensitivity (between 50 - 60%) and should be confirmed by PCR in areas where an infection in dogs with more of one member of the *Anaplasmataceae* family occur (Mylonakis et al., 2003). Blood smear on members of the *Rickettsiaceae* family is not useful because the cell target is endothelial cells, but the hemolymph test could be used to screen individual ticks by Gimenez staining (Oteo et al., 2014).

1.7.2 Serologic testing

Serological tests are the most frequently used and widely available methods for diagnosis of tick-borne diseases. The indirect immunofluorescence assay (IFA) is the gold standard test for diagnosing infection with *Ehrlichia*, *Anaplasma* and *Rickettsia* species; however, one of the main limitations of this method is the cross-reactivity that often exists among antigens of pathogens within the same genus and even in different genera such as *Ehrlichia* and *Anaplasma* (Parola et al., 2005). The characterization of major immune-reactive proteins in *E. canis* and *E. chaffeensis* has allowed the development of ELISA-based assays using specific peptides (p28, TRP19/TR32 and TRP36/TRP47) as antigen that provide an accurate serological tool to distinguish infection serologically or identify

coinfection by different genotypes (McBride and Walker, 2011). Some commercial in-clinic ELISA kits are available for detection of antibodies against outer membrane proteins of *A. platys, A. phagocytophilum, E. canis, E. chaffeensis* and *E. ewingii* (Stillman et al., 2014; Qurollo et al., 2014a).

The interpretation of the serological results is the greatest challenge for the clinician due to the timing of the immune responses and the presence of cross-reactive antibodies. The main problems of the serologic diagnosis that may obfuscate the interpretation of a test result are: (1) the immune response against rickettsial pathogens is developed after the clinical signs (1 week to 3 weeks post-exposure) and IgG antibodies may persist for months to years after infection. Therefore, a negative serological result may occur in a patient with acute infection (Gaunt et al., 2010), and (2) a positive serological result may indicate a past exposure or recent infection but does not always indicate an ongoing disease condition. This situation occurs in areas with high seroprevalence rates. Paired sera collected acutely, and 2 to 4 weeks late represent the preferred samples for serological evaluation. An rise of the titer between acute and convalescent phase by ELISA or a 4-fold increase in antibody titer by IFA is considered evidence of an active infection (Sainz et al., 2015). However, molecular tests are recommended to support the serological results, since the presence of bacterial DNA is a sign of active infection (Sainz et al., 2015). In the case of infection by a particular *Rickettsia* species, an IFA titer at least four-fold higher than that observed by any other *Rickettsia* species analyzed is considered to have been stimulated by the infecting *Rickettsia* species (Horta et al., 2004).

1.7.3 Molecular diagnosis by PCR

The polymerase chain reaction (PCR) is currently the most useful tool for detecting *Ehrlichia* spp and *Anaplasma* spp. The PCR compared with other techniques has some advantages: (1) PCR detection is more sensitive than other direct test such as blood smear examination. (2) The detection of DNA of a specific pathogen in an appropriate clinical should be considered evidence of an active infection. (3) PCR detection is particularly important for early stages of infection when antibody levels are very low or undetectable. (4) The continuous development of accurate molecular assays (qPCR) with even greater analytical sensitivity that allows determination of bacterial loads or simultaneously detect several related etiologic agents (Doyle et al., 2005; Qurollo et al., 2014b), and (5) Finally, sequencing of gene fragments amplified by PCR may reveal the identification of the specific *Ehrlichia/Anaplasma* species involved in the infection (Sainz et al., 2015). Several PCR targets have been used, including the 16S rRNA, *dsb* and *groESL* heat shock operon (Ismail and McBride, 2017).

Molecular diagnostic methods based on detection of *Rickettsia* DNA in blood are useful only during the late rickettsemic period when infected endothelial cells detach or contain sufficient quantities of bacteria for detection (Piranda et al., 2008; Eremeeva and Dasch, 2015), but PCR has shown poor sensitivity because SFG rickettsiae infect mainly endothelial cells and normally do not circulate in detectable quantities until the primary stage of acquired disease (Breitschwerdt et al., 1998). Even the presence of rickettsia in blood is intermittent (Labruna et al., 2009). Real-time PCR assays, when carefully designed and optimized, have improved sensitivity (Fournier and Raoult, 2004; Kidd et al., 2008). On the other hand, in the diagnosis of human rickettsioses, other specimens

that contain greater concentration of bacteria, such as crusts of eschars, swabs to obtain material from eschars an their ulcerated base, or biopsies of rash lesions have much better diagnostic yield by PCR (Parola et al., 2013). However, in fur-covered animals, such as dogs, eschars and a rash are not easy to find. In addition, *R. rickettsii* infections are not usually associated with inoculation eschars, which are rarely observed even in human patients with RMSF. Skin biopsies samples from rickettsial proliferation sites (like the ears) have improved detection of the DNA of *Rickettsia* spp by PCR during the acute stage of illness in guinea pigs experimentally infected (Levin et al., 2016). The most common targets used for rickettsial diagnosis include sca5 (OmpB), sca0 (OmpA) and *gltA* genes (Oteo et al., 2014).

1.7.4 Culture

The culture of tick-borne bacteria is generally difficult since they are fastidious organisms, and eukaryotic cell culture systems are required for obligate intracellular organisms (Shaw et al., 2001). In addition, isolation requires specialized expertise and BSL3 facilities, since the samples are considered potentially hazardous (Allison and Little, 2013). For the isolation of rickettsiae, blood with citrate or heparin is used but preferably specimens as skin biopsy (from rash and/or eschar) or attached ticks can be cultured in Vero cells or tick cell lines (Oteo et al., 2014). After inoculation onto cells, *Rickettsia* spp. may be detectable as early as 48 to 72 hours post-inoculation (Angelakis et al., 2012). The recovery of *Ehrlichia* and *Anaplasma* in antibiotic-free mammalian cell culture can also be used to definitively diagnose infection. *E. canis* and *E. chaffeensis* are usually cultivated in a canine histiocytic cell line (DH82 cells) and *A. phagocytophilum* in the

human promyelocytic leukemia cell line (HL-60). Unfortunately, *E. ewingii* and *A. platys* have not yet been successfully isolated in cell culture (Allison and Little, 2013). *Anaplasma* and *Ehrlichia* spp. can be recovered by culture of leukocyte fractions or whole EDTA-treated blood. Cultures are monitored once a week; the supernatant of the cell culture is taken every week, and detached cells are stained with Diff-Quik to search for morulae. A clinical sample may not present morulae observable in culture until 2 to 4 months after inoculation (Thomas et al., 2009). Similarly, *A. phagocytophilum* cultures should be monitored weekly, but the sensitivity of culture for detection of *A. phagocytophilum* can be equivalent to that of PCR and blood smear examination, yielding positive culture after two weeks after infection (Ismail and McBride, 2017).

1.8 Treatment

In dogs and humans, doxycycline is considered the treatment of choice for ehrlichiosis, anaplasmosis and RMSF (Nicholson et al., 2010; Breitschwerdt et al., 1999). For subclinical or chronic CME (without myelosuppression), a dose of 5 mg/kg, orally, twice daily, or at 10 mg/kg orally once daily for 28 days clears the infection in most dogs infected experimentally or naturally. The dose of doxycycline in dogs should not exceed 10 mg/kg (Mylonakis et al., 2019). The eradication of the microorganism must be demonstrated by PCR after completing the treatment. A similar regimen is used for the clinical disease associated with *A. phagocytophilum* and *R rickettsii*. Prompt treatment of a rickettsial disease, especially in the case of RMSF, is critical since delay in the administration of doxycycline can results in the patient's death. Clinical improvement is observed in most dogs within 1–2 days after starting antibiotic therapy (Sainz et al., 2015).

Doxycycline, unlike other tetracyclines, does not seem to cause tooth enamel discoloration in puppies; nevertheless, some dogs may not tolerate the administration of doxycycline due to anorexia, vomiting, diarrhea, or rapid post-treatment elevations of hepatic enzymes subsequent to the treatment (Villaescusa et al., 2015). In this case, the use of doxycycline should be reconsidered. Rifampicin may be an effective alternative to doxycycline for the treatment of CME and CGA with a dose of 15 mg/kg, PO, BID. Alternatively, *A. platys* infection can be treated with enrofloxacin at 5 mg/kg, every 12h for 14-21 days (Sainz et al., 2015). In more severely affected patients, concurrent supportive therapy such as blood transfusion, administration of parenteral fluids and/or prednisone may be of benefit, although corticosteroids may induce relapse and recrudescence in patients with subclinical infections (Allison and Little, 2013).

1.9 Prevention

The prevention of rickettsial infection in dogs must be focused on tick-bite prevention, and tick control in dogs and in the environment. The infection can be prevented by removing any attached tick immediately. It is also recommended to bathe or shower and undergo complete physical inspection shortly after exposure to a tick habitat to remove unattached ticks and locate those that are attached. To remove ticks, gently grasp the tick near to the skin with tweezers, forceps, or a commercially available tick removal device and retract using constant pressure. Prevention has been demonstrated by chemical treatment of animals and the environment for ticks (Otranto et al., 2008). For animals, a specific product can be selected according to the preference of use (collar, pour-on, or spot on). Some compounds, such as the pyrethroids or some

diazinon preparations, are registered as repellents. In urban areas, control of stray dogs may be a good means to control an outbreak and further spread of TBD (Dantas-Torres et al., 2012) .

***Annex 1**. Summary of Tick-borne rickettsial agents known to infect dogs with zoonotic risk in the Americas*

Agent	Disease(s)	Primary vector	Main vector host	Geographic distribution	In vivo-infected cells	Primary clinical manifestations in dogs	Primary clinical manifestations in humans	Severity dogs/humans
Ehrlichia canis	Canine monocytic ehrlichiosis (CME)	*Rhipicephalus sanguineus*	Dogs, wild canids	Southern USA, Central and South America, southern Europe, Africa, Middle East, Eastern Asia	Monocytes macrophages	Fever, weakness, lethargy, anorexia, pale mucous membranes, uveitis, epistaxis, lymphadenopathy, splenomegaly, thrombocytopenia, and weight loss	Fever, rash, headache, myalgia (asymptomatic in some cases)	++++/±
Ehrlichia chaffeensis	Human monocytic ehrlichiosis (HME)	*Amblyomma americanum*	White-tailed deer	USA, South America	Monocytes macrophages	Mild- to-moderate CME	fever, headache, lethargy, myalgia, maculopapular rash	++/++
Ehrlichia ewingii	Human ewingii ehrlichiosis (HEE)	*Amblyomma americanum* (Unconfirmed)	White-tailed deer, dogs	USA	Granulocytes	Mild- to-moderate CME with polyarthritis	Fever, thrombocytopenia	+/+
Anaplasma phagocytophilum	Human granulocytic anaplasmosis (HGA), canine anaplasmosis	*Ixodes persulcatus* complex	Rodents, birds, ruminants, dogs, horses, wild animals	North America, Europe, Asia	Granulocytes	Anorexia, lethargy, fever, epistaxis, vomiting, diarrhea, polyarthritis and thrombocytopenia	Malaise, low-back pain, fever, and/or gastrointestinal disorders	++/++
Anaplasma platys	Canine infectious cyclic thrombocytopenia (CICT)	*Rhipicephalus sanguineus* (Unconfirmed)	Dogs	America, Europe, Asia	Platelets	Less severe than CME with cyclically thrombocytopenia	Weakness, muscular pain, fatigue, decreased appetite, arthralgia and headaches	++/±
Rickettsia rickettsii	Rocky Mountain spotted fever (RMSF)	*Amblyomma cajennense* complex, *Dermacentor variabilis, Dermacentor andersoni, Rhipicephalus sanguineus, Amblyomma aureolatum*	Small mammals, dogs	Americas	Endothelial cells	Depression, anorexia, thrombocytopenia, neurological signs, cutaneous, ocular, and mucosal hemorrhages (ocular lesions and neurological signs are rare with	Fever, headache, myalgia, malaise, rash	++++/++++

						Brazilian strain infection)		
Rickettsia amblyommatis	N/A	*Amblyomma americanum, Amblyomma cajennense complex, Amblyomma spp.*	Birds, rodents, dogs, wildlife	Southeastern USA Central and South America	Endothelial cells	Do not exhibit evident clinical symptoms	Fever, headache, myalgia, rash	-/±
Rickettsia massiliae	Mediterranean spotted fever-like disease	*Rhipicephalus sanguineus*	Dogs	USA, Argentina	Endothelial cells	Mild- to-moderate canine rickettsioses clinical illness not described	Maculopapular rash including soles, headache, nausea, eschar presence	+/+
Rickettsia parkeri sensu stricto	Maculatum infection, tidewater spotter fever, American boutonneuse fever	*Amblyomma maculatum complex*	Birds, rodents, dogs, wildlife	Southern USA	Endothelial cells	Do not exhibit evident clinical symptoms	Fever, headache, malaise, myalgia, arthralgia, eschar, maculopapular rash on the body, palms and soles	-/++
Rickettsia parkeri strain Atlantic rainforest	Cutaneous-ganglionar rickettsiosis	*Amblyomma ovale complex*	Rodents, wild canids, dogs	Central and South America	Endothelial cells	Do not exhibit evident clinical symptoms	Fever, myalgia and arthralgia, macular rash, chills, muscle and joint pain, eschar	-/++

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Chapter II: Arroyave E, Cornwell ER, McBride JW, Díaz C, Labruna M, Rodas JD. Detection of tick-borne rickettsial pathogens in naturally infected dogs and dog-associated ticks from Colombia.

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

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

Short title:

Tick-borne pathogens in dogs and ticks of Colombia

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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 Medellin, 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*. Our results indicate that *E. canis* and *A. platys* are the main TBRP infecting dogs and ticks in Medellin, Colombia. 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 riquétsias 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 riquétsias. O intuito do 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*. Os resultados indicam que *E. canis* e *A. platys* são as principais RTC que infectam cães em Medellín, Colômbia. Porém, é evidente a exposição sorológica dos cães a riquétsias do grupo da febre maculosa.

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

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 and 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., 2016). *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 SFG rickettsiae 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 CICT 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 JW., 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 and 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 (Londono. 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; McCown et al., 2014b). 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 Medellin 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 Medellin 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./µl), mean (red) cell volume (MCV, fL), Hb (g/dL), red cell distribution width (RDW, %), platelets (no./µl), WBCs (no./µl), neutrophils (NE, no./µl), lymphocytes (no./µl), monocytes (no./µl), basophils (no./µl), and eosinophils (no./µl). 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 ≥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. amblyommatis* 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 µl 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 µl 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 $MgCl_2$, 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 µl. 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-Ruíz et al., 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 = (number of infected pools / total number of tested ticks) x 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, (http://genestudio.com/) 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 by the Maximum-Likelihood (ML) algorithms based on the Tamura 3-parameter model (T92 +G+I) using bootstrapping method (1000 bootstrap replicates). 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).

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.

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) (fig1).

The most common abnormal hematological findings in dogs with positive PCR for *A. platys* or *E. canis* were thrombocytopenia (*A. platys* median: 86 $x10^3$ platelets count, *E. canis* median: 98 $x10^3$ platelets count, reference value 290.8 ± 100), and low hematocrit (*A. platys* mean: 38.89% ± 9.46; *E. canis* mean: 36.55% ± 10.72; reference value 52.8 ± 6.5), however, there were no statistically significant differences between the hematologic parameters of canines with *A. platy* 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 and 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; Moraes-Filho et al., 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 GL 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, Paternina et al., 2017; 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., 2016).

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; McCown et al., 2014b). 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;

Lopes 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 and 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 (Londono. 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). Medellin 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 titters (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

The serologic and molecular findings of our study confirm that *E. canis* and *A. platys* are the main TBRP infecting dogs in Medellin, Colombia, 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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Table 1: Primers used for the amplification of some tick-borne pathogens.

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

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

Dog plasma	IFA serological endpoint titers according to *Rickettsia* species					Probable antigen-stimulating antibody response
	R. parkeri	***R. rickettsii***	***R. bellii***	***R. felis***	***R. amblyommatis***	
32	1024	256	-	-	-	*R. parkeri*
39	1024	256	-	64	-	*R. parkeri*
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	-	-	-	*R. parkeri*

-, <1:64

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	*E. canis*		*A. platys*	
			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)

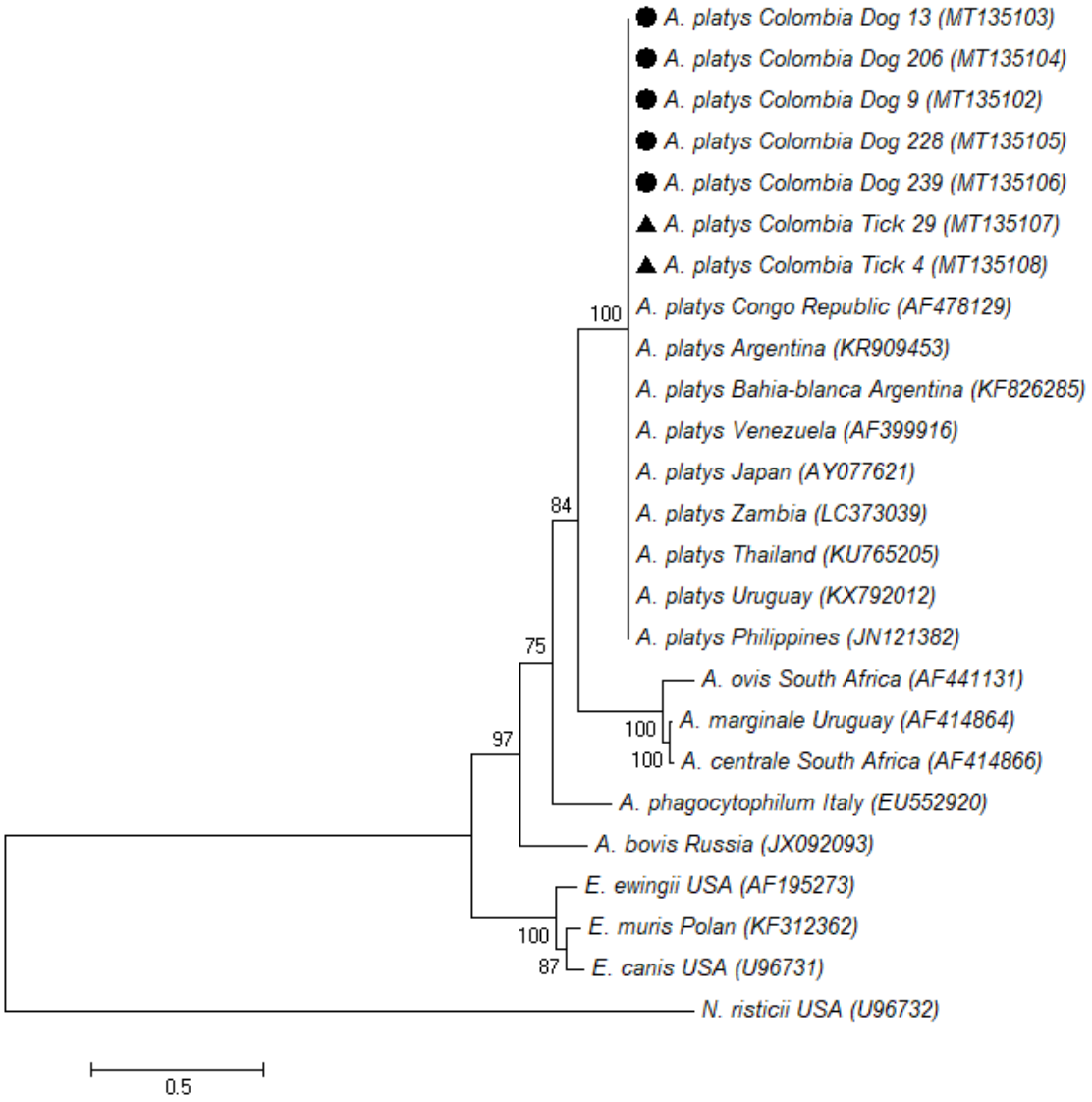

Fig. 1.

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.

Chapter III: Arroyave E, Rodas-González JD, Zhang X, Labruna M, González M, Fernández-Silva J, McBride JW. *Ehrlichia canis* TRP36 diversity in naturally infected-dogs from an urban area of Colombia.

Ehrlichia canis TRP36 diversity in naturally infected-dogs from an urban area of Colombia

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Abstract

Ehrlichia canis is the etiologic agent of a highly prevalent tick-borne disease, canine monocytic ehrlichiosis (CME). Four defined *E. canis* genotypes based on the *trp36* gene sequences have been reported, three of them identified in North or South America. The diversity of *E. canis* has been investigated using genetic and serologic approaches based on distinct 36 kDa tandem repeat protein (trp36) gene sequences that have been reported. The main objectives of this study were to determine the prevalence of *E. canis* infection in dogs from Medellín, Colombia by PCR and determine the *E. canis* diversity using molecular and serologic approaches. Blood was collected from dogs (n=300) with clinical signs of CME for PCR detection of *E. canis* 16S rRNA*, dsb* and *trp36* DNA. Phylogenetic analysis of *trp36* gene sequences was performed using MEGA. A serological evaluation was performed using immunofluorescence microscopy and ELISA with species-specific peptides from *E. canis* TRP19 and TRP36 (3 genotypes) and *E. chaffeensis* (TRP32). *E. canis* DNA (16S rRNA and/or *dsb)* was detected in 18% (53/300) of dogs by PCR amplification. The *trp*36 gene was amplified and sequenced from 35/53 16S rRNA/*dsb* PCR positive samples revealing three genotypes: United States (US; n=21), Costa Rica (CR; n=11), and Brazil (BR; n=3). All dogs with detectable *trp*36 DNA (n=35) had anti-*E. canis* TRP19 and TRP36 peptide antibodies that corresponded to the genotype detected by PCR. Dogs that had antibodies to the TRP19 peptide (82/300; 38%), also had antibodies to one or more genotype-specific TRP36 peptides. Based on TRP36 serology, the dogs exhibited highest frequency of infection with the US genogroup (US = 26), followed by the CR genogroup (CR = 19) and the BR genogroup (BR = 11). Notably, 26/53 *trp36* PCR positive dogs had detectable antibodies to multiple *E. canis*

genotypes (US/BR/CR=8, BR/CR=7, US/CR=6 and US/BR=5) suggesting coinfection or multiple sequential infections with different genotypes. Colombian dogs did not have antibodies to *E. chaffeensis* as determined by a TRP32 species-specific ELISA. Our results demonstrate the presence of three previously defined genotypes in North and South America in Colombian dogs (US, BR, CR). These results also demonstrate that TRP19 and TRP36 serology can provide valuable information regarding *E. canis* exposure and the potential genotype(s) involved in infection.

Keywords: *Ehrlichia canis; c*anine monocytic ehrlichiosis; tandem repeat protein; genotype; ELISA; Colombia.

Introduction

Ehrlichia canis is an obligately intracellular bacterium transmitted by the brown dog tick (*Rhipicephalus sanguineus* sensu lato) and the primary etiologic agent of canine monocytic ehrlichiosis (CME), a serious and sometimes lethal tick-transmitted rickettsial disease (Rar and Golovljova, 2011a). CME is reported worldwide but is more frequently observed in tropical and subtropical regions (Rar and Golovljova, 2011a). Although *E. canis* is primarily associated with canine disease, human infections have been reported in Venezuela (Perez et al., 2006) and more recently in Panama (Daza et al., 2018). Moreover, *E. canis* DNA has been detected in samples from human blood-bank donors in Costa Rica (Bouza-Mora et al., 2016).

Three clinical phases of CME have been described including acute, subclinical, and chronic. Acute *E. canis* infections are clinically characterized by anorexia, fever, weight loss, depression, lethargy, anterior uveitis, and retinal disease (Sainz et al., 2015). Infection can progress into a subclinical phase, in which the dog shows no obvious clinical signs and remains persistently infected for years in untreated or inappropriately treated dogs (Waner et al., 1997). In some cases, dogs may develop chronic severe disease, not easily distinguishable from the acute infections, because many clinical signs are similar; however, the prognosis is less favorable due to bone marrow hypoplasia (Mylonakis et al., 2004; Sainz et al., 2015; Waner et al., 1997). Co-infection of dogs with tick-borne pathogens such as *E. canis* and *Anaplasma platys* is common (Sainz et al., 2015; Santamaria et al., 2014), but the role of coinfection on the clinical course, disease severity, and treatment outcome in dogs infected with *E. canis* has not been investigated. Nevertheless, co-infections likely influence the severity of clinical disease (Mylonakis et

al., 2004).

Molecular characterization of *E. canis* has been accomplished using highly conserved genes such as 16S rRNA, disulfide oxidoreductase (*dsb*), and other immunoreactive protein gene sequences, including the OMP-1 family (p28/30). However, these genes provided little insight to the molecular differences among *E. canis* strains (Aguiar et al., 2013; Alves et al., 2014; Kamani et al., 2013; Siarkou et al., 2007; Zhang et al., 2008). Others have demonstrated that the *trp*36 gene provides more information regarding *E. canis* genetic diversity and can be used for genotyping *E. canis* strains based on amino acid tandem repeat sequences and/or on the numbers of tandem repeats (Zhang et al., 2008; Aguiar et al., 2013; Bouza-Mora et al., 2016; Cabezas-Cruz et al., 2014; Zweygarth et al., 2014). Additionally, phylogenetic analysis of *E. canis trp36* genes has identified 4 genogroups: United States (US) genogroup identified in North America, Brazil, Nigeria, Cameroon, Spain, Turkey and Israel (Ferreira et al., 2014; Kamani et al., 2013; Zweygarth et al., 2014), the Taiwan (TWN) genogroup identified in South Africa, Thailand, Turkey and Taiwan (Nambooppha et al., 2018; Hsieh et al., 2010), the Brazil (BR) genogroup identified in midwest, northern and southern regions of Brazil and recently in Turkey (Aguiar et al., 2013; Aktas and Özübek, 2019) and the Costa Rica (CR) genogroup recently detected in human blood donors from Costa Rica (Bouza-Mora et al., 2016) and described in canines from four Peruvian settlements (Geiger et al., 2018).

The antibody response to species-specific major epitopes located in the tandem repeat regions from two major immunoreactive proteins (TRP19 and TRP36) of *E. canis* has been investigated in experimentally and naturally infected dogs. The high sensitivity and specificity of TRP19 and TRP36 in serological tests, including early stages of

infection, have demonstrated the utility of these proteins for sensitive detection and ability to serologically distinguish infections caused by *E. canis* and specific *E. canis* genotypes (Cárdenas et al., 2007; Doyle et al., 2006; Aguiar et al., 2016).

CME is recognized as the most prevalent tick-borne disease in dogs from Colombia, especially in the urban and suburban areas. However, there is little genetic information available regarding *E. canis* diversity (McCown et al., 2014a; McCown et al., 2014b; Vargas-Hernández et al., 2012). The purpose of this study was to examine the diversity of *E. canis* in naturally infected-dogs from Medellin, Colombia. Our results provide serologic and molecular evidence of three *E. canis* genotypes circulating in the study area.

Materials and methods

Blood collection

Between February 2013 to January 2015, three hundred dogs referred to two veterinary teaching hospitals in Medellin city which had clinical signs of CME, including: fever, lethargy, anorexia and/or weight loss were included in this study. Blood was collected from the cephalic vein into sterile tubes with anticoagulant (EDTA), and then transported to the laboratory at 4 °C for further processing. Whole blood (1 ml) was aliquoted for DNA extraction, and the remaining sample was centrifuged at 2500 rpm for 10 min to obtain plasma. The whole blood and the plasma were stored at -80 °C.

PCR amplification and sequencing

Genomic DNA was extracted from 200 µl of whole blood samples using the DNeasy® Blood & Tissue Kit according to the manufacturer's protocol (Qiagen, Chatsworth, CA, USA). Nucleic acid was eluted into 100 µl of elution buffer and stored at -80 °C. For molecular diagnosis, PCR amplification was performed by targeting 16S rRNA gene of Anaplasmataceae family (Venzal et al., 2007b), and a fragment of *dsb* gene of *Ehrlichia* spp. (Doyle et al., 2005b) using the primers EHR16SD (5´-GGT-ACC-YAC-AGA-AGA-AGT-CC-3´) and EHR16SR (5´-TAG-CAC-TCA-TCG-TTT-ACA-GC-3´) and Dsb-330 (5´-GAT GAT GTC TGA AGA TATGAA ACA AAT-3´) and Dsb-728 (5´-CTG CTC GTC TAT TTT ACT TCTTAA AGT-3´), respectively. Each reaction was amplified using 1U of platinum Taq DNA polymerase (Invitrogen, Brazil), PCR buffer (20 mM Tris-HCl), 1.5 mM $MgCl^2$, dNTP mixture (0.2 mM each), 100∼200 ng of DNA template, and ultrapure Milli-Q water treated with DEPC (AMRESCO, USA), in a final volume of 25 µl. DNA from *E. canis* strain Jake (NCBI:txid269484) was used as a positive control, and DNA from uninfected dog blood was used as a negative control. The PCR was performed using a cycling protocol of 94°C for 5 min, and 35 cycles of 94°C for 30 s, 58°C (16S rRNA) and 54°C (*dsb*) for 30 s 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 GelRed (Biotium®, Fremont, CA, USA). Samples yielding visible PCR products compatible with 345 bp and 409 bp were considered positive for 16S rRNA or *dsb*, respectively.

Samples with detectable *E. canis* DNA through 16S rRNA or *dsb* genes were further investigated using a hemi-nested PCR to amplify the entire *trp*36 gene following

the conditions reported by Aguiar et al., 2014. The first reaction contained primers TRP36-F2 (5´-TTT-AAA-ACA-AAA-TTA-ACA-CAC-TA-3´) and TRP36-R1 (5´-AAG-ATT-AAC-TTA-ATA-CTC-AAT-ATT-ACT-3´) and the nested reaction primers TRP36-R1 and the TRP36-DF (5´-CAC-ACT-AAA-ATG-TAT-AAT-AAA-GC-3´). The thermocycling protocol were 94°C for 5 min, and 35 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 1 min.. Single PCR amplicons (ranging from 800 to 1000 bp) were visualized on 2% agarose gel electrophoresis.

DNA sequencing and phylogenetic analysis

The PCR products were sequenced by Macrogen (Seoul, Korea) and the sequences (forward and reverse) were aligned using GeneStudio (http://genestudio.com/) to obtain the consensus sequences. Alignments were made with MUSCLE (**MU**ltiple **S**equence **C**omparison by **L**og-**E**xpectation). The MEGA 7.0.21 program were used for the phylogenetic analysis, the distance neighbor-joining method was performed to build the phylogenetic tree using the Tamura 3-parameters model plus gamma distribution. The bootstrap test with 1000 replications was applied to estimate the confidence of branching patterns of the neighbor-joining tree. The GenBank accession numbers of the *E. canis trp36* sequences from central and North America were: United States: Jake (DQ085427) and Oklahoma (DQ085428); Mexico: isolates 37 and 97 (KT357369); South America: Brazil: Sao Paulo (DQ146154), Belem (JX429924), Londrina (JX312080), Monte Negro15 (JX312081), and Cuiaba1 (JX312079); Peru: Lima 26 (MF095618) and Paita 1 (MF095619); Central Africa: Nigeria-94 (JN982341); Cameroon-71 (DQ146155); South Africa: 171 (KC479020) and 222 (KC479021); Europe:

Spain-105 (KC479019); Middle East: Israel-Ranana (EU118961) and Israel 611 (EF636663); Taiwan: TWN1 (EF551366), TWN2 (EF560599), TWN3 (EF651794) and TWN4 (EU139491); Thailand: CM172 (MF771083) and CM180 (MF771084) and Turkey: ZKK53 (MG905712), DB51 (MG905713), B12 (MG905714) and mkk2 (MG905711). We used the orthologous sequence TRP47 of *E. chaffeensis* as outgroup. Thirty-two nucleotide sequences for the *E. canis trp36* gene reported here have been deposited in the GenBank database with the accession numbers MN159518 through MN159559.

Indirect immunofluorescence assay (IFA)

Canine plasma was tested by IFA using DH82 cells infected with *E. canis* strain Jake (USA) as previously described (McBride et al., 2001). Briefly, plasma was diluted with phosphate-buffered saline (PBS), starting in at 1:100 dilution and incubated on the slide for 30 min at 37°C. Slides were washed three times with PBS and incubated with FITC-labeled rabbit anti-dog IgG (whole molecule) (Sigma, St Louis, Mo, USA) at 1:100. Slides were rinsed and morulae visualized by immunofluorescent microscopy. Samples that were considered positive at the screening dilution (1:100) were further titrated using serial 2-fold dilutions to determine the endpoint titer. Sera from dogs experimentally infected with *E. canis* (GenBank Accession Number PMC153292) and negative dog plasma were used as controls.

Synthetic peptides and ELISA

All peptides were synthesized (Bio-Synthesis Inc., Lewisville, TX, USA) and the lyophilized peptides were resuspended in molecular biology grade water (1 mg/ml).

Synthetic peptides corresponding to mapped epitopes from *E. canis* TRP19 (HFTGPTSFEVNLSEEEKMELQEVS) (McBride et al., 2007), *E. canis* US TRP36 (TEDSVSAPATEDSVSAPA) (Doyle et al., 2006), and BR TRP36 (ASVVPEAEASVVPEAEASVVPEAE) (Aguiar et al., 2013) and CR TRP36 (EASVVPAAEAPQPAQQTEDEFFSDGIEA) (Bouza-Mora et al., 2016; Geiger et al., 2018). The TRP32 peptide (30 –mer, SDLHGSFSVELFDPFKEAVQLGNDLQQSSD) was used to detect *E. chaffeensis* antibodies (Luo et al., 2008).

ELISA plates (MaxiSorp; Nunc, Roskilde, Denmark) were coated (1.0 µg/well; 50 µl) with each respective synthetic peptide suspended in sterile phosphate-buffered saline (pH 7.4) and incubated overnight at 4°C. The next day, wells with antigen were washed three times with 200 µl tris buffered saline with Tween 20 (2%) (TBST; pH 7.6). The plasma diluted (1:100) in buffer dilution (TBST with 10% horse serum; Sigma H1270) (50 µl) was added to each well and incubated on a shaker at room temperature for 1 h. Plates were washed four times, and 50 µl of the secondary phosphatase-labeled ReserveAP goat anti-dog IgG (H+L) (Kirkegaard & Perry Laboratories, Gaithersburg, MD) antibody diluted in TBST (1:5,000) was added. Plates were incubated at room temperature with gentle shaking for 1 h, washed, and substrate (100 µl) (BluePhos; Kirkegaard & Perry Laboratories) was added to each well. Plates were incubated in the dark for 45 min, and color development was determined on an ELISA plate reader (Multiskan GO; Thermo Scientific, Finland) at a wavelength of A_{650}. Samples with an O.D. reading of 0.2 units were considered positive. A reading of 0.2 to 0.500 OD units was considered a weak positive, and a reading >0.500 OD units were considered a strong positive (Luo et al., 2010).

Results

Partial DNA fragments from 16S rRNA and/or *dsb* genes were amplified from 53/300 (18%) samples. The sequences obtained for both genes exhibited 100% identity with *E. canis*. The complete and partial *trp36* gene was amplified and sequenced only in 66% (35/53) of the 16S RNA/*dsb* PCR positive dogs. Eighteen of the samples remained negative for *trp36* gene, probably due to genetic variations in this gene or because of low sensitivity by the hemi-nested PCR. The complete *E. canis trp36* sequences varied in size (ranging from 615 to 1035 nucleotides), with nucleotide and amino acid identity ranging between 85.6-100% and 78.1-100%, respectively. The phylogenetic analysis on the *trp36* gene revealed three clusters in the Colombian dogs: the US genotype (TEDSVSAPA; 10 to 19 repeats) in 21 dogs, the CR genotype (EASVVPAAEAPQPAQQTEDEFFSDGIEA; 2 to 7 repeats) in 11 dogs, and the BR genotype (ASVVPEAE; 15 repeats) in 3 dogs (Fig. 1).

The length of the TRP36 N-terminal domain was identical among the *E. canis* genogroups (143 amino acids). The Colombian sequences associated to US genogroup (21 samples) were more divergent (between 1 to 5 amino acids substitution in this domain) unlike to the sequences related to the BR and CR genogroups that were highly conserved. Notably, the alignment of the N-terminal domain of the three TRP36 genotypes from Colombia and selected GenBank sequences revealed two variable regions between amino acids located in the positions 30-50 and 137-143 (shaded; Fig. 2). On the other hand, the comparison of the C-terminal domain among the different *E. canis* strain was highly divergent showing a variable length of amino acids ranged from 7 (CR) to 17 (BR) and 30 amino acids in the US genotype (Fig. 3).

Of the 35 dogs that had detectable *trp36* DNA by PCR, only 2 (dogs 167 and 259) had negative serology by ELISA or IFA, which suggest these dogs were recently infected and antibodies had not been developed yet. The remaining dogs (n=33) had antibody responses by IFA and the TRP19 peptide (*E. canis*-specific). Likewise, specific antibodies were detected against the TRP36 peptide consistent with the genotype detected by PCR. Notably, 14/35 (40%) dogs were *trp36* PCR positive and had antibodies that reacted with more than one genotype (US/CR=6, US/BR/CR=5, BR/CR=2 and US/BR=1) (Fig. 4).

We found that 82 (38%) of dogs had antibodies to TRP19 and at least one TRP36 peptide, where 56 dogs had antibodies against a single genotype (US = 26, BR = 11 and CR = 19) and 26 had antibodies to multiple genotypes (US/BR/CR = 8, BR/CR = 7, US/CR = 6 and US/BR = 5). All dogs were negative by ELISA to *E. chaffeensis* TRP32.

Discussion

E. canis is globally distributed and is the most common tick pathogen infecting dogs in Latin America (Ferreira et al., 2014; Montenegro et al., 2017; Saito et al., 2008). High seroprevalence by *E. canis* (ranging from 26-80%) based mainly on commercial serological tests have been previously reported from several cities throughout Colombia (McCown et al., 2014a; McCown et al., 2014b). However, the information regarding the diversity of *E. canis* is limited (Miranda and Mattar, 2015; Vargas-Hernández et al., 2012). The present study provides the first genetic and serological characterization of *E. canis* in Colombian dogs and demonstrates diversity involving 3 genotypes defined by the TRP36 gene/protein in natural infections.

The partial sequences of 16S rRNA and *dsb* genes obtained in this study were

consistent with previous reports that show complete identity with *E. canis* (Aguiar et al., 2013; Zhang et al., 2008). Conversely, the *trp36* has been described as a gene that has a significant divergence in different intercontinental geographic locations (Doyle et al., 2005a; Hsieh et al., 2010). Latin America stands out for having a several reports of different *E. canis* strains including the US genogroup present in Brazil and Venezuela; the CR genogroup reported in Peru and Costa Rica (Bouza-Mora et al., 2016; Geiger et al., 2018) and the BR genogroup described only in Brazil (Ferreira et al., 2014). Our results support previous knowledge about the significant genetic diversity of *E. canis* through the presence of three known genogroups (US, BR and CR) within Medellin city, Colombia and our results indicate that these genogroups are widely distributed in South America.

In this study, the amino acid sequence of the N-terminal domain of TRP36 is highly conserved within each genogroup (98-100%); however, among the US, BR, and CR genogroups there was significant divergence (Doyle et al., 2005a). The analysis on the alignment of the N-terminal region between the Colombian sequences with others sequences available in GenBank revealed that this divergence was located mainly in two variable regions (amino acids 30 to 50 and 137 to 143). The comparison on these variable regions (described in Fig. 2; highlighted in gray) show that each genogroup (USA, BR, CR and TWN) exhibits a different pattern. It suggests that these amino acid differences may have a geographical correlation that may be useful for phylogenetic and serologic analyses.

As previously reported, the comparison of the tandem repeat region suggest a recombination event occurred among BR and CR genotypes (Aguiar et al., 2013; Ferreira

et al., 2014). The BR genotype TR sequence (ASVVPEAE), is almost identical to a region within the CR genotype TR (EASVVPAAEAPQPAQQTEDEFFSDGIEA; underlined), except for a single amino acid substitution (E> A). Recombination has also been reported by others in related agents such as *E. ruminatium* and *Anaplasma marginale* (Graça et al., 2016; Allsopp and Allsopp, 2007; Cangi et al., 2016). Recombination is a mechanism commonly used by pathogens for immune evasion and it is also considered a major driver of genetic diversity in obligately intracellular pathogens (Palmer et al., 2016). This phenomenon could be derived by co-infection among the different genotypes in the same host (Aguiar et al., 2016). According to our serological findings, the dogs from Medellin city have been exposed to multiple genotypes of *E. canis*, which could precipitate a recombination event; however, future studies are needed to elucidate the role of recombination in the emergence of a new *E. canis* TRP36 genotypes.

Previous reports have demonstrated that TR sequences can be used to serologically distinguish infections by different *E. canis* genotypes (Aguiar et al., 2016). We expanded this approach by incorporating the immunoreactive TRP36 peptide based on the CR genotype. Thus, using TRP36 peptides for three genotypes, we determined that all the canines had detectable antibodies against the TRP36 peptide from the respective genotype detected by PCR. Similarly, all the dogs that were IFA positive had antibodies that reacted with the species-specific *E. canis* TRP19 peptide, demonstrating that TRP19 and TRP36 serology can provide valuable serologic information regarding *E. canis* exposure and the potential genotype(s) involved in infection.

Conclusion

This study is the first to investigate *E. canis* diversity in naturally infected Colombian canines. The 16S rRNA and *dsb* were highly conserved, while *trp36* suggests that three previously defined genotypes (US, BR, CR) are circulating in Colombia. These results also demonstrate that TRP19 and TRP36 peptide ELISA can provide valuable information regarding *E. canis* exposure and the potential genotype(s) involved.

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Appendices:

Figure 1: Phylogenetic tree of *E. canis trp36* nucleotide sequences

Figure 2: Comparison of *trp36* N-terminal and tandem repeat sequences of *E. canis*.

Figure 3: Comparison of *trp36* C-terminal domain amino acid sequences of different *E. canis* genogroups.

Figure 4: Reactivity of synthetic peptides derived from three genotypes of TRP36 (US, BR and CR) and TRP19 with antibody in plasma of naturally infected-dogs by ELISA.

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Fig 2: Comparison of TRP36 N-terminal and tandem repeat sequences of *E. canis*.

	E. canis genogroup	Amino acid position																																																	Tandem repeat region sequences[a]
		19	23	24	27	28	30	35	37	41	42	44	45	46	47	48	49	50	58	60	62	63	66	70	72	78	79	80	82	83	85	86	102	111	114	115	117	119	123	126	128	129	130	133	135	137	138	140	142	143	
Colombia Dog-324, 150	US	N	A	H	H	I	Q	G	S	L	E	D	I	E	N	H	P	G	K	N	H	V	D	V	A	N	M	K	H	S	N	V	N	M	Y	Q	G	D	H	P	R	P	A	N	A	V	L	P	G	V	TEDSVSAPA^{14}
Colombia Dog-354, 133, 236		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	TEDSVSAPA^{14,16,18}
Colombia Dog-101, 277, 99		.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	./T	.	.	.	.	.	K	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	TEDSVSAPA^{12,14,12}
Colombia Dog-275, 262		.	.	.	.	.	.	.	.	.	.	.	./V	.	.	.	.	.	.	.	.	.	.	.	.	.	I	N	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	TEDSVSAPA^{16,11}
Colombia Dog-251, 165		.	.	.	.	.	.	.	L	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	./A	.	.	./D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	TEDSVSAPA^{17,13}
Colombia Dog-72		.	.	.	.	.	.	.	.	.	Q	.	.	.	.	.	.	E	N	.	.	.	Y	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	TEDSVSAPA^{16}
Colombia Dog-240		.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	A	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	TEDSVSAPA^{19}
Colombia Dog-232, 70, 167		.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	TEDSVSAPA^{12,16,18}
Colombia Dog-163		.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	TEDSVSAPA^{10}
USA Jake (DQ085427)		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	D	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	TEDSVSAPA^{16}
Mexico 37y97 (KT357369)		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	TEDSVSAPA^{12,14,12}
Brazil Sao Paulo (DQ146154)		.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	TEDSVSAPA^{12,14,12}
Cameroon 71 (DQ146155)		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	TEDSVSAPA^{16}
Nigeria 94 (JN982341)		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	TEDSVSAPA^{12,14,12}
Spain 105 (KC479019)		.	.	N	.	.	.	.	.	.	Q	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	TEDSVSAPA^{8}
Thailand CM 172 (MF771083)		.	.	.	.	.	.	.	.	.	Q	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	TEDSVSAPA^{9}
Turkey ZKK53 (MG905712)		.	.	.	.	.	.	.	.	.	Q	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	TEDSVSAPA^{14}
Israel Ranana (EU118961)		.	.	.	.	.	.	.	.	.	Q	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	TEDSVSAPA^{11}
South Africa 171 (KC479020)	TWN	G	.	N	.	.	S	V	V	.	Q	E	V	G	.	S	G	.	.	.	.	.	.	.	.	.	.	.	R	N	.	A	K	.	E	G	-	E	.	S	L	L	P	S	.	.	.	.	.	.	TEDSVSAPA^{16}
Turkey MKK2 (MG905711)		G	S	N	.	.	S	V	V	I	Q	E	V	G	.	S	G	E	.	G	.	.	.	.	.	D	.	.	R	.	.	A	K	.	E	G	-	.	.	S	L	L	P	S	.	.	.	.	.	.	TEDSVSAPA^{8}
Taiwan TWN1-TWN4 (EF551366)		G	S	N	.	.	S	V	V	I	Q	E	V	G	.	S	G	E	.	G	.	.	.	.	.	D	.	.	R	.	.	A	K	.	E	G	-	.	.	S	L	L	P	S	.	.	.	.	.	.	TEDSVSAPA^{13,12,10,14}
Thailand CM 180 (MF771084)		G	S	N	.	.	S	V	V	I	Q	E	V	G	.	S	G	E	.	G	.	.	.	.	.	D	.	.	R	.	.	A	K	.	E	G	-	.	.	S	L	L	P	S	.	.	.	.	.	.	TEDSVSAPA^{8}
Colombia Dog-323, 321, 123	BR	G	.	N	.	.	S	V	V	.	Q	E	.	G	.	S	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	V	A	I	S	A	T	ASVVPEAE^{15}
Turkey DB51 (MG905713)		G	S	N	.	.	S	V	V	I	Q	E	V	G	.	S	G	E	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	V	A	I	S	A	T	ASVVPEAE^{13}
Brazil Cuiaba 1 (JX312079), Londrina,MN15		G	.	N	.	.	S	V	V	.	Q	E	.	G	.	S	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	V	A	I	S	A	T	ASVVPEAE^{12,15,10}
Colombia Dog-259	CR	.	.	.	.	.	.	.	.	.	Q	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	A	I	S	A	T	EASVVPAAEAPQPAQQTEDEFFSDGIEA^{5}
Colombia Dog-100, 189		.	.	.	.	.	.	.	.	.	Q	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	R	.	.	.	.	.	.	A	I	S	A	T	EASVVPAAEAPQPAQQTEDEFFSDGIEA^{4}
Colombia Dog-226, 197, 49, 25,8 ,3		.	.	.	.	.	.	.	.	.	Q	.	.	.	./D	.	.	.	./R	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	A	I	S	A	T	EASVVPAAEAPQPAQQTEDEFFSDGIEA^{4,7,2,7,7,2}
Colombia Dog-67, 61		.	.	.	.	M	.	.	.	.	Q	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	A	I	S	A	T	EASVVPAAEAPQPAQQTEDEFFSDGIEA^{7}
Peru Paita 1 (MF095619)		.	.	.	.	.	.	D	.	.	Q	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	A	I	S	A	T	EASVVPAAEAPQPAQQTEDEFFSDGIEA^{3}
Peru Lima 26 (MF095617)		.	.	.	.	.	.	D	.	.	Q	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	A	I	S	A	T	EASVVPAAEAPQPAQQTEDEFFSDGIEA^{2}
Turkey B12 (MG905714)		.	.	.	.	.	.	.	.	.	Q	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	A	I	S	A	T	EASVVPAAEAPQPAQQTEDEFFSDGIEA^{5}
Costa Rica CR-H (KU194227)		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	A	I	S	A	T	EASVVPAAEAPQPAQQTEDEFFSDGIEA^{5}

a Amino acids and number of tandem repeats (superscript) among different strains.
*Amino acids highlighted in grey represent two sites of divergence identified among strains.

Fig 3: Comparison of TRP36 C-terminal domain amino acid sequences of different *E. canis* genogroups.

Sequences	*E. canis* genogroup	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Colombia Dogs (15 sequences)	US	T	A	A	T	G	S	T	T	S	Y	N	H	N	T	G	L	E	F	L	D	L	D	S	D	I	L	N	M	L	Y
Colombia Dog-101,99		T	A	A	T	G	S	T	T	S	Y	N	H	N	T	G	L	E	F	L	D	L	**N**	S	D	I	L	N	M	L	Y
Colombia Dog-277		T	A	A	T	G	S	T	T	S	**T**	**T**	**-**	**-**	**-**	**-**	L	**D**	**L**	**S**	**F**	L	D	S	D	I	L	N	M	L	Y
USA Jake (DQ085427)		T	A	A	T	G	S	T	T	S	Y	N	H	N	T	G	L	**L**	D	L	D	**-**	**-**	S	D	I	L	N	M	L	Y
USA Oklahoma (DQ085428)		T	A	A	T	G	S	T	T	S	Y	N	H	N	T	G	L	E	F	L	D	L	D	S	D	I	L	N	M	L	Y
Mexico 37y97 (KT357369)		T	A	A	T	G	S	T	T	S	Y	N	H	N	T	G	L	E	F	L	D	L	D	S	D	I	L	N	M	L	Y
Brazil Sao Paulo (DQ146154)		T	A	A	T	G	S	T	T	S	Y	N	H	N	T	E	L	E	F	L	D	**-**	**-**	S	G	I	L	N	M	L	Y
Cameroon 71 (DQ146155)		T	A	A	T	G	S	T	T	S	Y	N	H	N	T	G	L	E	F	L	D	L	G	S	D	I	L	N	M	L	Y
Nigeria 94 (JN982341)		T	A	A	T	G	S	T	T	S	Y	N	H	N	T	G	L	E	F	L	D	L	G	S	D	I	L	N	M	L	Y
Spain 105 (KC479019)		T	A	A	T	G	S	T	T	S	Y	N	H	N	T	G	L	E	F	L	D	L	D	S	D	I	L	N	M	L	Y
Turkey ZKK53 (MG905712)		T	A	A	T	G	S	T	T	S	Y	N	H	N	T	G	L	E	F	L	D	L	D	S	D	I	L	N	M	L	Y
Israel 611 (EF636663)		T	A	A	T	G	S	T	T	S	Y	N	H	N	T	G	L	E	F	L	D	L	D	S	D	I	L	N	M	L	Y
South Africa 222 (KC479021)	TWN	T	A	A	T	G	S	T	T	S	Y	**D**	**R**	N	T	G	L	E	F	**-**	**-**	L	D	S	**N**	I	L	**K**	M	L	Y
Turkey MKK2 (MG905711)		T	A	A	T	G	S	T	T	S	Y	**D**	**S**	**D**	T	G	**F**	E	F	**-**	**-**	L	D	S	**N**	I	L	**K**	M	L	Y
Taiwan TWN2(EF560599)		T	A	A	T	G	S	T	T	S	Y	**D**	**S**	**D**	T	G	**F**	E	F	**-**	**-**	L	D	S	**N**	I	L	**K**	M	L	Y
Colombia Dog-323, 321 , 123	BR	E	A	L	A	Q	N	L	Q	Q	T	A	I	S	S	L	V	I	I	-	-	-	-	-	-	-	-	-	-	-	-
Brazil Cuiaba 1, Belem (JX429924)		E	A	L	A	Q	N	L	Q	Q	**K**	A	I	S	S	L	**S**	**D**	**N**	**I**	**E**	**D**	**I**	**L**	**S**	**V**	**L**	-	-	-	-
Brazil Londrina (JX312080)		E	A	L	A	Q	N	L	Q	**R**	**E**	**T**	I	S	S	L	V	I	I	-	-	-	-	-	-	-	-	-	-	-	-
Brazil Monte Negro 15 (JX312081)		E	A	L	A	Q	N	L	Q	Q	**E**	A	I	S	S	L	I	I	I	-	-	-	-	-	-	-	-	-	-	-	-
Turkey DB51 (MG905713)		E	A	L	A	Q	N	L	Q	**-**	T	A	I	S	S	L	V	I	M	-	-	-	-	-	-	-	-	-	-	-	-
Colombia Dog (9 sequences)	CR	E	V	L	S	A	F	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Colombia Dog-259, 61		**K**	V	L	S	A	F	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Peru: Paita 1 (MF095619), Lima26 (MF095617)		E	V	L	S	A	F	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Costa Rica CR-H (KU194227)		E	V	L	S	A	F	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Turkey B12 (MG905714)		**K**	V	L	S	A	F	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

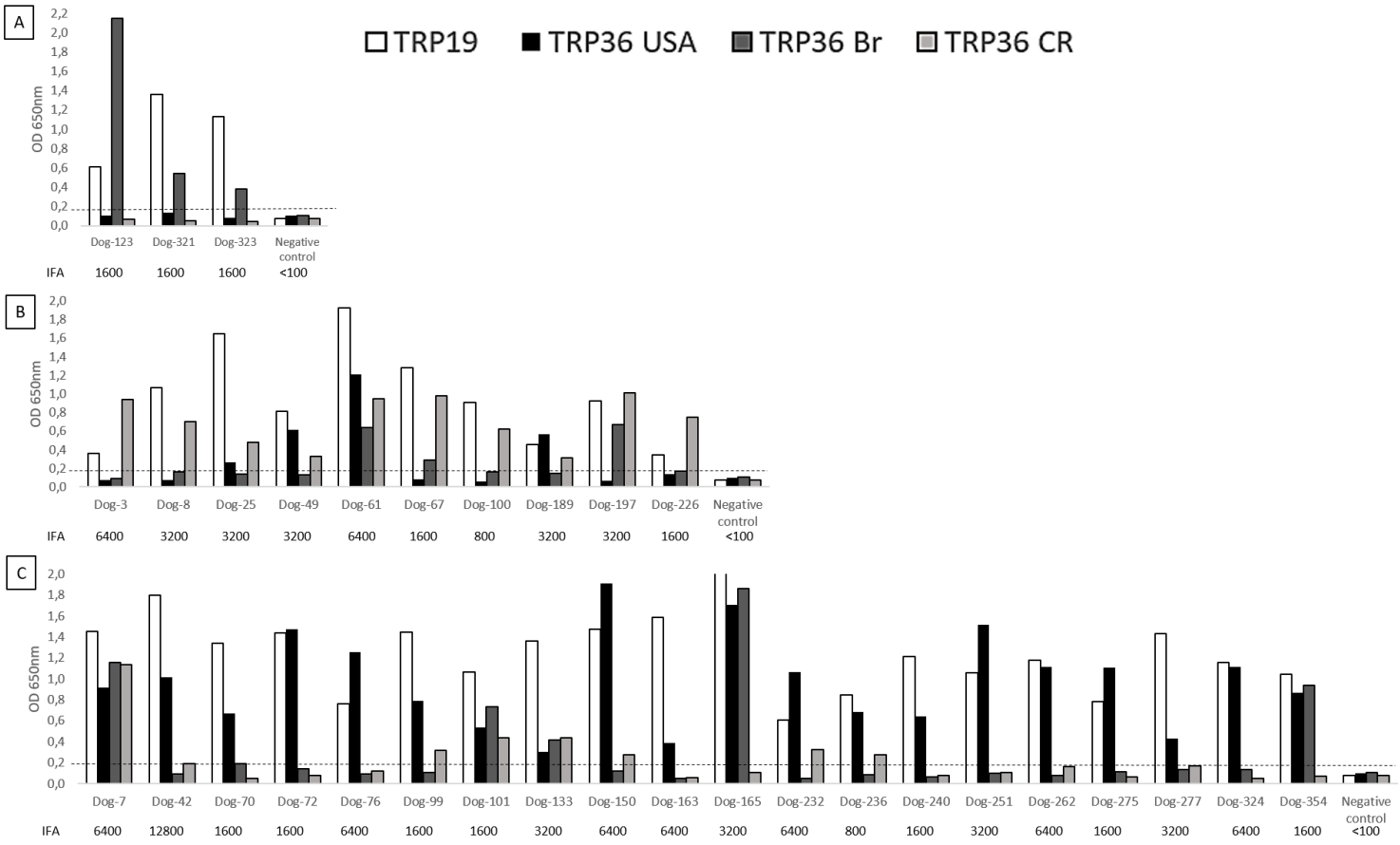

Fig 4: Reactivity of synthetic peptides derived from three genotypes of TRP36 (US, BR and CR) and TRP19 with antibody in plasma of naturally infected-dogs by ELISA. Anti-*E. canis* antibodies in dogs as determined by IFA and ELISA using synthetic TRP19 and TRP36 (US, Br and CR genotypes) peptides sequences for BR **(A)**, CR **(B)** and US genotypes **(C)**. The cutoff OD (0.2) denoted by a dotted line, indicates the threshold for a positive reading. IFA titers are shown below the ELISA data for each dog.

Chapter IV: General discussion and perspectives

1.1 General discussion

Tick-borne diseases are emerging in recent times due to ecological changes and the expansion of ticks to other niches increasingly recognized as a cause of disease in dogs and humans in urban environments. In countries like Colombia, with a significant absence of clinical and epidemiological data or rigorous epidemiological surveillance programs on tick-borne diseases (TBD), field surveillance for infected vectors and reservoir hosts can shed light on human and animal risk to tick-borne disease exposure. Dogs are considered important sentinel animals for human rickettsial infection, since they may suffer a similar clinical illness that humans such as Rocky Mountain spotted fever (RMSF) by *R. rickettsii* or may be asymptomatic (*R. parkeri*) and chronically infected, allowing them to be reservoirs of infection (*A. platys, A. phagocytophilum, E. chaffeensis, E. canis,* and *E. ewingii)*. Even if dogs might not necessarily be the main reservoirs or amplifying hosts they might serve as definitive feeding hosts for vector ticks or carry ticks infected by these pathogens to human dwellings (Sabatini et al., 2010; Nieri-Bastos et al., 2013; Silveira et al., 2007; Szabó et al., 2013).

The firsts evidence about TBD in Colombia started in an outbreak occurred between 1934 and 1936 in Tobia, Department of Cundinamarca, and was named “Tobia spotted fever”, which correspond to RMSF caused by *R. rickettsii* (Patiño et al., 1937*;* Patiño, 1941). This disease affected 20% of the population and had a lethality of 95% (62 out of 65 patients died in this outbreak). Seventy years later, two new fatal cases were confirmed in the same region in Cundinamarca involving to *R. rickettsii* again as the causal agent (Hidalgo et al., 2007). The second endemic area of RMSF in Colombia was recognized after three important SFG rickettsiosis

outbreaks in 2006 (Acosta et al., 2006), 2007 (Hidalgo et al., 2007) and 2008 (Pacheco et al., 2008) have occurred in northwestern of Colombia, in the Departments of Antioquia and Cordoba. In Latin America, RMSF outbreaks occur mainly in rural areas where ticks belong to the *Amblyomma cajennense* complex are the main vectors. We did not find evidence of *A. cajennense* ticks in this study, but our results showed *R. sanguineus* as the most important tick feeding in dogs in the metropolitan area of Medellin Colombia. Recent reports suggest this tick as a vector of *R. rickettsii* in urban areas in Baja California and Mexicali (Álvarez-Hernández et al., 2017) however, this study has no evidence of *R. rickettsii* neither in dogs or ticks. On the other hand, the role of *R. sanguineus* in the transmission of *R. rickettsii* in South America is still speculative, although experimental studies showed that *R. sanguineus* can acquire *R. rickettsii* by feeding on rickettsemic dog and *R. rickettsii* have been detected and isolated in cell culture from *R. sanguineus* collected on dogs in Brazil (Piranda et al., 2011; Pacheco et al., 2011).

The second pathogenic rickettsia was recognized in Colombia in 2014 through isolation of *R. parkeri* strain Atlantic rainforest in *A. ovale* tick retrieved on a dog in a region where evidence of a human case of mild rickettsiosis has been observed (Londoño et al., 2014; Acevedo-Gutiérrez et al., 2019) Ours results showed evidence of exposure to *R. parkeri* s.l. in three dogs, although *A. ovale*, the primary vector for this agent, was not found among the collected ticks on the dogs. In Brazil, others have shown that canines become exposed to *R. parkeri* when they have access to forest areas where the adult stage of *A. ovale* attaches to and feeds on dogs, and humans can be infected with rickettsia if bitten for an infected tick detached of a dog (Szabó et al., 2013). The same ecological scenario may be occurring in Colombia where the edges of urban areas resemble the ecological conditions of endemic areas

for *R. parkeri* strain Atlantic rainforest in Sao Pablo, Brazil (Sabatini et al., 2010; Spolidorio et al., 2010). Little is known about whether canines are susceptible host to *R. parkeri* strain Atlantic rainforest or their role as a reservoir, thus future studies should be conducted to elucidate the role of canines in the epidemiology of this disease.

Interestingly, in our study five canines showed low titers (1:64 to 1:128) suggesting that these dogs may have been infected with a non-pathogenic rickettsia (Breitschwerdt et al., 1988). No rickettsia DNA was found in ticks in our study but further work should to be conducted to determine whether the presence of non-pathogenic or less pathogenic rickettsia agents could prevent the establishment of *R. rickettsii* infection in these tick populations through an interference mechanism that could block a second rickettsia infection (Macaluso et al., 2002b). Otherwise, despite the serological evidence of *R. rickettsii* and *R. parkeri* in dogs in Colombia (Hidalgo et al., 2009, Londoño et al., 2014, Quintero et al., 2017), no clinical cases of rickettsiosis have been documented; nevertheless, cases may not be detected or misdiagnosed due to other more common tick-borne diseases such as ehrlichiosis and anaplasmosis that show undifferentiated clinical signs and respond to the same antibiotic treatment.

In this study, *E. canis* was the most common tick-borne pathogen found in dogs with a prevalence of 82% by IFA and 18% by PCR, this result is consistent with that of others that have been indicated that CME is the most important tick-borne disease in canines from urban areas of Latin America (Saito et al., 2008). The role of *E. canis* as a zoonotic agent is still questionable and should be elucidated. Although there are some reports of human monocytic ehrlichiosis (HME) caused by *E. canis* with moderate clinical signs with fever over 39°C, rash, headache, myalgia, cytopenia

(Perez et al., 2006), there is only one severe case with respiratory distress confirmed in a human immunocompetent patient (Daza et al., 2018). The genetic diversity of *E. canis* defined by the TRP36 gene/protein has been previously demonstrated, the present study provides the first genetic and serological characterization of this agent in Colombian dogs demonstrating the presence of 3 genotypes in dogs naturally infected (USA, Brazilian and Costa Rican genotypes). It is noteworthy that the more recently identified genotype described in Costa Rica associated with human infection, was also found in one of our studies, raising the question about the possible risk to humans by this *E. canis* genotype in Colombia (Bouza-Mora et al., 2016).

Canine infectious cyclic thrombocytopenia (CICT) caused for *A. platys* is the second more important tick-borne disease in dogs in Latin America. A notable increase in the number of reports of *A. platys* in dogs in the recent years suggests an emergence of this agent in Colombia (McCown et al., 2014a; McCown et al., 2014b; Vargas-Hernandez et al., 2016; Posada-Zapata et al., 2017). We found 8% (24/300) of plasma samples with anti-*Anaplasma* spp. antibodies detected by ELISA and 2.6% (8/300) canine blood samples with *A. platys* DNA. Interestingly, our result showed that most canines with antibodies against *A. platys* (16/24) also had antibodies against *E. canis*. CICT is less severe than CME or rickettsiosis, however, co-infection with other tick-borne pathogens increase the severity of a disease (Sainz et al., 2015; Santamaria et al., 2014; Gaunt et al., 2010). In this study, co-infection through DNA detection of *E. canis* and *A. platys* in one dog was demonstrated; however, we did not find hematological differences among the dogs infected with *E. canis* or *A. platys* compared to a dog infected with both agents. Huma anaplasmosis by *A. platys* is not well elucidated, only two human cases have been documented. Patients, both from Venezuela, experienced chronic, nonspecific clinical signs including headaches and

muscle pains and Intra-platelet inclusion bodies resembling *A. platys* in buffy coat smears, and *A. platys* DNA was amplified and sequenced in both patients. Two dogs belonging to one patient also had petechial lesions on the abdomen, thorax, and legs, thrombocytopenia, and antibodies against *Anaplasma* spp. (Arraga-Alvarado et al., 2014)

In our study, only two tick species were collected on dogs. the brown dog tick, *Rhipicephalus sanguineus* s.l. was the main tick specie identified with 92.2% (178/193) and *Rhipicephalus microplus* 7.8% (15/193) retrieved of two dogs from a peri-urban area. *R. sanguineus* s.l. is widely distributed around the world and is the most common tick species found in urban and rural environments where dogs and humans live (Dantas-Torres, 2008). This specie is a three-host tick that feeds primarily on dogs but occasionally on other hosts, including human when ticks populations are high or hosts are scarce (Dantas-Torres, 2008). In the New World, at least two lineages (tropical and temperate) of *R. sanguineus* have been reported showing different geographic distribution and vectorial competence (Moraes-Filho et al., 2011, Moraes-Filho et al., 2015, Nava et al., 2015). The temperate lineage is found in the regions of the United States, northern Mexico and the southern cone of South America (Argentina, Chile, and Uruguay) and the tropical lineage distributed from southern Brazil to northern Mexico, and some areas of the United States (Dantas-Torres et al., 2013; Labruna et al., 2017; Jones et al., 2017).

Rhicephalus sanguineus tropical lineage is the only lineage reported in Colombia (Paternina et al., 2017; Dantas-Torres et al., 2013). Experimental studies show that this lineage is the main vector of *E. canis* and according to previous evidence is also the probable vector of *A. platys* (Ramos et al., 2014; Carvalho et al., 2016; Cicuttin et al., 2015; De Almeida et al., 2012; Lopes et al., 2016), however, the

only study attempting to confirm the vector competence of *R. sanguineus* in the transmission of *A. platys* was unsuccessful (Simpson et al., 1991). Consistent with these results, we showed that *E. canis* was the most common agent found in *R. sanguineus* with 30.4% (21/69 pools); but also, we detected *A. platys* DNA in 6 pools (8.7%), supporting the hypothesis that *R. sanguineus* s.l. is the likely tick involved in the transmission of *A. platys*, since this was the only species found by us in the area where the infected dogs originated. In addition, the ticks with *A. platys* DNA were retrieved from dogs negative for *Anaplasma* spp. through serological and molecular assays, suggesting that these ticks may have acquired the bacteria in the previous life stage. Further studies are required to elucidate the role of *R. sanguineus* as a vector of *A. platys.* On the other hand, all *R. microplus* ticks collected in this study were negative by PCR for *16s rRNA*, *dsb*, *groEL*, and *gltA* genes.

Little is known about other tick-borne pathogens such as *E. chaffeensis, E. ewingii* and *A. phagocytophilum* in South America, although in North America these agents are often associated with canines and human infection. Some cases of ehrlichiosis have been diagnosed serologically in Colombia and Brazil; however, the *Ehrlichia* species associated with the infection was not determined due to the fact that serological methods exhibit cross-reactivity among other species belong to the *Anaplasmataceae* family (Hidrón Botero et al., 2014; da Costa et al., 2006; Calic et al., 2004). Similarly, two reports of potential infection to *A. phagocytophilum* have been reported in Colombia, but there was no DNA evidence that confirmed human cases (Faccini-Martínez et al., 2017; Máttar and Parra, 2006). Our study did not detected evidence of other agents such as *E. ewingii*, *E. chaffeensis* or *A. phagocytophilum* in dogs or ticks.

1.2 Perspectives

The goal of this study was to explore the tick-borne rickettsial pathogens (TBRP) in dogs and in dog-associated ticks and their potential risk to human infection in Medellin, Colombia. Our results proved evidence of infection in dogs for three genotypes of *E. canis* along with *A. platys*, and interestingly, we also showed evidence of exposure in canines with a specie close to *R. parkeri* and with a putative non-pathogenic rickettsia. These results raise new questions that should be solved in future studies.

This study showed that in the study area *R. sanguineus* tick is the main vector of *E. canis,* and is also likely involved in the transmission of *A. platys*, suggesting that individual ticks can be co-infected by both pathogens. Previous studies in *A. americanum* and *Ixodes scapularis* have recognized that co-infection with pathogenic, and even with no-pathogenic agents, may influence the physiology of the tick, and the vector competence, including the ability of the tick to acquire, harbor and transmit a pathogen (Cross et al., 2018). Further studies should be aimed to identify whether *R. sanguineus* has a vector competence for *A. platys* and also whether this tick can be co-infected and transmit both agents after feeding in a vertebrate host. This kind of work could provide a better understanding of the interaction among pathogens and vector.

Unfortunately, we were not successful in obtaining any isolate of *E. canis* in the blood samples collected from the canines that entered this study. The isolation of the Brazilian and Costa Rican genotypes of *E. canis* could allow us to understand the mechanisms of antigenic variation, recognize virulence factors and make progress in control strategies through the development of vaccines, thus, in future projects more effort will be dedicated to this goal.

In South America, cases of human ehrlichiosis have already been serologically diagnosed; however, the species associated with these diseases have not been identified. *E. canis* could be involved in these infections but their role in human cases has not been completed elucidated, even though *E. canis* was amplified in sera samples of patients that exhibit clinical signs but also in asymptomatic human. Further research on owners of dogs infected with *E. canis* could shed light regarding the risk of *E. canis* to public health.

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