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Two novel *Ehrlichia* strains detected in *Amblyomma tigrinum* ticks associated to dogs in peri-urban areas of Argentina

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ABSTRACT

The aim of this work was to describe two novel strains of *Ehrlichia* associated to *Amblyomma tigrinum* from Argentina. Molecular detection of agents belonging to the family Anaplasmataceae was performed targeting three different loci: 16S rRNA gene, *dsb* gene and a fragment of *groESL* heat shock operon. The results have shown that two different strains of *Ehrlichia* sp. associated to *A. tigrinum* are circulating in peri-urban areas of Argentina. The *Ehrlichia* strain detected in ticks from San Luis Province, named as *Ehrlichia* sp. strain San Luis, is closely related to the *Ehrlichia chaffeensis.* The novel *Ehrlichia* strain detected in Córdoba Province, named as *Ehrlichia* sp. strain Córdoba, is phylogenetically related to three *Ehrlichia* strains from Brazil, two of them isolated from wild carnivorous and the third one isolated from horse. Even though *Ehrlichia* sp. strain Córdoba was clustered with the three *Ehrlichia* strains from Brazil, the genetic similarity was too low to consider them as the same taxonomic entity. Blood samples of dogs were positive to *Anaplasma platys*. The association of these two novel strains with *A. tigrinum* has epidemiological relevance because adult stages of this tick species are common parasite of dogs in rural and peri-urban areas and they are aggressive to humans. The presence of these two novel *Ehrlichia* strains implies a potential epidemiological risk in Argentina because the species of the genus *Ehrlichia* are known to be pathogenic to both domestic mammals and humans.

1. Introduction

Bacteria of the genus *Ehrlichia* (Rickettsiales: Anaplasmataceae) are alpha-proteobacterial present in different regions of the world. They are obligate intracellular parasites with medical and veterinary importance that can infect monocytes, neutrophils, endothelial cells or neutrophils, depending upon the *Ehrlichia* species involved in the infection [1]. Although there are formally six recognized species that are tick-transmitted, namely *Ehrlichia canis, Ehrlichia chaffeensis*, *Ehrlichia ewingii*, *Ehrlichia ruminantium, Ehrlichia muris* and the recently described *Ehrlichia minasensis,* different strains of putative novel species of *Ehrlichia* were molecularly detected in the last years (e.g. *Ehrlichia* sp. TS37, *Ehrlichia* spp. from *Ixodes ovatus, Ehrlichia* sp. strain Anan, *Ehrlichia* sp. strain Jaguar, *Ehrlichia* sp. Fox-ES-1, Panola Mountain *Ehrlichia*, Daishan *Ehrlichia*, *Ehrlichia* sp. from *Rhipicephalus annulatus*, *Ehrlichia* sp. 3 from Australia, among others) [1–9], which strongly suggests that the number of *Ehrlichia* species are underestimated.

The only records based on PCR amplification of *Ehrlichia* spp. DNA reported to date in Argentina, correspond to the findings of *E. canis* in blood samples of dogs and infecting ticks belonging to the tropical lineage of *Rhipicephalus sanguineus* sensu lato [10–12], and to the report of *Ehrlichia* cf. *E. chaffeensis* infecting *Amblyomma parvum* ticks by Tomassone et al. [13]. However, the record of Tomassone et al. [13] is subject to confirmation because it was based on molecular markers without enough polymorphism to provide an accurate specific determination. The other mention on the presence of *Ehrlichia* sp. in Argentina was made by Ripoll et al. [14], who presented serology-based evidence of infection with *E. chaffeensis* or an antigenically related species in humans from Jujuy Province.

Amblyomma tigrinum is a tick widely distributed in South America [15]. This species has medical and veterinary relevance since its adult stages are common parasite of dogs in rural and peri-urban areas and because it is aggressive to humans [15]. In fact, in Argentina, *A. trigrinum* ticks were involved in the transmission of the human pathogen *Rickettsia parkeri* [16] and they were found naturally infected with high prevalence with *Coxiella burnetti* and ´*Candidatus* Rickettsia andeanae´ [17,18]. In view of the sanitary importance of *A. tigrinum,* this species was chosen in this work as the target for a study of *Ehrlichia* infection in ticks parasites of dogs in peri-urban areas of Argentina, where two novel *Ehrlichia* strains are reported.

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2. Materials and methods

Ticks were collected in four localities of Argentina belonging to the Chaco Seco Ecoregion as defined by Burkart et al. [19]: I) 63 questing adult ticks were collected from vegetation in Salsipuedes (31°09′S, 64°19′W), Córdoba Province; II) four adult ticks were collected on dogs in Nono (31°46′S, 64°59′W), Córdoba Province, and 17 questing adult ticks were also collected from vegetation in this locality; III) 11 adult ticks were collected on dogs in Merlo (32°20′S, 65°00′W), San Luis Province; IV) 32 adult ticks were collected on domestic dogs in Cortaderas (32°30′S, 65°00′W), San Luis Province. All ticks were determined as adults of *A. tigrinum* following Estrada-Peña et al. [20]. Additionally, blood samples of dogs were obtained from cephalic vein in Salsipuedes (n: 31) and Merlo (n: 65), in the same areas where ticks were collected. The sampled dogs were exposed to *A. tigrinum* infestation. All collection of ticks and blood of dogs in each locality were made in peri-urban zones characterized by a juxtaposition of urban and rural activities.

DNA extraction of ticks and from blood samples of dogs was carried out by using the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) following the manufacturer's instructions. Initial screening for Anaplasmataceae was performed with a PCR-amplified fragment of the 16S rRNA gene with the primers and protocols described by Parola et al. [21]. Samples showed to be positive to *Ehrlichia* were further used to amplify a ca. 400-bp fragment of the *dsb* gene with primers and protocols detailed in Aguiar et al. [22], and a ca.1100-bp fragment of *groESL* heat shock operon of *Ehrlichia* spp. with the primers and protocols showed by Liz et al. [23]. All the primers used in these procedures are detailed in Table 1. Finally, the samples positive to *Anaplasma* were used to amplify a ca. 700-bp fragment of the *groESL* gene by using the primers PLA-HS475F (AAGGCGAAAGAAGCAGTCTTA) and PLA-HS1198R (CATAGTCTGAAGTGGAGGAC) [24].

Before sequencing, 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 [25] with manual edition whenever it was necessary and aligned with the program Clustal W [26]. They were 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 both distance and character-based methods. Maximum-likelihood (ML) and Neighbor-joining (NJ) trees were constructed by using the program Mega 5.0 [27]. Support for the topologies was tested by bootstrapping over 1000 replications and gaps were excluded from the comparisons. To construct ML tree, best fitting substitution models were determined with the Akaike Information Criterion using the ML model test implemented in MEGA 5.0 [27]. The number of variable nucleotide positions was used to calculate pairwise estimates of percent sequence divergence. This analysis was also conducted with the program MEGA 5.0 [27]. All ambiguous positions were removed for each sequence pair.

Table 1
Primers used for detection of *Ehrlichia* in *Amblyomma tigrinum* ticks.

Target	Primer sequence	Reference
16S rRNA gene	EHR16SD 5′GGTACCYACAGAAGAAGTCC3′ EHR16SR 5′TAGCACTCATCGTTTACAGC3′	Parola et al. [21]
dsb gene	dsb-330 5′-GATGATGTCTGAAGATATGAAACAAAT-3′ dsb- 728 5′ CTGCTCGTCTATTTTACTTCTTAAAGT-3′	Aguiar et al. [22]
groESL heat shock operon	HS1a 5′-AITGGGCTGGTAITGAAAT* HS6a 5′-CCICCIGGIACIAIACCTTC* HS43 5′AT(A/T)GC(A/T)AA(G/A)GAAGCATAGTC** HSVR 5′-CTCAACAGCAGCTCTAGTAGC**	Liz et al. [23]

*First reaction; **Second reaction.

3. Results

A total of 127 adults of *A. trigrinum* were processed to detect infection with ehrlichial agents. Three of 63 unfed ticks collected from vegetation in Salsipuedes were positive after the first screening with 16S rRNA gene primers specific for Anaplasmataceae agents, and one of the 11 ticks collected on dogs in Merlo was also PCR positive for *Anaplasmataceae*. All the remaining ticks analyzed were negative. The 16S rDNA sequences of the positive ticks from Salsipuedes and Merlo matched those of *Ehrlichia* spp. These four samples were also positive when they were tested with the *dsb* and *groESL* PCRs.

The phylogenetic analysis of the *dsb* sequence of the positive specimen of *A. tigrinum* from Merlo in San Luis Province (Genbank accession number: KY413806) shows that this tick was infected with an *Ehrlichia* species closely related to *E. chaffeensis* (Figs. 1 and 2). The *Ehrlichia* detected in *A. tigrinum* from Merlo (named here as *Ehrlichia* sp. strain San Luis) was more closely related to an *Ehrlichia* strains (named as *E. chaffeensis*) detected in the marsh deer *Blastocerus dichotomus* in Brazil (Genbank accession number: JQ085942) than to *E. chaffeensis* from USA (Genbank accession number: AF403711) (see Figs. 1 and 2). The similarity of the *dsb* sequence of *Ehrlichia* sp. strain San Luis with *E. chaffeensis* from Brazil and *E. chaffeensis* from USA was 99.2% and 97.1%, respectively. The percent of similarity between *groESL* sequences of *Ehrlichia* sp. strain San Luis (Genbank accession number: KY425415) and *E. chaffeensis* from Brazil (Genbank accession number: JQ085941) was also 99%, but the similarity was lesser (98%) when the *groESL* sequences of *Ehrlichia* sp. strain San Luis and *E. chaffeensis* from USA (Genbank accession number: CP007480) were compared. The divergence among the *dsb* sequences of the *Ehrlichia* spp. included in the phylogenetic analyses is shown in Table 2.

According to the phylogenetic analysis performed with *dsb* sequences, the three positive *A. tigrinum* ticks from Salsipuedes were infected with an *Ehrlichia* sp. (named here as *Ehrlichia* sp. strain Córdoba) closely related to three *Ehrlichia* sp. strains from Brazil, namely *Ehrlichia* sp. strain Jaguar (isolated from the jaguar *Panthera onca*; Genbank accession number: HQ388287), *Ehrlichia* sp. Fox-ES-1 (isolated from the crab-eating Fox *Cerdocyon thous*; Genbank accession number: KC127678), and *Ehrlichia* sp. isolated from horse in Brazil (Genbank accession number: KT148966) (Figs. 1 and 2). The three *dsb* sequences of *Ehrlichia* sp. strain Córdoba obtained during this study were identical among each other (Genbank accession number: KY413807), and they showed the highest similarity with *Ehrlichia* sp. strain Jaguar (91.5%) and *Ehrlichia* sp. from horse in Brazil (92.3%). Two of the three *groESL* sequences of *Ehrlichia* sp. strain Córdoba detected in *A. tigrinum* from Salsipuedes were identical among each other (Genbank accession number: KY425416), and they differ in just one base with the third sequence. The analysis of these three *groESL* sequences confirms the results reached with *dsb* sequences indicating that *Ehrlichia* sp. strain Córdoba is an independent lineage within the genus *Ehrlichia.* The similarity between *groESL* sequences of *Ehrlichia* sp. strain Córdoba and those *groESL* sequences of *Ehrlichia* spp. deposited in Genbank was never higher than 90%. However, it is important to keep in mind that there are not available *groESL* sequences of *Ehrlichia* sp. strain Jaguar and *Ehrlichia* sp. Fox-ES-1 to infer their phylogenetic relationship with *Ehrlichia* sp. strain Córdoba by using sequences of this molecular marker.

All blood samples of dogs analyzed in this work were negative to *Ehrlichia* infection, but five samples from Salsipuedes and one sample from Merlo were positive to *Anaplasma* in the 16S rRNA PCR assay. These positive samples were used to amplify a partial sequence (ca. 700 bp) of the *Anaplasma platys groESL* gene. The five *groESL* sequences were identical among each other (Genbank accession number: KY425417), and they showed a degree of nucleotide sequence similarity with *groESL* sequences of *A. platys* from different countries, including Argentina, ranged from 99.7% to 100%.

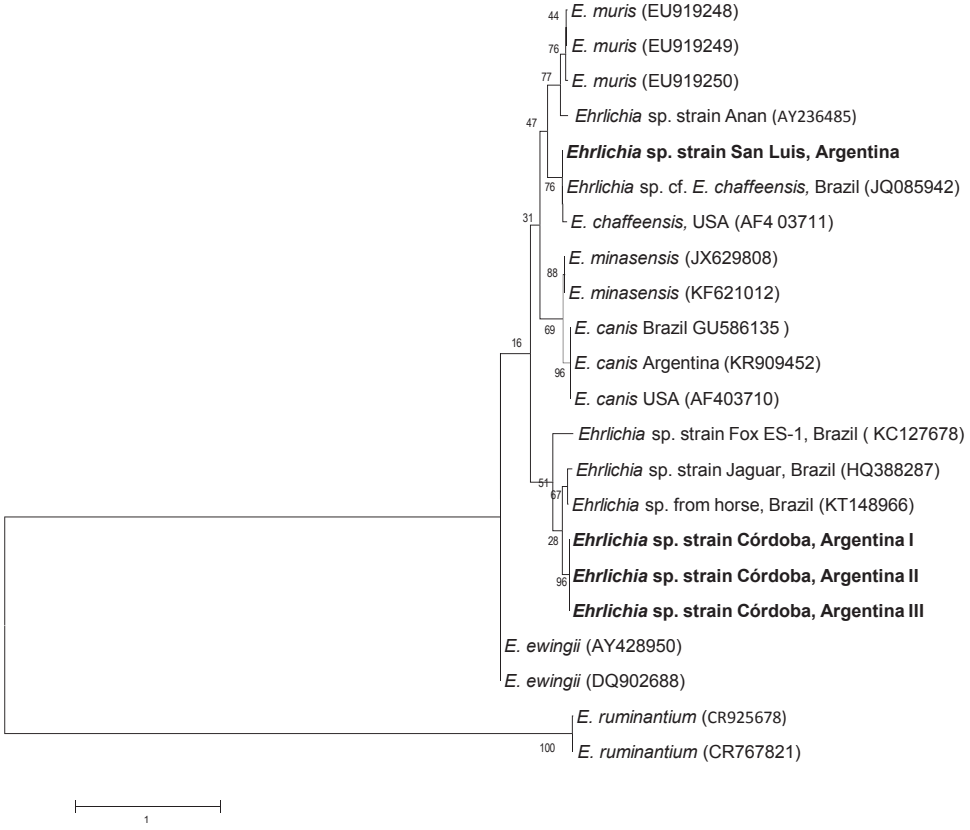

Fig. 1. Maximum-likelihood tree constructed from *dsb* sequences of *Ehrlichia* spp. (substitution model: Tamura 3 parameter + G). Numbers represent bootstrap support generated from 1000 replications. GenBank accession numbers are in brackets.

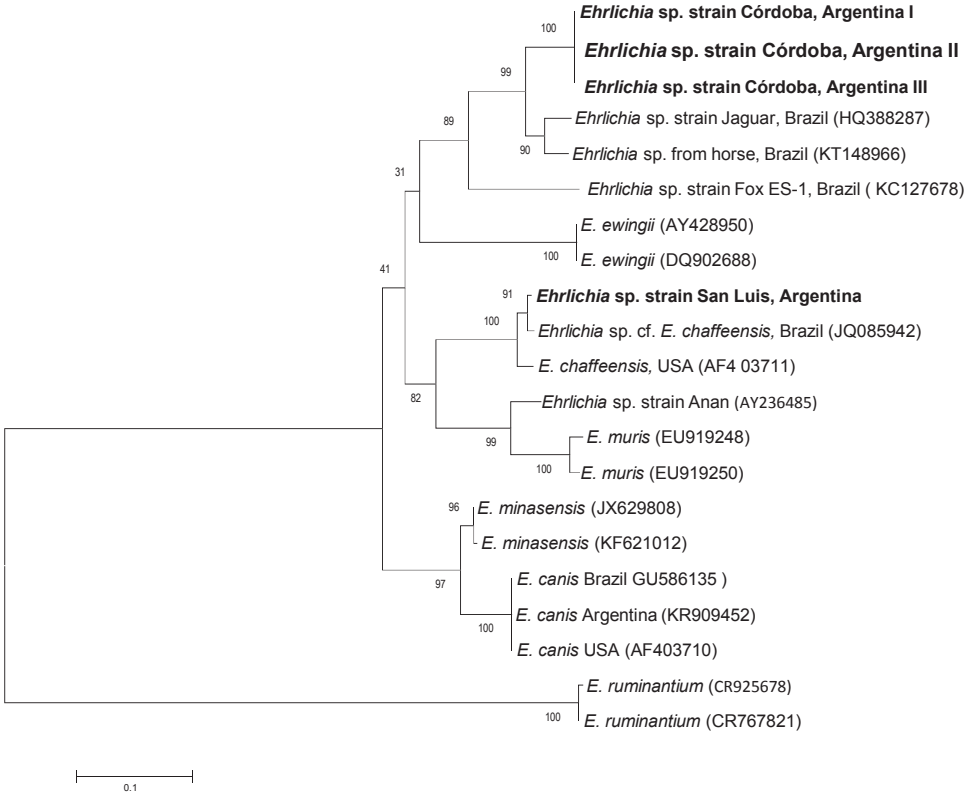

Fig. 2. Neighbor-joining tree constructed from *dsb* sequences of *Ehrlichia* spp. Numbers represent bootstrap support generated from 1000 replications. GenBank accession numbers are in brackets.

Table 2
Matrix of sequence divergence (% nucleotide difference) showing the pair-wise comparisons of the *dsb* sequences of *Ehrlichia*.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1-*Ehrlichia* sp. strain Córdoba 1	–																		
2-*Ehrlichia* sp. strain Córdoba 2	0	–																	
3-*Ehrlichia* sp. strain Córdoba 3	0	0	–																
4-*Ehrlichia* sp., horse, Brazil	7.7	7.7	7.7	–															
5-*Ehrlichia* sp. strain Jaguar, Brazil	8.5	8.5	8.5	4.3	–														
6-*Ehrlichia* sp. strain fox, Brazil	19.2	19.2	19.2	20.2	17.2	–													
7-*Ehrlichia* sp. strain San Luis	25.9	25.9	25.9	26.4	26	28.6	–												
8-*Ehrlichia* sp. *cf. E. chaffeensis*	25.4	25.4	25.4	25.9	25.5	29	0.8	–											
9-*E. chaffeensis* USA	25	25	25	25.5	25.1	30.8	2.9	2.6	–										
10-*E. muris*	33	33	33	34	32	31.5	22	20.7	21.1	–									
11- *E. muris*	29.2	29.2	29.2	29.3	30.9	30.4	20.2	20.7	21.1	2	–								
12. *E. muris*	28.2	28.2	28.2	28.4	29.9	29.5	19.7	20.2	20.6	1.6	1	–							
13-*Ehrlichia* sp. strain Anan	29.9	29.9	29.9	28	28.5	26.8	17.8	18.1	18.9	8.8	8.8	8.4	–						
14-*E. canis* Brazil	28.9	28.9	28.9	26.7	28.6	27.0	22.9	23.3	21.7	29.7	29.7	29.1	24.9	–					
15-*E. canis* Argentina	28.9	28.9	28.9	26.7	28.6	27.0	22.9	23.3	21.7	29.7	29.7	29.1	24.9	0	–				
16-*E. canis* USA	28.9	28.9	28.9	26.7	28.6	27.0	22.9	23.3	21.7	29.7	29.7	29.1	24.9	0	0	–			
17- *E. minasensis* 1	26.8	26.8	26.8	26.0	25.6	26.3	21.9	22.3	21.6	25.3	26.4	25.9	21.4	5.7	5.7	5.7	–		
18- *E. minasensis* 2	26.8	26.8	26.8	26.0	25.6	26.3	22.4	22.7	22.1	25.3	26.4	25.9	21.9	6	6	6	0.3	–	
19-*E. ewingii* 1	25.6	25,6	25.6	28.6	26.8	28.5	25.2	25.6	26.6	28.9	28.0	28.4	26.0	29.3	29.3	29.3	26.4	26.8	–
20-*E. ewingii* 1	25.6	25,6	25.6	28.6	26.8	28.5	25.2	25.6	26.6	28.9	28.0	28.4	26.0	29.3	29.3	29.3	26.4	26.8	0

4. Discussion

The results of this work have shown that at least two different strains of *Ehrlichia* sp. associated to *A. tigrinum* ticks are circulating in peri-urban areas of Argentina. With the exception of the well characterized findings of *E. canis* [10–12], the previous reports of ehrlichial agents in Argentina were based on non-specific serological tests [14] or in molecular markers without enough polymorphism [13]. Although Ripoll et al. [14] and Tomassone et al. [13] have mentioned that the ehrlichial agent that they found was related to *E. chaffeensis*, the evidences obtained in these works do not allow to accurately determining the evolutionary relationship of the *Ehrlichia* spp. at a specific level. In the current work, DNA sequences from three different loci were analyzed to infer the phylogenetic relationships of the *Ehrlichia* strains detected in *A. tigrinum* ticks. Two of these three molecular markers, *dsb* and *groESL,* have enough polymorphism to characterize the erhlichial agents at lower taxonomic levels.

The *Ehrlichia* strain found associated to *A. tigrinum* in San Luis Province, named here as *Ehrlichia* sp. strain San Luis, was molecularly almost identical to an *Ehrlichia* strain detected in the marsh deer *B. dichotomus* in Brazil by Sacchi et al. [28]. Both *Ehrlichia* strains present a similarity in both *dsb* and *groESL* sequences of 99% and they are clustered together in the phylogenetic trees (Figs. 1 and 2). Although *Ehrlichia* sp. strain San Luis is also phylogenetically closely related to *E. chaffensis* from USA (Figs. 1 and 2), the similarity in the *dsb* and *groESL* is lesser than 99%. Sacchi et al. [28] have assigned the name *E. chaffeensis* to the *Ehrlichia* strain that they found infecting *B. dichotomus* in Brazil, but further evidence is needed to determine whether the *Ehrlichia* strains detected in Argentina and Brazil should be named as a strain of *E. chaffeensis* sensu stricto or as a new species. But in any case, irrespective of formal nomenclatural aspects, it is evident that ehrlichial agents very similar to *E. chaffeensis* are circulating in these South American countries as demonstrated by Tomassone et al. [13], Sacchi et al. [28] and in this work. *Ehrlichia chaffeensis* is the causative agent of human monocytotropic ehrlichiosis in North America where the tick *Amblyomma americanum* is its principal vector [29]. Since *E. chaffeensis* has medical and veterinary significance in North America, the zoonotic relevance of *Ehrlichia* sp. strain San Luis in Argentina remains to be demonstrated.

Amblyomma tigrinum ticks from Córdoba Province were found to be infected with a novel *Ehrlichia* strain, denominated here as *Ehrlichia* sp. strain Córdoba. This novel *Ehrlichia* strain is phylogenetically related to three *Ehrlichia* strains from Brazil, two of them isolated from wild carnivorous *P. onca* [4] and *C. thous* [5], and the third one isolated from horse [30] (Figs. 1 and 2). Even though *Ehrlichia* sp. strain Córdoba was clustered with the three *Ehrlichia* strains from Brazil, the genetic similarity in *dsb* sequences (93%) was too low to consider them as the same taxonomic entity (*groESL* sequences of these three *Ehrlichia* strains from Brazil are not publically available). The phylogenetic trees constructed with *dsb* sequences (Figs. 1 and 2) show that the clade formed by *Ehrlichia* sp. strain Córdoba and the Brazilian *Ehrlichia* strains isolated from *P. onca, C. thous* and horse represent an independent lineage within the genus *Ehrlichia*. The pathogenicity to humans and domestic animals of all *Ehrlichia* strains belonging to this phylogenetic clade is unknown.

The principal hosts for the adults of *A. tigrinum* are wild carnivorous of the family Canidae and dogs, while small rodents of the families Caviidae and Cricetidae and birds are the principal hosts for immature stages [15]. The vertical transmission of bacteria of the genera *Ehrilchia* appears to be exclusively by transstadial route because definite transovarial transmission has not been demonstrated [1]. The infection with *Ehrlichia* is acquired during feeding of larvae or nymphs who pass the infection to adults by transstadial transmission. Therefore, in the particular case of *A. tigrinum*, some of the hosts for its immature stages as cricetid or caviid rodents are candidate to be the principal reservoirs hosts responsible for maintenance of the enzootic cycle of the *Ehrlichia* strains detected in Argentina during this work. In this sense, *E. chaffeensis* or closely related ehrlicial agents have been detected in rodents [29]. The role of rodents of the families Caviidae and Cricetidae as reservoirs hosts for the *Ehrlichia* stains associated to *A. tigrinum* ticks should be evaluated in future studies with both molecular and serological approaches in order to comprehend the enzootic cycle of these microorganisms with potential sanitary importance in Argentina.

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