Kafkas Univ Vet Fak Derg
24 (4): 557-562, 2018
DOI: 10.9775/kvfd.2018.19581

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
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Research Article

Anaplasma sp., *Ehrlichia* sp., and *Rickettsia* sp. in Ticks: A High Risk for Public Health in Ibagué, Colombia

Mónica OSORIO [1,a] Jorge MIRANDA [1,b] Marco GONZÁLEZ [1,c] Salim MATTAR [1,d]

[1] Instituto de Investigaciones Biológicas del Trópico, Facultad de Medicina Veterinaria y Zootecnia, Universidad de Córdoba, Montería, COLOMBIA

[a] ORCID: 0000-0003-4176-7550; [b] ORCID: 0000-0002-7110-7791; [c] ORCID: 0000-0001-6500-5601; [d] ORCID: 0000-0003-0526-4630

Article Code: KVFD-2018-19581 Received: 15.02.2018 Accepted: 11.05.2018 Published Online: 11.05.2018

How to Cite This Article

Osorio M, Miranda J, González M, Mattar S: *Anaplasma* sp., *Ehrlichia* sp., and *Rickettsia* sp. in ticks: A high risk for public health in Ibagué, Colombia. *Kafkas Univ Vet Fak Derg,* 24 (4): 557-562, 2018. DOI: 10.9775/kvfd.2018.19581

Abstract

The Order Rickettsiales comprises intracellular bacteria, including Rickettsiaceae and Anaplasmataceae; members of these families cause zoonotic diseases transmitted by ticks. The aim of this study was to make a detection of *Anaplasma, Ehrlichia* and *Rickettsia* in ticks of the Ixodidae (Acari: Ixodidae) family collected from domestic animals in Ibagué, Colombia. Ticks were collected from dogs, horses and cattle, classified taxonomically, and then subjected to DNA extraction. To detect *Anaplasma* sp., *Ehrlichia* sp., and *Rickettsia* sp. we carried out a conventional PCR to detect the *gltA* gene for *Rickettsia*, 16S rRNA for *Anaplasma* and *Ehrlichia*, and the *dsb* gene for *Ehrlichia*. The phylogenetic trees were constructed with the Neighbor-Joining method. A total of 1.247 ticks, mainly *R. microplus, R. sanguineus* and *D. nitens*, were collected. *Anaplasma* and *Ehrlichia* were detected in thirteen samples. The sequences showed a genetic similarity with *E. canis, E. mineirensis, A. phagocytophilum,* and *A. marginale*. No *Rickettsia* was found. This is the first time that active circulation of *Anaplasma* and *Ehrlichia* is demonstrated in Ibagué. Both pathogens are important because can produce economic losses in animals and humans disease. This finding will contribute to the implementation of early epidemiological alerts, as well as the design of measures to prevent and control diseases in the region.

Keywords: *Domestic animal, Environment and public health, Infectious disease vectors, Ixodidae, Molecular phylogeny, Tick*

Kenelerde *Anaplasma, Ehrlichia ve Rickettsia* Türleri: Ibagué, Kolombiya'da Halk Sağlığı Yönünden Yüksek Risk Faktörleri

Öz

Rickettsiales takımı, Rickettsiaceae ve Anaplasmataceae'nın da dahil olduğu hücre içi bakterileri içerir; bu ailelerin üyeleri keneler tarafından bulaştırılan zoonotik hastalıklara neden olurlar. Bu çalışmanın amacı, Colombia, Ibagué'de evcil hayvanlardan toplanan Ixodidae (Acari: Ixodidae) familyasına ait kenelerde *Anaplasma, Ehrlichia* ve *Rickettsia* türlerinin varlığını belirlemektir Köpek, at ve sığırlardan toplanan keneler taksonomik olarak sınıflandırıldı ve ardından DNA ekstraksiyonuna tabi tutuldu. *Anaplasma, Ehrlichia* ve *Rickettsia* türlerini belirlemek amacıyla, *Rickettsia* için *gltA* geni, *Anaplasma* ve *Ehrlichia* için 16S rRNA geni ve *Ehrlichia* için *dsb* genini tespit edecek konvansiyonel PCR yöntemi kullanıldı. Filogenetik yapılandırma için NJ (Neighbour-Joining) analizi kullanıldı. *R. microplus, R. sanguineus* ve *D. nitens* başta olmak üzere toplam 1.247 kene toplandı. On üç örnekte *Anaplasma* ve *Ehrlicha* tespit edildi. Sekanslar *E. canis, E. mineirensis, A. phagocytophilum* ve *A. marginale* ile genetik bir benzerlik gösterdi. *Rickettsia* bulunamadı. Bu çalışma ile Ibagué'de *Anaplasma* ve *Ehrlichia*'nın aktif sirkülasyonu ilk kez ortaya konuldu. Hayvan ve insan hastalıklarında ekonomik kayıplara neden olabileceğinden dolayı, her iki patojen de önemlidir. Bu bulgu, bölgedeki hastalıkların önlenmesi ve kontrol altına alınmasına yönelik önlemlerin oluşturulmasının yanı sıra, erken epidemiyolojik uyarıların uygulanmasına da katkıda bulunacaktır.

Anahtar sözcükler: *Çevre ve halk sağlığı, Enfeksiyöz hastalık vektörleri, Evcil hayvan, Ixodidae, Kene, Moleküler filogeni*

INTRODUCTION

Ticks are important vectors that may transmit pathogens such as viruses, bacteria, and protozoa and they may pose a significant problem in human and animal public health [1]. The Colombian tropic, climate, lush vegetation, high temperature, and the rich fauna in the area, promote the development of tick populations. In addition to these

İletişim (Correspondence)
+57 034 7569027
mattarsalim@hotmail.com; smattar@correo.unicordoba.edu.co

conditions, the animal production systems and human activities can aid in the risk of tick bites, which leads to the transmission of pathogenic agents in animals as well as in humans [2,3].

Genus *Anaplasma, Ehrlichia*, and *Rickettsia*, are Gram-negative coccobacilli of obligatory intracellular growth, and cannot survive outside a vector [4]; they infect endothelial cells, as well as cells in the immune system, monocytes or granulocytes, destroy the phagosome, multiply by binary fission in the cytoplasm, and some in the nucleus of infected cells [5].

The presence of *Ehrlichia, Anaplasma,* and *Rickettsia* in different ticks is closely related to reports of human [6,7] and animal disease cases throughout the world. Bovine anaplasmosis is one of the diseases that causes significant economic losses due to the high morbidity and mortality rates in tropical reported through serological tests in a Colombian army soldier who had been in contact with companion animals [8]. *Anaplasma phagocitophylum* was reported for the first time in serum of rural workers from Córdoba and Sucre [9]. In Colombia, the rickettsiosis is a disease that appears intermittently in tropical areas with some outbreaks [10]. In Latin America, during the last decades, has seen an increase in new species of *Rickettsia* of unknown pathogenicity, many of them detected in ticks at first, some were considered nonpathogenic, but later, their human pathogenicity was demonstrated, such as *Rickettsia massiliae* and *Rickettsia parkeri* [11].

There is no epidemiological information of circulation of *Anaplasma*, *Ehrlichia,* and *Rickettsia* in the city of Ibagué, located at the South-West of Colombia. This lack of epidemiological knowledge sets the ground for confusions, with wrong diagnoses, and therefore, wrong treatment. The purpose of this study was to detect *Anaplasma*, *Ehrlichia,* and *Rickettsia* in ticks collected in the province of Ibagué (Tolima).

MATERIAL and METHODS

Type of Study and Location

The province of Ibagué comprises 3.261 km^2 and eleven municipalities. The province is dedicated to agricultural and livestock activities. The sampled zones corresponded to the municipalities of Ibagué, located at 4º26´10¨N and 75º12´10¨W, Alvarado, El Espinal, Piedras, and Rovira *(Fig. 1)*. The mean annual temperature ranges between 18 and 22ºC, and its altitude ranges between 800 and 1000 meters above sea level.

Tick Gathering

Ticks were collected from dogs, cattle, and horses between August and November 2014. The dogs came from veterinary clinics and cattle and horses came from farms in the Ibagué area. The ticks were preserved in ethanol and identified through the taxonomic keys [12,13]. The ticks were arranged in groups of 2 to 5 ticks of the same genus, species, and host.

DNA Extraction

The protocols described by Miranda et al.[14] were followed with slight modifications. In the DNA extraction, we selected a 200 mL volume of the homogenized material to extract DNA from the commercial QIAamp™ DNA Mini kit (Qiagen, Chatsworth, CA). The extracted DNA was stored at -20°C until its PCR analysis.

Molecular Identification of Anaplasma, Ehrlichia and Rickettsia

For *Anaplasma* and *Ehrlichia,* we performed a conventional PCR using primers GE2` F2` (5´-GTT AGT GGC AGA CGG GTG AGT-3´, forward) and HE3 (5´-TAT AGG TAC CGT CAT TAT CTT CCC TAT-3´, reverse) to amplify a 360 bp fragment of the *16S rRNA* gene of the Anaplasmataceae family, and dsb-330 (5´-GAT GAT GTC TGA AGA TAT GAA ACA AAT-3´, forward) and dsb-728 (5´-CTG CTC GTC TAT TTT ACT TCT TAA AGT-3, reverse) to amplify a portion of 409 bp of gene *dsb*, for *Ehrlichia* sp.[15].

For *Rickettsia* sp., we used primers CS-78 (5´-GCA AGT ATC GGT GAG GAT GTA AT-3´, forward) and CS-323 (5´-GCT TCC TTA AAA TTC AAT AAA TCA GGA T-3´, reverse) to amplify a 401 bp fragment of gene *gltA* and 120.2788 (5´-AAA CAA TAA TCA AGG TAC TGT-3´, forward) and 120.3599 (5´-TAC TTC CGG TTA CAG CAA AGT-3´, reverse) for the 812 bp portion of the *ompB* gene [16]. PCR products were visualized in a 1.5% agarose gel, stained with ethidium bromide.

Sequence Analysis

The amplified products were sequenced with the dideoxy method in an automatic MegaBACE 750 (Amersham, Biosciences, Piscataway, NJ, USA) DNA sequencer. The nucleotide sequences of the positive samples underwent a BLAST to determine their similarity with other species of *Anaplasma, Ehrlichia,* and *Rickettsia*. The obtained sequences were aligned through the Geneious R9.1.2 and MEGA 7.0 software, the phylogenetic relations were inferred using the Molecular Evolutionary Genetics Analysis (MEGA 7.0) software [17]. For each analyzed gene, we built a phylogenetic tree with the Neighbor-Joining method, using the Kimura parameter as a nucleotide substitution model. The reliability of the phylogenetic tree analysis was determined by bootstrap with 1000 replications.

RESULTS

A total 1.247 adults ticks were collected from bovines, equines, and canines, and three species were identified: *Rhipicephalus sanguineus* (50.8%), *Dermacentor nitens* (27.3%) and *Rhipicephalus microplus* (21.9%). We found 685

ticks (54.9%) on 50 canines, 371 (29.7%) on 13 equines, and 191 ticks (15.3%) on 16 bovines. A total 119 groups were formed to extract DNA.

The 16S rRNA 4.2% (5/119) and *dsb* 6.7% (8/119) genes were amplified. Amplifications of gene 16S rRNA were from *R. microplus* collected from bovines; *dsb* gene amplifications were from *R. microplus* and *R. sanguineus* collected from canines in the areas of Alvarado and Ibagué. The conventional PCR was negative for *gltA* and *ompB* gene amplification in *Rickettsia*.

Sequence analysis with samples who amplified the two genes 16S rRNA and *dsb* showed 99% and 100% similarities with *Ehrlichia mineirensis* (KF621013.1, JX629805.1, and DQ379966 for 16S rRNA and KT314243.1, KM015219.1, JX629808.1 for *dsb*) in three bovine samples. Those for gene 16S rRNA in *R. microplus* DNA samples showed 99% similarity with *Anaplasma marginale* (KP877314.1, AB916498.1 and LC007100.1) and 99% with *A. phagocitophylum* (EU287434.1, KJ195692.1 and GU111741.1), collected from bovines in Alvarado and Ibagué, respectively.

The *dsb* gene analysis showed that, in four groups of canines, sequences with 99% and 100% similarity with *Ehrlichia canis* (KR732921.1, AF403710.1 and KU323869.1) were found; these groups were amplified from *R. sanguineus*. The phylogenetic trees with genes 16S rRNA and *dsb* show the various positions of the partial sequences of the genes with those available in GenBank *(Fig. 1, Fig. 2)*.

DISCUSSION

Anaplasma marginale and *E. canis* were detected in *R. microplus* and *R. sanguineus*; this is an important finding, since it is the first time they are detected in Ibagué, an area in the Colombian southwest. Finding *E. mineirensis* in Ibagué for the first time is also important, since it had only been reported in Córdoba (Colombia) [18].

In Colombia, many species of ticks have been described [12]. However, the diversity of ticks species collected in the present study could be explained by the number of canine, bovine, and equine hosts from where the ectoparasite samples were collected. On the other hand, despite finding *R. sanguineus* in two host species, it may also develop in rodents and other mammals, but the dog is the primary host and plays an important role in the development of high populations of this ectoparasite [19]. *R. microplus* is a primary pathogen in bovines [20,21]; however, it may appear in several hosts among which we can highlight buffalo, horses, donkeys, goats, sheep, deer, pigs, dogs, and some wild animals [22]. In this study, *R. microplus* was found in the three types of host vertebrates but mainly in bovines, which demonstrates its adaptability.

In this study, 119 groups of adult ticks were analyzed. Having only adults ticks limit the association of other stages with possible infection processes with *Anaplasma* and *Ehrlichia* and their vectorial capacity, since it has been identified

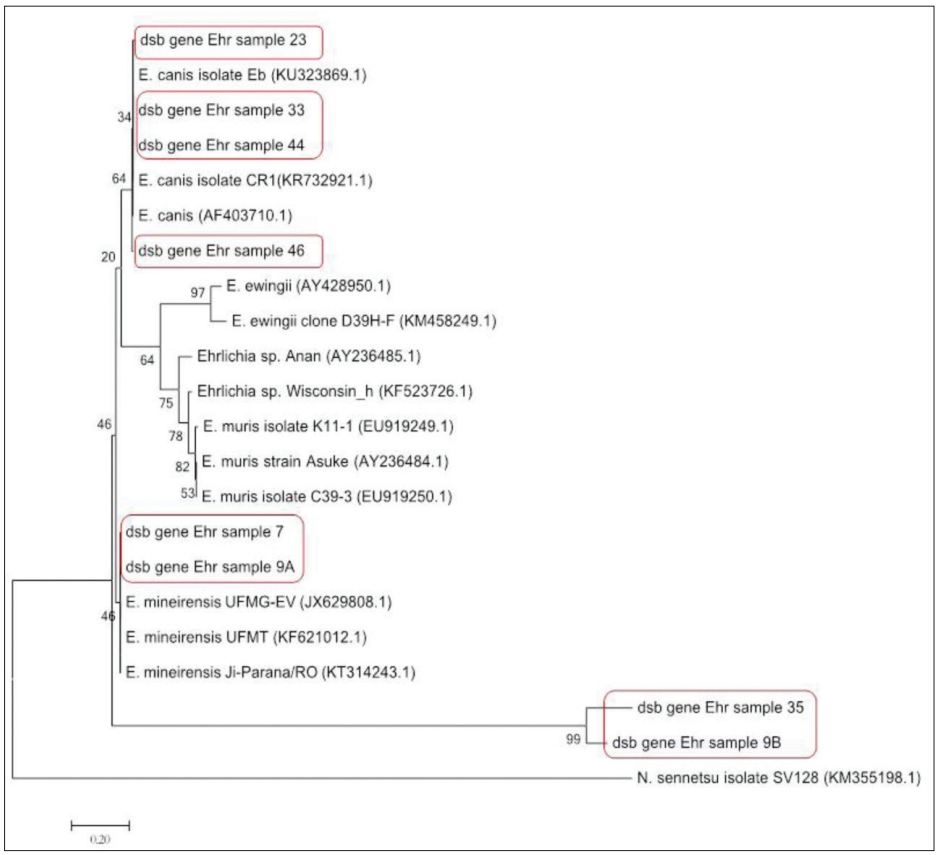

Fig 1. Phylogenetic tree showing samples positions, obtained by comparing the partial sequences of ribosomal 16S RNA gene (*16S rRNA*) with *Anaplasma* sp. and *Ehrlichia* sp. sequences available at GenBank (access number in parenthesis). The Neighbor-Joining method was used, based on the Kimura parameter model. The beginning of the branches show the bootstrap values (1000 replications). The analysis was carried out using the MEGA 7 program. The sample groups of the study are framed in a rectangle. *Neorickettsia sennetsu* was used as an external group

Fig 2. Phylogenetic tree showing samples positions, obtained by comparing the partial sequences of the *dsb* gene available at GenBank (access number in parenthesis). The Neighbor-Joining method was used, based on the Kimura parameter model. The beginning of the branches show the bootstrap values (1000 replications). The analysis was carried out using the MEGA 7 program. The sample groups of the study are framed in a rectangle. *N. sennetsu* was used as an external group

that larvae awaiting a host show more aggressive behavior in mass attacks to susceptible hosts; this description has been documented on human infestations of *Amblyomma cajennense* [23].

This study detected a group of ticks with a sequence 99% similar to *A. marginale* in a group of *R. microplus* ticks. The results of this study match with the research made by Wen et al.[24] in Tibet, who detected *Ehrlichia* and *Anaplasma* in *R. microplus* collected in cows. In Mongolia, Ybanez et al.[25] made the molecular characterization of *A. marginale* species detected in 8 (13.3% positivity) *R. microplus* from bovine cattle. *R. microplus* is important in veterinary medicine because it transmits *A. marginale*, which causes great economic losses to livestock farming in many subtropical and tropical countries [18]. *R. microplus* is not considered an ectoparasite that transmits pathogens to humans [20], however, *R. microplus* has been found bitten humans [19].

Argas persicus, *Rhiphicephalus annulatus*, *Rhiphicephalus decoloratus*, *R. microplus*, *Dermacentor albipictus*, *Dermacentor andersoni*, *Dermacentor occidentalis*, *Dermacentor variabilis*, *Ixodes ricinus*, *R. sanguineus,* and *Rhiphicephalus simus* are among the vector ticks that mechanically or biologically transmit *A. marginale*. Intra- or interspecies transmission is common in single-host *Rhiphicephalus* species; also, some authors have describe them as significant vectors of anaplasmosis in regions such as Central America, Latin America, the Caribbean, Australia, and South Africa [26]. *A. marginale* has been identified in differents regions in Colombia, farms animals in the Colombian Caribbean, found 31.7% (923 of 2909 animals) of positives to *A. marginale* in 101 of 104 farms studied [27]. Researchers in the Department of Córdoba report sequences with 99-100% identity with *A. marginale*, in the DNA of ticks collected from dogs, horses, and cows [18]. In Purificacion (Tolima), a 20% of *A. marginale* was reported with Giemsa stain on bovine coccygeal vein peripheral blood bovine [28]. This results in different regions of Colombia, confirm ours findings where the sequence detected showed a high genetic identity with *A. marginale*.

Anaplasma phagocytophilum has been confirmed in Asia and Europe and some countries in South America [29,30]. Serological studies in dogs from three cities of Colombia during 2011, reported *A. phagocytophilum* in 51%, 40% and 12% in Cartagena, Barranquilla and Medellin cities respectively [31]. Seroepidemiological data suggest that many human infections go unrecognized in Sucre and Cordoba (north of Colombia), where a prospective study in people with occupational risk factors (farmers and day workers) found a seroprevalence rate of 20% (15/75) for *A. phagocytophilum* [9]. There are a few reports of *A. phagocytophilum* in ticks from Colombia. This work has detected a sequence 99% identity with *A. phagocytophilum* in DNA of *R. microplus* collected in bovine from Ibagué area.

Five species of *Ehrlichia* have been identified, out of which three are known to cause human ehrlichiosis *(E. canis*, *E. chaffeensis* and *E. ewingii)* [32]. The phylogenetic characterization of *Ehrlichia* species isolated from ticks has identified *E. mineirensis* as a new species that has been reported in some parts of Latin America and Brazil [33]. It has been reported in Colombia by Miranda & Mattar [18]. New studies aimed at identifying this bacteria

are indispensable, in order to confirm the adaptation evidence just with *R. microplus* or with some other type of vectors. Our results agree with other studies, four ours sequences detected in *R. microplus* collected from bovine were 99 to 100% identical to *E. mineirensis*, further work to do a better characterization and to establish the current status of this strain are necessary, as well as needed to clarify the pathogenic potential, geographic distribution or host range of this agent.

Ehrlichia is one of the most reported species in South American vertebrates, and has been proven to circulate in canines in Colombian cities such as Cali and Medellín [34-36]. Dogs are the final hosts of ticks that transmit ehrlichioses, and therefore this disease must be considered as a public health issue due to its zoonotic potential [37].

Vargas-Hernandez et al.[36], detected *E. canis* in *R. sanguineus* ticks collected from dogs in Bogotá, Bucaramanga, and Villavicencio. *E. canis* is nowadays considered as a species that causes ehrlichiosis; not only has it been reported in canines, but also in felines and humans, and many of these cases have been detected from the transmission by *R. sanguineus* bites [38]. In this study, partial sequences 99-100% similar to *E. canis* were identified from the extraction of DNA from *R. sanguineus* captured on canines.

In the present work, the samples were amplified using conventional PCR, but yielded no positive results for *Rickettsia*. Studies conducted in Colombia have been identified the presence of *Rickettsia belli* [39], *Rickettsia rickettsii* [40], *Rickettsia felis* [41], *Candidatus Rickettsia colombianensis* [14] and *Rickettsia* sp. *Atlantic Rainforest strain* [42]. More ticks, especially from the *Amblyomma* genus, must be collected, and from other hosts different from those in this study, in order to detect species of *Rickettsia* in the zone.

In conclusion, the bacteria species reported in this study had not been previously reported in the department of Tolima, on the south-west of Colombia, and are of great interest for the region, since they are pathogens that cause diseases which entail economic losses in animals and humans. Detection of *Anaplasma* and *Ehrlichia* is fundamental in order to implement early prevention and control alerts by the epidemiological surveillance authorities in the region. All the cases evidenced in previous research and in this study represent a reappearance of such diseases, and must alert the epidemiological surveillance authorities of the country.

Acknowledgements

To the Mellitopalinological Research and Physico-chemical Properties of Foods Research Group of Universidad del Tolima for lending physical space and the equipment for the taxonomic classification of ticks. To Institute for Tropical Biological Research of Universidad de Córdoba, for all the financial and laboratory infrastructure support to undertake this study.

Conflicts of Interest

The authors hereby declare that we do not have any conflict of interest in regards to the information provided in this study.

Financiacing

Institute for Tropical Biological Research of Universidad de Córdoba.

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