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Short communication

Prevalence and infection intensity of *Rickettsia massiliae* in *Rhipicephalus sanguineus* sensu lato ticks from Mendoza, Argentina

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Abstract

Rickettsia massiliae belongs to the spotted fever group and in the New World is commonly associated with the brown dog tick, *Rhipicephalus sanguineus*. Herein we investigate the presence of *R. massiliae* in *Rh. sanguineus* sensu lato ticks in a location near the Andean foothills (Mendoza, Argentina), to provide a prevalence estimate and to assess the infection intensity of this pathogen. *Rickettsia massiliae* infection was found in 5.1% of the *Rh. sanguineus* s.l ticks analyzed, all with high infection intensities. Molecular analysis determined that all *R. massiliae*-infected *Rh. sanguineus* s.l. ticks belonged to the temperate lineage.

Keywords: *Rickettsia*; *Rhipicephalus sanguineus*; Temperate lineage; Vectorial competence; Infection intensity; Argentina

1. Introduction

Tick-borne rickettsioses are caused by obligate intracellular bacteria belonging to the spotted fever group of the genus *Rickettsia* [1]. The worldwide emerging pathogen *Rickettsia massiliae* belongs to this group, and in the New World it is commonly associated with the brown dog tick, *Rhipicephalus sanguineus* [1]. In the Southern Cone of South America, *R. massiliae* has only been reported in Buenos Aires city [2]. So far, although human cases of *R. massiliae* infection have been diagnosed by serological methods in Europe [3,4], only few cases of human disease caused by this pathogen were confirmed [see Ref. [5]]. The only confirmed case of *R. massiliae* infection in the New World was from Buenos Aires [6]; however, many cases of rickettsial infection may not be detected by the health system because it is still an unexpected disease [7]. In addition, *R. massiliae* may also be a health threat to domestic dogs [8].

Rhipicephalus sanguineus has medical and veterinary importance due to its role as vector of several pathogenic agents to both humans and dogs, such as *R. massiliae*, *Rickettsia rickettsii*, *Ehrlichia canis*, *Anaplasma platys*, among others [1,9]. Recent studies [10,11] determined the existence of different genospecies under the taxon *Rh. sanguineus* (from now on, *Rh. sanguineus* sensu lato), and at least two different lineages were determined in the New World: tropical and temperate [10]. Furthermore, these two lineages of *Rh. sanguineus* s.l. ticks showed marked differences in the vectorial competence to transmit *E. canis* [12,13].

The distribution of the levels of infection of rickettsial pathogens in naturally infected ticks is an important, yet understudied, aspect of the ecoepidemiology of *Rickettsia*. Recently, it was reported that *Rickettsia parkeri* infection intensity in *Amblyomma triste* ticks shows a bimodal distribution [14]. This distinct distribution could have important implications in the ecoepidemiology of *R. parkeri*, as it could result in

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differential exposure doses after a tick bite or it may indicate different transmission mechanisms adding to the long-term maintenance of this bacterium in nature. Consequently, the aim of this study was to investigate the presence of *R. massiliae* in *Rh. sanguineus* s.l. ticks in a location near the Andean foothills (Mendoza, Argentina), to provide a prevalence estimate and to assess the infection intensity of this pathogen in naturally infected *Rh. sanguineus* s.l. ticks. In addition, both *R. massiliae* and *Rh. sanguineus* s.l. ticks were molecularly characterized.

2. Materials and methods

Adult ticks were collected from domestic dogs (*Canis familiaris*) attending veterinary medical centers in Mendoza, Argentina (32°54′S, 68°49′W; Fig. 1) during October–November 2014. This site corresponds to the western extension of the Monte Province of the Chaco Phytogeographic Dominion near the limit with the pre-Puna Province. Collected ticks were brought alive to the laboratory and maintained in an incubator at 25 °C with 85% relative humidity until they were morphologically identified following standard taxonomic keys. The tick's surface was disinfected in iodine alcohol (10-min immersion), followed by several washes in sterile water.

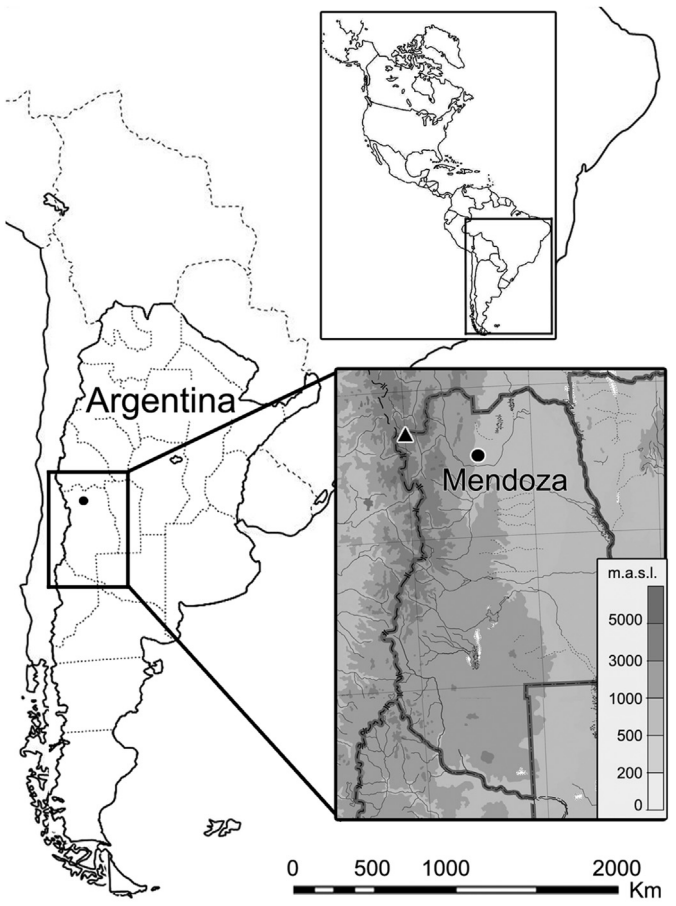

Fig. 1. Map of part of South America showing the sampling locality of the *Rhipicephalus sanguineus* sensu lato ticks (circle). Inset: altitudinal representation of Mendoza province. The triangle indicates the highest peak of the New World, Mt. Aconcagua (6962 m.a.s.l.). m.a.s.l.: meters above sea level.

One half of each tick (longitudinally divided into two halves with sterile forceps and scissors) was processed for DNA extraction in 500 μL of TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0) and heated at 95 °C for 20 min and held at −20 °C until tested by real-time PCR. Total DNA concentration was assessed using the SPECTROstar Nano and the MARS Data Analysis Software (BMG Labtech, Germany). The other half was placed in a sterile vial and immediately frozen at −80 °C.

All tick samples were screened for rickettsial infection by testing them individually by a real-time PCR assay using primers CS-5/CS-6 (Table 1) as previously described [15]. These primers were designed to amplify a 147-bp fragment of the citrate synthase gene (*gltA*) of *Rickettsia* spp. and have shown sensitivity down to one copy of the *glt*A gene of *R. rickettsii* [16]. DNA levels were normalized using tick 16S rRNA as previously described [14]. Product purity was confirmed by dissociation curves, and random samples were subjected to agarose gel electrophoresis. Ticks positive for *gltA* real-time PCR were subjected to further amplification of a larger fragment of *gltA* gene and *ompA* gene by routine PCR using primers CS-239/CS-1069 and RR190.70/RR190.701 respectively (Table 1), as described elsewhere [16]. In addition, the lineage and the respective haplotype of all the *Rickettsia*-positive ticks was determined by studying the tick 12S and 16S rRNA genes as previously described [11]. PCR products were checked by electrophoresis in a 1.5% agarose gel, column purified and sequenced directly in both directions using amplifying primers. A phylogenetic analysis of the rickettsial sequences obtained was performed with the Maximum-likelihood (ML) method by using the program Mega 5.0 [17]. Best fitting substitution models were determined with the Akaike Information Criterion using the ML model test implemented in MEGA 5.0. Support for the topologies was tested by bootstrapping over 1000 replications and all positions containing gaps and missing data were excluded from the comparisons. Isolation of rickettsiae was attempted on all the *gltA* real-time PCR positive *Rh. sanguineus* s.l. ticks by means of the shell vial technique as described previously [16]. DNA of infected Vero cells was tested by conventional PCR, checked by electrophoresis in a 1.5% agarose gel, column purified and sequenced directly in both directions using amplifying primers.

3. Results

3.1. Tick identification and determination of rickettsiae infection intensity

A total of 138 adults (26 males and 112 females partially or completely engorged) of *Rh. sanguineus* s.l. were removed from 42 dogs during October–November 2014. Seven *Rh. sanguineus* s.l. female ticks (5.1%) were found to contain DNA of the rickettsial gene *gltA* by real-time PCR, all of which presented high rickettsial concentration (from 3.27×10^5 to 1.36×10^8 *gltA* copies per tick). A larger fragment of *gltA* gene and *ompA* gene were amplified using

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Table 1
Sequences of primers used for amplification of rickettsial and tick genes.Table 1

Gene	Primer	Primer sequence (5′ → 3′)	Fragment size (bp)	Reference
gltA real-time	CS-5	GAGAGAAAATTATATCCAAATGTTGAT	147	Labruna et al., 2004
	CS-6	AGGGTCTTCGTGCATTTCTT		Labruna et al., 2004
gltA	CS-239	GCTCTTCTCATCCTATGGCTATTAT	788	Labruna et al., 2004
	CS-1069	CAGGGTCTTCGTGCATTTCTT		Labruna et al., 2004
ompA	RR190.70	ATGGCGAATATTTCTCCAAAA	592	Monje et al., 2014
	RR190.701	GTTCCGTTAATGGCAGCAT		Monje et al., 2014
12S rRNA	12S Fw	AAACTAGGATTAGATACCCTATTATTTTAG	399	Nava et al., 2012
	12S Rv	CTATGTAACGACTTATCTTAATAAAGAGTG		Nava et al., 2012
16S rRNA real-time	T16S5	GACAAGAAGACCCTA	215	Monje et al., 2014
	T16S3	ATCCAACATCGAGGT		Monje et al., 2014
16S rRNA	16S + 1	CCGGTCTGAACTCAGATCAAGT	452	Nava et al., 2012
	16S−1	GCTCAATGATTTTTTAAATTGCTGT		Nava et al., 2012

DNA obtained from infected ticks, and the resulting nucleotide sequences were 100% and 99.6% identical to the corresponding *gltA* (773/773) and *ompA* (571/573) sequences of *R. massiliae* strain AZT80 complete genome (CP003319), and 99.9% and 99.3% identical to the corresponding *gltA* (772/773) and *ompA* (569/573) sequences of *R. massiliae* strain MTU5 complete genome (CP000683). Complete identity was also observed with smaller overlapping segments of *gltA* (762/762) and *ompA* (459/459) genes of *R. massiliae* isolate CABA from Argentina (KT032119 and KT032121, respectively). Phylogenetic analysis based on *gltA* and *ompA* also placed the sequences obtained in this study in a clade with *R. massiliae* from other geographic regions (Fig. 2).

3.2. Isolation of rickettsiae

Isolation assays by the shell vial technique were attempted with all of the *gltA* real-time PCR positive ticks. *R. massiliae* was isolated from one of the *Rickettsia*-positive ticks; however, this isolate could not be established in Vero cell culture and it was lost after the third passage. DNA extracted from infected cells of the second and third passage was subjected to PCR targeting the *gltA* gene. A PCR product of the expected size was obtained only in the DNA sample from the second passage, which was 100% (773/773) similar to the *gltA* sequence obtained from the corresponding infected *Rh. sanguineus* s.l. tick (KU498299, this study). Isolation attempts from the other ticks with positive *gltA* real-time PCR were lost due to bacterial and fungal contamination.

3.3. Rh. sanguineus *sensu lato lineage determination*

All infected ticks were determined to belong to the temperate lineage. The 12S rRNA gene reported a single sequence of 339 bp for the seven ticks analyzed, which was 100% identical to the corresponding sequence of *Rh. sanguineus* s.l. haplotype VI [AY559841,11]. For the 16S rRNA gene, two different sequences of 405 bp were obtained. Of these, five ticks presented a sequence 100% identical to the corresponding sequence of *Rh. sanguineus* s.l. haplotype II

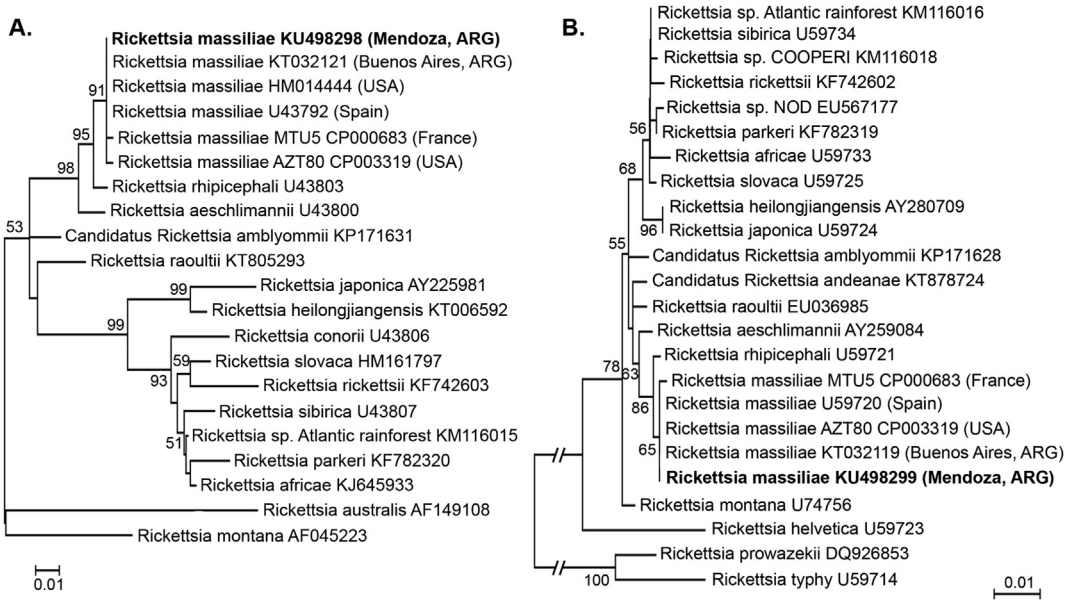

Fig. 2. Maximum likelihood trees inferred from comparison of the *Rickettsia ompA* (A) and *gltA* (B) partial sequences. The numbers at nodes are the bootstrap values. The scale bar represents the difference in nucleotide sequences. Sequences obtained in this study are identified as KU498298 (*ompA*) and KU498299 (*gltA*).

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[JX195168,11] and two presented a sequence 100% identical to the corresponding sequence of *Rh. sanguineus* s.l. haplotype I [JX195167,11]. Partial sequences from *R. massiliae* (*ompA*, *gltA*) and *Rh. sanguineus* s.l. (12S rRNA, 16S rRNA) generated in this study were deposited in GenBank (accession numbers KU498298-KU498302).

4. Discussion

The *R. massiliae* reported herein showed slight genetic differences with *R. massiliae* isolate AZT80 from the United States and *R. massiliae* isolate MTU5 from southern France. Previously, Cicuttin et al. [2] reported *R. massiliae* infecting 3.4% of the *Rh. sanguineus* s.l. ticks collected from domestic dogs in Buenos Aires city. In the present study, a similar *R. massiliae* infection prevalence was found in *Rh. sanguineus* s.l. ticks (5.1%) from a different eco-region.

Recently, Monje et al. [14] reported that *R. parkeri* infection intensity in questing *A. triste* ticks from the Parana River delta region display a bimodal distribution, with 60% of the infected ticks presenting high rickettsial loads and the remainder with low rickettsial levels. In the present study, *R. massiliae* infection intensity in engorged *Rh. sanguineus* s.l. ticks was high in all positive ticks, which suggests against the occurrence of a bimodal distribution in this system. However, the absence of low rickettsial intensities could be in part attributable to the "reactivation effect" of *rickettsia* in feeding ticks [18]. In this regard, a recent study demonstrated that blood feeding increases the bacterial load of *R. rickettsii* infecting its natural tick vector *Amblyomma aureolatum* [19]. Further research studying rickettsial loads in questing *Rh. sanguineus* s.l. ticks is warranted.

Recently, Moraes-Filho et al. [13] experimentally demonstrated that the two distinct lineages of *Rh. sanguineus* s.l. ticks present in South America [temperate and tropical lineages,10] had important differences in the vectorial competence to transmit *E. canis*. These authors reported that the tropical lineage of *Rh. sanguineus* s.l is a highly competent vector of *E. canis* but not the temperate lineage. Recent field surveys also support these differences in the vectorial competence of *Rh. sanguineus* s.l. lineages to transmit *E. canis* [12]. Nonetheless, the opposite seems to occur with the different lineages of *Rh. sanguineus* s.l. regarding the vectorial competence to transmit *R. massiliae*. Throughout the New World, the geographical distribution of *R. massiliae*-infected *Rh. sanguineus* s.l. overlaps the areas were the temperate lineage of *Rh. sanguineus* s.l. ticks is present. In North America, available reports of *R. massiliae*-infected *Rh. sanguineus* s.l. ticks are from above 32° of north latitude in both east and west coasts of the United States [8,20,21]. In South America, available reports of *R. massiliae*-infected *Rh. sanguineus* s.l. ticks are from below 32° of south latitude. These reports (only two) are from Argentina, one from Buenos Aires in the east coast [2] and one from Mendoza province in the foothills of the Andes (present study). Furthermore we determined that *R. massiliae*-infected *Rh. sanguineus* s.l. ticks from Mendoza indeed belonged to the temperate lineage. There are no reports of the presence of *R. massiliae* in geographical regions where the temperate lineage of *Rh. sanguineus* s.l. is absent. In contrast, all across the deduced distribution area of the tropical lineage other *Rickettsia* spp. (i.e. *R. rickettsii*, *Rickettsia amblyommii*, *Rickettsia andeneae*, *Rickettsia felis*, *Rickettsia* sp. strain Atlantic Rainforest) were associated with *Rh. sanguineus* s.l. ticks [1,22–25]. Of these reports, only Eremeeva et al. [25] molecularly identified *Rh. sanguineus* s.l. ticks as belonging to the tropical lineage, but due to the geographic distribution it is expected that tick populations surveyed in the other reports and reviewed in Parola et al. [1] also belonged to the tropical lineage [10].

The fact that in the New World *R. massiliae* has been associated only with *Rh. sanguineus* s.l. ticks belonging to the temperate lineage [8, this study] or was reported in areas where this lineage is distributed [2,20,21], suggests a difference in the vectorial competence to transmit this pathogen. However, experimental infestations on susceptible hosts with both temperate and tropical lineage populations must be done to confirm their vector competence/incompetence to transmit *R. massiliae*.

Conflict of interest

All authors declare no financial or commercial conflict of interest.

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