

Bacteria of the genera *Ehrlichia* and *Rickettsia* in ticks of the family Ixodidae with medical importance in Argentina

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Abstract The aim of this study was to get an overview about the occurrence of bacteria from the genus *Ehrlichia* and *Rickettsia* in ixodid ticks with medical importance in Argentina. Therefore, in 2013 and 2014, free-living ticks were collected in different provinces of northern Argentina. These ticks were determined as *Amblyomma sculptum*, *Amblyomma neumanni*, *Amblyomma parvum*, *Amblyomma triste*, *Amblyomma ovale*, *Amblyomma tonelliae* and *Haemaphysalis juxtakochi*. All samples were tested to determine the infection with *Ehrlichia* spp. and *Rickettsia* spp. by PCR assays. Rickettsial DNA was detected in all tested tick species, with the exception of *A. tonelliae*. ‘*Candidatus Rickettsia amblyommii*’, ‘*Candidatus Rickettsia andeanae*’, and *Rickettsia parkeri* were found in *A. neumanni*, *A. parvum*, and *A. triste*, respectively. Another rickettsial species, *Rickettsia bellii*, was found in *A. sculptum*, *A. ovale* and *H. juxtakochi*. None of the tested ticks showed infection with *Ehrlichia*. The results of the study demonstrate that *Rickettsia* species belonging to the spotted fever group are associated with various species of *Amblyomma* throughout a wide area of northern Argentina, where cases of *Amblyomma* ticks biting humans are common.

Keywords Ixodidae · *Rickettsia* · *Ehrlichia* · Argentina

Introduction

Bacteria of the genus *Rickettsia* are widespread in ticks and can cause a large range of diseases in humans (Parola et al. 2013). These obligate intracellular bacteria are divided in five groups: the spotted fever group (SFG), the typhus group (TG), the bellii group (TRG),

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the canadensis group, and ancestral groups (Gillespie et al. 2007; Weinert et al. 2009). The main vectors of these bacteria in South America are ticks of the genus *Amblyomma*. So far, 25 species of *Amblyomma* have been described for Argentina (Guglielmone and Nava 2006; Nava et al. 2009; Nava et al. 2014a, b). Also, most of the rickettsial infections in ticks of Argentina were found in species of this genus (Labruna et al. 2007a; Pacheco et al. 2007; Nava et al. 2008; Paddock et al. 2008; Tomassone et al. 2010; Cicuttin and Nava 2013; Romer et al. 2014; Monje et al. 2015; Tarragona et al. 2015; Bottero et al. 2015; Mastropaolo et al. 2016). Besides *Amblyomma* ticks, infection with the pathogenic *Rickettsia massiliae* was also reported in *Rhiphicephalus sanguineus* sensu lato ticks by Cicuttin et al. (2014). Because several species belonging to the genera *Amblyomma* and *Haemaphysalis* are aggressive to humans in Argentina (Guglielmone et al. 1991, 2006; Nava et al. 2006; Lamattina and Nava 2016), a high risk of transmission of rickettsial bacteria to human by these ticks exist.

Ehrlichiae are tick-transmitted obligate intracellular gram-negative bacteria and the etiological agents of emerging and life-threatening zoonoses (Doyle et al. 2005). *Ehrlichia canis*, *Ehrlichia chaffeensis*, *Ehrlichia ewingii*, and *Ehrlichia minascensis* can cause ehrlichioses in humans and domestic animals (Maeda et al. 1987; Breitschwerdt et al. 1998; Buller et al. 1999; Kocan et al. 2000; Liddell et al. 2003; Cabezas-Cruz et al. 2016; Carvalho et al. 2016). In Argentina ehrlichial DNA was detected in *Amblyomma parvum* (Tomassone et al. 2008) and *R. sanguineus* sl. (Cicuttin et al. 2015).

According to the aspect that some of the species from the genera *Ehrlichia* and *Rickettsia* are able to cause severe and also fatal human disease, and taking into account that human cases of rickettsioses and ehrlichiosis (confirmed and suspected) were reported in Argentina (Ripoll et al. 1999; Seijo et al. 2007; Paddock et al. 2008; García-García et al. 2010; Romer et al. 2011, 2014), the aim of this study was to get an overview about the occurrence of these tick-borne pathogens in ixodid ticks from different regions of Argentina.

Materials and methods

Ticks were collected on animals and by flagging in 2013 and 2014 in different regions of northern Argentina. Afterward the ticks were determined to species level following to Estrada-Peña et al. (2005), Barros-Battesti et al. (2006), Martins et al. (2014) and Nava et al. (2014a, b). Genomic DNA was extracted from each tick using a phenol/chloroform assay described by Mangold et al. (1998). For the detection of rickettsial DNA, the tested ticks were screened by a realtime-PCR (rt-PCR) method described by Labruna et al. (2004) and Guedes et al. (2005) (Primers; Table 1). The rt-PCRs were performed in a Rotor-Gene-Q6000 (Qiagen). PCR-positive samples were further tested by a battery of PCRs to amplify fragments of two rickettsial genes, those for citrate synthase gene (*gltA*) and 190-kDa outer membrane protein (*ompA*) (Table 1). All PCR runs were managed with two negative controls (water and none-template control) and a positive control (*Rickettsia massiliae* for *A. triste*; *R. parkeri* for the other ticks DNA). Ehrlichial DNA were detected by the usage of a PCR assay described by Doyle et al. (2005) which amplifies a fragment of the gene *dsb* (Table 1). Hereby, *Ehrlichia canis* was used as positive control. Positive PCR-amplicons were purified (Wizard® SV Gel and PCR Clean-Up System, Promega) and sequenced.

The sequences were edited using BioEdit Sequence Alignment Editor (Hall 1999) with manual edition whenever it was necessary, aligned with the program Clustal W (Thompson

Table 1 Used primers for the detection of *Ehrlichia* spp. and *Rickettsia* spp.

Pathogen	Target	Primer sequence	References
<i>Ehrlichia</i> spp.	<i>dsb</i>	dsb 330; 5'-GATGATGTCTGAAGATATGAAACAAAT-3'	Doyle et al. (2005)
		dsb 728; 5'-CTGCTCGTCTATTTTACTTCTTAAAG-3'	
<i>Rickettsia</i> spp.	<i>gltA</i> ^a	CS 239; 5'-GATGATGTCTGAAGATATGAAACAAAT-3-3'	Labruna et al. (2004)
		CS 1069; 5'-CTGCTCGTCTATTTTACTTCTTAAAGT-3'	
	<i>gltA</i> ^b	CS5; 5'-GAGAGAAAATTATATCCAAATGTTGAT-3'	Guedes et al. (2005), Labruna et al. (2004)
		CS6; 5'-AGGGTCTTCGTGCATTTCTT-3'	
<i>ompA</i>	Rr 190.70p; 5'-ATGGCGAATATTCTCCAAAA-3'	Regnery et al. (1991)	
	Rr 190.602n; 5'-AGTGCAGCATTCGCTCCCCT-3'		
<i>ompA</i>	190.70 F-A1; 5'-ATGGCGAATATTCTCCAAAA-3'	Eremeeva et al. (2006)	
	190.701 R-A3; 5'-GTTCCGTTAATGGCAGCATCT-3'		

^a Conventional PCR^b Real time PCR

et al. 1994) and compared with those sequences deposited in GenBank. Phylogenetic analyses were performed with Maximum-likelihood (ML) methods by using the program Mega 5 (Tamura et al. 2011). Best fitting substitution models were determined with the Akaike Information Criterion using the ML model test implemented in MEGA 5. Substitution models were GTR (G+I) for *gltA* and *ompA*. Support for the topologies was tested by bootstrapping over 1000 replications and gaps were excluded from the comparisons.

Results

A total of 1787 ticks were collected in 2013 and 2014 in different localities of northern Argentina (Table 2). These ticks were determined as adults and nymphs of *Amblyomma neumanni* (100), *A. parvum* (18), *A. triste* (3), *A. ovale* (19), *A. sculptum* (1414), *A. tonelliae* (160) and *Haemaphysalis juxtakochi* (73) (Table 2). All these ticks were analysed to test the presence of rickettsial DNA. In all tested species—except *A. tonelliae*—the rickettsial *gltA* gene could be amplified in some specimen of ticks by rt-PCR. Positive samples were used to amplify a fragment of *gltA* gene as described in “Materials and methods” section. *Amblyomma triste*, *A. parvum* and *A. neumanni* were found to be positive for *ompA* gene. These results are detailed in Table 2. Figure 1 shows the results of the phylogenetic analysis of the sequences of the *gltA* gene. The *gltA* partial sequence for *A. sculptum* from Reserva Ecológica El Bagual (Formosa province), *A. ovale* from Reserva Ecológica El Bagual (Formosa province), Parque Nacional Chaco (Chaco province), and *H. juxtakochi* Parque Nacional Chaco (Chaco province) were highly identical (>99.9%) to the corresponding sequences of *Rickettsia bellii* available at the Genbank (accession numbers U59716 and CP000087) (see Fig. 1). The *gltA* partial sequence of *A. triste* from Reserva Ecológica El Bagual (Formosa province) was identical to the *gltA* partial sequence of *Rickettsia parkeri* detected in *Amblyomma maculatum* (GenBank accession number: U59732). The *gltA* sequence of *A. neumanni* was identical to the corresponding sequence of ‘*Candidatus Rickettsia amblyommii*’ (GeneBank accession number: DQ517290) detected previously in *A. neumanni* from Argentina. Furthermore, the *gltA* sequence of *A.*

Table 2 Distribution of the tested ticks by province, species and stage

Species	Location/province	Latitude/longitude	Host	Stage	<i>Rickettsia</i> spp. ^a	<i>Ehrlichia</i> spp. ^a			
<i>Haemaphysalis juxtakochi</i>	PN Chaco, CHO	S26°51'/W59°37'	Free	F	2	n.d.			
				N	10	n.d.			
	RE El Bagual, FSA	S25°59'/W58°24'	Free	F	17	n.d.			
				M	16	n.d.			
<i>Amblyomma ovale</i>	PN Chaco, CHO	S26°51'/W59°37'	Free	N	28	n.d.			
				F	4	n.d.			
				M	4	n.d.			
	RE El Bagual, FSA	S25°59'/W58°24'	Free	F	4	n.d.			
<i>Amblyomma triste</i>	RE El Bagual, FSA	S25°59'/W58°24'	Free	M	3	n.d.			
				N	1	n.d.			
	Sebastian Elcano, CBA	S30°09'/W63°35'	Goat	F	1	n.d.			
				M	2	n.d.			
<i>Amblyomma parvum</i>	Quilino, CBA	S30°12'/W64°30'	Free	F	12	12			
				M	5	5			
				N					
	<i>Amblyomma sculptum</i>	PN Chaco, CHO	S26°51'/W59°37'	Free	F	2	2		
M					3	3			
N					155	155			
RE El Bagual, FSA		S25°59'/W58°24'	Free	F	17	17			
				M	29	29			
				N	678	678			
				Saladillo, JUJ	S24°01'/W64°15'	Free	N	4	4
				RN Privada Eco-Portal de Piedras, JUJ	S24°05'/W64°23'	Free	F	61	61
							M	55	55
La Ronda, JUJ		S24°05'/W64°14'	Free	N	361	361			
				Subteniente Perín, FSA	S25°39'/W60°07'	Free	N	6	6
							N	27	27
	Santa Clara, JUJ			S24°18'/W64°39'	Free	N	16	16	
	<i>Amblyomma tonelliae</i>			Saladillo, JUJ	S24°01'/W64°15'	Free	N	88	88
							El Tunal, SAL	S25°15'/W64°25'	Free
<i>Amblyomma neumanni</i>	Cruz Quemada, SAL	S25°01'/W64°59'	Horse	F	58	58			
				M	3	3			
				N	1	1			
<i>Amblyomma neumanni</i>	El Fuerte, JUJ	S24°15'/W64°24'	Horse	F	13	13			
				M	20	20			
	Jaime Peter, CBA	S30°24'/W64°32'	Horse	<i>Pecari tajacu</i>	M	36	36		
				M	3	3			
La Candelaria, SAL	S26°06'/W65°05'	Horse	M	18	18				

Table 2 continued

Species	Location/province	Latitude/longitude	Host	Stage	<i>Rickettsia</i> spp. ^a	<i>Ehrlichia</i> spp. ^a
	La Punta, SE	S28°22'/W64°47'	Horse	F	3	3
				M	3	3
	Rosario de la Frontera, SAL	S25°47'/W64°58'	Horse	F	1	1
				M	10	10
	Tapía, TUC	S26°35'/W65°16'	Horse	F	2	2
				M	1	1
Total				F	130	102
				M	208	183
				N	1449	1407
				Σ	1787	1692

CHO Chaco, FSA Formosa, CBA Córdoba, JUJ Jujuy, SAL Salta, SE Santiago del Estero, TUC Tucumán (provinces of Argentina), F female, M male, N nymph, n.d. not done, PN Parque Nacional, RN Reserva natural, RE Reserva Ecológica

^a Number of tested ticks

parvum from Quilino (Córdoba province) was identical to the *gltA* sequence of ‘*Candidatus Rickettsia andeanae*’ detected in *A. maculatum* and *Ixodes boliviensis* (GenBank accession number: GU169051). The results for *A. triste* and *A. parvum* obtained by the *gltA* sequences were confirmed by the *ompA* sequences (Fig. 2). Regarding *A. neumanni*, two different haplotypes of ‘*Candidatus R. amblyommii*’ were detected. One haplotype belonging to the ‘*Candidatus R. amblyommii*’ detected in *A. neumanni* from Cruz Que-mada (Salta province), and a second haplotype of ‘*Candidatus R. amblyommii*’ was found in *A. neumanni* from La Punta (Santiago del Estero province), Tapia (Tucumán province), Rosario de la Frontera (Salta province) and El Fuerte (Jujuy province) (Fig. 2). GenBank accession numbers for the detected strains are requested.

Besides the investigation on the presence of *Rickettsia* spp., 1692 ticks were tested in view of the occurrence of ehrlichial DNA [*A. neumanni* (100), *A. parvum* (18), *A. sculptum* (1414) and *A. tonelliae* (160); Table 2]. None of the tested ticks showed infection with *Ehrlichia* spp.

Discussion

This study confirms that different species of spotted fever group rickettsiae (SFGR) are widely distributed in northern Argentina, where they are associated to *Amblyomma* ticks. *Rickettsia parkeri* was found in *A. triste* from Formosa province. This human pathogenic SFGR was previously detected in *A. triste* and *A. tigrinum* ticks from the central area of Argentina in Buenos Aires and Córdoba Province, respectively (Nava et al. 2008; Cicuttin and Nava 2013; Romer et al. 2014), but the findings of this work show that *R. parkeri* is much more widespread in Argentina than previously known. This fact is relevant since it implies that human cases of rickettsioses by *R. parkeri* could also occur in the areas of northern Argentina where *A. triste* is present. In Brazil, 9.7% of the tested *A. triste* ticks were infected with *R. parkeri* (Silveira et al. 2007), in Uruguay the infection rate was 2.6% (Pacheco et al. 2006), and in Argentina the infection rate of *R. parkeri* in questing adults of

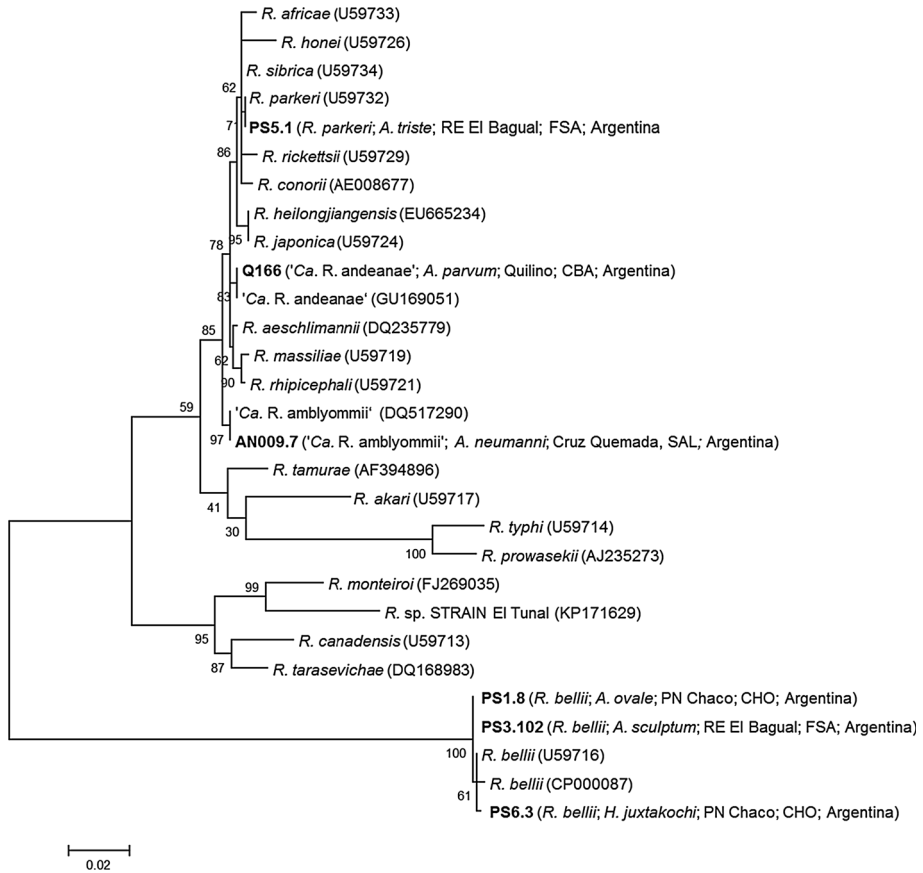


Fig. 1 Maximum-likelihood tree constructed from *gltA* partial sequences. Numbers represent bootstrap support generated from 1000 replications. GenBank accession numbers are in brackets (*A. Amblyomma*, *H. Haemaphysalis*, *Ca. Candidatus*, *R. Rickettsia*, *RE* Reserva Ecológica, *PN* Parque Nacional, *CBA* Córdoba, *CHO* Chaco, *FSA* Formosa, *SAL* Salta)

A. triste was 7.6% (Nava et al. 2008). Due to the small amount of *A. triste* ticks that were tested for *Rickettsia* in this study (three specimens) a precise statement about the prevalence of *R. parkeri* cannot be provided. However, a positive tick was found after analyse just three specimens, which suggest a high prevalence of infection in the area where the ticks were collected.

The other two members of the SFGR which were found in this study, namely '*Candidatus R. amblyommii*' and '*Candidatus R. andeanae*', were previously detected in different tick species of South America. In Argentina, '*Candidatus R. amblyommii*' was previously detected in *A. neumanni* and *A. hadanii* (Labruna et al. 2007a; Bottero et al. 2015; Mastropaolo et al. 2016). The results of this study clearly show that the association of '*Candidatus R. amblyommii*' and *A. neumanni* is a ubiquitous phenomenon along the distribution of this tick species, which is one of the more aggressive tick to humans in Argentina (Guglielmone et al. 1991; Nava et al. 2006). The prevalence of '*Candidatus R. amblyommii*' in this study could be estimated with 23.6%. Apperson et al. (2008) postulate the possibility that '*Candidatus R. amblyommii*' transmitted by *A. americanum* may cause

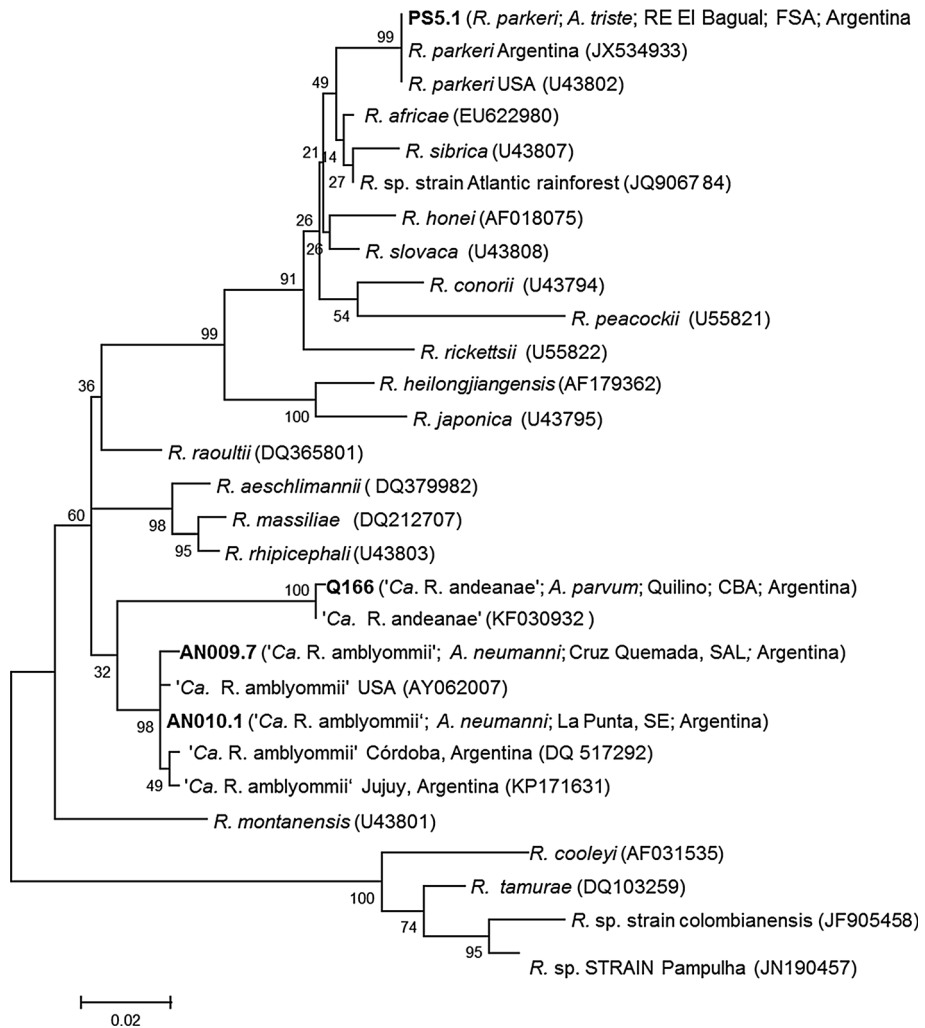


Fig. 2 Maximum-likelihood tree constructed from *ompA* partial sequences. Numbers represent bootstrap support generated from 1000 replications. GenBank accession numbers are in brackets (*A. Amblyomma*, *Ca. Candidatus*, *R. Rickettsia*, *RE* Reserva Ecológica, *CBA* Córdoba, *FSA* Formosa, *SE* Santiago del Estero, *SAL* Salta)

human cases of Rocky Mountain spotted fever in the United States of America, but this hypothesis was not confirmed so far. The association of '*Candidatus R. andeanae*' with *A. parvum* was previously reported in Argentina and Brazil (Pacheco et al. 2007; Nieri-Bastos et al. 2014). Also, infection with this SFGR was determined in *A. tigrinum* ticks from northwestern Argentina (Saracho Bottero et al. 2015), and Chile (Abarca et al. 2013). However, to date, this rickettsia species was not associated to human diseases.

Rickettsia bellii, which is a rickettsial bacterium with unknown pathogenicity (Parola et al. 2013), was found during this study in three species: *A. sculptum*, *A. ovale* and *H. juxtakochi*. The presence of this *Rickettsia* was previously reported in Argentina infecting *A. neumanni*, *A. tigrinum* and *A. dubitatum* ticks (Labruna et al. 2007a; Tomassone et al.

2010; Monje et al. 2015). In Brazil, Labruna et al. (2007b) and Pacheco et al. (2008) have reported the infection with *R. bellii* in *H. juxtakochi* and *A. ovale* ticks, respectively. The results of this work constitute the first report of *R. bellii* associated to *A. sculptum*, *A. ovale* and *H. juxtakochi* in Argentina.

The results of this study have demonstrated that rickettsial bacteria belonging to the spotted fever group are widespread in ixodid ticks with medical importance throughout a wide area of northern Argentina, which implies a risk for public health because all the tick species analysed in this work are aggressive to humans in this country.

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