Molecular characterization of novel *Ehrlichia* genotypes in *Ixodes auritulus* from Uruguay

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**ABSTRACT**

*Ehrlichia* are small intracellular Gram-negative bacteria transmitted by ticks. These microorganisms cause ehrlichiosis, a complex of life-threatening emerging zoonoses and diseases of global veterinary relevance. The aim of this study was to investigate the presence of *Ehrlichia* in free-living *Ixodes auritulus* collected in Uruguay. Ticks were collected from vegetation in five localities from the southeast and northeast of the country between 2014 and 2017. Detection of *Ehrlichia* DNA was performed in pools of adults or nymphs grouped according to the collection site and date. A total of 1,548 *I. auritulus* ticks were collected in four of the five locations sampled. Fragments of three loci (*16S rRNA*, *dsb* and *groEL*) were obtained by PCR, and phylogenies inferred using Bayesian inference analysis for each gene independently. DNA of *Ehrlichia* spp. was found in 15 out of 42 tick pools. Based on the topology of the phylogenetic trees, our sequences represent two novel genotypes for the genus named as *Ehrlichia* sp. Serrana and *Ehrlichia* sp. Laguna Negra. Both genotypes were closely related to *Ehrlichia* sp. Magellanica, a species detected in *Ixodes uriae* and Magellanic penguins. Considering that all stages of *I. auritulus* and *I. uriae* are parasites of birds, their phylogenetic relationships, and common eco-epidemiological profiles, it is reasonable to state that these genotypes of *Ehrlichia* spp. may represent a natural group likely associated with birds. Our results constitute the first characterization of *Ehrlichia* spp. in Uruguay. Future studies on birds reported as hosts for *I. auritulus* are needed to further understand the epidemiological cycles of both *Ehrlichia* genotypes in the country. Finally, *I. auritulus* does not feed on humans, so the two *Ehrlichia* species reported herein might have no implications in human health.

1. Introduction

The order Rickettsiales (Alphaproteobacteria) includes obligate intracellular parasites that infect a variety of invertebrate and vertebrate hosts. Rickettsiales comprises the families *Anaplasmataceae*, *Midichloriaceae* and *Rickettsiaceae* (Montagna et al., 2013; Szokoli et al., 2016). The family *Anaplasmataceae* is currently divided into five established and two *Candidatus* genera: *Anaplasma*, *Aegyptianella*, *Ehrlichia*, *Neorickettsia*, *Wolbachia*, “*Candidatus* Neoehrlichia”, and “*Candidatus* Xenohaliotis” (Thomas et al., 2016).

*Ehrlichia* are small Gram-negative tick-transmitted bacteria that form microcolonies within membrane-bound cytoplasmic vacuoles called morulae (Popov et al., 1998). These bacteria are the agents of ehrlichiosis, a complex of life-threatening emerging zoonoses and diseases of veterinary importance worldwide (Esemu et al., 2011). *Ehrlichia* species differ in their target cells both in mammals (monocytes, neutrophils or endothelial cells) and ticks (salia glands, intestinal epithelium, and hemolymph) (Brouqui & Matsumoto, 2007; Aguiar, 2017). In contrast to *Rickettsia* spp., there is no evidence of transovarial transmission in *Ehrlichia* spp. in ticks (Ismail & McBride, 2017). Instead,
ticks become infected with *Ehrlichia* spp. while feeding on infected vertebrate reservoirs and the infection is perpetuated transstadially (i.e. larva-nymph-adult) (Ismaïl & McBride, 2017). Human and animal ehrlichiosis are caused worldwide by six *Ehrlichia* species, namely *Ehrlichia canis, Ehrlichia chaffeensis, Ehrlichia muris, Ehrlichia ewingii, Ehrlichia ruminantium,* and *Ehrlichia minasensis* (see Aguiar, 2017). In the Southern Cone of South America, *E. canis*, the etiological agent of canine monocytic ehrlichiosis, is the most commonly reported species of this genus (Lopez et al., 2012; Lasta et al., 2013; Cicuttin et al., 2015).

Additionally, different strains closely related to *E. chaffeensis* were detected in *Amblyomma parvum, Amblyomma tigrinum* and in marsh deer (*Blasocaster dichotomus*) in Argentina (Tomassone et al., 2008; Cicuttin et al., 2017). *Amblyomma sculptum* (Sacchi et al., 2012), and some *Amblyomma* sp. strain Cordoba was detected in *A. tigrinum* ticks (Cicuttin et al., 2017). Recently, also in Argentina, *A. tigrinum*, *Amblyomma arboreum* and *Amblyomma neumannii* were found positive after molecular screenings targeting this bacterium, and three novel strains (*Ehrlichia* sp. strain Iberia, *Ehrlichia* sp. strain Delta, and *Ehrlichia* sp. strain La Dormida) were identified (Cicuttin et al., 2020; Eberhardt et al., 2020; Fargnoli et al., 2020).

In Brazil, reports include the detection of *Ehrlichia cf. chaffeensis* in *B. dichotomus* (Sacchi et al., 2012), and some *Ehrlichia* spp. strains pending further molecular characterization were detected in wild carnivores (Widmer et al., 2011; Almeida et al., 2013) and a horse (Vieira et al., 2016). Cattle and *Rhipicephalus microplus* ticks maintain *E. minasensis* infections in Brazil (Cabezas-Cruz et al., 2016; Aguiar, 2017) and recently, *Ehrlichia* sp. was detected in *Amblyomma scapularis* in the Brazilian Pantanal (Muraro et al., 2021).

In Chile, a novel genotype, *Ehrlichia* sp. Magellanica, seems to represent a bird-associated lineage within the genus, since it was

| Collection site | Date       | Stage | No. of ticks | Pools | Positive pools | Positive pools code | Ehrlichia genotype | GenBank accession numbers |
|-----------------|------------|-------|--------------|-------|----------------|-------------------|--------------------|--------------------------|
| Gruta de los Cuervos (T) | August 2016 | Nymph | 1             | 1     | 0             |                   |                    |                          |
|                 | December 2016 | Female | 1             | 1     | 0             |                   |                    |                          |
| Amarillo (Ri)  | October 2016 | Nymph | 1             | 1     | 1             | S12IaH14        | Serrana            | MW628647; MW650903      |
|                 | June 2017   | Female | 1             | 1     | 1             | S17laH1         | Serrana            | MW628649; MW650901; MW650909 |
|                 | May 2017    | Larva  | 6             | 3     | 0             |                   |                    |                          |
| Lunarejo (Ri)  | October 2016 | F-N-L | 0             | 0     | 0             |                   |                    |                          |
|                 | May 2016    | Nymph | 48            | 1     | 1             | S10laN9         | Serrana            | MW650902; MW650910      |
|                 | May 2017    | Nymph | 5             | 1     | 1             | S17laN33        | Serrana            | MW650904               |
| Reserva Natural Salus (La) | May 2016 | Nymph | 54            | 4     | 0             |                   |                    |                          |
|                 | December 2016 | Female | 6             | 1     | 0             |                   |                    |                          |
|                 | July 2017   | Nymph | 12            | 2     | 1             | S17laN33        | Serrana            | MW650904               |
| Laguna Negra (Ro) | March 2014 | Female | 4             | 1     | 1             | S5laH4          | Serrana            | MW650901               |
|                 | May 2014    | Nymph | 30            | 3     | 2             | S6laN9; S6laN11 | Serrana            | MW650902 (2)           |
|                 | August 2014 | Nymph | 144           | 2     | 2             |                   |                    |                          |
|                 | May 2014    | Female | 4             | 1     | 1             | S6laH12        | Laguna Negra       | MW628646; MW650906     |
|                 | November 2015 | Female | 4             | 1     | 1             | S6laH12        | Laguna Negra       | MW650908               |
|                 | August 2014 | Nymph | 47            | 5     | 1             | S7laN19         | Serrana            | MW650907               |
|                 | November 2014 | Female | 665          | 10     | 5             | S8laH27; S8laH28; S8laH31; S8laH33; S8laH34 | Laguna Negra (1); Serrana (4) | MW650907            |
|                 |             | Nymph | 16            | 2     | 1             | S8laN38        | Serrana            | MW650905               |
|                 |             | Larva | 30            | 0     | #             |                   |                    |                          |
| Total           |             |       | 1548          | 42    | 15            |                   |                    |                          |

**Abbreviations:** T, Tacuarembo; Ri, Rivera; La, Lavalleja; Ro, Rocha; #, larvae are not included in pools; F, Female; N, Nymph; L, Larva.

**Table 1**

| Primer name | Targeted gene | Sequence | Amplicon size (bp) | Reference |
|-------------|---------------|----------|--------------------|-----------|
| EHR165D    | 16S rRNA      | GATACCACTCATGTTCTAGCC | 345 | Parola et al. (2000) |
| EHR16SR    | 16S rRNA      | TAGACATCTCATGTTCTAGCC | 345 | Parola et al. (2000) |
| fD1         | groEL         | TAGGCTTGTGTTGTTCTAGCC | 1,500 | Weisburg et al. (1991) |
| R2          | groEL         | ACTGCCCTTCCTGTTCTCTATCCT | 1,500 | Weisburg et al. (1991) |
| HS1a        | groEL         | GATGCGGTGTTGTTGTTCTAGCC | 1,500 | Weisburg et al. (1991) |
| HS5a        | groEL         | CTACCAGGCAGCTCTCTGACGC | 1,500 | Weisburg et al. (1991) |
| HS43        | groEL         | TGCCAGCTCTGTTGTTGTTCTAGCC | 1,500 | Weisburg et al. (1991) |
| HS42        | groEL         | GGCGGCTCTGTTGTTGTTCTAGCC | 1,500 | Weisburg et al. (1991) |
| Dsb-330     | dsb           | GATGACAGGATTATTTGTTTAAAATT | 401 | Doyle et al. (2005); Almeida et al. (2013) |
| Dsb-720     | dsb           | CATTCTATGCTACGTTGTTGTTCTAGCC | 401 | Doyle et al. (2005); Almeida et al. (2013) |
| Dsb-380     | dsb           | ATTTTTAGGTTTTTACATCATTGCC | 401 | Doyle et al. (2005); Almeida et al. (2013) |

* Primers used in the initial PCR screening.

1 Primers used in the first and second round.
detected in *Ixodes uriae* collected on Magellanic penguins (*Spheniscus magellanicus*) (Muñoz-Leal et al., 2019).

While the occurrence of ehrlichiae in Uruguay is uncertain, Conti-Díaz (2001) referred to possible cases of ehrlichiosis in humans. Although the disease is listed as emergent in the country, infection in animals and vectors has not been investigated.

*Ixodes auritulus* is a cosmopolitan tick species distributed through the Afro-tropical, Australasian, Nearctic, and Neotropical Zoogeographic Regions and all stages parasitize mainly birds (Nava et al., 2017; Guglielmone et al., 2020). The presence of *Borrelia burgdorferi* (*sensu lato*) was recently reported in *I. auritulus* from Argentina and Uruguay (Cicuttin et al., 2019; Carvalho et al., 2020); however, information about the presence of other pathogens is scarce.

As *Ixodes* spp. also can transstadially maintain bacteria of the family *Anaplasmataceae*, the aim of this study was to investigate the presence of *Ehrlichia* in free-living *I. auritulus* ticks collected in Uruguay.

2. Materials and methods

2.1. Study area

Fieldwork was conducted at five localities in Uruguay between 2014 and 2017. Three localities, Gruta de los Cuervos (31.618888, -56.046389), Tacuarembó Department; Amarillo (31.663611, -55.050555) and Lunarejo (31.141388, -55.900277), Rivera Department, are located in the northeast region of the country, within the

Fig. 1. Bayesian phylogenetic analysis inferred for partial fragments of the 16S rRNA gene. Bayesian posterior probabilities > 0.95 are indicated above or below the nodes. The positions of *Ehrlichia* sp. genotype Serrana and *Ehrlichia* sp. genotype Laguna Negra are highlighted within red and blue boxes, respectively. The scale-bar indicates the number of substitutions per nucleotide position. Genbank accession numbers are in parentheses.
Uruguayan ecoregion Gondwanic Sedimentary Basin (Brazeiro et al., 2012). The other two localities, Reserva Natural Salus (−34.421111, −55.315000), Lavalleja Department, and Laguna Negra (−34.085833, −53.738055), Rocha Department, are in the southeast part of the country, and belong to the ecoregion Sierras del Este sensu Brazeiro et al., (2012).

2.2. Tick collection and identification

Questing ticks were collected from vegetation using the flagging method. The collection was carried out for a period of 2 h in the samplings in each visit to the sites. Ticks were picked up from the cloth at 5–10-m intervals and stored in plastic tubes with 95% ethanol. In the laboratory, each arthropod was identified using a stereomicroscope following the morphological keys for larval, nymph and adult stages by Kohls (1960), Webb et al. (1990), and Nava et al. (2017).

2.3. DNA extraction and detection of Ehrlichia spp.

Only free-living nymphs and adult ticks were used in this study. Since Ehrlichia spp. are not maintained by transovarial transmission, larvae were not analyzed.

Detection of Ehrlichia DNA was performed into pools of adult or nymphs (1–10 ticks per pool) separated according to site and collection date. Briefly, ticks were rinsed with distilled water to remove ethanol and bisected longitudinally using sterile scalpels and forceps. Finally, each pool was homogenized cutting thoroughly the ticks with dissecting scissors. DNA was extracted using the commercial kit GeneJET Genomic DNA Purification Kit (Thermo Scientific, Lithuania) following the manufacturer’s instructions. The concentration and purity of DNA was determined using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Molecular screening was carried out using primers EHR16SD/EHR16SR with PCR conditions described by Parola et al. (2000). This PCR enables detection of a 16S rRNA gene fragment of members of the family Anaplasmataceae including the genera Anaplasm, Ehrlichia, Neorickettsia and Wolbachia. Positive samples were subjected to four additional PCR protocols. Two overlapping fragments were obtained using primers fD1/EHR16SR and EHR16SD/Rp2 (Weisburg et al., 1991; Inokuma et al., 2001) to amplify nearly full-length sequence of the 16S rRNA gene. In addition, a semi-nested and nested PCRs targeting dsb (disulfide oxidoreductase) and groEL (60 kDa chaperonin) genes, respectively, were performed (Sumner et al., 1997; Lotric-Furlan et al., 1998; Nicholson et al., 1999; Doyle et al., 2005; Almeida et al., 2013). All primers used and fragment sizes are listed in Table 1. Distilled water and an Ehrlichia canis DNA-positive sample were included as negative and positive controls in all runs. Five microliters of PCR products were analyzed by electrophoresis into 1.5% agarose gels, stained with GoodView™ Nucleic Acid Stain (Beijing SBS Genetech Co., Ltd.), and examined under UV transillumination. Amplicons were purified using GeneJET PCR purification kit (Thermo Scientific, Lithuania) and sent for sequencing to Macrogen (Seoul, Korea). BLASTn analyses (www.ncbi.nlm.nih.gov/blast) were performed with E. canis DNA sequences from Sin et al. (2005).
performed in order to infer closest identities with microorganisms available on GenBank database (Altschul et al., 1990), and to include these sequences into a phylogenetic analysis. Nucleotide identities of obtained sequences were calculated using the Sequence Identity and Similarity (SIAS) calculator (http://imed.med.ucm.es/Tools/sias.html).

2.4. Phylogenetic analyses

Independent alignments using obtained sequences for 16S rRNA, dsb and groEL loci were constructed with CLUSTAL W (Thompson et al., 1994) including homologue sequences downloaded from GenBank. Three phylogenetic trees were inferred using Bayesian inference analysis as implemented in MrBayes 3.2.5. (Huelsenbeck & Ronquist, 2001), using 1,000,000 generations. The general time reversible (GTR) model was chosen to run all the trees. Each tree was sampled every 100 generations, begun with random seeds and ran four times. The first 25% of the trees was discarded as “burn-in”, and the remaining subset of trees was used to calculate Bayesian posterior probabilities. Sequences of “Candidatus Neoehrlichia mikurensis” (EU810406; AB213021) and *Ehrlichia ruminantium* (AF308669) were used to root the phylogenetic trees.

3. Results

3.1. Tick collection

A total of 1,548 *I. auritulus* ticks (73 females, 185 nymphs and 1,290 larvae) were collected on vegetation at four of the five locations sampled (Table 2). Other tick species such as *Amblyomma aureolatum*, *Haemaphysalis juxtakochi*, and *Ixodes fuscipes* were also collected but not included in this study.

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Table 3

| Pairwise comparison matrix for 16S rDNA sequences of *Ehrlichia* sp. Serrana, *Ehrlichia* sp. Laguna Negra, and *Ehrlichia* sp. Magellanica |
|---------------------------------------------------------------------------------------------------------------|
| **Species**                                                                                                             | 1 | 2 | 3 | 4 | 5 | 6 |
| *Ehrlichia* sp. Laguna Negra S6laH12 (LN)                                                                                 | – | – | – | – | – | – |
| *Ehrlichia* sp. Serrana S10laN9 - Salus (MW650902)                                                                           | 99.79 | – | – | – | – | – |
| *Ehrlichia* sp. Serrana S17laH1 - Amarillo (MW650901)                                                                         | 99.78 | 100 | – | – | – | – |
| *Ehrlichia* sp. Serrana S17laN33 - Salus (MW650904)                                                                           | 99.68 | 99.89 | 99.89 | – | – | – |
| *Ehrlichia* sp. Serrana S12laH4 - Amarillo (MW650903)                                                                           | 99.78 | 100 | 100 | 99.89 | – | – |
| *Ehrlichia* sp. Magellanica (MK049840)                                                                                       | 97.12 | 97.12 | 90.8 | 92.24 | 92.52 | – |

Fig. 3. Bayesian phylogenetic analysis inferred for partial fragments of the dsb gene. Bayesian posterior probabilities > 0.95 are indicated above or below the nodes. The positions of *Ehrlichia* sp. genotype Serrana and *Ehrlichia* sp. genotype Laguna Negra are highlighted within red and blue boxes, respectively. The scale-bar indicates the number of substitutions per nucleotide position. GenBank accession numbers are in parentheses.
Table 4
Pairwise comparison matrix for *dsb* sequences of *Ehrlichia* sp. Serrana, *Ehrlichia* sp. Laguna Negra, and *Ehrlichia* sp. Magellanica

|      | 1              | 2              | 3              | 4              | 5              | 6              | 7              |
|------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| 1    | Ehrlichia sp. Laguna Negra S6LaH12 (LN) | –              | –              | –              | –              | –              | –              |
| 2    | Ehrlichia sp. Serrana S8Lan38 (LN)     | 88.47          | –              | –              | –              | –              | –              |
| 3    | Ehrlichia sp. Serrana S10LaN9 (Salus)  | 88.47          | 100            | –              | –              | –              | –              |
| 4    | Ehrlichia sp. Serrana S12LaH4 (Amarillo) | 88.47          | 100            | 100            | –              | –              | –              |
| 5    | Ehrlichia sp. Serrana S17LaH1 (Amarillo) | 88.16          | 99.68          | 99.68          | 99.68          | 100            | –              |
| 6    | Ehrlichia sp. Serrana S17LaN33 (Salus) | 88.16          | 99.68          | 99.68          | 99.68          | 98.02          | 91.83          |
| 7    | Ehrlichia sp. Magellanica (MK049838)   | 88.78          | 87.22          | 87.22          | 87.22          | 86.91          | 86.91          |

Table 5
Pairwise comparison matrix for *groEL* sequences of *Ehrlichia* sp. Serrana, *Ehrlichia* sp. Laguna Negra, and *Ehrlichia* sp. Magellanica

|      | 1              | 2              | 3              | 4              | 5              |
|------|----------------|----------------|----------------|----------------|----------------|
| 1    | Ehrlichia sp. Laguna Negra S6LaH12 (LN) | –              | –              | –              | –              |
| 2    | Ehrlichia sp. Serrana S8Lan38 (LN)     | 99.78          | –              | –              | –              |
| 3    | Ehrlichia sp. Serrana S10LaN9 (Salus)  | 96.05          | 95.83          | –              | –              |
| 4    | Ehrlichia sp. Serrana S17LaH1 (Amarillo) | 95.61          | 95.39          | 98.02          | –              |
| 5    | Ehrlichia sp. Magellanica (MK049839)   | 92.85          | 92.51          | 92.17          | 91.83          |

3.2. Detection of *Ehrlichia* DNA in ticks

Ticks were processed and analyzed in 42 pools (17 containing females and 25 containing nymphs). Of these, 15 (9 containing females and 6 containing nymphs) were positive for DNA of *Ehrlichia* spp. Geographically, *Ehrlichia* sp. DNA was detected in Amarillo (two pools of females), Reserva Natural Salus (two pools of nymphs) and Laguna Negra (seven pools of females and two of nymphs) (Table 2).

3.3. Phylogenetic analyses and nucleotide comparisons of sequences

Trees inferred by Bayesian analysis were constructed to define the phylogenetic position of the generated sequences. With the exception of the *groEL* tree, our sequences clustered into a monophyletic group including *Ehrlichia* sp. Magellanica from Chile (Figs. 1–3). However, sequences for all three loci were obtained from only two pools (S6LaH12 and S17LaH1) (Table 2). Based on the topology of the phylogenetic trees, two genotypes of *Ehrlichia* with different haplotypes appear in all processed pools; these are referred to as *Ehrlichia* sp. genotype Serrana and *Ehrlichia* sp. genotype Laguna Negra. The genotype Serrana was identified in 13 pools (86.7%), and the genotype Laguna Negra in 2 (13.3%) positive pools of ticks. The sequences generated in this study were deposited in the GenBank database and the accession numbers are listed in Table 2. The percent nucleotide identities among 16S rDNA, *dsb* and *groEL* sequences between *Ehrlichia* sp. genotype Serrana, *Ehrlichia* sp. genotype Laguna Negra and the most similar sequence available on GenBank, *Ehrlichia* sp. Magellanica (MK049840, MK049838, MK049839), are shown in Tables 3–5.

4. Discussion

In the present study, we performed molecular screening of *Ehrlichia* spp. in *I. auritulus* and revealed the occurrence of two genotypes of *Ehrlichia* provisionally named Serrana and Laguna Negra. Genetic comparisons conducted for the three studied genes are consistent to consider them as separate organisms. This assumption was confirmed by the topologies of phylogenetic trees since Serrana and Laguna Negra genotypes formed well-defined and separated clades within the genus *Ehrlichia*. Interestingly, *Ehrlichia* sp. Magellanica seems to correspond to a closely related species, because it grouped with both genotypes in all phylogenies. In addition, *Ehrlichia* sp. Magellanica and both genotypes herein characterized were similar when performing nucleotide pairwise comparisons, and all three were detected in ticks that parasitize birds.
Methodology, Resources, Formal Analysis, Investigation, Writing - Original Draft, Writing - Review & Editing, Methodology, Formal Analysis, Investigation, Writing - Original Draft, Writing - Review & Editing. All authors read and approved the final manuscript.

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Declaration of competing interests

The authors declare that they have no competing interests.

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