



Molecular detection and characterization of *Anaplasma platys* and *Ehrlichia canis* in dogs from northern Colombia

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ABSTRACT

Ehrlichia canis and *Anaplasma platys* are intracellular tick-transmitted bacteria that infect dogs; there is evidence for limited zoonotic potential as well. The prevalence of *E. canis* in Colombia has been evaluated in different regions; however little is known about the prevalence or distribution of *A. platys*. Neither pathogen has been studied in the Magdalena region, thus the purpose of our study was to assess the prevalence of these pathogens in dogs attending veterinary clinics from the cities of Santa Marta and Ciénaga, and to assess possible associated risk factors for infection. *A. platys* and *E. canis* infections in blood were evaluated by Taqman PCR assays. *E. canis* was detected in 26/170 (15.3%, 95% CI 10.4%–21.8%) and *A. platys* in 34/168 (20.2%, 95% CI 14.6%–27.3%) of all dogs tested. Eleven dogs (6.5%, 95% CI 3.4–11.7%) were coinfecting with both pathogens. Sequencing results showed low diversity within *E. canis* and within *A. platys* strains, however a strain of *E. canis* detected in our study area is genetically distinct from strains reported in another city of Colombia. Our results suggest that for *A. platys*, Santa Marta dogs were at greater risk than Ciénaga dogs, and that purebred dogs were at slightly lower risk in both areas. The confirmation of these pathogens in northern Colombia should cause concern for the possible co-transmission of these agents to humans or animals in the region.

1. Introduction

The pathogenic bacteria *Ehrlichia canis* and *Anaplasma platys* cause canine monocytic ehrlichiosis and canine cyclic thrombocytopenia, respectively, and are both transmitted by the brown dog tick (*Rhipicephalus sanguineus sensu lato*), which is widely distributed around the world (Dantas-Torres, 2010). These diseases mainly affect canines, in the case of ehrlichiosis causing potentially fatal, chronic multi-systemic disease with possible hemorrhage, pancytopenia, and lymphadenopathy. Molecular testing of symptomatic human patients detected DNA of these bacteria in Venezuela (Perez et al., 2006; Arraga-Alvarado et al., 2014), Mexico (Silva et al., 2014), and Costa Rica (Bouza-Mora et al., 2017). Therefore, testing dogs for these pathogens is very important to support management of the health of the domestic dog population, and also allows dogs to act as sentinels for human health (Jones et al., 2018).

Although both of these pathogens have been reported in Colombia, studies have been limited to particular regions, and differences in methodologies used make the results difficult to compare. As a consequence, eco-epidemiological aspects and distribution of these pathogens are unclear. Very little is known about *A. platys* in Colombia: one

study documented an *A. platys* PCR prevalence of 2.2% in blood from 91 dogs in the cities of Bogotá, Villavicencio and Bucaramanga (Vargas-Hernandez et al., 2016), and a 53% seroprevalence was found among dogs in Barranquilla (McCown et al., 2014).

In contrast, there is more evidence for *E. canis* from various regions of Colombia including the Caribbean coastal areas. The Caribbean studies confirmed *E. canis* in ticks in the department of Cordoba using DNA sequencing (Miranda and Mattar, 2015); and studies in Barranquilla reported seroprevalence of 74–83% (n = 223) (McCown et al., 2014, 2015). Prevalences of 28% and 6% of *E. canis* and *Anaplasma* sp. respectively were observed by immunochromatography combined with blood smear analyses among 184 dogs attending veterinary clinics (Badillo-Viloria et al., 2017).

No such studies are available for the department of Magdalena in northern Colombia although environmental and socio-economic conditions of this region could support these tick-borne diseases (TBDs). The purpose of this study was to detect and characterize *A. platys* and *E. canis* from dog blood samples from the cities of Santa Marta and Ciénaga in the department of Magdalena, Colombia, using molecular techniques.

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2. Methods

2.1. Samples

Dogs were included in this study by convenience sampling of patients visiting two different veterinary clinics, one in the city of Santa Marta (11°15'56"N, 74°12'09"W) and one in the city of Ciénaga (11°00'12"N, 74°15'33"W) in the department of Magdalena, northern Colombia between January and November 2017. Both cities are located 2 m above sea level and close to the Sierra Nevada mountains. The climate is warm and dry, with an annual rainfall of 362 mm and 622 mm respectively and an average temperature of 28 °C. All animals included in this study were privately owned dogs with an outdoor or mixed indoor-outdoor lifestyle. Some dogs appeared healthy, while others had different clinical signs suggestive of TBDs. Most of the owners were not aware of TBDs and no tick control measures had been used on these dogs. Data on age, sex, breed, and locality were recorded for each dog. Blood samples were drawn from the jugular vein into ethylenediaminetetraacetic acid (EDTA) tubes and kept at –20 °C until DNA extraction.

2.2. DNA extraction

DNA was extracted from blood using the DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, USA) following manufacturer instructions.

2.3. Amplification and sequencing

Real-time PCR amplification was performed using 1 uL of extracted DNA in a 12 uL reaction of Maxima Probe/ROX qPCR Master Mix (ThermoFisher, Waltham, MA, USA) with previously published *dsb* primers for the detection of *E. canis* (Doyle et al., 2005) and proprietary 16S rRNA primers for *A. platys* based on GenBank ID EU004823.1 designed and validated by the Real-time PCR Research and Diagnostics Core Facility at UC Davis (www.vetmed.ucdavis.edu/taqmanservice). For both assays, the thermal cycling protocol consisted of 50 °C for 2 min, then 95 °C for 10 min followed by 50 cycles at 95 °C for 15 s and 60 °C for 1 min. Each reaction contained a positive sample (a sample that had been amplified and for which DNA sequencing confirmed presence of pathogen-specific DNA) and molecular-grade water as negative controls. Results of real-time PCR were considered positive if they had a cycle threshold (CT) value < 40 and a characteristic amplification curve. A CT value > 40 was only considered positive if confirmed with sequencing.

For phylogenetic analyses, the 16S rDNA region of *A. platys* and *E. canis* were amplified from positive dog blood by conventional PCR using 3–5 uL of extracted DNA in 25 uL reactions of AmpliTaq Gold DNA Polymerase (ThermoFisher, Waltham, MA, USA) as previously described by Pinyoowong et al. (2008) and Inayoshi et al. (2004), respectively. Using the same reagents, the *dsb* gene for *E. canis* was also amplified with primers from Labruna et al. (2007). All reactions included molecular-grade water as a control. Amplifications were followed by 1% agarose-gel electrophoresis and were visualized with GelRed® (Biotium, Hayward, CA, USA) under UV-light. PCR products were purified with either ExoSAP-IT PCR Product Cleanup Reagent (ThermoFisher, Waltham, MA, USA) or QiaQuick Gel Extraction (Qiagen, Valencia, CA, USA) and sequenced in both forward and reverse directions on an ABI 3730 sequencer (Davis Sequencing). Sequences were verified using BLAST search of GenBank (NCBI; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Manual edits and alignments were performed in the program CLC Main Workbench v.7.6.2 (Qiagen, Valencia, CA, USA).

2.4. Phylogenetic analysis

The sequences obtained in this study and others available in

GenBank were aligned using the program MEGA 7.0 with the ClustalW algorithm (Thompson et al., 1994).

For phylogenetic reconstruction, Bayesian inference and maximum likelihood analyses were performed in the MrBayes 3.2.2 (Ronquist et al., 2012) and RAxML 8.0.24 (Stamatakis, 2006) programs, respectively. The best nucleotide substitution model for each of the datasets was selected using the Partition Finder program (Lanfear et al., 2012), with the Bayesian Information Criterion. The GTR + G was selected as the best model for the 16S gene of the *E. canis* sequences. The GTR model was selected for the 16S gene of the *A. platys* sequences.

2.5. Data analysis

Data were maintained in Excel (Version 15.34, Microsoft, Redmond, WA, USA) and all statistical analyses were performed in R (Version 3.4.3, R Core Team, 2017). Prevalence and 95% confidence intervals were calculated with the function *prop.test*. Univariate and multivariable logistic regression analyses, as well as Spearman's rank coefficient correlation, were used to assess potential risk factors for either *E. canis* or *A. platys*. The strengths of association were assessed through odds ratios, p-values (≤ 0.05) and 95% confidence intervals. Factors included in multivariable analyses were location, sex, age, and breed. Dogs were first grouped by months of age (0–4, 5–14, 15–60, and > 60) and later as dependent juveniles (< 4 months) or independent adults (> 4 months). Dog breeds were recorded categorically as either purebred (e.g. Golden Retriever, Jack Russell, etc.) or mixed breed. The best model was chosen based on lowest AIC score after a backwards stepwise approach. A chi-square test was used to determine whether coinfection occurred more frequently than would be expected by chance.

3. Results

3.1. Real-Time PCR

Blood from 170 dogs (n = 34 Ciénaga, n = 136 Santa Marta) successfully yielded DNA for screening of TBD (Table 1). Forty-nine dogs were PCR positive for at least one pathogen for an overall TBD prevalence of 28.8% (95% CI 22.2–36.3%) across sites in the department of Magdalena. PCR-positive results included 17 samples (12 *E. canis* and 5 *A. platys*) with CT values above 40 that were confirmed as genus *Ehrlichia* or *Anaplasma* through DNA sequencing. *E. canis* was detected in 26/170 dogs (15.3%, 95% CI 10.4%–21.8%) and *A. platys* in 34/168 dogs (20.2%, 95% CI 14.6%–27.3%). Eleven dogs (6.5%, 95% CI 3.4–11.7%) were coinfecting with both pathogens, but coinfection was less prevalent than expected (p = 0.005). Prevalence of both TBD was higher in Santa Marta than Ciénaga (Table 1).

3.2. Risk factor assessment for TBD

Dogs had 5.0 times greater odds of being PCR positive for *A. platys* if they resided in Santa Marta, but slightly lower odds if they were purebred in the univariate logistic regression model (Table 2). The adjusted odds ratio for *A. platys* was 4.7 for purebred dogs in Santa

Table 1

Sample sizes, demography, and real-time PCR results of privately-owned domestic dogs from two cities in the district of Magdalena, northern Colombia that were screened for *Ehrlichia canis* and *Anaplasma platys* between January and November 2017.

Location	N	Sex F/M	Breed		<i>E. canis</i> Pos/N (%)	<i>A. platys</i> Pos/N (%)
			Mixed	Pure		
Ciénaga	34	16/18	9	25	0/34 (0%)	2/34 (5.8%)
Santa Marta	136	75/61	52	84	26/136 (19.1%)	32/134 (23.8%)
Totals	170	91/79	61	109	26/170 (15.3%)	34/168 (20.2%)

Table 2

Univariate and adjusted multivariable analysis of risk factors that were significantly associated with being PCR positive for *Anaplasma platys* among privately-owned domestic dogs from two cities in the district of Magdalena, Colombia (n = 168).

Risk Factors	Odds Ratio	P-value	95% CI
Location			
Ciénaga	Ref		
Santa Marta	5.0	0.01	1.1–22.8
Breed			
Mixed	Ref		
Pure	0.46	0.01	0.21–1.0
Location + Breed	4.7	0.01	1.0–21.6

Marta in the multivariable logistic regression model (Table 2). Neither sex nor age were significantly associated with *A. platys*. There were no significant predictors for infection with *E. canis* using either univariate or multivariable logistic regression models.

3.3. Amplification, sequencing and Molecular characterization of *A. platys* and *E. canis* from Colombian dogs

Approximately 1.4 kb amplicons corresponding to the targeted 16S rRNA gene fragment were obtained from two *A. platys* PCR-positive and three *E. canis* positive dogs in Santa Marta and were deposited in GenBank under accession numbers: [MK121782](#), [MK138362](#), [MK138376](#), [MK138377](#), [MK138374](#). Amplicons of 410 bp corresponding to the targeted *dsb* gene fragment were obtained from four *E. canis* positive dogs in Santa Marta and were deposited in GenBank under accession numbers: [MK783023](#), [MK783024](#), [MK783025](#), [MK783026](#).

Two *A. platys* Santa Marta 16S rDNA amplicons were sequenced and a complete sequence of 1371 bp was obtained for each one. Both sequences were confirmed as *A. platys*, then aligned and compared to each other as well as 14 other *A. platys* strains reported from France, Thailand, India, Zambia, Cuba, Japan, Spain, China, Venezuela, USA, Croatia, Italy, and Colombia (Sup Table 1). The two *A. platys* isolates were 100% identical to one another and differed by only one single nucleotide polymorphism at position 7 from *A. platys* from France (AF303467), Thailand (EF139459), India (KT982643), Zambia (LC269822), and Cuba (KX792089). Most of the remaining *A. platys* strains showed very close identity ranging from 99.27 to 99.85%. Our 16S fragment had very little sequence overlap with a strain previously reported from Colombia (KF576217, Vargas-Hernandez et al., 2016)

but of the 7 bp that did align, 5 were polymorphic (29% identity). The lack of overlap among sequences generated between these two Colombian studies can be attributed to the use of different primers targeting different regions within 16S.

Three *E. canis* Santa Marta 16S rDNA amplicons were sequenced and a complete sequence of 1360 bp was obtained for each one. All sequences were confirmed as *E. canis* and then aligned and compared to each other as well as 18 other *E. canis* strains reported from Brazil, Turkey, Thailand, India, Venezuela, Italy, Greece, Taiwan, South Africa, Japan, China, USA, Spain, Peru, Israel, and Colombia (Sup Table 2). Two of the three *E. canis* isolates were 100% identical to one another and to *E. canis* from Brazil (EF195135). The third *E. canis* isolate differed by a single nucleotide polymorphism from cytosine to thymine at nucleotide position 859. Santa Marta sequences differed from isolates from Brazil (EF195134), Italy (EU439944), Venezuela (AF373612-3), Greece (EF011110-1), India (JX861392), Turkey (KJ513197), Thailand (EU263991), and Taiwan (GU810149) by a single nucleotide polymorphism at position 905. Several of the remaining *E. canis* strains showed very close identity (99.71–99.85%) but more polymorphisms were observed between *E. canis* Santa Marta and Lima (94.18%), Israel (93.96%), China “Gxht67” (70.77%). Again there was little sequence overlap between our fragment and the fragment from a strain previously reported from Colombia (JN368080, Vargas et al., 2012), yet 2 of 6 bp were polymorphic (67% identity) (Sup Table 2). Both this Colombian isolate and the Chinese isolate were substantially shorter in length, just 362 bp and 967 bp respectively, limiting comparability.

All *dsb* sequences were confirmed as *E. canis*, then aligned and compared to each other as well as 11 other *E. canis* strains available in GenBank: USA ([AF403710](#)), Thailand ([KY576856](#)), Brazil ([GU586135](#), [KP167596](#), [DQ460716](#), [DQ460715](#)), Costa Rica ([KR732921](#)), Cameroon ([DQ124254](#)), Argentina ([MF805005](#), K253450), and Mexico ([KU323869](#)). The four Santa Marta isolates were 100% identical to one another. Similarly, Santa Marta strains were identical to 10 of the comparative *E. canis* strains and differed only from Mexico by 2 bp (data not shown). Due to this lack of variability in the *dsb* region, these sequences were not used in downstream phylogenetic analyses.

3.4. Phylogenetic analysis of *Ehrlichia* and *Anaplasma*

Phylogenetic analyses were performed using a complete 1360–1371 bp fragment of *Ehrlichia* or *Anaplasma* 16S rDNA from this study along with type or reference sequences from 16 *E. canis* or 10 *A. platys* isolates, respectively, for which this fragment was also available in GenBank. The closely related species of *E. chaffeensis*, *E. ewingii*, and *A.*

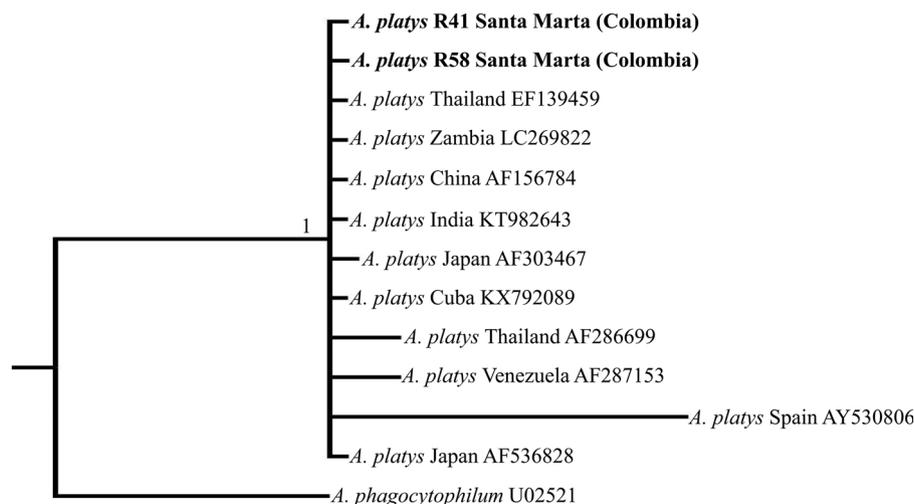


Fig. 1. Tree topology of phylogenetic analyses by Maximum Likelihood and Bayesian Inference including sequences of the 16S gene of *Anaplasma platys* obtained in this work and others from GenBank. The numbers correspond to the values of posterior probabilities, bootstrap values were not included but were < 50%.

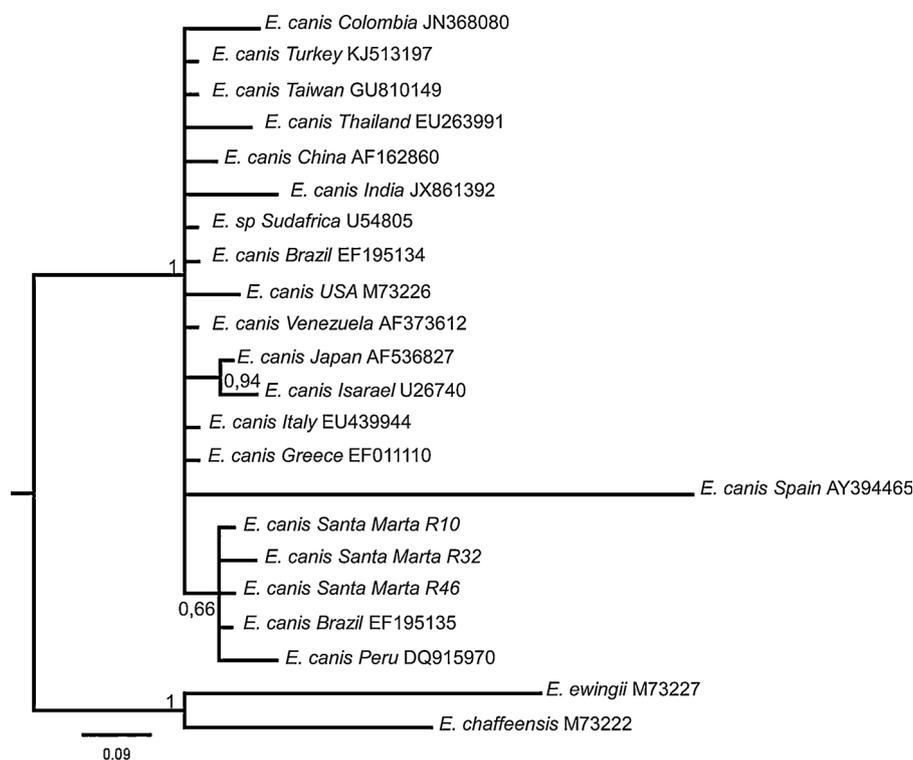


Fig. 2. Bayesian Inference phylogenetic analyses including 16S genes of *Erlichia canis* obtained in this work and others from GenBank. The numbers correspond to the values of posterior probabilities.

phagocytophilum were used as outgroups. The resulting phylogenetic tree for *A. platys* (Fig. 1) revealed that there is global mixture forming a monophyletic clade with no general geographic trend. The resulting phylogenetic tree for *E. canis* (Fig. 2) similarly revealed a worldwide mixture. Our sequences group with sequences from Peru and Brazil, but with low support.

4. Discussion

We have molecularly identified *A. platys* and *E. canis* for the first time in dogs in Magdalena, Colombia, with prevalences of 20.2% and 15.3%, respectively. Although ehrlichiosis and anaplasmosis are frequently diagnosed and treated by veterinarians in the region, the frequencies of these pathogens which we detected were low relative to findings in other regions of Colombia. Possibly explanations could be differing detection methodologies or differing underlying ecologies, possibly related to climatic conditions. Studies in which samples were obtained from feral dogs (McCown et al., 2014, 2015; Vargas et al., 2012) generally showed higher prevalence of infection in comparison to studies like ours in which samples came from owned dogs seen at veterinary clinics (e.g. Badillo et al., 2017). Typically, serostudies would yield higher prevalence than PCR (McCown et al., 2014, 2015; Vargas et al., 2012). In relation to weather conditions, PCR-prevalence detected by McCown et al. (2015) was higher in Barranquilla and Cartagena, when compared to Medellín, with the first two cities being tropical and associated with wetland ecosystems, while Medellín is located approximately 1500 m above sea level, and has a cooler tropical weather. Santa Marta has similar environmental conditions to Cartagena and Barranquilla.

E. canis and *A. platys* share the same tick vector, and dogs may become infected with both pathogens, either simultaneously or sequentially. We found 11 dogs coinfecting with both *E. canis* and *A. platys*, as has been reported in Brazil, with coinfection in 3.4% of dogs in Espírito Santo (Vieira et al., 2018), and 16.1% of dogs in Recife (Ramos et al., 2010). The clinical implications in individuals coinfecting

with *A. platys* and *E. canis* range from pronounced anemia to a reduction in circulating platelets (thrombocytopenia) (Gaunt et al., 2010). In coinfections, one of the two hemopathogens usually predominates, which causes most of the conditions and clinical signs in individuals (Al Izzi et al., 2013). Gaunt et al. (2010) found that *A. platys* infection tended to be more persistent in coinfecting dogs than in dogs lacking *E. canis* infection. They also concluded that coinfections should be considered in dogs with atypically severe or unusual clinical presentations.

Our data and analyses supported the low diversity within *E. canis* and *A. platys* strains already reported in other studies using the 16S rRNA gene (Pinyoowong et al., 2008; Unver et al., 2001). Interestingly, although there was little sequence overlap between our strains and the only 16S sequence previously reported in Colombia, there were polymorphisms in the overlapping section. The phylogenetic analysis also placed the previously reported Colombian strain in a different clade than our Santa Marta sequences. A similar result was recently reported by Daramola et al., 2018, who found molecularly different strains of *E. canis* within Nigeria also using 16S, and by Namboopphaa et al. (2018) who also reported two different genogroups of *E. canis* in Thailand.

Our results also corroborated the low diversity in the *dsb* gene as previously reported (Cicuttin et al., 2016; Namboopphaa et al., 2018), making it useful for molecular detection and corroboration of *E. canis* but not informative for phylogenetics (Namboopphaa et al., 2018). Due to these reasons, resolution of the trees is poor and with low values of bootstraps/posterior probabilities suggesting that more data, and also other more variable regions, such as the *gp36* gene (Namboopphaa et al., 2018), are needed to increase confidence of these relationships.

Infection with *E. canis* or *A. platys* in dogs is associated with different risk factors, with studies reporting higher prevalence among males than females and among older dogs than young ones (Vieira et al., 2013; Barrantes-Gonzalez et al., 2018). We did not find significant predictors for infection with *E. canis*. For *A. platys*, Santa Marta dogs were at greater risk than Ciénaga dogs, and purebred dogs were at slightly lower risk in both areas. Ciénaga is a smaller, more rural city compared to Santa Marta. However, we have no explanation for why

our results contradict other studies that report that regions with less urbanization and a lower socioeconomic status had higher prevalence of pathogens such as *E. canis*, when compared to urban areas (Vieira et al., 2013; Barrantes-Gonzalez et al., 2018; Dantas-Torres et al., 2018). Barrantes-Gonzalez et al. (2018) also found greater risk of infection in mixed-breed dogs, suggesting this was due to ecological factors rather than immunological factors. Owners of purebred dogs might be more cautious or restrictive of their dogs' movements, and mixed-breeds are more likely to be kept outside. However, at least one study has shown that certain breeds are more susceptible to heavy infestations by the vector, *R. sanguineus* s.l. (Louly et al., 2009), so the possibility of some influence of physiological differences between breeds or purebreds and mixed breeds cannot be discounted.

All our samples are from dogs with owners attending veterinary clinics. The proximity between humans and dogs has been suggested as a possible risk factor for human infection with *E. canis* and *A. platys* (Jones et al., 2018). Although *A. platys* is typically considered pathogenic only in animals, it has been detected in the blood of humans and dogs of the same household in Chicago (Breitschwerdt et al., 2014), and in two women with chronic symptoms in Venezuela (Arraga-Alvarado et al., 2014). *E. canis* is distributed worldwide and is considered a serious, potentially fatal canine pathogen. Although initially human ehrlichiosis was thought to be caused by *E. canis* with which it cross-reacts on serology, most cases are now known to be associated with *E. chaffeensis*. Nevertheless, *E. canis* infections have been confirmed in human patients from Venezuela (Perez et al., 2006) and Costa Rica (Bouza-Mora et al., 2017). The molecular identification of *E. canis* and *A. platys* in Santa Marta and Ciénaga, two cities of northern Colombian reinforces the importance of alerting the veterinary community, dog owners, and public health authorities to prevent the risk of transmission of these vector-borne pathogens among dogs and other hosts.

5. Conclusion

Our study confirms for the first time the presence of *E. canis* and *A. platys* in dogs from Magdalena in northern Colombia. Ehrlichiosis is frequently diagnosed and treated by veterinarians in the region, however, we found low frequencies of these pathogens in comparison to other regions of Colombia. This result indicates that unless confirmatory or diagnostic tests are performed, practitioners should not assume a case to be canine ehrlichiosis, even if some signs may appear to be indicative of this disease. We reported new sequences of *E. canis*, molecularly different from the only sequence available in GenBank for Colombia. Also, we report the first 16S sequences for *A. platys* from Colombia, which, as expected, are very conserved with others from around the world. We also found dogs to be coinfecting with both pathogens, which should be considered by veterinarians, as it has been reported that coinfection with two or more tick-borne pathogens may make it difficult to associate a specific clinical sign to a particular canine vector-borne disease. Our results raise questions about possible co-transmission of these agents to other animals in the region.

Declarations of interest

The authors declare that they have no conflict of interest.

Ethical approval

Permission for manipulating the animals was approved by Universidad del Magdalena Ethical Committee (Acta 001–18).

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.vetmic.2019.05.002>.

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