Hemogregarine and Rickettsial infection in ticks of toads from northeastern Colombia

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ARTICLE INFO

Keywords:
Amblyomma dissimile
Rhinella spp.
Hemolivia spp.
Amphibian host

ABSTRACT

The toads Rhinella spp. are in constant contact with humans and domestic animals and are commonly parasitized by ticks, which are also potential vectors of pathogenic microorganisms, such as apicomplexans and rickettsia. However, little is known about microorganisms associated with toad ticks. In this work, we molecularly evaluated the presence of Rickettsia spp. and hemogregarines in ticks of Rhinella horribilis and R. humboldti in the Colombian Caribbean, finding two different species of Rickettsia: the colombianensi strain and one close to R. bellii. In the case of hemogregarines, since only 18S gene sequences are available, it is difficult to define species and place them correctly in a phylogeny, but most of our samples show a 99% identity with Hemolivia stellata, while others identical to each other seem to form another clade within this genre. All collected ticks were identified as Amblyomma dissimile, representing the first time that H. stellata was recorded in this tick. The prevalence of both microorganisms was very high, which makes it necessary to generate robust phylogenies to clarify their taxonomic diversity and to correctly define their ecological role and pathogenicity, which should be taken into account in amphibian conservation plans and veterinary medicine.

1. Introduction

There are several types of ectoparasites that transmit pathogens to anurans, among which are leeches, mosquitoes and ticks. Within the latter, nine species of the family Ixodidae and one of Argasidae are known; they parasitize toads of the genus Rhinella (Burridge, 2011; Bermúdez et al., 2013), and Amblyomma dissimile and Amblyomma rotundatum are the most common. Both are tri-host ticks that generally use amphibians and reptiles as hosts during their life cycle, although they can accidentally parasitize birds and mammals, including humans (Lampo et al., 1997; Guglielmone et al., 2006); Amblyomma dissimile has a greater diversity of hosts, but within anurans, it is only recorded in toads of the Bufonidae family (Guglielmone and Nava, 2010).

The parasitism of these ticks leaves clear cutaneous lesions in the toads, weakening the animal and directly increasing the risk of subsequent infections, whether primary infections, starting from cutaneous lesions, or secondary infections through vector transmission of microorganisms, which is considered a mechanism of population regulation for the hosts (Lampo and Bayliss, 1996; Smith et al., 2008). Although these infections often show no symptoms in wild vertebrates, there is evidence of pathologies, such as anemia, erythrocytic hypertrophy and loss of hemoglobin, along with the possibility of causing phenotypic and phenological changes in the vectors (Desser et al., 1995; Wozniak et al., 1996; Ferguson et al., 2013), especially in infections by hemogregarines (Gupia et al., 2012; Rodrigues-Calil et al., 2017).

This artificial group is composed of six Apicomplexa genera (Barta et al., 2012), of which only Hepatozoon and Hemolivia parasitize the anurans (Maia et al., 2016). In fact, the latter was described in its natural hosts Rhinella marina (s.l.) and the tick Amblyomma rotundatum, with the species Hemolivia stellata (Petit et al., 1990). Like this one, its congeners use heterothermic vertebrates as intermediate hosts and ticks as definitive hosts (Karadjian et al., 2015) and can also be transmitted through the consumption of other infected vertebrates (Davies and Johnston, 2000).

At the same time, ticks are important vectors of bacteria to multiple vertebrate groups. The genus Rickettsia (Rickettsiales: Rickettsiaceae) has been reported more frequently in Latin America, given its importance in public and veterinary health (Witter et al., 2016); despite this incidence, in the country, the knowledge of transmission to amphibians is scarce. Studies focused on vectors have resulted in the discovery of several species of unknown pathogenicity, thanks to the progressive improvement of detection techniques. Such is the case of the Rickettsia sp. Colombianensis strain in reptile ticks (Miranda et al., 2012; Santodomingo et al., 2018); this species is phylogenetically related to R. monacensis, which was considered nonpathogenic when it was discovered but is currently known to cause disease in humans, as...
has occurred with other species, such as R. aeschlimannii, R. massiliae and R. slovaca in the old world (Parola et al., 2005; Jado et al., 2007). Despite the great advances in the study of these bacteria and other microorganisms associated with ticks, very little has been reported about the roles played by amphibians and their ticks in the epidemiology of human and animal diseases. In Colombia, there has been no report of pathogens associated with amphibian ticks, with Bufonidae being one of the most abundant and diverse anuran families in the country, where species such as Rhinella marina and R. horribillis are present in urban, rural and wild lowland environments, very close to humans and domestic animals (Acevedo-Rincón et al., 2016; Acosta-Galvis, 2017). Therefore, the objective of this work was to evaluate the presence of Rickettsia spp., Hepatozoon spp. and Hemolivia spp. in ticks and some tissues of toads in the department of Magdalena (Colombia).

2. Materials and methods

2.1. Samples studied

The ticks were taken directly from toads from seven localities located in the department of Magdalena in northern Colombia. The toads were captured under the visual encounter search method and through pitfall traps at two locations. Samples taken from each individual were placed in 1.5-ml vials with 96% ethanol and were maintained at −20 °C until their identification. For the taxonomic identification of ticks, the keys from Voltzit (2007), Osorno-Mesa (1940) and Jones et al. (1972) were used for adult specimens, and the keys from Martins et al. (2010) were used for the nymphs. Larvae were identified using the key from Osorno-Mesa (1940) and were corroborated using molecular methods (the COI gene). Additionally, six individuals of Rhinella horribillis collected at the Universidad del Magdalena were sacrificed, from which ticks, blood, liver, heart and spleen samples were also extracted. Permission for trapping and manipulating the animals as well as collecting the ectoparasites for this study was given by ANLA (Autoridad Nacional de Licencias Ambientales) under the permit no. 1293, and was approved by Universidad del Magdalena Ethical Committee (Acta 001–15).

2.2. DNA extraction

For the adult ticks, the anterior half was used to extract the DNA; for the larvae and nymphs, the complete individual was used. The DNA was extracted individually or in pools, depending on the number of ticks of each stage found in each toad, using the MasterPure™ DNA Puriﬁcation Kit (Epicenter, USA). Each pool included up to 5 individuals. DNA extraction of the liver, heart and spleen samples was performed following the instructions of the ISOLATE II Genomic DNA kit, and the MasterPure™ DNA Puriﬁcation Kit for Blood was used for the blood samples. DNA extraction and its quality were confirmed by means of electrophoresis in agarose gel and with GelRed (Biotium) staining.

2.3. Amplification of COI

Amplification of COI was performed through conventional PCR in an Eppendorf Mastercycler™ Pro thermocycler, using the universal primers for invertebrates that amplify the gene: LCO1490 (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′) and HCO2198 (5′TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) (Folmer et al., 1994). Amplifications were designed with a volume of 25 μL, containing 3 μL of extracted DNA, 0.5 μL of Taq Polymerase (5 U/μL, Bioline), 1 μL of MgCl₂ (50 mM), 2.5 μL of PCR Buffer (10X), 0.5 μL of dNTPs (10 mM) and 1 μL of each primer (10 pmol). The conditions of the amplifications were as...
follows: an initial denaturation of 95 °C for 1.5 min, followed by 35 cycles of 94 °C for 30 s, annealing at 45 °C for 1 min, extension at 72 °C for 1 min and a final extension at 72 °C for 7 min.

2.4. Amplification of GltA/16S rRNA/Omp A// from Rickettsia

Amplification was performed by conventional PCR in an Eppendorf Mastercycler® Pro thermal cycler, using the primers with the following sequences: CS-78 (GCAAGTATCGGTGAGGATGTAAT) and CS-323 (GCTTCCTTAAAATTCAATAAATCAGGAT) (Labruna et al., 2004). For the PCR, 4 μL of extracted DNA was used, and the final reaction volume was 25 μL, containing 0.5 μL of Taq Polymerase (BIOLASE TM, Bioline), 1 μL of MgCl (50 mM), 2.5 μL of PCR Buffer (10X), 0.5 μL of dNTPs (10 mM) and 1 μL of each primer (10 pmol). The conditions of the amplifications were as follows: an initial denaturation of 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s, annealing at 48 °C for 30 s, extension at 72 °C for 30 s and a final extension at 72 °C for 7 min.

Table 1

Sequences of Rickettsia downloaded from GenBank and generated in this study (in bold) that were included in the phylogenetic analyses shown in Fig. 1.

| Species                          | Genes                  | GenBank accession numbers |
|----------------------------------|------------------------|---------------------------|
| Rickettsia sp. clone RA143       | gltA                   | KY996395                  |
| Rickettsia sp. Strain Colombiensi | gltA/ompA/16S          | JF905456/KF691749/        |
|                                  |                        | KF891750                  |
| Rickettsia sp. clone Necocli 190 | gltA                   | JX91583                    |
| Rickettsia tamurae strain AT-1   | gltA/ompA/16S          | AF394896/QI03259/         |
|                                  |                        | NR_042727                 |
| Rickettsia monacensis            | gltA/ompA/16S          | DQ100163/LN794217/        |
|                                  |                        | LN794217                  |
| Rickettsia sibirica sibirica     | gltA/ompA/16S          | KM288711/KM288712/        |
|                                  |                        | 16S                       |
| Rickettsia mongolitomana         | gltA/ompA/16S          | DQ097081/DQ097082/        |
| Rickettsia slovaca               | gltA/ompA/16S          | U59725/CP002428/          |
| Rickettsia conori                | gltA                   | U59728                     |
| Rickettsia parkeri               | gltA/ompA/16S          | KJ158742/KJ158741/        |
| Rickettsia africar               | gltA/ompA/16S          | U59733/CP001612/          |
| Rickettsia japonica              | gltA/ompA/16S          | AY743327/AD01533/         |
|                                  |                        | AP015533                  |
| Rickettsia beliangiensis         | gltA/ompA/16S          | AY205776/AH012829/        |
|                                  |                        | 16S                       | NR_041770                 |
| Rickettsia honei                 | gltA/ompA/16S          | AF018074                  |
| Rickettsia raoulhii              | gltA/ompA/16S          | KU13590/KR668783/         |
| Rickettsia aeszlimannii          | gltA/ompA/16S          | AY259084/AY259083/        |
|                                  |                        | 16S                       | KJ331974                  |
| Rickettsia massillae             | gltA/ompA/16S          | HM050293/QI94551/         |
|                                  |                        | 16S                       | LI6016                    |
| Rickettsia rhipeophidi           | gltA/ompA/16S          | DQ865206/DQ865208/        |
| Rickettsia australis             | gltA/ompA/16S          | U59716/U12459/            |
| Rickettsia sp. clone Turbo 31    | gltA/ompA/16S          | JX915976                  |
| Rickettsia sp. clone Necocli 181 | gltA/ompA/16S          | JX915577                  |
| Rickettsia typbi                 | gltA/ompA/16S          | U59714/NR_074394/         |
| Rickettsia bellii                | gltA/ompA/16S          | QJ59684/UI1014/           |
| Rickettsia rickettsii            | gltA/ompA/16S          | CP006009                  |
| Rickettsia bellii                | gltA/ompA/16S          | CP015010                  |
| Rickettsia felis                 | gltA/ompA/16S          | CP00005/                  |
| Rickettsia sp. strain Colombiensi| gltA/ompA/16S          | MH196484/MH196501/        |
|                                  |                        | MH196505                  |
| Rickettsia sp. strain Colombiensi| gltA/ompA/16S          | MH196496                  |
| Rickettsia bellii-like (RJ41)    | gltA                   | MH196501                  |

(caption on next page)
of denaturation at 95 °C for 30 s, annealing at 58 °C (16S rRNA)/52 °C (ompA) for 1 min, extension at 72 °C for 2 min and a final extension at 72 °C for 7 min.

### 2.5. Amplification of 18S gene of Hepatozoon

The amplification was performed by conventional PCR in an Eppendorf Mastercycler® Pro thermocycler, using primers HepF300 (GGTTCCTGACCTATCGCTTCCAGGAC) and Hep900 (CAATCTAAGAATTTCACCTCTGAC) (Ujvari et al., 2004). For the PCR, 4 μL of extracted DNA was used, and the final reaction volume was 25 μL, which contained 0.5 μL of Taq Polymerase (5 U/μL, Bioline), 1 μL of MgCl₂ (50 mM), 2.5 μL of PCR Buffer (10X), 0.5 μL of dNTPs (10 mM) and 1 μL of each primer (10 pmol). The conditions of the amplifications were as follows: an initial denaturation at 94 °C for 3 min, followed by 35 cycles at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 1 min and a final extension at 72 °C for 10 min. The products obtained from the amplification of COI, gltA, 16S, ompA and 18S were purified with SureClean Plus (Bioline, USA) following the supplier’s instructions. These products were sequenced in both directions.

### 2.6. Analysis of the sequences

The sequences were checked using the NCBI BLAST tool (www.ncbi.nlm.nih.gov) and were then edited with ProSeq V3 software (Filatov, 2009). Using the program MEGA 7.0 (Kumar et al., 2016) with the ClustalW algorithm (Thompson et al., 1994), the sequences obtained in this study and others available in GenBank were aligned.

### 2.7. Phylogenetic analyses

For phylogenetic reconstruction, Bayesian inference and maximum likelihood were used 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 (BIC; Schwarz, 1978). The GTR + G model was implemented for the 1st and 2nd codon positions of the gltA gene and for 2nd and 3rd codon positions of OmpA; the GTR model was used for the 3rd codon position of gltA, and the GTR + I + G model was used for the 16S gene (Rickettsia spp.). The 18S (hemogregarines) was analyzed using the GTR + G model.

Two independent runs of 10,000 generations, discarding 25% of the trees. The standard deviation of the independent sequences (< 0.01) was used to validate the convergence, grouping the likelihood values over time and using the SumP command in MrBayes. The posterior probability of each clade was measured based on the percentage of trees that recovered that particular clade (Huelsenbeck and Ronquist, 2001). For the maximum likelihood analyses, the fast-scaling algorithm was used with 1000 Bootstrap (BP) pseudoreplicates, taking BP values greater than 70% as a high statistical support (Hillis and Bull, 1993).

### 3. Results

A total of 208 ticks were collected (16 adults, 90 nymphs and 102 larvae), which were identified as Amblyomma dissimile and Amblyomma cf. dissimile. 4 nymphs and 6 larvae were extracted from 5 R. horribilis toads, whereas the rest were collected from 44 R. horribilis individuals. Specifically, 1 to 3 ticks were collected per R. horribilis toad, whereas we found an average of 4.5 ticks per R. horribilis toad. We analyzed 9 adults, 41 pools of nymphs and 32 pools of larvae using molecular methods, including 4 pools of nymphs and 4 pools of larvae from R. horribilis and 5 females, 4 males, 37 pools of nymphs and 28 pools of larvae from R. horribilis.

Rickettsia DNA was detected in 5/9 (55%) adults, 24/41 (57%) pools of nymphs and 28/32 (88%) pools of larvae, including 2 females, 3 males, 22 pools of nymphs and 24 pools of larvae from R. horribilis, and 2 pools of nymphs and 4 pools of larvae from R. humboldti. None of the tissues was positive for Rickettsia. The percentage of positivity for both Rickettsia and for hemogregarines was expressed as a minimum infection rate (MIR), assuming that one tick was positive in each positive pool.

Hemogregarine DNA was detected in 6/9 adults (66%), 33/41 (80%) pools of nymphs and 18/32 (56%) pools of larvae including 3 females, 3 males, 31 pools of nymphs and 16 pools of larvae from R. horribilis and 2 pools of nymphs and 2 pools of larvae from R. humboldti. Tissues from the six toads were also found positive for hemogregarine DNA; however, we successfully sequenced tissues from only two. The first was a blood sample (cod HSS3) of an individual who also had a positive female tick (cod NH114). The second individual was found positive for liver, blood and spleen tissues (cod HH7, HSS7, HH87 respectively). Among the infected ticks, 5 adults, 13 pools of nymphs and 18 pools of larvae from R. horribilis and 2 pools of nymphs and 4 pools of larvae from R. humboldti were coinfected with both Rickettsia and hemogregarine DNA.

All COI sequences showed 99% identity with A. dissimile. The sequences of the genes gltA and ompA showed identities of 100% with Rickettsia sp. strain Colombianensis (e.g., JF905456.1/MF034497.1) and 99% with R. tamurae sequences (e.g., KT753265.1) and R. monacensis (e.g., KU586332.1). GlA sequence corresponding to the J41 sample (pool of larvae from R. horribilis) had 99% identity with R. bellii (e.g., NR_074484.2). The phylogenetic analyses also showed the proximity between our sequences with the group conformed by R. monacensis, Rickettsia sp. Colombianensis and R. tamurae, and the sample J41 with R. bellii (Fig. 1, Table 1).

The sequences of the fragments amplified with the Hepatozoon primers showed identities of 99% with Hemolivia stellata sequences (e.g., KP881349.1) and 98% with Hemolivia sp. (e.g., KX347435.1). Bayesian Inference and Maximum Likelihood analyses grouped 6/7 of the samples obtained from ticks with H. stellata and one taken from sample blood. By Bayesian Inference, the remaining sample taken from ticks and the others obtained from the tissues appeared within the clade of Hemolivia as sisters to all of the others (Fig. 2); by Maximum Likelihood, they appeared to be sisters to all of the others except to the group of Hemolivia marie. All our sequences were deposited in GenBank under accession nos. MH196473-MH196505.

### 4. Discussion

This is the first report of parasitism by Amblyomma dissimile in Rhinella horribilis; a previous report described A. dissimile using R. horribilis and R. marina as hosts (Osorno-Mesa, 1946; Guglielmone and Nava, 2010). Likewise, this report represents the first time that Hemolivia stellata has been detected in Amblyomma dissimile and the third time that Rickettsia spp. has been reported (Miranda et al., 2012; Santodomingo et al., 2018), with two possible different species detected: Rickettsia sp. strain Colombienesi and another close to Rickettsia bellii. The latter was detected in only one pool of larvae, in contrast, R. bellii had been found in A. rotundatum taken from Rhinella spp. from Brazil and Panama, with high infestation rates (Labruna et al., 2004; Andoh et al., 2015; Horta et al., 2015; Silva et al., 2016).

Despite the high prevalence of Rickettsia spp. in the tick samples,
not present or not detectable in some tissues of these hosts. Therefore, future studies should focus on elucidating whether or not the toads may be infected with these bacteria to understand the ecological relationship that exists between these bacteria and the toads. In addition, it is imperative that sequences from regions other than the 16S gene be obtained for the phylogenetic identification and location of hemogregarines and to clarify the true vectors and intermediate hosts for these species, their transmission dynamics and their life cycles, to clarify the true diversity in these taxa and their role in the population dynamics of their hosts, which are fundamental for the conservation of a group that is particularly sensitive to climate change, habitat fragmentation and habitat loss.

Conflicts of interest

The authors declare no conflicts of interest.

Financing

This study has been funded by the patrimonial fund for research (Fonciencias) of Universidad del Magdalena [VIN2016104] and the scholarship of young researchers and innovators 706–2015 of the administrative department of science, technology and innovation (COLCIENCIAS) [FP44842-561-2015].

Acknowledgements

We thank Gustavo López Valencia for guiding us in the taxonomic identification of the ticks. To Jorge Eguis, Jefferson Villalba, Sintana Rojas, Harold Cuello, Juan Carlos Dib, Cristhian Cotes, Sebastian Contreras and Juan David Jiménez for their collaboration in the collection of samples. To German Blanco and his students for help in taking tissue samples.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ijppaw.2018.06.003.

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Barta, J.R., Ogedengbe, J.D., Martin, D.S., Smith, T.D., 2012. Phylogenetic position of the lizard Téara dugesi (Lacertidae) and its Ixodes ricinus ticks. Therefore, it cannot be ruled out that heterothermic hosts such as amphibians and reptiles are sentinels or reservoirs of Rickettsia spp., since there are some studies that demonstrate the presence of Rickettsia in amphibians, though they are not very precise in their identification (Desser and Barta, 1984, 1989; Desser, 1987; Bataille, A., Lee-Cruz, L., Tripathi, B., Waldman, B., 2018). It is possible that the molecular detection of Rickettsia spp. is not sensitive enough, though, as proposed by Levin et al. (2016), this may vary depending on the stage in which the infection is found.

In contrast, infection by hemogregarines was found in all types of tissue analyzed, and there was also a high prevalence in ticks. Our phylogenetic analyses showed that the samples analyzed corresponded to the genus Hemolivia, with the majority being grouped with H. stellata and the remaining ones forming an independent clade within this genus, using the phylogenetic framework suggested by Kvičerová et al. (2014) and including the only sequence of H. stellata available in GenBank (KP881349). This species of hemogregarine has only been described in Amblyomma rotundatum as the definitive host, R. marina as an intermediate host and, more recently, in Ameiva ameiva (Petit et al., 1990; Lainson et al., 2007).

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