

Detection of “*Candidatus Rickettsia* sp. strain Argentina” and *Rickettsia bellii* in *Amblyomma* ticks (Acari: Ixodidae) from Northern Argentina

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Abstract Ixodid ticks were collected from vegetation and from humans, wild and domestic mammals in a rural area in the semi-arid Argentine Chaco in late spring 2006 to evaluate their potential role as vectors of Spotted Fever Group (SFG) rickettsiae. A total of 233 adult ticks, identified as *Amblyomma parvum*, *Amblyomma tigrinum* and *Amblyomma pseudoconcolor*, was examined for *Rickettsia* spp. We identified an SFG rickettsia of unknown pathogenicity, “*Candidatus Rickettsia* sp. strain Argentina”, in *A. parvum* and *A. pseudoconcolor* by PCR assays targeting *gltA*, *ompA*, *ompB* and 17-kDa outer membrane antigen rickettsial genes. *Rickettsia bellii* was detected in a host-seeking male of *A. tigrinum*. *Amblyomma parvum* is widespread in the study area and is a potential threat to human health.

Keywords *Amblyomma* spp. · *Candidatus Rickettsia* sp. strain Argentina · *Rickettsia bellii* · Argentina · Spotted fever · Tick-borne disease

Introduction

Spotted Fever Group (SFG) rickettsiae cause human illness in several countries in South America, where new tick-borne rickettsial diseases have been discovered in the past few

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years (Parola et al. 2009). Indeed, human cases of SFG rickettsiosis were reported in Brazil, Colombia, Peru, Argentina and Uruguay (Galvão et al. 2003). Familial outbreaks can occur with high percentage of fatal cases, thus constituting a public health problem (Galvão 2004). Moreover, ecological transformations and vulnerable socio-economic conditions likely determine rickettsial emergence.

In Argentina, fatal cases of Rocky Mountain Spotted Fever occurred in Jujuy province where *Rickettsia rickettsii* was identified in *Amblyomma cajennense* ticks (Ripoll et al. 1999; Paddock et al. 2008). A case of rickettsial spotted fever was reported in the Paraná Delta region of Buenos Aires province (Seijo et al. 2007). Thereafter, *Rickettsia parkeri* was detected in *Amblyomma triste* collected in the same region (Nava et al. 2008). In addition, *Rickettsia massiliae* was identified in *Rhipicephalus sanguineus* collected in a low-income area of Buenos Aires city (Cicuttin et al. 2004), *Rickettsia amblyommii* and *Rickettsia bellii* were detected in *Amblyomma neumanni* ticks (Labruna et al. 2007) in Córdoba province. Finally, a new spotted fever group rickettsia designated as ‘*Rickettsia* sp. strain Argentina’ was detected in *Amblyomma parvum* (Pacheco et al. 2007a).

Taking advantage of our field work on the eco-epidemiology of Chagas disease in a well-defined rural area in the semiarid Argentine chaco (Santiago del Estero province), we collected ticks on vegetation by dragging and from mammal hosts, including humans. The aim was investigating tick species composition and host range, to assess their infection with *Rickettsia* spp. and to determine their potential role as vectors of tick-borne pathogens to humans.

Materials and methods

Study area

Field work was carried out in the area of Amamá ($27^{\circ}12'30''S$, $63^{\circ}02'30''W$) and neighboring rural villages (Trinidad, Mercedes, San Luis) and in an isolated settlement (Lote S), situated in Moreno Department, Province of Santiago del Estero, Argentina. The area is part of the semiarid southern Chaco, with a dry season from April to October (Ceballos et al. 2006).

Tick collection

Sampling was conducted in November–December 2006 (late spring). Sites chosen for the collection of questing ticks were representative of the main habitat types in the study area (secondary wood, exploited wood, pastures, peridomestic areas, burned pastures). Dragging was conducted along 100 m transects by two operators using a 1 m² cotton cloth during 25 sessions over a 2 weeks period in December 2006. A dragging cloth was also put around the trousers when walking in the woods and ticks were collected from it.

Feeding ticks were gathered from animals when visiting houses during the Chagas disease project field work (convenience sampling). Wild mammals examined for ticks (four armadillos and three fox puppies) were animals captured by the villagers on the same day or a few days before; moreover, we collected ticks from one fox which was found dead on the road. Sampled cows belonged to a single farm and were chosen randomly before being subjected to acaricide dipping. Tick-infested dogs lived in 10 different houses and goats in three houses. Two families (one in Trinidad and one in Amamá) were asked to collect ticks on themselves; they were given tubes with 70% alcohol that were returned to the research team on the next visit.

Ticks were put in 70% alcohol and identified with existing keys (Estrada-Peña et al. 2005; Guglielmone and Viñabal 1994; Guglielmone et al. 1990; Onofrio et al. 2006). One male and one female from each collected tick species were kept as reference specimens for accurate tick identification.

Molecular diagnostic tests

Adult ticks were individually homogenized with a pestle in microcentrifuge tubes and DNA was extracted using the DNeasy tissue kit (QIAGEN Valencia, CA, USA). Negative controls (distilled water) were added to verify potential contamination of samples during this phase.

PCR was used to amplify a fragment of the citrate synthase gene (*gltA*, 401 bp product) common to all *Rickettsiae* (Labruna et al. 2004). Positive ticks were further tested by a PCR assay targeting the 190-kDa outer membrane protein gene *ompA*—specific for the Spotted Fever Group (SFG). We used primers Rr190.70F and Rr190.602R (530 bp product; Regnery et al. 1991), and designed the new primers OmpArg-F (AGCCGCTTATTCAACCTCA) and OmpArg-R (AATTAGTCAGCATTGCTCCC) that give a 484 bp product (cycling conditions: 1 cycle 3' 94°C; 35 cycles 45" 95°C, 30" 54°C, 1' 72°C; extension 7' 72°C).

A subset of samples was tested by other PCR assays targeting fragments of the 17-kDa gene (549 bp; Labruna et al. 2004) and *ompB* gene (856 bp; Roux and Raoult 2000) to better characterize the rickettsial species. In all PCR reactions, 5 µl of DNA sample were amplified; distilled water was used as negative control, and DNA from a *R. conorii* isolate used as positive control.

Sequencing was performed using Big Dye Terminator vs. 3.1 and 3130xl Genetic analyzer (Applied Biosystem, Foster City, CA). Sequences were analyzed with Vector NTI 9.0.0. Individual genes and concatenated DNA sequences were aligned using the computer program ClustalX (Thompson et al. 1997). The corresponding sequences of *R. parkeri*, *R. conorii*, *R. rickettsii*, *R. honei*, *R. massiliae*, *R. rhipicephali*, *R. montanensis*, *R. peacockii* and *R. amblyommii* were used as reference sequences and *R. canadensis* as outgroup. Phylogenetic analyses were conducted using the program MEGA version 3.1 (Kumar et al. 2004). Maximum-parsimony and neighbor-joining trees were generated; confidence values were determined by bootstrap analysis with 1,000 replicates.

Statistical analysis

Prevalence of PCR-positive results was calculated for each pathogen, host and tick species. Exact binomial 95% confidence intervals (CI) for infection prevalence were calculated. Fisher's exact test was used to study the association between categorical variables. Analyses were performed using R software (R Development Core Team 2009). Given the non-random sampling, we did not compare the numbers of ticks and prevalence of infection between different sites.

Results

A total of 247 ticks was collected, 226 on mammal hosts and 21 host-seeking (Table 1). All ticks belonged to the genus *Amblyomma*. Nymphs ($n = 8$) were all collected on *Chaetophractus* sp. armadillos. Adult ticks ($n = 239$) were identified to species level and classified as *Amblyomma parvum* (83.7%), *Amblyomma tigrinum* (10.9%) and *Amblyomma*

Table 1 Stage and species-specific numbers of *Amblyomma* spp. ticks collected on mammal hosts and host-seeking

Source of ticks			Number of collected ticks				
Host common name	Host scientific name	No. of examined hosts	Adults			Nymphs	Total
			<i>A. parvum</i>	<i>A. pseudoconcolor</i>	<i>A. tigrinum</i>		
Human	<i>Homo sapiens</i>	19	82	1	5	0	88
Dog	<i>Canis lupus familiaris</i>	12	34	0	14	0	48
Grey fox	<i>Lycalopex gymnocercus</i>	4	28	0	4	0	32
Goat	<i>Capra hircus</i>	6	16	0	0	0	16
Cow	<i>Bos taurus</i>	5	17	0	0	0	17
Three-banded armadillo	<i>Tolypeutes matacus</i>	1	5	0	0	0	5
Hairy armadillo	<i>Chaetophractus villosus</i>	1	0	4	0	7	11
Armadillo	Not determined	1	0	8	0	0	8
Small armadillo	<i>Chaetophractus vellerosus</i>	1	0	0	0	1	1
Host-seeking	–	–	18	0	3	0	21
Total			200	13	26	8	247

pseudoconcolor (5.4%). Overall, 91.2% of the adult ticks were collected on hosts, especially on humans (36.8%) (Table 1). Female ticks constituted 54.0% of the adult tick sample.

Amblyomma parvum infested all host species apart from *Chaetophractus* spp. armadillos and one non identified armadillo, whereas *Amblyomma tigrinum* and *A. pseudoconcolor* had a narrower host range (Table 1). *Amblyomma parvum* was by far the most abundant tick on humans (93.2%), and infested significantly more humans than other host species (Fisher's test; $P = 0.0014$). *Amblyomma tigrinum* was significantly more abundant on canids than on other hosts ($P < 0.001$), whereas *A. pseudoconcolor* prevalence was greater on armadillos ($P < 0.001$). Among host-seeking ticks, only 3 *A. parvum* females were collected by dragging during two sessions in two exploited forest sites, and 15 *A. parvum* and 3 *A. tigrinum* adults were collected from the researchers' clothes.

A total of 233 adult ticks (198 *A. parvum*, 11 *A. pseudoconcolor* and 24 *A. tigrinum*) was examined for *Rickettsia* spp. by PCR. Expected PCR products for the gltA rickettsial gene were obtained from 82.8% of the ticks. The prevalence of rickettsial infection was 93.9% in *A. parvum* and 54.5% in *A. pseudoconcolor*, whereas only one of 24 *A. tigrinum* was positive (Table 2). All gltA positive samples were also positive to *ompA* gene PCR, except for the positive *A. tigrinum* (Table 2).

The prevalence of SFG rickettsiae was significantly higher in *A. parvum* than in other species (Fisher's test; $P < 0.0001$). Host-specific prevalences of SFG *Rickettsiae* are reported in Table 3; no significant difference in *A. parvum* infection prevalence among different host species was detected ($P = 0.78$). Rickettsial infection prevalence did not differ significantly between male and female ticks (Fisher's test; $P = 0.6$).

Table 2 Prevalence of *Rickettsia* spp. infection (*gltA* and *ompA* genes) in different *Amblyomma* species

Tick species	No. of ticks	No. of ticks infected (% prevalence; 95% CI)	
		<i>gltA</i> gene	<i>ompA</i> gene
<i>A. parvum</i>	198	186 (93.9; 89.6–96.8)	186 (93.9; 89.6–96.8)
<i>A. pseudoconcolor</i>	11	6 (54.5; 23.4–83.2)	6 (54.5; 23.4–83.2)
<i>A. tigrinum</i>	24	1 (4.2; 0.1–21.1)	0 (0; 0–14.2)
Total	233	193 (82.8; 77.4–87.4)	192 (82.4; 76.9–87.1)

Table 3 Prevalence of *Rickettsia* spp. infection (*ompA* gene) in *Amblyomma* spp. ticks collected on different hosts and host-seeking; armadillos species were grouped together

Source of ticks	No. of ticks tested	No. of SFG infected ticks (% prevalence; 95% CI)
Human	85	76 (89.4; 80.8–95.0)
Dog	47	31 (66.0; 50.7–79.1)
Grey fox	32	28 (87.5; 71.0–96.5)
Goat	16	15 (93.7; 69.8–99.8)
Cow	17	13 (76.5; 50.1–93.2)
Armadillos	15	11 (73.3; 44.9–92.2)
Host-seeking	21	18 (85.7; 63.7–96.9)

A subset of *ompA*-positive *A. parvum* and *A. pseudoconcolor* was further tested by PCR targeting 17-kDa ($n = 8$) and *ompB* genes ($n = 11$). Products from the *gltA*, 17-kDa, *ompB* and *ompA* PCR revealed 100% identical DNA sequences to each other for each gene. The GenBank accession numbers for each gene are: EU826506 and EU826507 (17-kDa outer membrane antigen); EU826509 and EU826509 (*gltA*); EU826512 and EU826513 (*ompA*); EU826514 and EU826515 (*ompB*).

Local and global nucleotide sequence alignment (Blastn and Clustal X, respectively) revealed 100% nucleotide similarity to the ‘*Rickettsia* sp. strain Argentina’ detected in Northern Córdoba province (Pacheco et al. 2007a). Gene sequence-based criteria (Fournier et al. 2003) were applied for further species validation of the rickettsial organism. Individual and concatenated DNA sequences fragments were used for phylogenetic analysis. The resulting phylogenetic trees were very similar, showing that the fragments under study cluster with *R. montanensis* fragments. The available sequences allowed us to calculate the percentage of pairwise nucleotide sequence similarity with *R. montanensis* *gltA* (99.3%), *ompA* (91.9%) and *ompB* (95.3%) genes.

The only *A. tigrinum* testing *gltA*-positive was also positive to 17-kDa and negative to the other genes; both fragments had 100% nucleotide similarity to *Rickettsia bellii*.

Discussion

Our study confirms the finding of a separate SFG species in *A. parvum* in Northeastern Argentina, as recently reported by Pacheco et al. (2007a). The percentage of pairwise similarity of three gene fragments with their homologues from the phylogenetically closest validated species (*R. montanensis*) were below the cutoff values proposed by Fournier et al.

(2003) ($\geq 99.9\%$ for *gltA*; $\geq 98.8\%$ for *ompA*; $\geq 99.2\%$ for *ompB*), suggesting that the detected rickettsia is a new species. We propose the name “*Candidatus Rickettsia* sp. Argentina” for this organism. However, *rrs* and *geneD* sequences need to be further characterized to better determine its taxonomic status.

Primers Rr190.70F and Rr190.602R are apparently not suitable to amplify the *ompA* gene of “*Candidatus R. sp. strain Argentina*” (see also Pacheco et al. 2007a), therefore we designed more specific primers.

The prevalence of infection by this SFG rickettsia in questing and on-the-host ticks, including ticks collected on humans, was very high, especially in *A. parvum* (>90%). Such high prevalence is comparable to the prevalence of endosymbiont rickettsial species that are not pathogenic to vertebrate hosts and maintain natural infections in ticks via transstadial and transovarial transmission. Remarkably, when comparing “*Candidatus R. sp. strain Argentina*” *ompA* and 17-kDa gene fragments against the rickettsial endosymbiont of *Amblyomma maculatum* (accession numbers EF689729 and EF689728, respectively), a 100% pairwise sequence similarity was obtained. Moreover, the absence of a significant difference in the infection prevalence among *A. parvum* feeding on different host species reinforces the endosymbiont hypothesis. Non-pathogenic rickettsiae have shown to affect the distribution and dynamics of pathogenic rickettsial species and have important public health consequences, as in the case of *R. peacockii* competing with *R. rickettsii* in *Dermacentor andersoni* in the USA (Burgdorfer et al. 1981). In addition, interference between *Rickettsia rhipicephali* and *Rickettsia montana* in *Dermacentor variabilis* has been documented (Macaluso et al. 2002). Nevertheless, the role of “*Candidatus R. sp. strain Argentina*” as a human pathogen cannot be ruled out, given that several rickettsial species of unknown pathogenicity have been recognized as zoonotic pathogens in recent years (Parola et al. 2005; Walker 2007).

Amblyomma parvum is a common tick of domestic animals and frequently feeds on humans in Argentina and Brazil, where it is considered a potential vector of zoonoses (Guglielmone et al. 2006). *A. parvum* was found on all host species; it was the most abundant tick on humans, and was found infected by SFG rickettsiae. Moreover, *Ehrlichia chaffeensis*, the agent of human monocytotropic ehrlichiosis, was recently detected in *A. parvum* in the same geographic area (Tomassone et al. 2008); this tick species is therefore a potential threat to human health.

The finding of “*Candidatus R. sp. strain Argentina*” in *A. pseudoconcolor* appears to be the first evidence of a rickettsial infection in this tick species. *A. pseudoconcolor* commonly feeds on several genera of armadillos (*Dasypus*, *Chaetophractus*, *Euphractus*, *Tolypeutes*, *Zaedyus*), and accidentally feeds on domestic animals (Guglielmone et al. 2003; Guglielmone and Nava 2006). However, we collected one specimen on a human host.

Our study also provides the first evidence of *R. bellii* in *A. tigrinum* ticks. *R. bellii*, a rickettsia whose pathogenicity is unknown, is the most frequent rickettsial species infecting ticks in Brazil, and its natural infection in vertebrate hosts has been demonstrated (Pacheco et al. 2007b). In Argentina *R. bellii* has been detected only in *Amblyomma neumannii* (Labruna et al. 2007). *A. tigrinum* is usually collected on carnivores (Guglielmone and Nava 2006), but in our study area a few specimens were also collected on humans.

Host-seeking ticks were collected by dragging in forest under current exploitation. Major environmental changes that include massive deforestation for cattle-ranching or agriculture were taking place at the time of our surveys and during the previous decade (Ceballos et al. 2006). Most of the local villagers collaborating with us in tick collection were men employed in logging operations, and reported frequent contact with ticks. The degradation of sylvatic habitats may augment the contact rate of wild animals with

peridomestic or domestic sites and with domestic animals. Therefore, rickettsial diseases could increase their prevalence and public health impact in the study region over the coming years.

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