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High prevalence of "*Candidatus* Rickettsia amblyommii" in Amblyomma ticks from a Spotted Fever Endemic Region in North Argentina

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ABSTRACT

Ticks from an endemic Spotted Fever region in Argentina were analysed by PCR for Spotted Fever Group Rickettsiae. DNA of "*Candidatus* Rickettsia amblyommii" was found in 21.3% of *Amblyomma hadanii* and in 44.0% of *A. neumanni*. *Amblyomma sculptum* (formerly *A. cajennense*) and *Haemaphysalis juxtakochi* were negative for rickettsial DNA. DNA of *Rickettsia rickettsii*, the etiological agent of the clinical cases reported within the studied region was not detected in the analysed sample.

1. Introduction

Endemic areas for transmission of rickettsial spotted fever caused by *Rickettsia rickettsii* in Argentina are located in the northern provinces of Jujuy and Salta (Fig. 1), associated with the bio-geographic region of the Yungas (Cloud Forest), where several human cases have been diagnosed and characterized [1,2]. However, until today, *R. rickettsii* has been detected only in a pool of 5 *Amblyomma cajennense* nymphs collected in the area [2]. A recent study concluded that ticks previously included under this name in Argentina in fact belong to two related species, *A. sculptum* and *A. tonelliae* [3]. These species are sympatric in the ecotone of two contrasting environments, the Yungas, a mountainous cloud forest, and Chaco, a dry thorny semi-deciduous forest strongly modified by human activity [4]. Furthermore, *A. neumanni* and a recently described tick species, *A. hadanii*, are also prone to bite humans in the Cloud Forest [5,6]. These circumstances result in uncertainty as to which tick species could be involved in transmission. The aim of this study was to examine ticks for spotted fever group rickettsiae by molecular methods in order to expand our knowledge of the species of rickettsiae and ticks which may be involved in the transmission of Spotted Fever in this endemic area.

2. Materials and methods

During 2011 and 2012, four seasonally distributed sample collections were made in the El Rey National Park, a protected area located in the Department of Anta, Salta Province, Argentina (24°15′ S, 64° 40′ W). It covers a surface of 44162 ha. The area is mountainous with altitudes varying between 700 and 2300 m above sea level (masl). The climate is mountainous subtropical with marked seasons; 80% of a year's rain occurs during summer (November–March), with an average of 1500 mm in the mountain zone, and less rainfall in the mountain base zone. Mean temperatures are 21 °C in summer and 8 °C in winter. Temperatures below 0 °C are recorded during the latter season. Two large biomes are represented in the Park: Yungas and West Chaco. Yungas are divided into four altitude strata: Transitional Forest, Mountain base forest (400–700 masl), Mountain Forest (700–1500 masl), Mountain woods (1500–2000 masl) and High grasslands (above 2000 masl). Chaco biome refers to Mountain Chaco, comprising open and forested environmental settings that in the Park are extended between 800 and 900 masl. Ixodid tick species with confirmed

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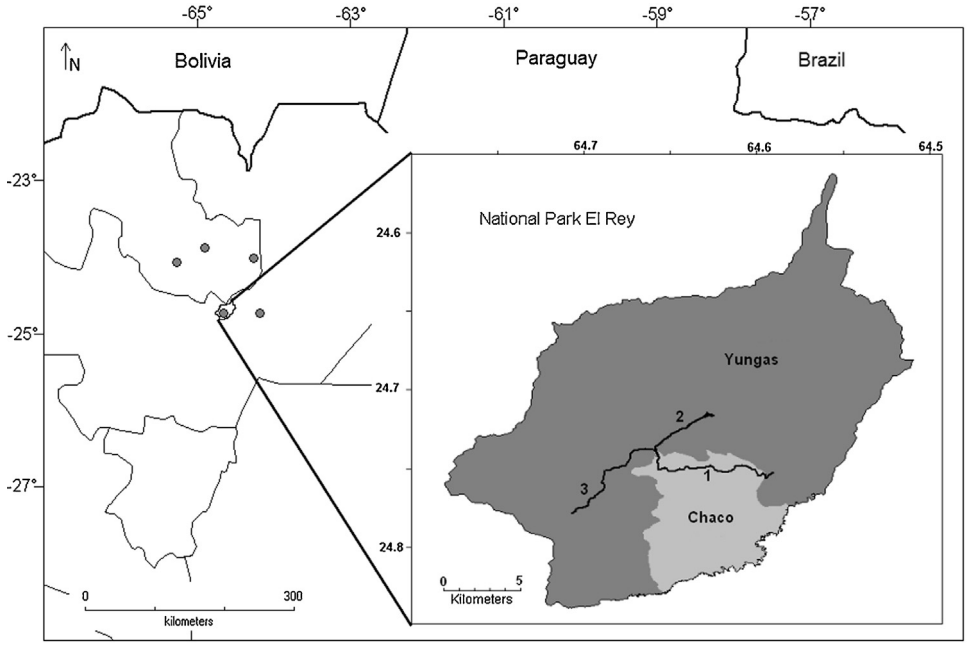

Fig. 1. Spotted Fever outbreaks localities in North Argentina (gray circles) with detail of National Park El Rey. Sampled paths are named with numbers: (1) Mountain Chaco, (2) Transitional Forest, (3) Mountain Forest.

presence in the National Park are *A. sculptum, A. hadanii, A. neumanni, Haemaphysalis juxtakochi, H. leporispalustris, Ixodes luciae, I. pararicinus,* and *I. longiscutatus* [5,7–9].

Questing adult ticks were collected from vegetation by dragging a piece of cloth of 1 × 1.5 m and were preserved in 96% ethanol. During each campaign, three paths comprising Mountain Forest, Transitional Forest and Chaco environments were sampled (Fig. 1). The ticks were identified following standard taxonomic keys [10] or original descriptions [3,5] and were individually homogenized in 2 mL Eppendorf tubes (Eppendorf, Hamburg, Germany) with one 5-mm steel bead, 80 μL phosphate buffered saline, in a Tissue Lyser (QIAGEN, Hilden, Germany) for 5 min at 30.000U/min; DNA was extracted from each individual with the Roche High Pure PCR Template Preparation Kit (Roche) by using the protocol for animal tissue. Quality and quantity of extracted DNA were tested with a NANO DROP ND-1000 spectrophotometer (Peqlab). After the DNA extraction, every sample was screened for SFG Rickettsiae by PCR with specific primers for a fragment of the *gltA* gene [11]. The positive samples were further analysed for a fragment of the *ompA* gene [11]. The latter PCR products were sequenced. Phylogenetic analysis was carried out using maximum-likelihood (ML) method with the program MEGA 5 [12]. The ML tree was generated with the GTR model by using a discrete Gamma-distribution (+G). The best-fitting substitution models were determined with the Bayesian Information Criterion using the ML model test implemented in MEGA 5 [12]. Support for the topologies was tested by bootstrapping over 1000 replications, and gaps were excluded. Representative sequences of ompB were obtained according to Roux and Raoult [13] and compared by BLAST analysis with those deposited in GenBank. Association among PCR results (positive/negative) and environment, season and tick gender was explored by contingency tables and Chi square test.

3. Results

A total of 478 adult ticks from the following species were analysed: *A. sculptum* (n 271), *A. hadanii* (n 127), *A. neumanni* (n 18) and *H. juxtakochi* (n 63). DNA from Ca. R. amblyommiií was detected in 27 out of 127 (21.3%) specimens of *A. hadanii* and in 8 out of 18 (44%) specimens of *A. neumanni*. All specimens of *A. sculptum* and *H. juxtakochi* resulted negative. Positive specimens of *A. hadanii* were collected in every season and in all the sampled environments. The statistical analysis showed no association between positive results and tick gender or season. The association between a PCR results and environment was significant, being the prevalence of infected ticks higher in the Mountain Forest as in Transitional Forest or Chaco (P = 0.009). All infected *A. neumanni* ticks were found in winter in Chaco environment. In the phylogenetic analysis, ompA sequences corresponding to three different haplotypes (*A. hadanii*: FR02322782 and FR02322785; *A. neumanni*: FR02322811) (GenBank accession numbers: KX198769 (haplotype FR02322782); KX198770 (haplotype FR02322785); KX198771 (haplotype FR02322811)), were distributed in two clades, although with a difference of not more than 1.1% in reference to other sequences of 'Ca. R. amblyommii' (Fig. 2).

In order to confirm the results obtained with gltA and ompA sequences, a representative sequence of ompB of the "Ca. R. amblyommii" detected in *A. hadanii* (Genbank number: KX198772) was compared by BLAST analysis with sequences in the NCBI gene database. This sequence presented a similarity of more than 99% with the ompB sequence of "Ca. R. amblyommii" from USA (Genbank number HM446490). The same result was obtained when a representative sequence of ompB of the "Ca. R. amblyommii" detected in *A. neumanni* (genbank number: KX198773) was compared with the sequence of "Ca. R. amblyommii" from USA (Genbank number HM446490).

4. Discussion

All tick species analysed in this study frequently bite humans [6]. National Park El Rey is a confirmed locality of transmission of Rickettsial Spotted Fever in North Argentina [1] and *R. rickettsii* is the etiological agent of Spotted Fever within this region [2]. However no DNA of *R. rickettsii* was found among the samples analysed in this study. The only *Rickettsia* species detected in this work was "Ca. R. amblyommii" and a high proportion of *A. hadanii* and *A. neumanni* were found infected. This *Rickettsia* species is of unknown pathogenicity, although it has been sus-

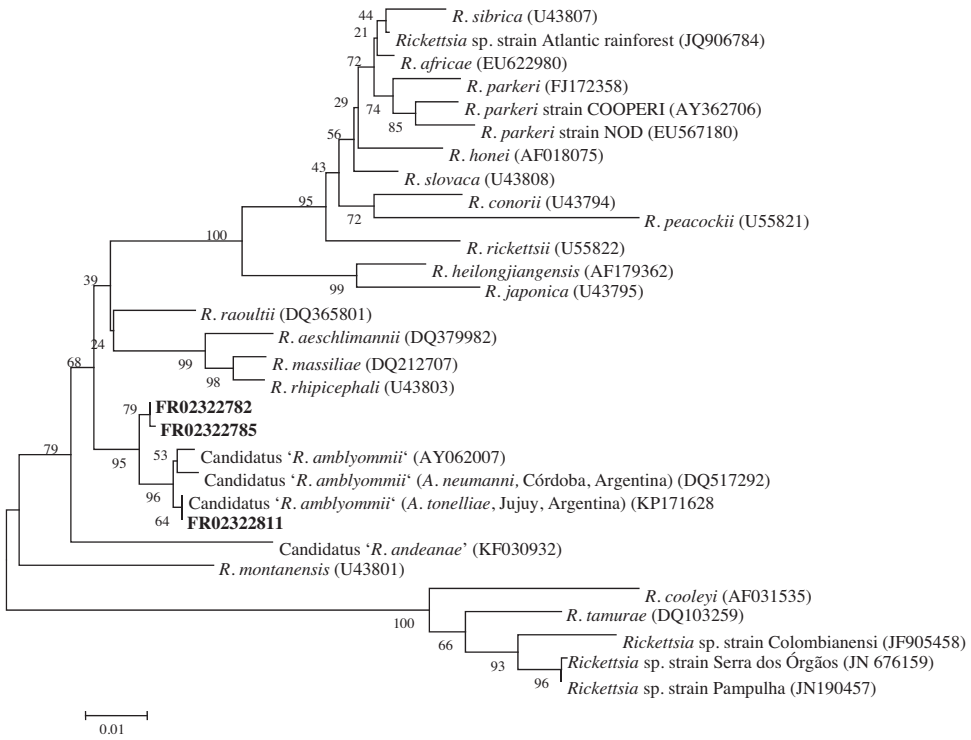

Fig. 2. Maximum-likelihood tree constructed from ompA partial sequences. Numbers represent bootstrap support generated from 1000 replications. GenBank accession numbers are in brackets.

pected responsible for Spotted Fever cases in the United States [14] and it has recently been experimentally determined that it can be highly pathogenic to guinea pigs [15]. In Argentina this bacterium has previously been found in populations of *A. neumanni* [16,17] and *A. tonelliae* [18] both in the semiarid Chaco. *Amblyomma tonelliae* (previously determined in Argentina as *A. cajennense*), has an ecological preference for dry environments [4]. Sequences of "Ca. R. amblyommii" from *A. neumannii* formed a clade together with sequences of "Ca. R. amblyommii" that were reported previously in *A. neumamni* and *A. tonelliae* ticks from Argentina, and in *A. americanum* from USA. Sequences of "Ca. R. amblyommii" detected in *A. hadanii* grouped in a different clade, although with differences among haplotypes that are too small (1%) to consider them a priori different species. *Amblyomma hadanii*, formerly confused with *A. coelebs*, is restricted to the Yungas environment were nymphs and adults have been found biting people [5,7]. Most of the *A. hadanii* specimens collected in this work were found also in the Mountain Forest. Although the proportion of infected ticks in this environment was higher as in the Transitional Forest or Chaco, it would be necessary a larger ecoepidemiological study to confirm the causality of this association. *Amblyomma sculptum* (previously considered *A. cajennense*), has been incriminated as a vector of spotted fever in South America [19]. Indeed, it is the main vector of Brazilian Spotted Fever by *R. rickettsii* in endemic areas of Brazil [19]. No specimens of *A. sculptum* were found infected in the present study, despite the fact that the analysed sample would allow the detection of a positive with 95% confidence if the prevalence were 1% [20], which is the reported prevalence in other endemic areas [19]. Neither was found evidence of infection with rickettsiae in *H. juxtakochi.* This tick has been previously found infected with *R. belli* and *R. rhipicephali* in Brazil. While there are no records for the last Rickettsia species in Argentina, the former one has been already detected in other tick species in the country. The results of this study suggest that *R. rickettsii*, if present, has a very low prevalence in the analysed tick species but also that there is a high proportion of ticks infected with "Ca. R. amblyommii", what could be indicative of a high rate of circulation of this organism among the natural hosts of *A hadanii* and *A neumannii*. This should be taken in account when interpreting results of serologic trails or serologic diagnostic tests, due to the possibility of cross reactions.

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