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A novel *Ehrlichia* strain (Rickettsiales: Anaplasmataceae) detected in *Amblyomma triste* (Acari: Ixodidae), a tick species of public health importance in the Southern Cone of America

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ABSTRACT

The aim of this work was to report the detection of a putative novel *Ehrlichia* strain associated with the tick *Amblyomma triste*. Free-living adult ticks determined as *A. triste* were collected by drag-sampling in Argentina and Uruguay. Molecular detection of *Ehrlichia* agents was performed targeting three different loci: 16S rRNA gene, *dsb* gene and a fragment of *groESL* heat shock operon. In total, 164 adults of *A. triste* (38 from INTA E.E.A Delta del Paraná in Argentina and 126 from Toledo Chico in Uruguay) were analyzed. One tick (0.6%) collected in INTA E.E.A. Delta del Paraná (Argentina) was positive. The phylogenetic analyses show that the *Ehrlichia* strain found in this study (named *Ehrlichia* sp. strain Delta) represents an independent lineage within the genus *Ehrlichia*, close to *E. chaffeensis* and *E. muris*. This is also the first report of an *Ehrlichia* agent infecting the tick *A. triste*. The medical and veterinary significance of *Ehrlichia* sp. strain Delta remains to be demonstrated. However, it is important to mention that adults of *A. triste* are aggressive to humans and domestic mammals. Therefore, the potential role of *A. triste* in the transmission of *Ehrlichia* agents to humans or domestic animals across its distributional range should be highlighted, even more considering that *Ehrlichia* sp. strain Delta is phylogenetically related to the zoonotic *E. chaffeensis*, which is recognized as pathogenic to both humans and animals.

KEYWORDS

Ehrlichia; ticks; *Amblyomma*; South America

Introduction

Bacteria of the genus *Ehrlichia* (Rickettsiales: Anaplasmataceae) are alpha-proteobacterial, tick-transmitted, obligate intracellular parasites with medical and veterinary importance that can infect monocytes, neutrophils, endothelial cells or neutrophils [1,2]. Formally there are six recognized species: *Ehrlichia canis, Ehrlichia chaffeensis, Ehrlichia ewingii, Ehrlichia ruminantium, Ehrlichia muris and Ehrlichia minasensis*, but different strains of putative novel species of *Ehrlichia* have been molecularly detected in the last 20 years [1–14, among others].

Amblyomma triste is part of the *Amblyomma maculatum* group, which also includes to *Amblyomma maculatum* sensu stricto and *Amblyomma tigrinum* [15]. This complex is widely distributed throughout the Neotropical and Nearctic regions, from southern U.S.A. to Argentina and Uruguay [1,15,16]. These three species have medical and veterinary relevance since their adult stages are aggressive to humans and domestic mammals, and because they transmit the human pathogen *Rickettsia parkeri* [16]. In the Southern Cone of America, specifically in Argentina and Uruguay, *A. triste* adults are prone to infest humans, dogs and cattle, and it is the principal vector of *R. parkeri* [16–19].

In Argentina, the records of *Ehrlichia* spp. correspond to *E. canis* detected in blood samples of dogs and in ticks from the *Rhipicephalus sanguineus* group [20–24], *Ehrlichia* sp. strain San Luis (closely related to *E. chaffeensis*) infecting *A. tigrinum* and *Amblyomma parvum* ticks [11,25], *Ehrlichia* sp. strain La Dormida associated to *Amblyomma neumanni* [14], others *Ehrlichia* spp. infecting *A. tigrinum* [11,12], and two reports of *Ehrlichia* cf. *E. chaffeensis* infecting *A. parvum* [26] and the marsh deer *Blastocerus dichotomus* [27]. Furthermore, Ripoll et al. [28] presented serology-based evidence of human infection with *E. chaffeensis* (or an antigenically related species) in Jujuy Province, and Halac [29] reported a case of human disease in Cordoba Province attributable to infection with *Ehrlichia*, although the evidence presented by this author is not enough to confirm it. No *Ehrlichia* species have been detected in Uruguay so far.

Due to the relevance of *A. triste* in terms of public health and veterinary issues, the knowledge of the potential role that this tick play in the transmission of

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tick-borne pathogens constitutes a relevant trait. Therefore, the aim of this work was to report the detection of a putative novel *Ehrlichia* strain associated with the tick *A. triste* and to describe its phylogenetic relationship with other species and strains of the genus *Ehrlichia*.

Materials & methods

Questing adults ticks (unfed) were collected by drag-sampling of the vegetation using a 1.50 m white cloth flag between September, October and November of 2017 at the Estación Experimental Agropecuaria Delta del Paraná, Instituto Nacional de Tecnología Agropecuaria (INTA E.E.A. Delta del Paraná), Campana (34°11´S, 58°50´W), Buenos Aires Province, Argentina, and Toledo Chico (34°44´S, 56°06´W), Canelones Department, Uruguay. Both localities belong to the Pampa Biogeographic Province as described in Morrone et al. [30]. Ticks were determined following Nava et al. [16].

DNA extraction of each tick was carried out by using the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) following the manufacturer's instructions. Molecular diagnosis by PCR was performed according to methods previously described by the authors cited in Table 1. Initial screening for Anaplasmataceae was performed with a PCR-amplified fragment of the 16S rRNA gene following conditions described in Parola et al. [31] and *Anaplasma centrale* was used as a positive control. Samples showed to be positive to *Ehrlichia* were further used to amplify a ca. 350-bp fragment of the *dsb* gene following the methods described in Aguiar et al. [32] and Almeida et al. [6], and a ca. 1100-bp fragment of *groESL* heat shock operon of *Ehrlichia* according to Liz et al. [33], with *E. canis* as a positive control. Nuclease-free water was used as a negative control for all PCRs.

PCR-products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, USA) and sequenced with a 3500 Genetic Analyzer sequencer (Applied Biosystems, Foster City, USA). Sequences were edited using BioEdit Sequence Alignment Editor [34] with manual edition whenever it was necessary, aligned with the program Clustal W [35] and compared with those sequences of *Ehrlichia* and *Anaplasma* deposited in GenBank by using BLAST (www.ncbi.nlm.nih.gov/blast). Phylogenetic analyses were performed with Maximum-likelihood (ML) and best-fitting substitution models were determined with the Akaike Information Criterion using the ML model test implemented in MEGA 5.0 [36]. Support for the topologies was tested by bootstrapping over 1000 replications and gaps were excluded from the comparisons. Sequences of *Anaplasma marginale* (*dsb*) and *Neorickettsia risticii* (*groESL*) were included as outgroup.

Table 1. Used primers for the detection of *Ehrlichia*.

Target	Name	Primer sequences (5´-3´)	Reference
16S rRNA	EHR16SD	GGTACCYACAGAAGAAGTCC	[30]
	EHR16SR	TAGCACTCATCGTTTACAGC	
dsb	*dsb-330*	GATGATGTCTGAAGATATGAAACAAAT	[6,31]
	dsb-380	ATTTTTAGRGATTTTCCAATACTTGG	
	dsb-728	CTGCTCGTCTATTTTACTTCTTAAAGT	
groESL	*HS1a*	AITGGGCTGGTAITGAAAT	[32]
	HS6a	CCICCIGGIACIAIACCTTC	
	HS43	ATWGCWAARGAAGCATAGTC	
	HSVR	CTCAACAGCAGCTCTAGTAGC	

Results

In total, 218 adults of *A. triste* (38 from INTA E.E.A. Delta del Paraná and 180 from Toledo Chico) were analyzed for *Ehrlichia* and *Anaplasma* infection. One tick (0.45%) was positive for the initial PCR of the Anaplasmataceae family. The positive tick was a female collected in INTA E.E.A Delta del Paraná. The sequence obtained from a fragment of the 16S rRNA gene (GenBank accession number: MT672744) matched with a high similarity with different 16S sequences belonging to species and strains of the genus *Ehrlichia*.

The positive sample of the 16S rRNA fragment was also positive for the heminested *dsb* and nested *groESL* PCRs. Both amplicons were sequenced (GenBank accession number of *dsb* sequence: MT681331; GenBank accession number of *groESL* sequence: MT681330) and phylogenetically analyzed because they have enough polymorphism to differentiate *Ehrlichia* spp. at the species level. The ML phylogenetic trees for the *dsb* fragment (Figure 1) show that the *Ehrlichia* strain found in this study, named as *Ehrlichia* sp. strain Delta, represent an independent lineage within the genus *Ehrlichia*, close to *E. chaffeensis* and *E. muris*. They form a clade with 70% bootstrap support. The phylogenetic analysis of *groESL* sequences (Figure 2) also indicates that *Ehrlichia* sp. strain Delta is genetically different from the remaining *Ehrlichia* spp. and that it also constitutes a clade (89% bootstrap support) with *E. chaffeensis* and *E. muris*, and other strains not yet formally described. The *dsb* sequence of *Ehrlichia* sp. strain Delta differed by more than 10% with the remaining sequences of *Ehrlichia* spp. available in GenBank (an alignment of 231 bp was analyzed), and that of *groESL* by more than 4.75% (an alignment of 980 bp was analyzed).

Discussion

This is the first report of the genus *Ehrlichia* in the tick *A. triste*. In the current work, DNA sequences from three different loci were studied to infer the phylogenetic relationships of the *Ehrlichia* sp. detected in *A. triste*. Specifically, *dsb* and *groESL* molecular markers have enough polymorphism to characterize the erhlichial agents at lower taxonomic levels. Phylogenetic

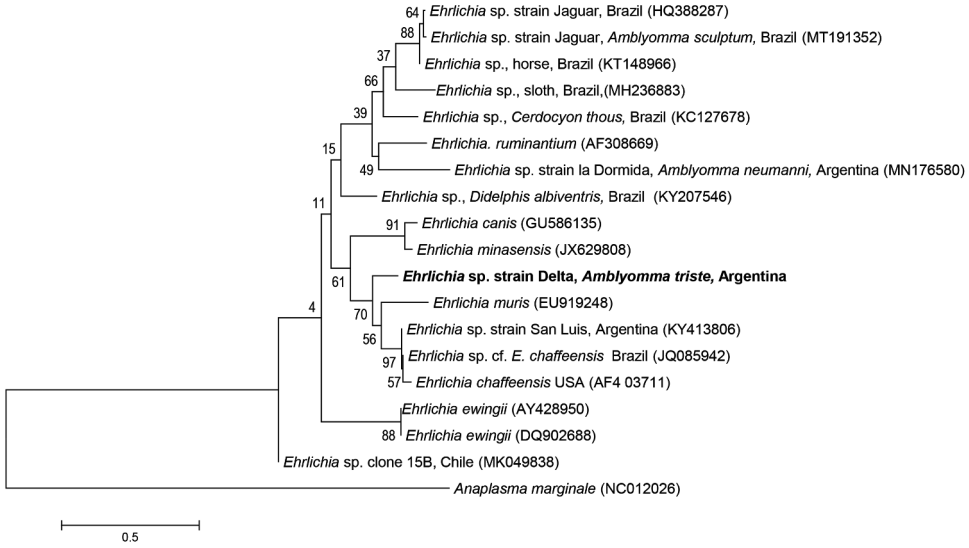

Figure 1. Maximum-likelihood tree constructed from *dsb* sequences of *Ehrlichia* spp. (substitution model: Tamura 3 parameter + G). Numbers represent bootstrap support generated from 1,000 replications. GenBank accession numbers are in brackets.

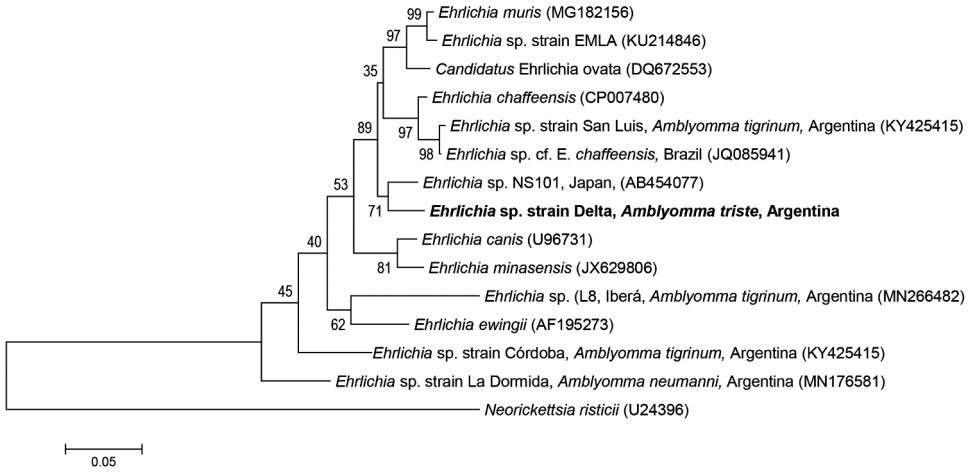

Figure 2. Maximum-likelihood tree constructed from *groESL* sequences of *Ehrlichia* spp. (substitution model: GTR + G). Numbers represent bootstrap support generated from 1,000 replications. GenBank accession numbers are in brackets.

analyses showed that *Ehrlichia* sp. strain Delta is closely related to *E. chaffeensis* and *E. muris* (Figures 1 and 2).

In Argentina, the previous reports of ehrlichial agents close to *E. chaffeensis* were in *A. tigrinum* and *A. parvum* ticks from the north-central area of the country [11,25], where *A. triste* is not distributed. Interestingly, Guillemi et al. [27] report *Ehrlichia* cf. *E. chaffeensis* infecting the marsh deer *B. dichotomus* from Paraná Delta Area and Iberá Wetland from Argentina, but no sequences of *dsb* and *groESL* genes were obtained from that *Ehrlichia* detected in *B. dichotomous*. Therefore, a comparison between both *Ehrlichia* strains is currently not feasible. Previously, Venzal et al. [37] found no positive ticks for *Ehrlichia* in an analysis of 51 specimens of *A. triste* from Uruguay. The *A. triste* ticks from Uruguay analyzed during this work were also negative for *Ehrlichia* infection.

Amblyomma triste, A. maculatum and A. tigrinum are phylogenetically very close [15]. *Amblyomma triste* sensu stricto is found in Argentina, Paraguay, Uruguay and southern Brazil, ecologically associated with wetlands and flood areas [15,16]. In the Southern Cone of America, this tick is distributed in the sub-basins of the Paraná and Uruguay rivers, and in the Samborombón Bay in the province of Buenos Aires, including Buenos Aires city [18,38,39]. The principal hosts for the adults of *A. triste* are the marsh deer *B. dichotomus*, wild and domestic carnivorous (dogs, *Panthera onca, Puma concolor, Herpailurus yagouaroundi, Chrysocyon brachyurus, Lycalopex vetulus*), cattle and *Hydrochoerus hydrochaeris* [16,18]. Adults of this tick are also aggressive to humans (16). Small rodents of the families Cricetidae (subfamily Sigmodontidae) and Caviidae are the principal hosts for immature stages [16,18,40].

Vertical transmission of bacteria of the genus *Ehrlichia* appears to be exclusively transstadial because the transovarial transmission has not been demonstrated for this genus [1]. Thus, infection with *Ehrlichia* is acquired by a tick during feeding of larvae or nymphs, and then the infection pass to adults ticks by transtadial transmission. Sigmondontid or caviid rodents, principal hosts for immature stages of *A. triste*, are candidate to be reservoir hosts responsible for maintenance of the enzootic cycle of *Ehrlichia* sp. strain Delta detected in this work, as it was also hypothesized for the *Ehrlichia* sp. strain San Luis detected in *A. tigrinum*, which is very closely related to *E. chaffeensis* [11]. In this sense, it is important to highlights that *E. chaffeensis, E. muris* and other closely related ehrlichial agents have been detected in rodents [41,42].

The medical and veterinary significance of *Ehrlichia* sp. strain Delta remains to be demonstrated. However, it is important to mention that *A. triste* adults are aggressive to humans and domestic mammals as cattle and dogs [16]. Therefore, the potential role of *A. triste* in the transmission of *Ehrlichia* agents to humans or domestic animals across its distributional range should be highlighted, even more considering that *Ehrlichia* sp. strain Delta is phylogenetically related to the zoonotic *E. chaffeensis*, which is recognized as pathogenic to both humans and animals [40]. The finding of this work plus those data obtained in the last years in South America regarding the circulation of *Ehrlichia* spp. other than *E. canis* (i.e. *E. minasensis, Ehrlichia* sp. strain Córdoba, *Ehrlichia* sp.strain San Luis, *Ehrlichia* sp. strain L8 (Iberá), *Ehrlichia* sp. strain La Dormida, *Ehrlichia* sp. cf. *E. chaffeensis, Ehrlichia* sp. clone 15B, *Ehrlichia* sp. from *Didelphis albiventris, Ehrlichia* sp. from *Cerdocyon thous, Ehrlichia* sp. from sloth, *Ehrlichia* sp. from horse, *Ehrlichia* sp. strain Jaguar and *Ehrlichia* sp. strain Delta [5,6,8,11–14,26,27,43,44, this work]), clearly highlight the need to consider as targets these microorganisms when serological, molecular and clinical studies on tick-borne pathogens in humans and domestic and wild mammals are conducted in countries of this continent.

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Disclosure statement

The authors of the current study declare no conflict of interest.

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