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**ORIGINAL ARTICLE**

# *Rickettsia amblyommii* Infecting *Amblyomma auricularium* Ticks in Pernambuco, Northeastern Brazil: Isolation, Transovarial Transmission, and Transstadial Perpetuation

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## Abstract

This study investigated rickettsial infection in *Amblyomma auricularium* ticks from the state of Pernambuco, northeastern Brazil. An engorged female of *A. auricularium* collected from a skunk (*Conepatus semistriatus*) was sent alive to the laboratory, where the female was found through molecular analysis to be infected by *Rickettsia amblyommii*. This engorged female oviposited, and its offspring was reared through three consecutive generations, always using tick-naïve rabbits to feed the ticks. PCR performed on five egg pools, 10 larvae, 10 nymphs, and 10 adults of each of the three generations always yielded rickettsial DNA, indicating maintenance of rickettsial infection in the ticks by transstadial and transovarial passages. DNA sequences of random PCR products from eggs, larvae, nymphs, and adults were identified as *R. amblyommii.* All infested rabbits seroconverted to *R. amblyommii* antigens at the 21$^{st}$ day after infestation, indicating that larvae, nymphs, and adults transmitted *R. amblyommii* through parasitism. However, no infested rabbit presented fever or any clinical alteration during the experimental period. Rickettsiae were successfully isolated from the two *A. auricularium* females, and the isolates were established in Vero cell culture. Molecular characterization of the isolates confirmed *R. amblyommii* by sequencing partial *gltA, ompA,* and *ompB* genes. From another sample of 15 *A. auricularium* adult ticks collected from two armadillos (*Euphractus sexcinctus*), eight (53.3%) were infected by *R. amblyommii.* This study reports *R. amblyommii* infecting the tick *A. auricularium* for the first time. This is also the first report of rickettsia infecting ticks in the northeastern region of Brazil.

**Key Words:** *Rickettsia amblyommii*—*Amblyomma auricularium*—northeastern Brazil.

## Introduction

The hard tick *Amblyomma auricularium*is widely distributed in the neotropical region, from Mexico to Argentina. Although there have been reports in southern United States (Lord and Day 2000), it is not known if *A. auricularium* is established in that country (Guglielmone et al. 2003). Within its distribution area, all parasitic stages of *A. auricularium* are found feeding mainly on armadillos (Dasypodidae), although immature stages are also found feeding on small rodents (Guglielmone et al. 2003, Horta et al. 2011). Despite its wide geographical distribution, there has been no report of rickettsial infection in *A. auricularium.*

Until 2003, *Rickettsia rickettsii* was the only rickettsia that was reported infecting ticks in South America. During the last 10 years, there has been an increasing number of tick-associated rickettsiae in this continent, with reports of *R. parkeri, R. rhipicephali, R. monteiroi, R. massiliae, R. bellii,* and *R. amblyommii* infecting South American ticks for the first time (for review, see Labruna et al. 2011a).

Herein, we investigated rickettsial infection in *A. auricularium* ticks from two localities in the northeastern region of Brazil, a geopolitical region that comprises an area of 1,548,803 km$^2$, 18% of the Brazilian land, where no *Rickettsia* species has ever been reported infecting ticks.

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## Materials and Methods

In June, 2010, an engorged female tick was collected from a striped hog-nosed skunk (*Conepatus semistriatus*) in Ibimirim (08°32′S, 37°41′W), Pernambuco State, northeastern Brazil. This female was identified as *A. auricularium* according to Onofrio et al. (2006) and sent alive to the laboratory, where it was held in an incubator set at 25°C and 85% relative humidity for oviposition and larval eclosion. Molecular analyses (techniques described below) showed that both this female (at the end of oviposition) and a sample of its egg mass contained *Rickettsia* DNA. Therefore, this female offspring was reared in the laboratory through three consecutive generations. For this purpose, each feeding stage of each tick generation was allowed to feed on a tick-naïve white New Zealand rabbit (*Oryctolagus cuniculus*). Infestations consisted of ~2000 larvae, ~500 nymphs, or 20 adult couples per rabbit. Larvae and nymphs were allowed to feed inside cotton sleeves glued to rabbit ears, whereas adult ticks fed inside cotton sleeves glued to the shaved skin of the rabbit's dorsum, as previously described (Pinter et al. 2002). Off-host developmental stages (molting, oviposition, egg incubation) occurred inside the same incubator cited above.

From each of the three tick generations, five egg pools (each containing ~20 eggs from one engorged female), 10 unfed larvae, 10 unfed nymphs, and 10 unfed adult ticks (five males, five females) were randomly selected to be processed by molecular analysis for rickettsial infection. For this purpose, each egg pool or an individual tick was submitted to DNA extraction by the guanidine isothiocyanate-phenol technique, as previously described (Sangioni et al. 2005), and subsequently tested by PCR targeting a 617-bp fragment of the *ompA* gene of spotted fever group *Rickettsia,* using primers Rr190.70F and Rr190.701R, as previously described (Roux et al. 1996). In both DNA extraction and PCR assays, "blank" tubes containing no tick sample or DNA template were used as negative controls. At the same time, *R. parkeri* DNA was used as positive control in all PCR assays. PCR products from five larvae, five nymphs, and five adults from the first tick generation underwent DNA sequencing, and the resultant sequences were compared with GenBank data by BLAST analysis (Altschul et al. 1990).

Each infested rabbit had its rectal temperature measured daily from the infestation day (day 0) to the 21$^{st}$ day after infestation. Rabbits were considered febrile when rectal temperature was >40.0°C (Monteiro 1933). These animals were also observed for possible clinical alterations, such as scrotal or skin lesions. Blood was collected through the ear vein from each infested rabbit at days 0 and 21 after tick infestation, and the blood serum was tested by the immunofluorescence assay (IFA) following previously described protocols (Labruna et al. 2007a) using Vero cells infected with *R. amblyommii* strain Ac37 as crude antigen. Titers ≥64 were considered positive. Positive sera were titrated to the end point titers by dilution in two-fold increments.

With the purpose of isolateing rickettsiae from *A. auricularium* ticks, two unfed adult female ticks from the first generation were individually inoculated into shell vials containing a monolayer of confluent Vero cells, as previously described (Labruna et al. 2004). Rickettsial isolation was checked by Gimenez staining of scrapped cells (Gimenez 1964). If *Rickettsia*-like organisms were observed, cells were harvested and inoculated into a 25-cm$^2$ flask containing a monolayer of confluent uninfected Vero cells. After the establishment of each isolate in the laboratory (*i.e.*, at least three cell passages, with the prevalence of Vero-cell infection exceeding 90% in each), DNA was extracted from the infected cells and tested in a battery of different PCR protocols, with primers Rr190.70F and Rr190.701R targeting a portion of the rickettsial *ompA* gene, primers 120.M59 and 120-807 targeting a portion of the rickettsial *ompB* gene (Roux and Raoult 2000), and two primer pairs (CS-78 and CS-323; CS-239 and CS-1069) targeting two overlapping fragments of the rickettsial *gltA* gene (Labruna et al. 2004). All PCR products were sequenced, and the resultant sequences were compared with GenBank data by BLAST analysis.

In November, 2011, a total of 15 *A. auricularium* adult ticks (12 males, three females) were collected from two yellow armadillos (*Euphractus sexcinctus*) in Petrolina (09°23′S, 40°30′W), Pernambuco State, preserved in 70% ethanol, and sent to the laboratory. Each tick was submitted to DNA extraction and PCR amplification of a portion of the rickettsial *ompA* gene, as described above. PCR products were sequenced, and the resultant sequences were compared with GenBank data by BLAST analysis.

To confirm the taxonomic identification of the tick colony evaluated in the present study, DNA from an adult female of the third laboratory generation of *A. auricularium* from Ibimirim was submitted to PCR targeting a fragment of the tick mitochondrial 16S rRNA gene (Mangold et al. 1998). The PCR product was sequenced, and the resultant consensus sequence was compared with GenBank data by BLAST analysis.

## Results

The *A. auricularium* colony was successfully maintained in the laboratory for three consecutive generations, with 50–90% recovery rates, molting, and oviposition success of engorged ticks. PCR performed on the original engorged female collected in the field, its egg sample, and on five egg pools, 10 larvae, 10 nymphs, and 10 adults of each of the three generations always yielded a single amplicom of the expected size, indicating that the colony, originated from an infected female, remained 100% infected by rickettsia for three consecutive generations. In all reactions, PCR product was never obtained from the negative control tubes. DNA sequences of the PCR products obtained from the original engorged female at the end of oviposition, its egg sample, and from five larvae, five nymphs, and five adults from the first tick generation were 99.7% (585/587 bp) similar to *R. amblyommii* strain TX051 from the United States (EF689731).

A total of nine tick-naïve rabbits were infested by ticks, one rabbit for each tick feeding stage of each generation. All rabbits were seronegative at day 0, and seroconverted to *R. amblyommii* antigens at the 21$^{st}$ day after infestation, with end point titers of 64 or 256 for larval and nymphal infestations, and of 128 or 256 for adult infestations. No infested rabbit presented fever or skin lesion during the 21-day period of clinical examinations.

Rickettsiae were successfully isolated from the two *A. auricularium* females, and the isolates were established in Vero cell culture. DNA extracted from infected cells yielded PCR products of the expected size for the three *Rickettsia* genes tested. Partial *gltA* sequence showed 99.9% (1066/1067 bp)

similarity to *R. amblyommii* strain AaR/SC from United States (JN378401), and 99.8% (1065/1067 bp) similarity to *R. amblyommii* strain AcaIII from northern Brazil (AY375163). Partial *ompA* sequence showed 99.7% (585/587 bp) similarity to *R. amblyommii* strain TX051 from the United States (EF689731), and 99.6% (547/549 bp) similarity to *R. amblyommii* from Panama (HM582436). Partial *ompB* sequence showed 99.9% (756/757 bp) similarity to *R. amblyommii* strain AcaIII from northern Brazil (AY375164), and 99.7% (755/757 bp) similarity to *R. amblyommii* strain AaR/SC from United States (JN378402). Partial sequences (*gltA, ompA, ompB)* from *R. amblyommii,* here designated as strain AaPE, generated in this study were deposited into GenBank and assigned nucleotide accession nos. JX867425–JX867427, respectively.

From the 15 *A. auricularium* adult ticks collected from two yellow armadillos in Petrolina, eight (53.3%) yielded PCR products that generated identical DNA sequences that were 100% similar to the *ompA* sequence obtained for strain AaPE.

A partial sequence of the 16S rRNA gene generated from an *A. auricularium* female of the tick colony of the present study was shown by BLAST analysis to be 99.3% identical (398/401 bp) to the corresponding sequence of *A. auricularium* from central-western Brazil (FJ627952).

## Discussion

This study reports *R. amblyommii* infecting the tick *A. auricularium* for the first time. Rickettsial infection was confirmed by isolating the organism in cell culture. This study adds another *Amblyomma* species to a growing list of *Amblyomma* species that have been found infected by *R. amblyommii* in nature. Since its first reports infecting *Amblyomma americanum* ticks in the United States (Burgdorfer et al. 1981, Goddard and Norment 1986), *R. amblyommii* has been reported infecting *Amblyomma cajennense* in Brazil, Costa Rica, and Panama (Labruna et al. 2004, Bermudez et al. 2011, Hun et al. 2011), *Amblyomma coelebs* in Brazil and Fench Guyana (Labruna et al. 2004, Parola et al. 2007), and *Amblyomma neumanni* in Argentina (Labruna et al. 2007b). In addition, *R. amblyommii*–like strains have been reported infecting the ticks *Amblyomma longirostre* and *Amblyomma geayi* in Brazil (Ogrzewalska et al. 2008, 2010). Therefore, it seems that *R. amblyommii* is widespread among *Amblyomma* ticks in the New World. Our findings are the first report of a *Rickettsia* species infecting ticks in the Northeastern region of Brazil.

Because all tested ticks (eggs, larvae, nymphs, and adults) were PCR positive for three consecutive generations, our results indicate that *R. amblyommii* was efficiently maintained by 100% transovarial transmission and transstadial perpetuation in *A. auricularium* ticks. Because 100% of the individual larvae from the three generations were PCR positive, this result also indicates that transovarial transmission was characterized by 100% filial infection rates. In a previous study, Burgdorfer et al. (1981) showed that *R. amblyommii* (strain WB-8-2) infected all tissues of the tick *A. americanum,* with the ovary more heavily infected; these authors reported transstadial and transovarial passage of strain WB-8-2 in *A. americanum,* with filial infection rates between 30% and 100%. Together, these results indicate that *R. amblyommii* is primarily maintained in nature by transstadial and transovarial passage in ticks. However, because infection rates under natural conditions are highly variable, many times below 50%, further studies are needed to test if the *R. amblyommii* infection has any adverse on ticks. In this regard, Labruna et al. (2011b) reported 100% transovarial and transstadial passage, and 100% filial infection rates of *Rickettsia rickettsii* in *Amblyomma aureolatum* ticks. However, the *R. rickettsii* infection was deleterious to part of the engorged females, a fact that explained the low infection rate (around 1%) usually found for *A. aureolatum* ticks under natural conditions (Labruna et al. 2011b).

In the present study, all infested rabbits seroconverted to *R. amblyommii,* indicating that *R. amblyommii* was efficiently transmitted to rabbits through tick parasitism. However, infested rabbits presented no clinical alteration, indicating that *R. amblyommii* is not pathogenic to laboratory rabbits. This result corroborates Burgdorfer et al. (1981), who showed that infection by *R. amblyommii* (strain WB-8-2) was not pathogenic for meadow voles (*Microtus pennsylvanicus*) and guinea pigs (*Cavia porcellus*) under laboratory conditions. The pathogenicity of *R. amblyommii* to humans is controversial. While Burgdorfer et al. (1981) presented epidemiological evidence that it is not pathogenic, Apperson et al. (2008) provided serological evidence that *R. amblyommii* was associated with spotted fever cases in the United States. Regardless of that, the present study confirms that *R. amblyommii* is transmitted by ticks and infects laboratory rabbits, corroborating previous studies that provided serological evidence that *R. amblyommii*–infected domestic animals such as horses and dogs (Labruna et al. 2007a, Bermudez et al, 2011, Melo et al. 2011). These findings indicate that *R. amblyommii* could at least interfere in serological diagnosis of spotted fever, because it is known that *R. amblyommii* antigens cross react with antigens of pathogenic agents such as *R. rickettsii* and *R. parkeri* (Piranda et al. 2008, Horta et al. 2010). Finally, because *A. auricularium* has never been reported biting humans, its role in transmitting *R. amblyommii* to humans should be irrelevant.

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## Author Disclosure Statement

Authors declare no conflict of interest.

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