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Research Paper

Detection of *Rickettsia bellii* and *Rickettsia amblyommii* in *Amblyomma longirostre* (Acari: Ixodidae) from Bahia state, Northeast Brazil

Douglas McIntosh[1], Rodrigo Alves Bezerra[2], Hermes Ribeiro Luz[1], João Luiz Horacio Faccini[1], Fernanda Amato Gaiotto[2], Gastón Andrés Fernandez Giné[2], George Rego Albuquerque[3]

[1]Departamento de Parasitologia Animal, Universidade Federal Rural do Rio de Janeiro, Seropédica, RJ, Brazil.
[2]Departamento de Ciências Biológicas, Universidade Estadual de Santa Cruz, Ilhéus, BA, Brazil.
[3]Departamento de Ciências Agrárias e Ambientais, Universidade Estadual de Santa Cruz, Ilhéus, BA, Brazil.

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Abstract

Studies investigating rickettsial infections in ticks parasitizing wild animals in the Northeast region of Brazil have been confined to the detection of *Rickettsia amblyommii* in immature stages of *Amblyomma longirostre* collected from birds in the state of Bahia, and in immatures and females of *Amblyomma auricularium* collected from the striped hog-nosed skunk (*Conepatus semistriatus*) and armadillos (*Euphractus sexcinctus*) in the state of Pernambuco. The current study extends the distribution of *R. amblyommii* (strain Aranha), which was detected in *A. longirostre* collected from the thin-spined porcupine *Chaetomys subspinosus* and the hairy dwarf porcupine *Coendou insidiosus*. In addition, we report the first detection of *Rickettsia bellii* in adults of *A. longirostre* collected from *C. insidiosus* in the state of Bahia.

Key words: *Rickettsia amblyommii*, *Rickettsia bellii*, porcupine, Bahia, Brazil.

Introduction

Tick-borne rickettsioses are zoonotic infections, with a global distribution, caused by intracellular bacteria belonging to genus *Rickettsia* (Parola *et al.*, 2013). Prior to 2004, the only tick-associated *rickettsia* species documented in Brazil was *Rickettsia rickettsii*, the etiological agent of Brazilian spotted fever (BSF). However, during the last decade an additional seven species (*Rickettsia amblyommii*, *Rickettsia bellii*, *Rickettsia monteiroi*, *Rickettsia parkeri*, *Rickettsia rhipicephali*, *Rickettsia* sp. Pampulha strain, and *Candidatus* Rickettsia andeanae) have been detected in a variety of tick species, parasitizing a range of hosts including wild birds and mammals (Labruna *et al.*, 2011; Parola *et al.*, 2013; Nieri-Bastos *et al.*, 2014).

The tick, *Amblyomma longirostre* is widely distributed throughout Central and South America (Guglielmone *et al.*, 2003; Barros-Battesti *et al.*, 2006). Regarding the life cycle, adults are reported as predominantly infesting arboreal, neotropical porcupines of the family, Erethizontidae (Silveira *et al.*, 2008; Nava *et al.*, 2010), while larval and nymphal stages have been found almost exclusively in association with passeriform birds, suggesting that it is an arboreal tick which inhabits the forest canopy (Labruna *et al.*, 2007a; Nava *et al.*, 2010; Luz and Faccini, 2013; Sanches *et al.*, 2013; Torga *et al.*, 2013).

Studies investigating rickettsial infections in ticks parasitizing wild animals in the Northeast region of Brazil are scarce and have been restricted to the description of *R. amblyommii* in immature stages of *A. longirostre* collected from birds in the state of Bahia (Ogrzewalska *et al.*, 2011a), and in immatures and adults of *Amblyomma auricularium* collected from the striped hog-nosed skunk (*Conepatus semistriatus*), and armadillos (*Euphractus sexcinctus*) in the state of Pernambuco (Saraiva *et al.*, 2013).

Send correspondence to H.R. Luz. Departamento de Parasitologia Veterinária, Universidade Federal Rural do Rio de Janeiro, Seropédica, RJ, Brazil. E-mail: hermesluz@globomail.com.

The current study reports tick infestations in two species of porcupines cohabiting remnants of Atlantic Forest in Southern Bahia, and provides details of molecular detection of rickettsial infections in adult *A. longirostre*.

Material and Methods

Ticks were collected, manually and/or using tweezers, from a total of 7 thin-spined porcupines (*Chaetomys subspinosus*) and from 6 hairy dwarf porcupines (*Coendou insidiosus*), captured in the Una Biological Reserve (Rebio-Una) and the Una Wildlife Refuge (Revis-Una) located in the municipality of Ilhéus (14°47'20" S, 39°02'56" W), Southern Bahia state, Northeast Brazil. All procedures were conducted with the legal approval and consent of the Brazilian Federal Authority (ICMBio, license numbers: 25184-1; 23468-2 and 27021-1).

Each tick was placed in an individual plastic flask containing 92% ethanol and transported within 24 hours to the laboratory in order to perform taxonomic identification procedures as proposed by Barros-Battesti *et al.* (2006). The DNA was extracted from ticks as follows: Individual ticks were washed twice with 1mL of ice-cold phosphate buffered saline (PBS; pH 7.2), then re-suspended in 250 μL of PBS in a screw capped 1.5 mL microcentrifuge tube containing 50 mg of acid-washed, 425-600 microns glass beads (Sigma-Aldrich; product # G8772). The tubes were placed on a mini-beadbeater-16 apparatus (Biospec; Bartlesville, USA), and the ticks were disrupted by a single cycle of agitation (60 seconds). Cell lysis was completed by the addition of 250 μL of cell disruption solution (20 mM Tris-HCl, 20 mM EDTA, 400 mM NaCl, 1% sodium dodecyl sulfate, 10 mM $CaCl_2$) and 20 μL of proteinase K (20 $\mu g/mL^{-1}$; Sigma-Aldrich). Lysates were incubated for 3 hours at 56 °C, and DNA was extracted by single rounds of phenol and phenol chloroform treatment, followed by precipitation with an equal volume of isopropanol for 30 min at room temperature. Precipitated (16,000x g) DNA pellets were desalted twice with 70% ethanol and re-suspended overnight at 4 °C in 50 μL of Buffer AE (10 mM Tris-HCl, 0.5 mM EDTA, pH 9.0), and stored at -20 °C.

Individual DNA samples were examined by PCR using the primers CS-239 and CS-1069, which amplify an 834-bp fragment of the *gltA* gene of all known *Rickettsia* species and the primers 17k-5 and 17k-3, which amplify a 549-bp fragment of the rickettsial *htrA* gene (Labruna *et al.*, 2007b). Samples which were positive for these two assays were further tested by additional PCR protocols in order to undertake genetic characterization of the *Rickettsia* isolates. Additional protocols employed the primers Rr190.70p and Rr190.602n, which amplify a 530-bp fragment of the rickettsial *ompA* gene (Labruna *et al.*, 2007b), and primers 120-M59 and 120-807, which amplify an 865-bp fragment of the rickettsial *ompB* gene (Roux and Raoult, 2000).

The PCR mixtures (25 μL) contained 2.5 μL of Platinum Taq DNA polymerase buffer (Life Technologies, Brasil), 2.5 mM $MgCl_2$, 200 μM dNTPs, 20 *p*moles of each primer, 0.5 units of Platinum Taq DNA polymerase, and 2 μL of DNA template. Samples were initially heated to 95 °C for 5 min to denature the template and activate the polymerase, followed by 40 repeated cycles of denaturation at 95 °C for 20 seconds, annealing at 52 °C for 20 seconds, and extension at 72 °C for 30 seconds (*htrA* and *ompA*) or for 40 seconds (*gltA* and *ompB*), followed by a final extension at 72 °C for five minutes. PCR products were analyzed by gel electrophoresis (1.5% agarose), and the amplicon sizes were determined by comparison with a DNA molecular weight marker (GeneRuler 100 bp DNA Ladder, product # SM024, Thermo Scientific).

Nucleotide sequencing of PCR products was performed as follows: 10 μL of PCR products were treated with Exo-Sap-IT (GE Healthcare), according to the manufacturer's protocol and sequenced in both directions, employing the amplification primers, using the BigDye Ready Reaction mix (ABI Corp), and reaction products were analyzed on a 3500 automated genetic analyzer (ABI Corp). Sequence alignments were performed using Sequencher (Version 5.2, Genecodes Corporation, CA). Aligned sequences were entered into the BLAST search algorithm (Altschul *et al.*, 1990) and the NCBI nucleotide database to determine gene identity.

Results

A total of 10 adult ticks (8 males, 2 females) from *C. subspinosus* and nine male ticks from *C. insidiosus* were obtained (Table 1). DNA was extracted from all the ticks and examined employing PCR assays targeting the rickettsial genes *gtlA*, encoding citrate synthase and *htrA*, encoding a 17 kDa outer membrane antigen. Six *A. longirostre* ticks produced amplicons of the expected molecular weight

Table 1 - Hosts and tick species examined for rickettsial DNA by PCR followed by nucleotide sequencing. MM (males), FF (females).

Host	Ticks (wstage)	Nº tested	No. positive for *R. bellii*	No. positive for *R. amblyommii*
C. subspinosus	*A. longirostre* (8MM/2FF)	10	0	4
C. insidiosus	*A. longirostre* (9MM)	9	1	1
Total		19	1	5

for both target sequences. Comparative sequence analysis of the *htrA* amplicon generated from one of the ticks collected from *C. insidiosus* revealed 100% nucleotide sequence similarity (484 of 484 bases sequenced) with sequences deposited as the *htrA* sequence of *R. bellii* (GenBank accession numbers, CP00849 and CP00087). In addition, the *gltA* amplicon derived from the same tick showed 99.9% nucleotide sequence similarity (790/791 nucleotides), with sequences deposited as the *gltA* gene of *R. bellii* (GenBank accession numbers, CP00849 and CP00087). As such, the tick was considered to be infected with *R. belli* and represented the first recorded detection of this species in *A. longirostre*. The novel *R. bellii* sequences were deposited in the GenBank with the accession numbers KJ534308 (*htrA*) and KJ534309 (*gltA*).

Sequencing of the *htrA*, *gltA*, *ompA,* and *ompB* amplicons obtained from the five other PCR positive ticks (four collected from *C. subspinosus* and one from *C. insidiosus*) revealed them to be identical to each other and showed similarity levels of 99.4% (477/480 for *htrA*), 100% (788/788 for *gltA*), 99.6% (485/487 for *ompA*), and 99.4% (812/817 for *ompB*) to the corresponding sequences of *R. amblyommii* strain Aranha (GenBank accession numbers, AY360215, AY360216, AY360213, and AY360214, respectively). The novel sequences were deposited in the GenBank with the following accession numbers KJ534311 (*htrA*), KJ534310 (*gltA*), KJ534312 (*ompA*), and KJ534313 (*ompB*).

Discussion

The lack of amplification of *ompB* and *ompA* from the DNA extracted from one of the ticks collected from *C. insidiosus* suggested the presence of a non-spotted fever group (SFG) rickettsial agent. Subsequent sequence analysis confirmed the suspicion and identified the bacterium as *R. bellii.* This is the first report of *R. bellii* infecting adults of *A. longirostre* and the first report of this species of *Rickettsia* in the state of Bahia. *R. bellii* represents a rickettsial agent of undetermined pathogenicity (Labruna *et al.*, 2009; Labruna *et al.*, 2011), which has been detected in a variety of tick species throughout the New World (Labruna *et al.*, 2004a; Labruna *et al.*, 2007a; Pacheco *et al.*, 2008; Barbieri *et al.*, 2012). In the specific case of Brazil, Labruna *et al.* (2004a) reported *R. bellii* as the species most frequently encountered in *Amblyomma* ticks collected from eight areas of the Amazon forest, and Pacheco *et al.* (2009) related the presence of *R. bellii* in 634 (23.8%) of 2,666 *A. dubitatum* ticks examined, in the state of São Paulo, Brazil. To date, *Ixodes loricatus, Haemaphysalis juxtakochi,* and 13 species of the genus *Amblyomma (A. ovale*, *A. oblongoguttatum*, *A. scalpturatum*, *A. humerale*, *A. rotundatum*, *A. aureolatum*, *A. dubitatum*, *A. incisum*, *A. nodosum*, *A. varium*, *A. neumanni, A. tigrinum,* and *A. sabanerae* (Barbieri *et al.*, 2012; Labruna *et al.*, 2011; Ogrzewalska *et al.*, 2012a), have been found to be infected with *R. belli* in Latin America. The elevated occurrence of infection observed in *Amblyomma* ticks with this rickettsial agent, has led some authors to suggest the existence of a symbiotic co-evolution of these species (Labruna *et al.*, 2004a).

Nucleotide sequencing of the PCR products generated from the five additional *A. longirostre* demonstrated that all of them were infected with a genetic variant of *R. amblyommii*, which showed an elevated level of nucleotide similarity (99.4% to 100%) to sequences originating from *R. amblyommii* strain Aranha. These findings extend the geographic distribution of this species of *Rickettsia* to southern Bahia, and provide additional evidence for the role of adult *A. longirostre* as a source of this bacterium. In this context, the strain Aranha was originally identified in DNA pooled from two *A. longirostre* adult male ticks recovered from the arboreal Brazilian porcupine, *C. prehensilis,* in the Amazonian state of Rondonia (Labruna *et al.*, 2004b).

The bacterium *R. amblyommii* is a SFG rickettsial agent with a broad distribution throughout the Americas (Labruna *et al.*, 2011), having been initially recorded in the lone star tick, *Amblyomma americanum* from the USA (Burgdorfer *et al.*, 1981). Recent data (Zhang *et al.*, 2012) revealed that *R. amblyommii* infection occured in 40-60% of *A. americanum* collected in the USA. In the case of Brazil, five species of the genus *Amblyomma* i.e., *A. cajennense*, *A. coelebs*, *A. longirostre*, *A. geayi* (Labruna *et al.*, 2011), and *A. auricularium* (Saraiva *et al.*, 2013), have been identified as being infected with this bacterium.

Despite being classified as a SFG agent, *R. amblyommii* is generally considered to be of undetermined pathogenicity, with some authors considering it to be non-pathogenic (Burgdorfer *et al.*, 1981; Parola *et al.*, 2013; Saraiva *et al.*, 2013). Nevertheless, it is pertinent to note that possible human infections with this agent have been reported in the USA (Apperson *et al.*, 2008). Further support for a potential role of this bacterium in human disease was provided by the findings of Jiang *et al.* (2010), which demonstrated, via quantitative real-time PCR, that *R. amblyommii* was present in 80.5% (58 of 72) of pools of lone star ticks and in 66.5% (244 of 367) of individual *A. americanum* ticks recovered from humans in the USA during the period 2002 to 2005. Data from serological surveillance of dogs in the Pantanal region of Brazil provided evidence for infection by *R. amblyommii* most likely via the tick *A. cajenennse* (Melo *et al.*, 2011). Moreover, a subsequent study of birds and other wild animals from the Atlantic Forest in the state of São Paulo, Brazil (Ogrzewalska *et al.*, 2012b) also recorded high levels of sero-conversion for *R. amblyommii* in small mammals and detected *R. amblyommii* by PCR in 41.7% of *A. longirostre* ticks col-

lected from birds. Such data clearly demonstrate that this species of *Rickettsia* is widely distributed and is circulating within wild and domestic animal populations in Brazil. Studies carried out by Saraiva *et al.* (2013) showed that *R. amblyommii* can be perpetuated transstadially and transmitted transovarially in *A. auricularium* ticks, and that such behavior is important for the maintenance of *R. amblyommii* in nature.

Studies of rickettsial infections in Brazilian ticks in forest environments have traditionally focused on terrestrial hosts, *e.g.*, capybaras, anteaters, and horses, and more recently on wild birds (Ogrzewalska *et al.*, 2012b). In contrast, arboreal mammals have received limited attention as hosts for ticks, and even less is known regarding their role as reservoirs for tick borne pathogens. This dearth of information most likely reflects the difficulties associated with the capture of these animals. The predominance of *R. amblyommii*, the species of *Rickettsia* most frequently detected in immature forms of *A. longirostre* ticks associated with Brazilian wild birds, provides evidence of a role for porcupines in the maintenance of rickettsial infections within populations of *A. longirostre*. Clearly, further studies are warranted to fully elucidate the function of these hosts in the ecology of *A. longirostre* and the rickettsia species present therein.

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