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Molecular detection of *Candidatus* Rickettsia colombianensi in ticks (Acari, Ixodidae) collected from herpetofauna in San Juan de Carare, Colombia

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ABSTRACT

Knowledge about ticks and Rickettsiae in Colombia is still limited and the areas of the country where studies have been conducted are scarce. In this study, ticks (Acari, Ixodidae) associated with reptiles and amphibians in San Juan de Carare, Santander department, were morphologically and molecularly identified and tested for the presence of Rickettsia. For the molecular characterization of ticks, CO1, 12S and 16S sequences were generated and compared with other sequences available in genbank. Our analyses confirmed that the collected ticks were *Amblyomma dissimile*, and we provide the first report of this species parasitizing the snake *Leptodeira septentrionalis*. Of the samples analyzed, 69% were positive for *Rickettsia* sp. using the gltA, ompA and sca1 genes. Rickettsia sequences generated in this study were also compared to sequences downloaded from genbank by Bayesian inference (BI) and maximum likelihood (ML) phylogenetic analyzes. The presence of a single Rickettsia species, *Candidatus* Rickettsia colombianensi, was identified. This study expands the knowledge regarding the distribution of *A. dissimile* ticks and Rickettsiae in Colombia.

1. Introduction

The genus *Rickettsia* has been the causal agent of epidemics in different areas of the world, and biological groups such as mites, ticks, lice, and fleas are the main vectors (Vélez et al., 2012). One of the diseases that this group of bacteria transmits is rickettsiosis, which has taken a leading role as an emerging and re-emerging disease affecting both animals and humans (Hidalgo et al., 2013). *Rickettsia* species have been found in various taxa, such as amphibians, reptiles, birds, mammals, and arthropods, the latter acting as vectors (Bernabeu and Segura, 2005).

Herpetofauna has been studied more in recent years from an epidemiological perspective, evaluating its possible role in the epidemiology of various zoonotic diseases (Aguillón et al., 2007). In some scenarios, the rapid settlement of human communities in rural areas makes it possible for them to become part of the epidemiological cycle of zoonotic pathogens and alters the existing relationships between vectors and reservoirs, causing the emergence and resurgence of diseases (Monsalve et al., 2009).

Different tick species have been associated with reptiles and amphibians in the region. *Amblyomma dissimile* was reported as a parasite of herpetofauna, especially reptiles in the Caribbean, Andean and Orinoquia regions of Colombia (Carrascal et al., 2009; Cotes-Perdomo et al., 2018; Mora-Rivera et al., 2020; Osorno-Mesa, 2006; Santodomingo et al., 2018), while *A. rotundatum* was found in the Pacific region (Benavides-Montaño et al., 2018; Osorno-Mesa, 2006).

There are reports of *Rickettsia* associated with ticks that have been collected in reptiles and amphibians in northern regions of Colombia such as La Guajira, Magdalena, and Cordoba. These studies report the same *Rickettsia* strain, Candidatus *Rickettsia colombianensi*, a Ricketssia of unknown pathogenicity, but closely related to old world pathogenic species such as *R. monacencis* and *R. tamurae* (Miranda et al., 2012; Cotes-Perdomo et al., 2018; Santodomingo et al., 2018, 2019). *Rickettsia monacencis* and *R. tamurae*, together with *R. slovaca*, *R. aeschlimannii*, and *R. massiliae* were initially considered non-pathogenic, but today they are known to cause disease in humans (Parola et al., 2005; Jado et al., 2007). This makes it imperative to have a record of the presence of Rickettsiae in the region, its distribution and possible hosts, in order to

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be able to design adequate prevention and control strategies.

The objective of this work was to evaluate the presence of *Rickettsia* spp. in ticks associated with reptiles and amphibians in San Juan de Carare region in the department of Santander, Colombia, a region in where such studies have not been previously performed.

2. Material and methods

2.1. Study area and herpetofauna sampling

The samples were collected in the locality of San Juan de Carare, Cimitarra municipality, Santander Department, Colombia; located at 6° 42 ′58 0.20″N and 74° 08 ′02 0.71″W during five visits between March 2017 and May 2018. This locality exhibits temperature ranges from 23.4 °C to 32.9 °C, an approximate altitude of 92 m above sea level (Alfonso, 2009), and comprises a series of perpetual and seasonal wetlands along the San Juan River. We carried out vertebrate collections in two types of environments: around villages, hereafter referred to as the "urban environment", and inside forest patches, hereafter referred to as the "sylvatic environment". Hosts were captured by visual encounter search method, physically restrained, placed in cloth bags individually and identified morphologically at species level. We identified the specimens based on Rengifo and Lundberg (1999), Páez et al. (2002), and Castro-Herrera. and Vargas-Salinas (2008). We followed the taxonomy proposed by Uetz (2016: www.reptile-database.org) for reptiles. Ticks were removed with forceps from each individual host and stored in 1.5 ml vials with 96% ethanol until processing in the laboratory.

2.2. Morphological identification of ticks

The morphological identification of the adult ticks was done using the taxonomic keys of Voltzit (2007), Osorno-Mesa (1940) and Jones et al. (1972). For nymphs, the key developed by Martins et al. (2010) and for the larvae the key of Osorno-Mesa (1940) was used. Ticks were separated by stage and host.

2.3. Molecular characterization of ticks

Fourteen tick samples, including larvae analyzed individually and in pools of five, 4 nymphs (analyzed individually), and 6 adults, collected in three different host were molecularly confirmed (Supplementary Table 1). The DNA was extracted following the "ISOLATE II Genomic DNA" Kit (BIOLINE, USA) protocol. DNA extractions and its quality were confirmed by performing 2% agarose gel electrophoresis stained with "GelRed" (Biotium). CO1, 12S and 16S genes were amplified using the primers shown in Supplementary Table 2. Amplifications were carried out in a thermocycler (Eppendorf Mastercycler. Pro), in a final volume of 25 µl, including in each reaction 2 µl of DNA, 0.25 µl of Taq polymerase (5 u/µL BIOLASE ™, Bioline USA), 1 µl of MgCl (50 mM), 2.5 µl of buffer PCR (10X), 0.5 µl of dNTP (10 mM) and 0.5 µl of each primer (10 µM). The products were visualized by electrophoresis on 2% agarose gels with GelRed staining (Biotium). Each of the samples obtained in the amplification was purified using the SureClean purification kit (Bioline USA). The PCR products were sequenced in both directions.

2.4. Molecular detection of Rickettsiae

Amplifications of the genes gltA, ompA and sca1 were performed using a conventional PCR in an "Eppendorf Mastercycler Pro" thermal cycler, using the primers and specifications shown in Supplementary Table 2. The final reaction volume corresponded to 25 µL, which contained 1 µL of MgCl (50 mM), 2.5 µL of buffer (10X, PCR buffer), 1 µL of dNTPs (10 mM), 1 µL of each primer (10 pmol, forward and reverse), 15 µL of water, 0.5 µL of Taq polymerase (5 U/µL, BIOLASE TM, Bioline) and 3 µL of the extracted DNA. Positive amplification products were purified using the kit "E.Z.N.A Cycle Pure Kit" (Omega Bio-tek), and sequenced in both directions.

2.5. Analyses of tick and Rickettsia sequences

Tick sequences were verified using the BLAST tool (www.ncbi.nlm.nih.gov/Blast.cgi). Rickettsia sequences were also verified using BLAST (www.ncbi.nlm.nih.gov) and subsequently edited with the ProSeq V3 software (Filatov, 2009) and Geneious Prime 2022.0.2 (https://www.geneious.com). The ClustalW algorithm (Thompson et al., 1994), implemented in MEGA11 program (Tamura et al., 2021), was used for the alignment of the Rickettsia sequences obtained in the present study and others available in GenBank (Supplementary Table 3). A phylogenetic reconstruction by Bayesian inference and maximum likelihood was performed using the programs MrBayes 3.2.2 (Ronquist et al., 2012) and RAxML 8.0.24 (Stamatakis, 2006) respectively, using a concatenated data set of the three genes. The best partition schemes and the most suitable substitution models were chosen using the Partition Finder program (Lanfear et al., 2012), according to the Bayesian Information Criterion (Schwarz, 1978). The GTR + G model was used for all the positions in the three genes.

3. Results

3.1. Diversity of herpetofauna species and taxonomic identification of ticks

A total of 47 individuals from 11 species were recorded, of which 1 was amphibian and 10 reptiles. In total, reptiles belonged to 10 genera of the order Squamata (n = 45) and the amphibian was *Rhinella horribilis* (n = 2). Fourteen of the 47 individuals were captured in the urban environment, while 33 were captured in the sylvatic environment. Ten individuals were found infested with ticks, six from the urban and four from the sylvatic environment (Table 1). The prevalence of tick infestation in the herpetofauna population examined was 21.3%. A total of 247 ticks were collected in five hosts species. All the samples were taxonomically identified as *Amblyomma dissimile* and were categorized between larvae, nymph, and adult stages (Table 2).

3.2. Molecular characterization of ticks and molecular detection of Rickettsiae

Verifications with the NCBI BLAST tool showed that all the analyzed tick sequences belong to the species *A. dissimile* with 99–100% identity. Our second match was with *A. rotundatum* with a much lower percentage of identity (92–94%). On the other hand, a total of 71 samples were tested for Rickettsia, of which 49 samples (29/43 larvae, 7/11 nymphs, and 13/18 adults) were positive, which is equivalent to 69% (Table 2). The largest number of individuals collected corresponded to larvae, which were processed individually or in pools of five specimens (just the ones collected on *Iguana iguana*), while all nymphs and adults were processed individually. From the positive samples, 15 gltA, 10 sca1 and 11 ompA sequences were generated (Supplementary Table 1 includes detailed information of the samples from which those sequences were obtained). The success of the PCRs with the gltA primers was high, however, the sequences were not long or had bad quality. All positive samples were corroborated with the ompA and sca1 primers. In the Blast analyses, most of the gltA sequences showed an identity percentage from 99,68–100% with *Rickettsia tamurae* (MN947696) and one of them (the longest one) of 100% with *Candidatus* Rickettsia colombianensi (MW384861); the ompA sequences showed 98,74–100% identity with *Candidatus* Rickettsia colombianensi (MN058024); and for sca1, most of the sequences showed a 100% of identity with uncultured *Rickettsia* sp. (MG020421 and MG020420), and one of them a 99,39% with *Rickettsia monacensis* (LN794217), mainly because there are not sca1 sequences named as *Rickettsia colombianensi*. Further analyzes using Bayesian inference and maximum likelihood grouped our samples into the same

Table 1
Number of amphibians and reptiles captured and infested with ticks in San Juan de Carare, Colombia (6° 42′58 0.20″N/74° 08′02 0.71″W), between March 2017 and May 2018.

		Urban environment		Sylvatic environment		
Order	Species	Abundance	Number of hosts infested with ticks	Abundance	Number of hosts infested with ticks	Prevalence (%)
Squamata	*Basiliscus basiliscus*	3	–	5	–	0
Squamata	*Boa constrictor*	1	1	–	–	100
Squamata	*Chironius spixii*	–	–	1	–	0
Squamata	*Corallus ruschenbergerii*	4	–	16	2	10
Squamata	*Iguana iguana*	5	4	4	–	44.4
Squamata	*Leptoderia septemptrionalis*	–	–	1	1	100
Squamata	*Leptophis ahaetulla*	–	–	2	–	0
Squamata	*Mastigodryas pleei*	–	–	1	–	0
Squamata	*Pseudoboa neuwiedii*	–	–	1	1	100
Anura	*Rhinella horribilis*	2	1	–	–	50
Squamata	*Tupinambis teguixin*	–	–	1	–	0
TOTAL		14	6	33	4	21.3

Table 2
Number of ticks collected per host species and number of ticks positive for Rickettsia.

Host species	Total of ticks collected (larvae/nymphs/adults)	No. of larva or larvae pools Rickettsia positive/total tested for Rickettsia	No. of nymphs Rickettsia positive/ total tested for Rickettsia	No. of adults Rickettsia positive/ total tested for Rickettsia
Leptodeira septentrionalis	21 (18/2/1)	11/12 (92%)	2/2 (100%)	1/1 (100%)
Iguana iguana	220 (179/8/33)	18/31 (58%)	4/6 (57%)	10/14 (72%)
Corallus ruschenbergerii	3 (0/1/2)	–	0/1 (0%)	1/2 (50%)
Boa constrictor	1 (0/0/1)	–	–	1/1 (100%)
Rhinella horribilis	2 (0/2/0)	–	1/2 (50%)	–
Total	247	29/43 (67%)	7/11 (64%)	13/18 (72%)

clade with *Candidatus* Rickettsia colombianensi, *Rickettsia* sp strain colombianensi, and Rickettsia endosymbiont *Amblyomma dissimile* (Supplementary Fig. 1).

Sequences generated in this study were uploaded to GenBank under accession numbers: ON138709-ON138719 (16S Amblyomma), ON138623-ON138635 (CO1 Amblyomma), ON149661-ON149672 (12S Amblyomma), ON159963 – ON159972 (Sca 1 Rickettsia), ON159973 – ON159987 (gltA Rickettsia), ON159988 – ON159998 (ompA Rickettsia).

4. Discussion

Reptiles may serve as a suitable host for various tick species and contribute to transmission of pathogenic organisms (Mendoza-Roldan et al., 2021a). However, despite the great diversity of amphibian and reptile species distributed in Colombia, tick-reptile associations are still poorly documented for most of the country. This is the first study related to the molecular detection of *Rickettsia* in ticks associated with herpetofauna in Santander, Colombia.

The ectoparasite found in the samples collected in this study corresponded exclusively to *Amblyomma dissimile*. This tick is commonly found as an ectoparasite of reptiles and some amphibians like toads. Its distribution goes from Florida to South America and the Caribbean, except Chile and Uruguay (Voltzit, 2007; Jones et al., 1972). In Colombia, other records correspond to the departments of Cesar, La Guajira, Magdalena and Córdoba (Miranda et al., 2012; Cotes-Perdomo et al., 2018; Santodomingo et al., 2018, 2019). *Amblyomma dissimile* was identified parasitizing *Boa constrictor*, *Corallus ruschenbergerii*, *Iguana iguana*, *Leptodeira septentrionalis* and *Rhinella horribilis*. These hosts, with the exception of *L. septentrionalis*, were also identified as hosts of *A. dissimile* in other studies (Álvarez-Calderón et al., 2005; Guglielmone and Nava, 2010; Santodomingo et al., 2018).

The species *L. septentrionalis*, locally known as Mapaná or false Mapaná, has a distribution that overlaps that of *A. dissimile* (Dávila and Buelvas, 2009; McKelvy et al., 2013). Also sharing a variety of habitats such as the dry forest, rainforest, riverside forest, savannah, and others (Medina-Rangel, 2011). However, its wide distribution led to this species having divergency processes, for which currently three clades of this biological group are recognized, two of these in Central America and one in South America (Daza et al., 2009), which makes it necessary to evaluate whether Central American clades would be parasitized by the same ticks. This snake, like the other species collected in the sylvatic environment during our surveys, was collected in the rainforest understory, within a forest patch of primary vegetation. It had high levels of tick infestation harboring all parasitic stages of *A. dissimile* (18 larvae, two nymphs, and one adult), with ticks scattered in its ventral area. These infestation levels could reflect the great host-tick adaptation, explaining why they don't appear to have any deleterious effects (Bower et al., 2019).

Of the samples infesting *L. septentrionalis*, 93% were positive for *Rickettsia*. In general, we found high percentages of *Rickettsia* positivity in ticks from hosts collected in both the sylvatic and the urban areas, but parasitic loads were lower in the sylvatic environments. Urban expansion can represent a wider range of host availability for ticks, which can be related to higher parasitic loads in urban areas (Calegaro-Marques and Amato, 2014). In our study, one of the urban reptiles that presented the highest tick infestations was *Iguana iguana*. Four out of the five iguanas collected in the urban environment had 213 ticks, while none of the iguanas collected in the sylvatic environment were found with ectoparasites. Additionally, 63% of the iguana ticks tested were positive to *Rickettsia*. Iguanas are one of the most trafficked reptiles in Colombia, mainly for their meat and eggs (Martinez and Gomez, 2013; Janssen, 2021). This is of concern as illegal animal trade and traffic has been associated with the dispersion of ticks and their associated pathogens (Erster et al., 2015; Mihalca, 2015). Moreover, as this species was captured in both environments, the connectivity between its populations remains a risk for the movement of pathogens between wild and urbanized environments (Corn et al., 2011).

Despite the sampling effort in each field trip, the number of individuals collected and infested with ticks was limited, largely because

searching for, manually capturing, and handling these vertebrate species is physically challenging. However, this data remains relevant from the One Health perspective, as pathogenic organisms for humans such as *Rickettsia monacensis* and *Rickettsia helvetica* had been found in reptile tissue suggesting that these vertebrates could have a role as amplifiers of these bacteria (Mendoza-Roldan et al., 2021b). *Ca.* Rickettsia colombianensi is phylogenetically close to *R. monacensis* and *Rickettsia tamurae*, and it has been previously evidenced in several studies in the departments of Córdoba and Magdalena in the Colombian Caribbean region (Miranda et al., 2012; Cotes-Perdomo et al., 2018; Santodomingo et al., 2018), and now in the Santander department, which suggest this rickettsia is widely distributed and probably abundant in the country. The high prevalence of *Ca.* R. colombianensi in *A. dissimile* ticks indicates a well-established arthropod-bacteria relationship, which makes it necessary to evaluate if this bacterium could be harmful and/or confers and advantage for *A. dissimile* over other tick species.

Although in the Blast searchers only the ompA sequences match with *Ca.* R. colombianensi, mainly due to the lack of samples named as *R. strain colombianensi* for sca1 and the short sequences obtained for the gltA, the concatenated phylogenetic analyses grouped the *Rickettsia* sequences reported in this study in a clade with *Ca.* Rickettsia colombianensi sequences. As Quintero et al. (2017) points out, the detection of *Ca.* R. colombianensi in ticks that are potential ectoparasites to humans, as is the case for *A. dissimile*, is of concern, as the pathogenic potential of *Ca.* R. Colombianensi has not been discarded. Oteo et al. (2014), also proposes that any species of *Rickettsia* described in ticks, despite the lack of knowledge regarding its ecology, should be considered a potential pathogen for humans.

The wide host range of *A. dissimile* has been acknowledged in previous works, as well as ours, all the parasitic stages of this tick are mostly found on reptiles and amphibians, but as seen in our results, adult stages are most likely to appear on bigger hosts such as turtles, boas or iguanas, as well as mammals including humans (Guglielmone and Nava, 2010). The ecology of these generalist hematophagous arthropods is of special interest as they may serve as a bridge for the spread of pathogens from sylvatic to urban environments and vice versa (Rosenberg and Beard, 2011). In addition to *Rickettsia*, *A. dissimile* has also been reported infected with a wide variety of microorganisms belonging to well-known pathogenic groups such as *Anaplasma*, *Borrelia*, and *Hepatozoon* among others (Ogrzewalska et al., 2018). Although many of these strains remain of unknown pathogenicity, preventing their spread, through strict control measures on hosts traffic (such as iguanas), agricultural frontier expansion, and changes in land use, to wild, and native populations should be a priority.

5. Conclusion

The ticks collected in San Juan de Carare in the department of Santander correspond entirely to the *Amblyomma dissimile* species. In this study, *A. dissimile* is recorded for the first time as an ectoparasite of *Leptodeira septentrionalis*. Likewise, the amplification and sequencing of the gltA, ompA and sca1 genes allowed the identification of *Candidatus* Rickettsia colombianensi, a Rickettsia of unknown pathogenicity, but closely related to old world pathogenic species that should remain of concern.

Declaration of competing interest

We have no conflicts of interest to disclose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2022.08.004.

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