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Detection of *Ehrlichia* sp. strain San Luis and *Candidatus* Rickettsia andeanae in *Amblyomma parvum* ticks

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ABSTRACT

Owing to the sanitary importance of the tick *Amblyomma parvum*, this study evaluated the infection by *Ehrlichia*, *Anaplasma* and *Rickettsia* species of questing *A. parvum* collected in northwestern Argentina. Our results showed that *A. parvum* ticks in this region are infected with the recently reported *Ehrlichia* sp. strain San Luis, closely related to *Ehrlichia chaffeensis*. A high prevalence of *Candidatus* Rickettsia andeanae was observed. Most of the infected ticks presented rickettsial loads lower than those previously reported for other spotted fever group rickettsiae. The presence of *Ehrlichia* sp. strain San Luis in *A. parvum* is a potential risk for public health as the principal hosts of this tick are domestic mammals in rural areas and humans are frequently bitten by this tick species.

1. Introduction

Ticks are the arthropod vectors that transmit the largest number of pathogens, affecting humans, livestock and companion animals (de la Fuente et al., 2008). In Argentina, the principal bacterial pathogens transmitted by ticks belong to the genera *Ehrlichia, Anaplasma* and *Rickettsia* (Nava et al., 2008; Paddock et al., 2008; Cicuttin et al., 2015, 2017). Ehrlichiosis is a disease caused by obligate intracellular Gram-negative bacteria belonging to the order Rickettsiales, family Anaplasmataceae, that primarily infect leukocytes and endothelial cells of mammals, infected through attachment and feeding of their respective tick vectors (Brouqui and Matsumoto, 2007). Some *Ehrlichia* species are pathogens of veterinary importance, and some others are important zoonotic agents in the northern hemisphere, such as *Ehrlichia chaffeensis* and *Ehrlichia ewingii* (Dumler et al., 2001). In Argentina, the existing records of *Ehrlichia* spp. infection correspond to the findings of *Ehrlichia canis* in dog blood and in *Rhipicephalus sanguineus* sensu lato ticks (Cicuttin et al., 2015, 2016), *Ehrlichia cf. E. chaff ;eensis* infecting *Amblyomma parvum* ticks (Tomassone et al., 2008) and two novel *Ehrlichia* strains detected in *Amblyomma tigrinum* ticks denominated *Ehrlichia* sp. strain San Luis and *Ehrlichia* sp. strain Cordoba (Cicuttin et al., 2017), being the former closely related to *E. chaffeensis.* Albeit Tomassone et al. (2008) reported *E. chaffeensis* infecting *A. parvum* ticks, this assumption was based on the sequence of the 16S rRNA and the variable length PCR target (known as VLPT or TRP32) genes, which are not adequate to accurately determine the evolutionary relationship of *Ehrlichia* spp. at a specific level (Sumner et al., 1999; Yu et al., 2007).

Rickettsiosis is caused by Gram-negative bacteria belonging to the order Rickettsiales that primarily infect endothelial cells, being the principal etiological agents of spotted fever in Argentina *Rickettsia rickettsii*, *Rickettsia parkeri* and *Rickettsia massiliae* (Paddock et al., 2008; Garcia-Garcia et al., 2010; Romer et al., 2011, 2014). *Candidatus* Rickettsia andeanae is a recently recognized bacterium that belongs to the spotted fever group of rickettsiae (Jiang et al., 2005), but its pathogenicity is unknown. This *Rickettsia* was previously reported infecting *A. parvum, A. tigrinum* and *Amblyomma pseudoconcolor* in Argentina (Pacheco et al., 2007; Tomassone et al., 2010[1]; Saracho Bottero et al., 2015).

Amblyomma parvum is a tick distributed from southern Mexico to Argentina (Nava et al., 2017). This species has medical and veterinary relevance since its adult stages are common parasite of domestic mammals in rural areas (cattle, goat, horse, pig) and because it also bites humans in some parts of Argentina and Brazil (Nava et al., 2017). This study evaluated the infection by *Ehrlichia*, *Anaplasma* and *Rickettsia* species of *A. parvum* questing ticks collected in northwestern Argentina.

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[1] In the works of Pacheco et al. (2007) and Tomassone et al. (2010) *Candidatus* R. andeanae is named as '*Candidatus* Rickettsia sp. strain Argentina'.

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2. Material and methods

Questing adult ticks were collected from the vegetation by drag flagging in a field near Los Milagros, Santiago del Estero province, Argentina (S27°13′, W62°42′) during December 2016 (late spring). This site is located in the Western Chaqueño District belonging to the phytogeographical Chaco Domain according to Cabrera (1976). Ticks were determined by using standard taxonomic keys and morphological descriptions (Nava et al., 2017) and then processed for DNA extraction by a boiling technique (Monje et al., 2014).

All tick samples were screened for *Ehrlichia* and *Anaplasma* infection through testing them individually by a real-time PCR assay using primers AE2-Fw CGCAAGGYTKAGCTAATCCRTAAAAGT and AE-Rv RCACCAGCTTCGAGTTAAGCCAAT, which were designed to amplify a 177-bp fragment of the 16S rRNA gene of *Ehrlichia* and *Anaplasma* genera. Real-time PCRs were performed in an Applied Biosystems (CA, USA) Step One™ thermocycler with 20 µl per reaction, which contained 4 µl of 5 × Phire reaction buffer, 250 µM dNTP, 0.2 pM of each primer, 2 µl of 10 × SYBR Green I (Invitrogen, USA), 2 mM Cl_2Mg, 100 ng of total DNA and 0.4 µl of Phire Hot Start II DNA polymerase (Thermo Scientific, USA). Cycling conditions consisted of 3 min at 98 °C for initial denaturation, and 40 cycles of 5 s at 98 °C, 20 s at 55.5 °C and 20 s at 62.5 °C. *Ehrlichia/Anaplasma* PCRs included in all cases a positive control (*Ehrlichia canis*) and two negative controls, one using molecular-grade water and another using DNA from *Rickettsia parkeri* strain NOD. Samples positive for *Ehrlichia* spp. were further tested using primers that amplify *Ehrlichia dsb* gene as previously described (Aguiar et al., 2007). For the detection of rickettsial DNA samples were tested by real-time PCR amplification of citrate synthase (*gltA*) gene using primers CS5/CS6, as previously described (Monje et al., 2016a). These primers have shown sensitivity down to one copy of *gltA* gene (Labruna et al., 2004), however the region amplified is highly conserved and not proper for molecular characterization of rickettsiae. Previous studies reported primers RR190.70 F/RR190.602R as not suitable for amplification of the *ompA* gene of *Candidatus* R. andeanae (Pacheco et al., 2007; Tomassone et al., 2010), therefore we used primers 107 F/299R to amplify a highly polymorphic region of *ompA* gene (Kidd et al., 2008). DNA levels were normalized using tick 16S rRNA and quantified as previously described (Monje et al., 2014). *Rickettsia* PCRs included in all cases a positive control (*Rickettsia parkeri* strain NOD) and a negative control using molecular-grade water. Product purity was confirmed by dissociation curves, and random samples were subjected to agarose gel electrophoresis. PCR products (*Ehrlichia/Anaplasma* 16S rRNA, *dsb*, *ompA*) were column purified and sequenced directly in both directions using amplifying primers.

3. Results

A total of 58 adult ticks were collected. Of these, 49 were identified as *Amblyomma parvum* (19 males, 30 females), five as *Amblyomma argentinae* (2 males, 3 females) and four as *Amblyomma tigrinum* (1 male, 3 females).

Two *A. parvum* ticks were positive for *Ehrlichia/Anaplasma* 16S rRNA PCR. BLAST analysis revealed that these sequences were 100% identical to the 16S rRNA sequences of several *Ehrlichia* species such as *E. chaffeensis* (NR_074500), *Ehrlichia ruminantium* (NR_074513) and *Ehrlichia muris* (NR_121714). The 399-bp fragment of the ehrlichial *dsb* gene obtained from these ticks was 100% identical to the corresponding sequence of *Ehrlichia* sp. strain San Luis (KY413806) and 97% identical to the corresponding sequence of *E. chaffeensis* from U.S.A. (CP007480, CP007478, CP007476).

Of the 49 *A. parvum* ticks, 44 were positive for *gltA* and *ompA* rickettsial genes (89.8%), including the two *A. parvum* ticks also positive for *Ehrlichia*, all of which presented rickettsial concentrations ranging from 2.3×10^3 to 2.1×10^6 copies per tick (Fig. 1). The 209-bp fragments of *ompA* gene amplified from *A. parvum* ticks were identical among each other, and 100% identical to the corresponding sequence of *Candidatus* R. andeanae (KY628370).

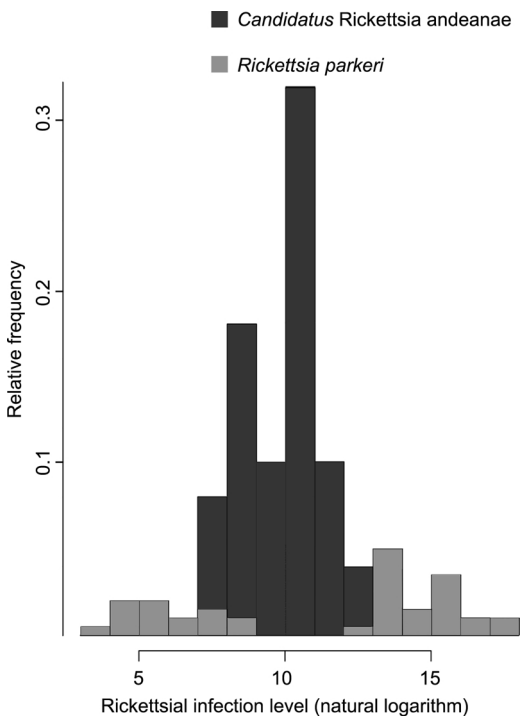

Fig. 1. Distribution of the infection intensity in *Amblyomma parvum* ticks positive for *Candidatus* Rickettsia andeanae (dark grey). For comparison purposes, distribution of infection intensity in *Amblyomma triste* ticks positive for *Rickettsia parkeri* (Monje et al., 2014) is depicted in light grey.

All *A. argentinae* and *A. tigrinum* ticks were negative for *Ehrlichia/Rickettsia*.

Consensus sequence of *dsb* gene from *Ehrlichia* sp. strain San Luis obtained in this study was deposited in GenBank (accession number MH261375).

4. Discussion

Herein, the presence of ehrlichial agents was screened by targeting the ribosomal 16S rRNA gene and was further characterized by sequencing of the protein-coding gene *dsb*. Previously, Tomassone et al. (2008) reported the presence of *E. chaffeensis* in Argentina by using the 16S rRNA and VLPT genes as molecular markers. These authors reported 16S rRNA sequences similar to *E. chaffeensis* but divergent VLPT sequences, and thus concluded that genetic variants of *E. chaffeensis* circulate in Argentina (Tomassone et al., 2008). While the 16S rRNA gene is highly conserved, and therefore not suitable for phylogenetic positioning (Yu et al., 2007), the VLPT gene is highly polymorphic and was originally described to identify genetic variations of *E. chaffeensis* isolates from U.S.A. (Sumner et al., 1999). In contrast, the *dsb* gene was demonstrated to be polymorphic and highly informative for the genus *Ehrlichia* (McBride et al., 2002; Aguiar et al., 2007; Cicuttin et al., 2017). The *dsb* gene partial sequences generated in our study showed that the ehrlichial agent infecting *A. parvum* ticks in northwestern Argentina is identical to the newly reported *Ehrlichia* sp. strain San Luis, also from Argentina in San Luis province (Cicuttin et al., 2017). Ticks in our study were collected in a field located 33 km from the site were Tomassone et al. (2008) reported the presence of *E. chaffeensis*. Due to the small distance and to the absence of geographical barriers between the two sites, we assume that the *Ehrlichia* strain associated to *A. parvum* detected by Tomassone et al. (2008) is the same as the one reported here. Hence, we infer that the ehrlichial agent present in northwestern Argentina is in fact *Ehrlichia* sp. strain San Luis.

Infection by *Candidatus* R. andeanae were reported at high rates in populations of *A. parvum* in Brazil (Nieri-Bastos et al., 2014) and Argentina (Pacheco et al., 2007; Tomassone et al., 2010; this study). Furthermore, high prevalence of *Candidatus* R. andeanae infecting *A. tigrinum* and *Amblyomma maculatum* from Argentina and U.S.A., respectively, was recently reported (Saracho Bottero et al., 2015; Paddock et al., 2015). Although very pertinent for our understanding of the eco-epidemiology of rickettsial pathogens, studies on the distribution of the levels of infection in ticks are scarce. In this respect we reported that in *Amblyomma triste* adult ticks *R. parkeri* infection intensity presents a bimodal distribution with 60% of the infected ticks presenting high rickettsial loads and the remainder with low rickettsial levels (Monje et al., 2014), meanwhile *R. massiliae* presented only high infection intensities in engorged *Rhipicephalus sanguineus* s.l. adult ticks (Monje et al., 2016b). Interestingly, the infection intensity of *Candidatus* R. andeanae observed in *A. parvum* adult ticks presented a distribution with most of the infected ticks presenting intermediate rickettsial loads, right within the range of values reported for *R. parkeri* infection intensity (Fig. 1). Others attempted isolation and propagation of *Candidatus* R. andeanae in cell culture using different cell lines and reported in all cases slow growth and low numbers of this *Rickettsia* (Luce-Fedrow et al., 2012; Ferrari et al., 2013). Furthermore, our results showed that even when quantified in questing *A. parvum* ticks, the infection intensity of *Candidatus* R. andeanae is moderate. Although the 16S rRNA *Ehrlichia/Anaplasma* real-time PCR used in this study was not tuned for quantitative purposes, the Ct values (~33) obtained for *Ehrlichia* sp. strain San Luis are compatible with low levels of infection.

Ehrlichia sp. strain San Luis was recently described infecting *A. tigrinum* ticks in the central region of Argentina. Herein we report a new tick species as a possible vector of *Ehrlichia* sp. strain San Luis in northwestern Argentina, *A. parvum*. In North America *E. chaffeensis* has veterinary and medical significance as it is the causative agent of human monocytotropic ehrlichiosis, where the tick *Amblyomma americanum* is its principal vector (Yabsley, 2010). However, little is known about the presence of ehrlichial agents infecting ticks of the genus *Amblyomma* in southern South America. Adults of *A. parvum* present low host specificity, feeding on large wild and domestic mammals of different orders (Nava et al., 2017), while immature stages appear to depend on the presence of the medium-sized rodent *Galea musteloides* (Rodentia: Caviidae), at least in Argentina (Nava et al., 2006a). Similarly, immature stages of *A. tigrinum* are hosted by rodents of the families Caviidae and Cricetidae being *G. musteloides* extremely prone to be infested by *A. tigrinum* nymphs (Nava et al., 2006b). Since it is assumed that there is no transovarial transmission of bacteria of the genus *Ehrlichia* in ticks (Brouqui and Matsumoto, 2007), infection with *Ehrlichia* must be acquired during feeding of immature stages, which then pass the infection to adults by transstadial transmission. Therefore, the use of *G. musteloides* as a host for their immature stages seems to be a junction between the ecology of *A. parvum* and *A. tigrinum*,arising this rodent as a candidate to be the principal reservoir host responsible for the maintenance of the enzootic cycle of *Ehrlichia* sp. strain San Luis in Argentina. In this respect, further research is necessary to reveal the role of Caviidae rodents in the ecology of *Ehrlichia* sp. strain San Luis in Argentina as well as its zoonotic relevance. The presence of this ehrlichial agent closely related to *E. chaffeensis* in *A. parvum* is a potential risk for public health as the principal hosts of *A. parvum* are domestic mammals (cattle, goat, horse) in rural areas and humans are frequently bitten by this tick species.

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