Experimental and Applied Acarology (2022) 86:271–282
https://doi.org/10.1007/s10493-022-00684-0

Rickettsia spp., *Ehrlichia* sp. and *Candidatus* Midichloria sp. associated to ticks from a protected urban area in Buenos Aires City (Argentina)

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Received: 27 May 2021 / Accepted: 9 January 2022 / Published online: 13 January 2022

Abstract
The aim of this study was to determine the infection with Rickettsiales in ticks and birds from the main protected urban area of Buenos Aires City (Argentina). One *Amblyomma aureolatum* (0.2%) and one *Ixodes auritulus* (0.1%) were positive by PCR targeting *Rickettsia* 23S-5S rRNA intergenic spacer. Phylogenetic analysis shows to findings in *A. aureolatum* are closely to *Rickettsia bellii* and for *I. auritulus* are related to '*Candidatus* Rickettsia mendelii'. One *I. auritulus* (0.1%) and three *A. aureolatum* (0.6%) were positive by PCR for a fragment of the 16S rRNA gene of the Anaplasmataceae family. The sequences obtained from *A. aureolatum* were phylogenetically related to Midichloriaceae endosymbionts. The sequence from *I. auritulus* s.l. had 100% identity with *Ehrlichia* sp. Magellanica from Chile and two genotypes of *Ehrlichia* sp. from Uruguay. The results of our study show that *Rickettsia* and *Ehrlichia* are present in ticks in the main protected urban area of Buenos Aires City.

Keywords *Rickettsia* · *Ehrlichia* · Midichloriaceae · Ticks · Buenos Aires City · Argentina

Introduction

Ticks (Acari: Ixodida) are associated as potential vectors to a considerable diversity of microorganisms, some of which are pathogens to humans and animals (Sonenshine and Roe 2014). From a veterinary and public health perspective, bacteria belonging to the families Rickettsiaceae and Anaplasmataceae are among the most relevant tick-borne microorganism (Sonenshine and Roe 2014).

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Members of the genus *Rickettsia* (family Rickettsiaceae, order Rickettsiales, phylum Proteobacteria) are obligate intracellular bacteria that are etiological agents of diseases in humans and animals, with tropism for endothelial cells. The pathogenesis caused by *Rickettsia* is related its injury, causing vasculitis, microbleeds, increased vascular permeability, edema and activation of inflammation and coagulation mechanisms (Merhej and Raoult 2011). Phylogenetically, the genus *Rickettsia* is divided into four groups: (1) spotted fever group (e.g., *Rickettsia rickettsii*), mainly transmitted by hard ticks; (2) transitional group, which includes *Rickettsia felis* and *Rickettsia akari*, transmitted by fleas and mites, respectively; (3) typhus group: *Rickettsia typhi* and *Rickettsia prowazekii*, transmitted by fleas and lice, respectively; and (4) an ancestral group that includes *Rickettsia bellii* and *Rickettsia canadensis*, mainly transmitted by ticks (Merhej and Raoult 2011).

The family Anaplasmataceae (order Rickettsiales, phylum Proteobacteria) includes the genera *Ehrlichia*, *Anaplasma*, *Neorickettsia* and *Wolbachia* (Dumler et al. 2001). Obligate intracellular bacteria of the genera *Ehrlichia* and *Anaplasma* reside within cytoplasmic vacuoles, separately or more frequently in compact inclusions (morulae), present in mature or immature hematopoietic cells, in peripheral blood, or in host tissues. These bacteria are vectored by ticks and they are etiological agents of diseases of dogs and other canids, humans and ruminants (Dumler et al. 2001).

The family Midichloriaceae (order Rickettsiales, phylum Proteobacteria) is an emerging novel group of intracellular bacteria associated with a wide range of hosts, such as ticks, fleas, stink bugs, ciliates, amoebae, cnidarians, sponges, fish, and various vertebrates (Montagna et al. 2013; Szokoli et al. 2016). '*Candidatus* Midichloria mitochondrii', the first member described, presents an unusual lifestyle inside the tick mitochondria (Montagna et al. 2013; Szokoli et al. 2016).

There is an increase in reports about ticks and their pathogens in small natural areas in urban environments (LaDeau et al. 2016; Cicuttin et al. 2019). Humans and animals inhabiting these small areas may even have a high risk of exposure to tick-borne pathogens due to a high density of ticks, related to an imbalance in host availability (LaDeau et al. 2016). Rickettsiales previous reports for Buenos Aires City correspond to the findings of *Rickettsia massiliae* in *Rhipicephalus sanguineus* s.s. ticks and one human case for this pathogen (García-García et al. 2010; Romer et al. 2014), *Ehrlichia canis* in dogs (Cicuttin et al. 2016) and *Anaplasma platys* in dogs and in *R. sanguineus* s.s. (Romer et al. 2014; Cicuttin et al. 2015). The aim of this study was to determine the infection with members from Rickettsiales in different tick species and birds present in the main protected urban area of Buenos Aires City.

Methods

Study area

The protected urban area Reserva Ecológica Costanera Sur (RECS; 34°36′S, 58°21′W) is characterized by different environments of artificial origin, such as marshes, lagoons, pastures, thickets and forests, in addition to the beaches of the river. Birds represent the most diverse group of vertebrates. Regarding reptiles, the lizard *Salvator merianae* is a typical inhabitant of the Reserve. Mammals mainly include rodents from the families Muridae, Cricetidae and Caviidae, and opossums (family Didelphidae). Furthermore, stray dogs (*Canis lupus familiaris*), which circulate throughout the reserve and surrounding

Table 1 Primers used for *Rickettsia* spp.

Target	Name	Sequence (5′–3′)	References
23S-5S rRNA intergenic spacer	RCK/23-5-F	GATAGGTCRGRTGTGGAAGCAC	Jado et al. (2006)
	RCK/23-5-R	TCGGGAYGGGATCGTGTGTTTC	
gltA	CS-239	GCTCTTCTCATCCTATGGCTATTAT	Labruna et al. (2004)
	CS-1069	CAGGGTCTTCGTGCATTTCTT	

poor neighborhoods, constitutes an important component of the fauna in the area (Wais de Badgen 2013). RECS area borders with two crowded neighborhoods, Puerto Madero and Rodrigo Bueno, with contrasting socioeconomic characteristics, and the La Plata River (Wais de Badgen 2013). The study was conducted with permissions of the authorities of Reserva Ecológica Costanera Sur (numbers 30/09/2010, 01/2014, 20/2016, 32/2016 and 17/2018).

Samples

Free-living ticks were monthly collected from vegetation in 2013 and 2014, and September and October 2018 by using cloth flags and carbon dioxide traps. Furthermore, a total of 340 birds were caught between winter of 2016 and autumn of 2017, on seasonal sampling. Ticks attached to head and neck of 47 birds were collected. By last, more ticks were also collected by RECS staff from an undetermined number of stray dogs, one human, and a working hut.

In addition, approximately 100 µl of blood was collected from the jugular vein from mainly large Passeriformes birds in good physical condition caught for tick collection. Furthermore, birds found dead in the area and derived for diagnosis of zoonoses at Instituto de Zoonosis Luis Pasteur (Buenos Aires City) between 2011 and 2017, were also included in this study. The sample collected from each individual dead bird was a pool of organs (spleen and liver).

The detailed procedures for the tick sampling on hosts and taxonomic determination have been published elsewhere (Cicuttin et al. 2017, 2019).

DNA extraction and PCR amplification

Tick larvae were grouped in pools of 1–10 specimens for DNA extraction according to species, date and host of collection; DNA of nymphs and adults were extracted individually. DNA extraction from ticks, blood and tissues was performed using the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) following the manufacturer's instructions.

The detection of *Rickettsia* spp. was initially performed by a simple PCR to amplify a fragment of variable size from the 23S-5S rRNA intergenic spacer (Jado et al. 2006). The molecular characterization of the findings was carried out by a PCR for a *gltA* gen (Labruna et al. 2004) (Table 1).

Initial PCR with primers for a 16S rRNA fragment were used for the Anaplasmataceae family (Parola et al. 2000) (Table 2). This pair of primers has been used routinely to detect bacteria of this family; however, several studies have shown that they also detect a group of

Table 2 Primers used for the Anaplasmataceae family

Target	Name	Sequence (5′–3′)	References
16S rRNA	EHR16SD	GGTACCYACAGAAGAAGTCC	Parola et al. (2000)
	EHR16SR	TAGCACTCATCGTTTACAGC	
dsb	*dsb-330*	GATGATGTCTGAAGATATGAAACAAAT	Aguiar et al. (2007)
	dsb-380	ATTTTTAGRGATTTTCCAATACTTGG	Almeida et al. (2013)
	dsb-728	CTGCTCGTCTATTTTACTTCTTAAAGT	Aguiar et al. (2007)
groESL	*HS1a*	AITGGGCTGGTAITGAAAT	Liz et al. (2000)
	HS6a	CCICCIGGIACIAIACCTTC	
	HS43	ATWGCWAARGAAGCATAGTC	
	HSVR	CTCAACAGCAGCTCTAGTAGC	

closely related α-proteobacteria within the order Rickettsiales like Midichloriaceae family (Parola et al. 2003; Venzal et al. 2008).

The positive samples for *Ehrlichia* were further characterized by a PCR for fragments of two different genes: *dsb* (heminested) and *groESL* (nested) (Liz et al. 2000; Aguiar et al. 2007; Almeida et al. 2013) (Table 2).

For all PCR reactions, nuclease free water was used as negative control. DNA of the *Rickettsia conorii* (kindly provided by the Laboratorio de Espiroquetas y Patógenos Especiales, Instituto de Salud Carlos III, Spain) and *Anaplasma centrale* served as positive control for screening PCRs of *Rickettsia* and Anaplasmataceae, respectively.

Sequence comparison and phylogenetic analysis

Amplified PCR-products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and sequenced with a 3500 Genetic Analyzer sequencer (Applied Biosystems, Foster City, CA, USA). The obtained sequences were edited using BioEdit Sequence Alignment Editor (Hall 1999) with manual edition whenever it was necessary and aligned with the program Clustal W (Thompson et al. 1994). Sequences obtained in this work were compared with those sequences deposited in GenBank by using BLAST (www.ncbi.nlm.nih.gov/blast). Phylogenetic analysis was performed with the maximum-likelihood (ML) method. The best-fitting substitution model was determined with the Bayesian Information Criterion using the ML model test implemented in MEGA 6 (Tamura et al. 2013). Gaps were excluded in the pairwise comparison, and support for the topology was tested by bootstrapping over 1000 replications.

Results

In total, 1282 ticks were analyzed: 1091 free-living ticks (420 *Amblyomma aureolatum*, 606 *Ixodes auritulus* s.l.[1] and 65 *Amblyomma triste*), 100 collected on birds (88 *I. auritulus* s.l. and 12 *A. aureolatum*), 89 collected on dogs (86 *A. aureolatum*, 2 *Rh. sanguineus* s.s.

[1] According to Guglielmone et al. (2020).

Table 3 Molecular diagnosis of Rickettsiales microorganism in various species and stages of ticks

Species	Stage	n	Pool	PCR (%)		
				Genus *Rickettsia*	Family Anaplasmataceae	Family Midichloriaceae
Amblyomma aureolatum	Larva	369	47	0	0	0
	Nymph	63	–	0	0	0
	Female	83	–	1 (1.2)	0	3 (3.6)
	Male	4	–	0	0	0
	Total	519	–	1 (0.2)	0	3 (0.6)
Amblyomma triste	Female	46	–	0	0	0
	Males	21	–	0	0	0
	Total	67	–	0	0	0
Ixodes auritulus s.l.	Larva	628	92	0	0	0
	Nymph	58	–	1 (1.7)	1 (1.7)	0
	Female	8	–	0	0	0
	Total	694	–	1 (0.1)	1 (0.1)	0
Rhipicephalus sanguineus s.s.	Female	1	–	0	0	0
	Male	1	–	0	0	0
	Total	2	–	0	0	0
Total		1282	–	2 (0.2)	1 (0.1)	3 (0.2)

and 1 *A. triste*), 1 *A. triste* from a human and 1 *A. aureolatum* on a working hut. Details of tick stage per host are shown in Table 3.

In addition, 144 blood samples from birds were studied, including 30 birds with tick infestation (15 *Turdus rufiventris*, 11 *Turdus amaurochalinus*, 2 *Saltator aurantiirostris*, 1 *Stephanophorus diadematus* and 1 *Furnarius rufus*). Also 168 pools of organs (spleen and/or liver) of birds found dead were analyzed. Detailed information is presented in supplementary material (Online Resources 1 and 2).

Genus *Rickettsia*

One female of *A. aureolatum* (0.2% of the total) collected on dog and one nymph of *I. auritulus* s.l. (0.1% of the total) collected on *T. amaurochalinus* were positive by PCR for a fragment of the intergenic spacer of rRNA 23S-5S. The female of *A. aureolatum* was collected in April, while the nymph of *I. auritulus* s.l. was collected in October. The remaining ticks and all bird samples (including the *T. amaurochalinus* blood that had the *Rickettsia*-positive tick) were negative for *Rickettsia*.

The sequence obtained from the 23S-5S rRNA fragment of *A. aureolatum* (350 bp; GenBank acc. nr. MW824653) had 99.1-99.7% identity with different findings of *Rickettsia bellii* in ticks, whereas that the sequence from *I. auritulus* s.l. (204 bp; GenBank acc. nr. MW824654) presented 86.8–86.9% with different species of *Rickettsia* such as *R. amblyommatis*, *R. felis*, *R. massiliae* and *Candidatus* Rickettsia andeanae, among others.

The positive sample of *I. auritulus* s.l. in the PCR rRNA 23-5S of the genus *Rickettsia* was also positive in the PCR of the *gltA* gene and could be sequenced (GenBank acc. nr.

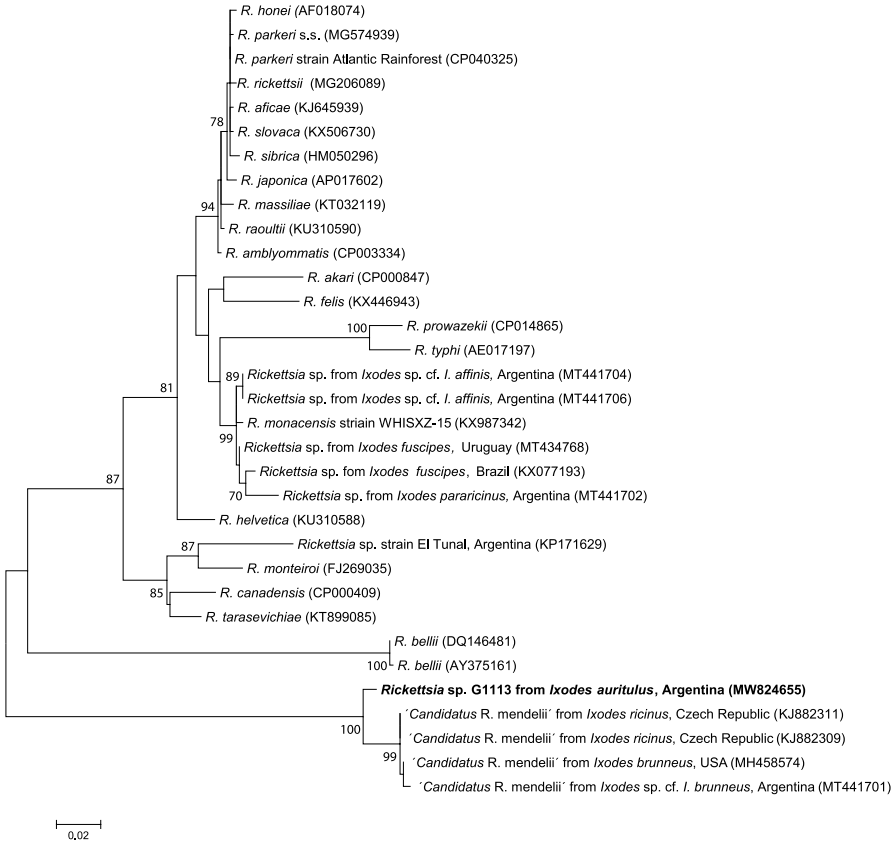

Fig. 1 Phylogenetic tree generated by the maximum likelihood method (GTR+G) for a fragment of the *gltA* gene of the genus *Rickettsia*. The numbers on the nodes represent the resampling support generated by 1000 replications (only bootstrap support >70 is shown). GenBank accession numbers are shown in parentheses

MW824655). This *gltA* sequence was phylogenetically related to sequences of '*Candidatus* Rickettsia mendelii' isolated from *Ixodes ricinus* in Czech Republic (KJ882311 and KJ882309), from *Ixodes brunneus* in USA (MH458574) and from *Ixodes silvanus* (named as *Ixodes* sp. cf. *I. brunneus*[2]) in Argentina (MT441701) (Fig. 1). The similarity of the *gltA* sequence obtained from *I. auritulus* in this work with these four sequences of '*C.* R. mendelli' ranged from 97.8 to 98.8%.

The positive sample of *A. aureolatum* to PCR rRNA 23S-5S of the genus *Rickettsia* was negative to PCR for a fragment of the *gltA* gene of the genus *Rickettsia*.

[2] See Saracho-Bottero et al. (2021) about the taxonomic status of this tick.

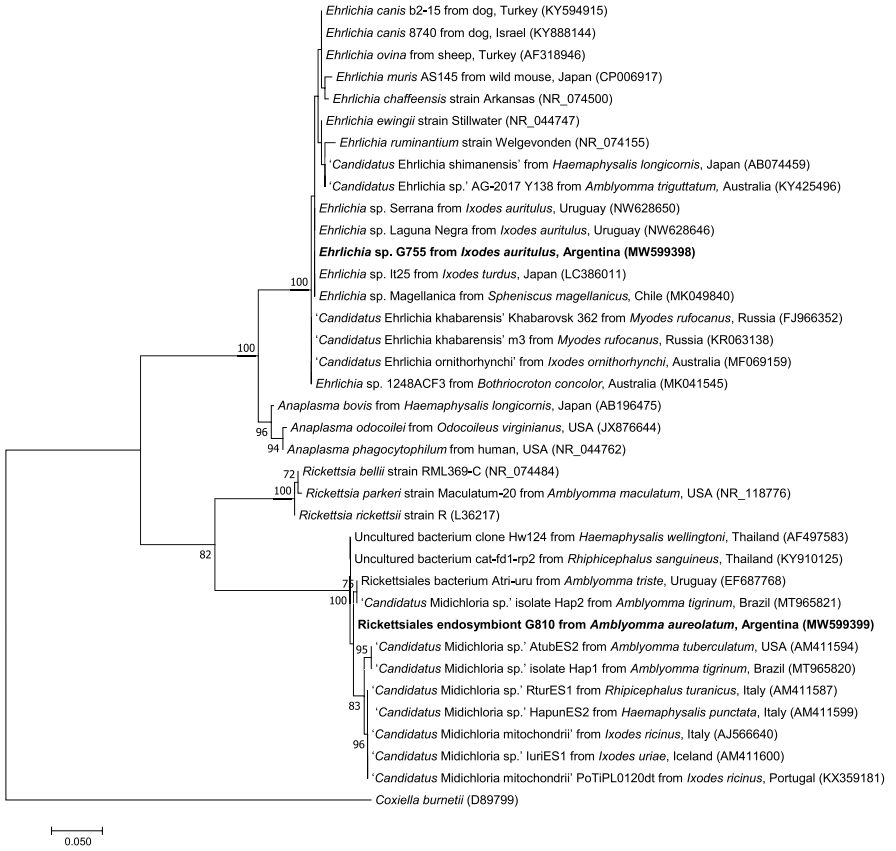

Fig. 2 Phylogenetic tree generated by the maximum likelihood method (T92+G) for a fragment of the 16S rRNA of Anaplasmataceae, Midichloriaceae and Rickettsiaceae families. The numbers on the nodes represent the resampling support generated by 1000 replications (only bootstrap support >70 is shown). GenBank accession numbers are shown in parentheses

Family Anaplasmataceae

One free-living nymph of *I. auritulus* s.l. (0.1% of the total) collected from the vegetation were positive by PCR for a fragment of the 16S rRNA gene of the Anaplasmataceae. The nymph of *I. auritulus* s.l. was collected in December. The remaining ticks and all bird samples were negative.

The sequence obtained from *I. auritulus* s.l. (GenBank acc. nr. MW599398) had 100% identity with *Ehrlichia* sp. Magellanica detected in penguins (*Spheniscus magellanicus*) from Chile (MK049840), two genotypes of *Ehrlichia* sp. in *I. auritulus* from Uruguay (NW628646, NW628650) and *Ehrlichia* sp. for *Ixodes turdus* from Japan (LC386011). These three sequences are phylogenetically related to *Ehrlichia* spp. from *Bothriocroton concolor* (MK041545) and *Ixodes ornithorhynchi* (MF069159), both from Australia, and '*Candidatus* Ehrlichia khabarensis' detected in the rodent *Myodes rufocanus* in Russia (KR063138 and FJ966352) (Fig. 2). The similarity of the 16S rRNA sequence obtained from *I. auritulus* s.l. in this work with these four sequences was 99.7%.

Unfortunately, it was not possible to amplify the sample of *I. auritulus* s.l.-*Ehrlichia* positive by PCR for fragments of the *dsb* and *groESL* genes.

Family Midichloriaceae

Three *A. aureolatum* females (0.6% of the total) collected between October and February on dogs were positive by PCR for a fragment of the 16S rRNA gene. The three sequences obtained from *A. aureolatum* (GenBank acc. nr. MW599399) had 100% identity with each other and were phylogenetically related to sequences of α-proteobacteria endosymbionts found in different tick species in Thailand (*Rh. sanguineus* and *Haemaphysalis wellingtoni*) and Uruguay (*A. triste*), '*Candidatus* Midichloria sp.' Hap2 from Brazil (*Amblyomma trigrinum*) and '*Candidatus* Midichloria mitochondrii' isolated from *I. ricinus* (Fig. 2). The similarity of the 16S rRNA sequence obtained from *A. aureolatum* in this work with these five sequences ranged from 98.7 to 99.7%.

Discussion

This study reports the finding of *Rickettsia* sp. and *Ehrlichia* sp. in *I. auritulus* s.l. and *R. bellii* in *A. aureolatum* from Argentina. In addition, the finding in *A. aureolatum* of a group of Midichloriaceae endosymbionts of ticks is reported, being the third evidence in ticks for South America and the first for *A. aureolatum*.

Different species of *Rickettsia* have been found in ticks from the genus *Ixodes* throughout the world (Merhej and Raoult 2011; Akl et al. 2019; Tokarz et al. 2019), including some pathogenic for humans such as *Rickettsia australis* (Merhej and Raoult 2011). In South America there are few reports of *Rickettsia* spp. associated to *Ixodes* ticks. One *Rickettsia* sp. was detected in *Ixodes pararicinus* (larvae and nymphs) collected on birds from Salta (Argentina) (Flores et al. 2016). Sebastian et al. (2020) reported the finding of a *Rickettsia* sp. closely related to *Rickettsia buchneri* in *I. pararicinus* and *Ixodes* sp. cf. *Ixodes affinis*, which were collected from vegetation and on birds in different regions of Argentina. Sebastian et al. (2020) also found this strain of *Rickettsia* in free-living *Ixodes fuscipes* from Uruguay, and a *Rickettsia* sp. closely related to '*C.* R. mendelii' in *I. silvanus* (named as *Ixodes* sp.) collected on birds in Tucumán (Argentina). Finally, Blanco et al. (2016) reported *Rickettsia* sp. in *I. fuscipes* (named as *Ixodes aragoi*) collected from rodents in Brazil.

'*Candidatus* R. mendelii' was previously detected in *I. ricinus* from the Czech Republic (Hajduskova et al. 2016) and in *Ixodes brunneus* from USA (Cumbie et al. 2020). In South America, the strain related to '*C.* R. mendelii' previously detected in *I. silvanus* from Argentina is related to the *Rickettsia* sp. found in our study (Sebastian et al. 2020).

The only antecedent of *R. bellii* in *A. aureolatum* correspond to findings in ticks collected on dogs in Brazil (Pinter and Labruna 2006). In Argentina, *R. bellii* was reported in different species of ticks such as *Amblyomma neumanni*, *Amblyomma dubitatum*, *Amblyomma trigrinum*, *Amblyomma sculptum*, *Amblyomma ovale* and *Haemaphysalis juxtakochi* (Sebastian et al. 2016). *Rickettsia bellii* is classified within the group called ancestral and has been found in ticks and insects, being considered a symbiont species (Merhej and Raoult 2011; Krawczak et al. 2018). There is no evidence that it causes disease in humans or animals (Merhej and Raoult 2011; Krawczak et al. 2018).

In this study, we did not detect *Rickettsia parkeri* in *A. triste*. This result is unexpected considering that the association between *R. parkeri* and *A. triste* appears to be a ubiquitous phenomenon. Furthermore, *R. parkeri* has been found in different regions of South America, and even in regions close to the RECS such as the Paraná Delta (Nava et al. 2008; Romer et al. 2011) and Ensenada (Villalba-Apestegui et al. 2018). This situation could be explained by the relative recent anthropomorphic conformation of the Reserva Ecológica Costanera Sur, which was created in the year 1986 (Wais de Badgen 2013). Therefore, it is probably the founder population of *A. triste* was not infected and remained isolated during this time. However, it is necessary to continue researching this highly important tick species.

Ticks of the genus *Ixodes* have been recognized as potential vectors of *Ehrlichia* spp. in different regions of the world (Pritt et al. 2017). The 16S rRNA sequence of the *Erhlichia* sp. found in *I. auritulus* s.l. in this study was identical to the sequences of the *Ehrlichia* spp. found in *I. auritulus* from Uruguay, in *I. turdus* from Japan and in the penguin *S. magellanicus* from Chile (Muñoz-Leal et al. 2019; Taira et al. 2019; Félix et al. 2021). Unfortunately, the *Ehrlichia* sp. found in our study could not be characterized with more polymorphic molecular markers such as *dsb* or *groESL*, therefore, the current phylogenetic information is limited.

Rickettsiales organisms found in *A. aureolatum* are related to Midichloriaceae family, a group of endosymbionts bacteria (Montagna et al. 2013; Szokoli et al. 2016). In Uruguay, Venzal et al. (2008) detected α-proteobacteria in 33% of adult pools of *A. triste*. And more recently in Brazil, Arrais et al. (2021) determined two haplotypes (Hap 1 and Hap2) of '*C.* Midichloria sp.' in 47.6% of pools of *A. tigrinum* collected on *Chrysocyon brachyurus*. In comparison, it was only detected 0.6% of the *A. aureolatum* ticks analyzed during this study to be positive to a '*C.* Midichloria sp.' phylogenetically related to that found in *A. triste* from Uruguay by Venzal et al. (2008).

Different *Rickettsia* species are efficiently transmitted both transstadially and transovarianly in ticks (Merhej et al. 2014). However, in some species of Rickettsiae the participation of amplifying mammalian hosts is observed (Merhej et al. 2014). The role of birds as amplifiers is much more debated, although various studies have detected *Rickettsia* spp. (mainly *Rickettsia helvetica*) in blood of birds from Europe (Hornok et al. 2014; Berthová et al. 2015). There are no bibliographic reports for America of findings in birds. All reports correspond to detection of *Rickettsia* in ticks collected on birds, but not in bird themselves. On the other hand, hard ticks are vectors of *Ehrlichia* spp., but transovarial transmission does not appears to occur. A vertebrate host is required to maintain the infection within tick populations (Dumler et al. 2001). Historically, mammals have been considered the natural vertebrate hosts of the genus *Ehrlichia* (Dumler et al. 2001). However, recent studies in reptiles and birds and their associated ticks, reveal a more complex scenario (Machado et al. 2012; Andoh et al. 2015; Muñoz-Leal et al. 2019) detected *Ehrlichia* sp. in spleen of penguins *S. magellanicus* in southern Chile, whereas Machado et al. (2012) and Sacchi et al. (2021) report *Ehrlichia* spp. in whole blood of different birds (*Falcos sparverius*, *Coragyps atratus*, *Neochen jubata Megascops choliba*, *Speotyto cunicularia*, *Rupornis magnirostris*, *Tyto alba* and *Asio clamator*). With the exception of a study in Brazil that studied two *Caracara plancus* (being negative) (Machado et al. 2012), there are no previous studies of *Ehrlichia* spp. on the bird species analyzed in our study. By last, Midichloriaceae has been associated with fish and mammals, but not with birds (Montagna et al. 2013; Szokoli et al. 2016).

Amblyomma aureolatum has significance from a public health perspective because adults of this tick species are known to bite humans, but there is no evidence that *R. bellii*

causes disease in humans or animals and the Midichloriaceae found in *A. aureolatum* are of pathogenicity unknown. On the other hand, the epidemiological risk that implies the infection with *Rickettsia* and *Ehrlichia* species associated with *I. auritulus* s.l. seems to be low because this tick is not aggressive to humans; furthermore, the *Rickettsia* and *Ehrlichia* strains associated to this tick are of pathogenicity unknown to humans.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10493-022-00684-0.

Acknowledgements We gratefully acknowledge to Juan Carlos Sassarolli (Instituto de Zoonosis Luis Pasteur), Lorena Zapata (Reserva Ecológica Costanera Sur) and residents of Veterinary Public Health Residency (Instituto de Zoonosis Luis Pasteur). This work was partially supported by research fellowship 'Ramón Carrillo-Arturo Oñativia' 2015 (Ministry of Public Health, Argentina) and by grant for Clinical and Epidemiological Research 2016-2018 (Roemmers Fundation, Argentina). SN acknowledges the financial support of INTA, Asociación Cooperadora Inta Rafaela and CONICET.

Declarations

Conflict of interest None.

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