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Candidatus Rickettsia andeanae, a spotted fever group agent infecting *Amblyomma parvum* ticks in two Brazilian biomes

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Adult ticks of the species Amblyomma parvum *were collected from the vegetation in the Pantanal biome (state of Mato Grosso do Sul) and from horses in the* Cerrado *biome (state of Piauí) in Brazil. The ticks were individually tested for rickettsial infection via polymerase chain reaction (PCR) targeting three rickettsial genes,* gltA, ompA *and* ompB. *Overall, 63.5% (40/63) and 66.7% (2/3) of* A. parvum *ticks from Pantanal and Cerrado, respectively, contained rickettsial DNA, which were all confirmed by DNA sequencing to be 100% identical to the corresponding fragments of the* gltA, ompA *and* ompB *genes of* Candidatus *Rickettsia andeanae. This report is the first to describe* Ca. *R. andeanae in Brazil.*

Key words: ticks - *Amblyomma parvum* - *Candidatus* Rickettsia andeanae

The bacterial genus *Rickettsia* (Rickettsiales: Rickettsiaceae) includes Gram-negative coccobacilli organisms in obligatory associations with eukaryote cells. A number of *Rickettsia* species genetically classified into the spotted fever group (SFG) are agents of tick-borne diseases in the world (Parola et al. 2005). In Brazil, the most important SFG agents are *Rickettsia rickettsii,* the causative agent of Brazilian spotted fever, which is the most deadly rickettsiosis globally (Guedes et al. 2005, Labruna 2009), and the strain Atlantic rainforest, an *Rickettsia parkeri*-like agent that was only recently shown to cause spotted fever in humans (Spolidorio et al. 2010, Silva et al. 2011). Other tick-associated rickettsiae, namely *Rickettsia rhipicephali, Rickettsia amblyommii, Rickettsia monteiroi* and *Rickettsia bellii,* have also been reported in Brazil, but have not been associated with human illness (reviewed by Labruna et al. 2011). It is noteworthy that during the last three decades, approximately 10 SFG *Rickettsia* species or subspecies previously known to infect only ticks were further identified as emerging agents of tick-borne rickettsioses throughout the world (Parola et al. 2005, Shapiro et al. 2010). An outstanding example is *R. parkeri*, which has been known to infect *Amblyomma* ticks for more than 60 years, but was recognised as a human pathogen only during the last 10 years (Paddock et al. 2004). Based on these findings, it is prudent to consider any new tick-associated *Rickettsia* species as a potential human pathogen until further studies are conducted.

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The tick species *Amblyomma parvum* is distributed from southern Mexico to Argentina (Guglielmone et al. 2003). While the *A. parvum* adult stage has a preference for parasitising medium to large-sized mammals (ruminants, horses and carnivores), larvae and nymphs seem to prefer small mammals (Aragão 1936, Nava et al. 2008). Recent studies in Argentina and Brazil have indicated that rodents of the genera *Galea* and *Trichomys* are important hosts for *A. parvum* sub-adults (Nava et al. 2008, Horta et al. 2011). Of note, the *A. parvum* adult stage is an important human-biting tick in Argentina and Brazil (Guglielmone et al. 2006); therefore, it is a potential pathogen vector for humans.

To evaluate rickettsial infection in *A. parvum* ticks from Brazil, tick specimens were collected in two Brazilian biomes. In the Pantanal biome, free-living ticks were collected by CO_2 traps set on the vegetation in the Nhumirim Farm (18º59′S 56º39′W) at the Nhecolândia Pantanal, state of Mato Grosso do Sul, central-western Brazil, from March 2006-January 2007, as detailed previously (Cançado et al. 2008). In the *Cerrado* biome, ticks were collected from horses on a farm (04º76'91''S 42º58'44''W) in the municipality of Teresina, state of Piauí (PI), northeastern Brazil, during August 2010. The ticks were preserved in absolute ethanol until they were processed in the laboratory.

Each adult *A. parvum* tick was subjected to DNA extraction by the guanidine isothiocyanate-phenol technique, as previously described (Sangioni et al. 2005). Purified DNA was stored at -20ºC until it was used as template for polymerase chain reaction (PCR) amplifications. Five microlitres of each tick DNA template (approximately 500 ng of DNA) was used for PCR using the primers CS-78 (forward) GCAAGTATCGGTGAGGATGTAAT and CS-323 (reverse) GCTTCCTTAAAATTCAATAAATCAGGAT, which amplify a 398-bp fragment of the citrate synthase gene (*gltA*) of, most likely, all

known *Rickettsia* species (Labruna et al. 2004). If an expected product was observed following gel electrophoresis, the tick was tested using two other PCR protocols: one targeting a fragment of the rickettsial 190-kDa outer membrane protein gene (*ompA*) using primers Rr190.70F ATGGCGAATATTTCTCCAAAA and Rr190.701R GTTCCGTTAATGGCAGCATCT (Eremeeva et al. 2006) and the other targeting a fragment of the rickettsial 135-kDa outer membrane protein gene (*ompB*) using primers 120-M59 CCGCAGGGTTGGTAACTGC and 120-807 CCTTTTAGATTACCGCCTAA (Roux & Raoult 2000). Ten microlitres of each PCR product was electrophoretically separated in a 1.5% agarose gel stained with ethidium bromide and examined using ultraviolet transillumination. PCR products compatible with the expected band size were purified using ExoSap (USB Corporation, Cleveland, OH, USA) and sequenced in an automated sequencer (model ABI Prism 310 Genetic, Applied Biosystems, Perkin Elmer, CA, USA) according to the manufacturer's protocol. The partial sequences that were obtained were subjected to BLAST analyses (ncbi.nlm.nih.gov/blast) to determine the closest similarities to other *Rickettsia* species available in GenBank.

Sixty-three (37 males, 26 females) out of 81 *A. parvum* adult specimens collected from the vegetation in the Pantanal were tested in this study. From the *Cerrado* area of PI, 3 *A. parvum* specimens (2 males, 1 female) were collected from three out of 27 examined horses; other tick species (not tested in this study) collected from these horses were *Amblyomma cajennense* (8 adults) and *Dermacentor nitens* (570 adults). The *gltA*-PCR indicated that 40 (63.5%) *A. parvum* specimens (20 males, 20 females) from Pantanal and two *A. parvum* males (66.7%) from PI were infected by *Rickettsia*. From these 42 *gltA*-PCR positive ticks, 23 were positive in the *ompA*-PCR and 17 were also positive in the *ompB*-PCR. High-quality DNA sequences were obtained from 19 *gltA*-positive ticks, 12 *ompA*-positive ticks and 11 *ompB*-positive ticks, including the two males from PI. For each rickettsial gene, the sequences from different individual ticks were identical to each other and were also 100% identical to the corresponding sequences of *Candidatus* Rickettsia andeanae from the United States of America (USA), Peru and Argentina, (e.g., GU169051, EF451001 for *gltA*; EU826513, EF372578 for *ompA*; GU395297, EF451003 for *ompB*) when submitted to BLAST analyses. The sequences generated in this study have been submitted to GenBank under the accessions KF030931-KF030933.

This study reports the identification of the SFG rickettsial agent *Ca*. R. andeanae for the first time in Brazil. Taxonomic identification of the agent was confirmed by sequencing fragments of one conserved housekeeping gene (*gltA*) and two highly polymorphic outer membrane protein genes (*ompA* and *ompB*). Among the 42 ticks that were PCR-positive for the *gltA* gene, only 23 were positive in the *ompA*-PCR and 17 in the *ompB*-PCR. Because fragments of approximately 398, 630 and 820 bp were targeted by the *gltA, ompA* and *ompB*-PCR protocols, respectively, the different results obtained with these protocols are possibly related to different PCR sensitivities, as it is well known that the shorter the PCR fragment, the more efficient the PCR reaction (Bustin 2000).

Ca. R. andeanae has been reported to infect ticks in Peru, USA, Argentina and Chile (Blair et al. 2004, Pacheco et al. 2007, Paddock et al. 2010, Abarca et al. 2012). More recently, Luce-Fedrow et al. (2012) and Ferrari et al. (2013) reported evidence for the continuous in vitro cultivation of *Ca.* R. andeanae. A number of different tick species have been reported to harbour *Ca.* R. andeanae *in nature*. These species include *Amblyomma maculatum, Ixodes boliviensis* and *Rhipicephalus sanguineus* in Peru (Blair et al. 2004, Flores-Mendoza et al. 2013), *A. maculatum* in USA (Paddock et al. 2010, Luce-Fedrow et al. 2012), *A. parvum* and *Amblyomma pseudoconcolor* in Argentina (Pacheco et al. 2007, Tomassone et al. 2010) and *Amblyomma triste* in Chile (Abarca et al. 2012). According to these references, different designations have been given to *Ca.* R. andeanae, such as *Rickettsia* sp. strain Argentina or the rickettsial endosymbiont of *A. maculatum*. Because the DNA sequences representing these different designations were 100% identical to each other, they are all considered to represent *Ca.* R. andeanae (Paddock et al. 2010, Luce-Fedrow et al. 2012, Ferrari et al. 2013), which was first reported in Peru by Blair et al. (2004). Because *Ca* R. andeanae-infected ticks were found in distant areas of two Brazilian biomes, it is possible that the distribution of this organism within Brazil is much broader than is reported here.

The majority of the ticks in this study were infected with *Ca.* R. andeanae. This finding is in accordance with previous studies that reported that most of the *A. parvum* ticks from Argentina (69.2%) were infected with *Ca.* R. andeanae. However, infection rates by this agent among *A. maculatum* ticks in USA have been much lower, usually between 1-10% (Paddock et al. 2010, Jiang et al. 2012). Regardless, the role of *Ca.* R. andeanae as a human pathogen is unknown. Because *A. parvum* is an important human-biting tick in South America (Guglielmone et al. 2006), further studies are needed to evaluate the capacity of *Ca.* R. andeanae to be tick transmitted and to infect humans or other vertebrate hosts.

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