Ticks and Tick-borne Diseases 10 (2019) 1109–1112

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Short communication

Strain diversity of *Rickettsia amblyommatis* in ticks infesting birds in the North Huetar conservation area of Costa Rica

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ARTICLE INFO

Keywords:
Multilocus analysis
Amblyomma geayi
Amblyomma longirostre
resident birds
Migrating birds
Central America

ABSTRACT

Although the presence of rickettsial agents in ticks infesting wild birds in Costa Rica has been recently reported, information on strain diversity is limited to selected rickettsial species. In order to mine deeper into rickettsial agents of ticks infesting Costa Rica wild birds a total of 399 birds from the North Huetar Conservation Area of Costa Rica were captured, and 134 immature ticks (76 larvae and 58 nymphs) were recovered from 61 birds. Ticks were tested for the presence of *Rickettsia* spp. by conventional PCR and sequencing of the *gltA*, *ompA*, *ompB*, 17 kDa, and *groEL* genes. Six (11.3%) *Amblyomma longirostre* and *Amblyomma geayi* ticks collected from passeriform birds, yielded amplicons of the expected size. Amplicons were sequenced, and BLAST results collectively showed that all sequences had 99–100% nucleotide identity with *Rickettsia amblyommatis* (formerly, *'Candidatus* Rickettsia amblyommii'*)*. Three different *R. amblyommatis* strains were identified. Four new tick species–host associations and the first detection of *R. amblyommatis* in *A. geayi* in Costa Rica are also reported.

1. Introduction

Birds play an important epidemiological role in tick-borne diseases by transferring and disseminating pathogenic bacteria around the world. Migratory species can cover long distances favoring the establishment of these pathogens in new areas and in novel hosts. Extensive research efforts were put on bird-tick-bacterium associations in the American continent, mainly in Brazil (Ogrzewalska et al., 2013; Santolin et al., 2013; Ramos et al., 2015). However, several studies revealed associations also in other countries, such as the United States (Mukherjee et al., 2014), Honduras (Novakova et al., 2015), and Peru (Ogrzewalska et al., 2012).

Recently, the presence of three rickettsial agents infecting ticks sampled from wild birds were reported in Costa Rica: *Rickettsia amblyommatis* (formerly, '*Candidatus* Rickettsia amblyommii') in *Amblyomma longirostre*, *Rickettsia bellii* in *Amblyomma sabanerae,* and a novel *Rickettsia* sp. agent in *Ixodes minor* (Ogrzewalska et al., 2015). Considering that Costa Rica´s avian biodiversity is one of the highest in the world (900 bird species), it can be postulated that most bird-tick-bacterium associations are still to be uncovered. The aim of the present study was to evaluate the strain diversity of *Rickettsia* spp. found in ticks parasitizing wild birds in Northern Costa Rica throughout a multilocus analysis.

2. Material and methods

Ethical statement: The present study was conducted according to the legal requirements of Costa Rica.

Birds from the North Huetar Conservation Area of Costa Rica were captured during a sampling campaign conducted from February 2008 to June 2010, examined for ectoparasites, and released. In total, 134 immature ticks (76 larvae and 58 nymphs) were recovered. Tick specimens were identified at genera level following taxonomic keys (Fairchild et al., 1966). Genomic DNA was extracted from tick pools with Blood and Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer`s instructions. The detection of *Rickettsia* spp. was performed by conventional polymerase chain reaction (PCR) and sequencing. Genes *gltA* and *ompA* were amplified following the protocols described by Labruna et al. (2004) and Regnery et al. (1991), respectively. Three additional genes were amplified for *Rickettsia* spp.-positive samples: *ompB* (Choi et al., 2005), *17 kDa* (Webb et al., 1990), and *groEL* (Chisu et al., 2016), as well as the molecular determination of the tick species, amplifying mitochondrial 16S rDNA (Norris et al., 1996).

PCR reactions contained a final concentration of 1X DreamTaq Polymerase Master Mix (ThermoScientific), 0.2 u M of each primer, 20 ng/ul DNA sample and water until 25 ul final volume was reached.

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https://doi.org/10.1016/j.ttbdis.2019.06.007
Received 28 August 2018; Received in revised form 25 May 2019; Accepted 9 June 2019
Available online 10 June 2019
1877-959X/

Rickettsia felis DNA-positive control was kindly donated by Centro de Investigaciones Regionales Dr. Hideyo Noguchi, Universidad Autónoma de Yucatan, Merida, Mexico. Molecular biology grade water (Fermentas®) was used as negative control in all the cases.

The expected fragment size of each gene was 401 bp for *gltA*, 532 bp for *ompA*, 407 bp for *ompB*, 434 bp for 17 kDa, 508 bp for *groEL,* and 462 bp for 16S rRNA. Amplicons were visualized in 1% agarose gels stained with GelRed™ Nucleic Acid Gel Stain (Biotium, 5 µg/ml), and positive samples were sent to Macrogen (Seoul, Korea) for sequencing. Partial sequences were edited with BioEdit (Hall, 1999) to remove primer sequences. Alignments were made by MUSCLE (Edgar, 2004) and phylogenetic trees were constructed with MEGA7 (Kumar et al., 2016) using the Maximum-Likelihood method. Bootstrap of 1000 replicates were used to determine statistical strength of relationships between the clades.

3. Results

A total of 399 birds belonging to 45 species and 17 families were captured, and 134 ticks (76 larvae and 58 nymphs) were recovered from 61 (15.3%) animals. Ticks were identified down to genus level as *Amblyomma* spp. (Supplemental Table 1). A total of six (11.3%) out of 54 tick pools analyzed yielded amplicons of the expected size in the different PCRs. BLAST results showed that all sequences were 99–100% identical to *R. amblyommatis* and determined the ticks as *A. longirostre* (n = 4) and *Amblyomma gaeyi* (n = 2). Three distinct *R. amblyommatis* strains were identified by multilocus approach. Analyses of the five genes (*gltA, ompA, ompB*, 17 kDa and *groEL*) of the three identified strains (strain 1 [S1], strain 2 [S2], strain 3 [S3]) by bird and tick species are shown in Table 1. The first strain was identified in *A. longirostre* collected from *Phaenostictus mcleannani.* The second strain was found in *Amblyomma geayi* recovered from *Glyphorhynchus spirurus* and *Thamnophilus atrinucha.* The third strain was detected in three *A. longirostre* ticks infesting *Dendrocincla fuliginosa, Attila spadiceus* and *Catharus ustulatus*. All these bird species belong to the order *Passeriformes* and are resident species, except *C. ustulatus*. Except for *D. fuliginosa* and *P. mcleannani* all these tick-host associations were never been observed to date.

Sequences were deposited at the National Center of Biotechnology Information (NCBI) GenBank database, under the following accession numbers: *gltA* (MG712728, MG712729 and MG712730); *ompA* (MG787411, MG787412 and MG787413); *ompB* (MH567002); *17 kDa* (MH567004); and *groEL* (MH567003). Only one representative sequence was submitted for the last three genes, since sequences were identical between strains. Additionally, mitochondrial 16S rDNA gene sequences of *A. longirostre* (MK605928, MK605929, MK605930 and MK605931) and *A. geayi* (MK605932 and MK605933) were deposited at the NCBI GenBank. Sequences of *A. longirostre* were 98–100% identical to sequence KF702347 from Costa Rica and sequences of *A. geayi* were 96% identical to sequence KM042851 from Brazil.

Amplified fragments from *gtlA* gene strain 1 were found to be 100% (350/350) identical to corresponding sequence KX099898 recovered from southeast Brazil, the strain 2 was 100% (350/350) identical to the sequence KF179351 from Paraguay, and the strain 3 showed 100% (350/350) identity with KF7023310 from Costa Rica. Furthermore, sequence from *ompA* gene strain 1 was 100% (509/509) identical to KM262194 from the northeast Brazil and strain 2 showed 100% (488/488) identity with EU274656 from Sao Paulo, while strain 3 showed 100% (489/489) identity with MF034496 from Colombia. Analyses of *17 kDa* gene showed 99% (366/367) identity with KJ534311 from Brazil. The *ompB* and *groEL* sequences were 100% (403/403) and 99% (468/470) identical with their corresponding genes in a *R. amblyommatis* complete genome record isolated from Argentina (CP015012).

Phylogenetic analyses were based on a 695 bp-concatenated sequence of *gltA* (315 bp) and *ompA* (380 bp), the two most informative genes representative of *Rickettsia* spp. BLAST results and phylogeny confirmed the presence of three strains of *R. amblyommatis* in ticks harbored by birds in Costa Rica (Fig. 1). Furthermore, *R. amblyommatis* is reported for the first time in *A. geayi* in Costa Rica.

4. Discussion

Strain diversity of *R. amblyommatis* was investigated in tick from birds in Costa Rica, and the evolutionary relationships with other South American strains were investigated through a multilocus approach. Results provide additional evidence on the expansion of areal distribution of rickettsial pathogens through ticks hosted by birds, and increase our knowledge on the role of ticks as relevant vector for spreading bacterial diseases. Four new rickettsial hosts, and novel tick species-host associations (*A. longirostre* on *A. spadiceus* and *C. ustulatus*, *A. geayi* on *G. spirurus* and *T. atrinucha)* were identified. The association of *A. longirostre* with *C. ustulatus*, has particular relevance in the epidemiological scenario, for *C. ustulatus* being a migrating species (Cohen et al., 2015).

Rickettsia amblyommatis had been reported infecting many species of *Amblyomma* ticks in the Western Hemisphere, showing ability to circulate between different tick species and animal hosts (Labruna et al., 2011). In this study, most of the *R. amblyommatis* infected ticks were found on resident passerine birds (Barrantes et al., 2016; Garrigues et al., 2018), this diminishing their role on dispersing infected ticks over long distances. However, finding an infected tick on *C. ustulatus*, a medium-sized thrush that lives in North America, and migrates as far as to Argentina, suggests that this wild bird species could play a role in dispersing *R. amblyommatis* infected ticks (Wilson et al., 2008; Mukherjee et al., 2014; Cohen et al., 2015). It is important to stress that *R. amblyommatis* is considered a human pathogen that can cause spotted fever illness, as established based on serological evidence in the United

Table 1
BLAST results comparing the sequences obtained in this study by bird and tick species with the GenBank database.

Sample	Bird species	Tick species	Ticks	Strain	GenBank sequences				
					gltA	ompA	ompB	17kDA	groEL
47	*Phaenostictus mcleannani*	*Amblyomma longirostre*	2L	S1	KX099898 (100)	KM262194 (100)	CP015012 (100)	KJ534311 (100)	CP015012 (99)
43	*Glyphorhynchus spirurus*	*Amblyomma geayi*	1L	S2	KF179351 (100)	EU274656 (100) JN126316 (99)	CP015012 (100)	KJ534311 (100)	CP015012 (99)
44	*Thamnophilus atrinucha*	*Amblyomma geayi*	1L						
51	*Attila spadiceus*	*Amblyomma longirostre*	2N	S3	KF702331 (100)	MF034496 (100)	CP015012 (100)	KJ534311 (100)	CP015012 (99)
52	*Catharus ustulatus*	*Amblyomma longirostre*	1L						
16	*Dendrocincla fuliginosa*	*Amblyomma longirostre*	3L						

L: Larva, N: Nymph.

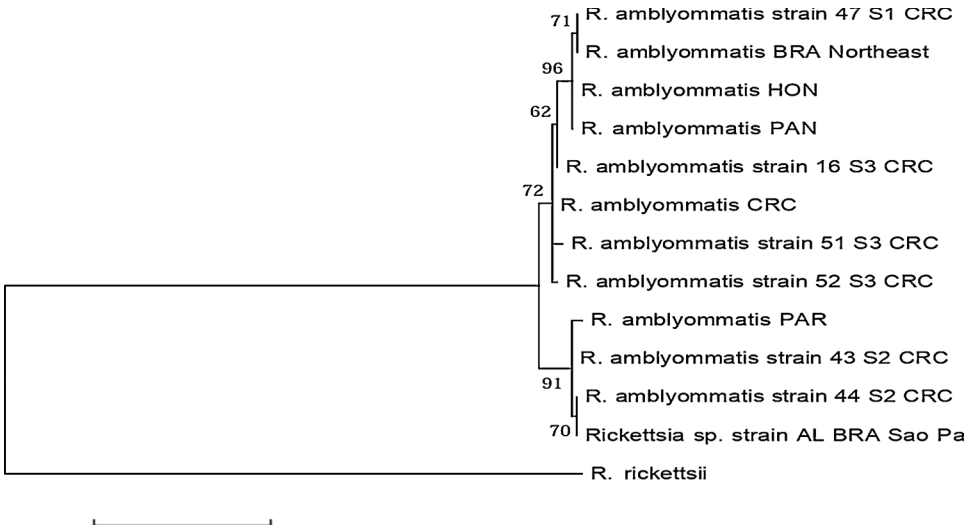

Fig. 1. Phylogenetic tree of *R. amblyommatis* detected in ticks removed from birds in different countries. The analyses were based on a concatenated sequence of *gltA* (315bp) and *ompA* (380 bp) genes of 695 bp size and inferred by the maximum-likehood method. Molecular phylogenetic analysis by Maximum Likelihood method on the Tamura-3-parameter model. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. GenBank accession numbers of both genes for all taxons included in this tree were: *R. amblyommatis* BRA Northeast (*gltA* KM262197, *ompA* KM262194), *R. amblyommatis* HON (*gltA* KP835792, *ompA* KP835795), *R. amblyommatis* PAN (*gltA* HM582335, *ompA* HM582336). *R. amblyommatis* CRC (*gltA* KF702331, *ompA* KF702333), *R. amblyommatis* PAR (*gltA* KF179351, *ompA* KF179350), *Rickettsia* sp. strain AL BRA Sao Pa (*gltA* EU274654, *ompA* EU274656), and *R. rickettsii* (*gltA* KC469610, *ompA* KC763629). BRA: Brazil, HON: Honduras, PAN: Panama, CRC: Costa Rica, PAR: Paraguay.

States (Apperson et al., 2008; Vaughn et al., 2014). Furthermore, two of six guinea pigs infected with a Costa Rican strain of *R. amblyommatis* developed orchitis (Rivas et al., 2015). It has been hypothesized that people in Latin America infected with this bacterium do not show clinical signs or develop disease due to infections with other spotted fever group rickettsiae that provide cross-immunity and protection (Troyo et al., 2016).

Bacteria belonging to the spotted fever group have been molecularly characterized by using several genes as molecular probes; it has been reported that *gltA* and *17 kDa* represent less informative genes than *ompA* and *ompB* (Labruna et al., 2004). Also, *groEL* gene, which encodes a heat shock protein, has been used for diagnosis and phylogenetic analysis of rickettsial agents (Lee et al., 2003; Chisu et al., 2016). In our study *ompA* and *gltA* proved to be the most informative genes to distinguish between strains of *R. amblyommatis*, while *ompB*, *17 kDa* and *groEL* amplicons yielded identical results between strains. For this reason, we consider that it is important to use a multilocus approach when performing intraspecific analysis, not only because it increases the available genetic information of the species studied, but also, because it is possible that the variation found is present in genes that are normally considered highly conserved.

Phylogenetic analyses with a concatenated sequence of *ompA* and *gltA* genes were supported by high bootstrap values (˜70) confirming the association between Costa Rican and South American strains. Especially, the second strain (S2), which was identified in *C. ustulatus,* seems to be related with South American strains, while the third strain (S3) was related to a previously reported Costa Rican isolate (GenBank KF702333) and strain one (S1) clustered with isolates from Central and South American countries, seeming to be a more widespread strain. A recent study reported new *R. amblyommatis* sequences in Costa Rica (Troyo et al., 2016), however we could not make any comparison as only one gene (*gltA* or *ompA)* was sequenced. However, *gltA* sequences KX544805, KX544806, KX544812 shared 99.7%, 99.1% and 99.4%, of identity with our strain one (S1), respectively; while *ompA* sequence KX544815 was also 99.7% (462/463) identical with our strain one (S1).

5. Conclusions

Strain diversity of *R. amblyommatis* was assessed in bird ticks in Costa Rica indicating intraspecific variation.

Acknowledgements

This investigation was supported by the Fondo Especial para el Financiamiento de la Educacion Superior, Consejo Nacional de Rectores (FEES-CONARE). We wish to thank Area de Conservación Huetar Norte for the research permits, Angie Sánchez Nuñez and Carol Sánchez Nuñez for sample collection and the farm owners who provided access to their properties.

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.ttbdis.2019.06.007.

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