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ISOLATION OF *Rickettsia bellii* FROM *Amblyomma ovale* AND *Amblyomma incisum* TICKS FROM SOUTHERN BRAZIL

AISLAMIENTO DE *Rickettsia bellii* A PARTIR DE GARRAPATAS *Amblyomma ovale* Y *Amblyomma incisum* PROCEDENTES DEL SUR DE BRASIL

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ABSTRACT

Objective. To isolate and characterize rickettsiae from the ticks *Amblyomma ovale* and *Amblyomma incisum* collected in the state of São Paulo. **Materials and methods.** Adult, free-living *A. ovale* and *A. incisum* were collected in an Atlantic rainforest area in the state of São Paulo, Brazil. Each tick was tested using the hemolymph assay; samples from positive ticks were placed in shell vials in order to isolate rickettsiae and subsequently grown in Vero cells. Amplification of three rickettsial genes (*gltA*, *htrA* and *ompA*) was attempted using polymerase chain reaction (PCR) for each isolate obtained. Amplicons were subsequently sequenced. **Results.** A total of 388 *A. incisum* and 50 *A. ovale* were collected. Only one *A. incisum* and one *A. ovale* were hemolymph-test positive. *Rickettsiae* were successfully isolated from these ticks; however establishment in Vero cell culture was successful only for the isolate from *A. ovale*. Bacterial contamination in the first cell passage of the *A. incisum* isolate precluded successful isolation of the organism. PCR products were obtained with the *gltA* and *htrA* primers for the two isolates, but no product was obtained with the *ompA* primers. By BLAST analysis, partial *gltA* and *htrA* sequences of isolates from *A. ovale* and *A. incisum* were similar to the corresponding sequences of *R. bellii*. **Conclusions.** This is the first report of *R. bellii* infecting *A. incisum* and the first successful isolation from *A. ovale*.

Key words: *Rickettsia bellii*, *Amblyomma ovale*, *Amblyomma incisum*, Brasil.

RESUMEN

Objetivo. Aislar *Rickettsias* mediante cultivo celular a partir de muestras de garrapatas *Amblyomma ovale* y *Amblyomma incisum* del estado de São Paulo **Materiales y métodos.** *A. ovale* y *A. incisum* adultas de vida libre fueron colectadas en una área de selva tropical Atlántica en el estado de São Paulo, Brazil. Cada garrapata fue sometida a la prueba de hemolinfa, las garrapatas positivas en esta prueba fueron evaluadas con la técnica de *shell vial* con el propósito de aislar rickettsias en cultivo de células Vero. Pasajes celulares de los aislados fueron identificados genotípicamente por la reacción en cadena por la polimerasa (PCR) dirigidos a fragmentos de tres genes de rickettsias (*gltA*, *htrA* y *ompA*), seguido por secuenciación de ADN. **Resultados.** Un total de 388 *A. incisum* y 50 *A. ovale* fueron colectadas. Por la prueba de hemolinfa, únicamente una *A. incisum* y una *A. ovale* fueron positivas. Las *Rickettsias* fueron exitosamente aisladas de estas garrapatas. Sin embargo, el cultivo continuo en células Vero fue posible sólo para la garrapata *A. ovale*, debido a contaminación bacteriana en el primer pasaje celular de la muestra de *A. incisum*. Los productos de PCR fueron obtenidos con los *primers* *gltA* y *htrA* para los dos aislados, no obstante, ningún producto fue obtenido con los *primers* *ompA*. Por análisis BLAST, secuencias parciales de *gltA* y *htrA* procedentes de los aislados de *A. ovale* y *A. incisum* fueron similares a las secuencias correspondientes a *R. bellii*. **Conclusiones.** Este es el primer reporte de *R. bellii* infectando *A. incisum* y el primer establecimiento exitoso de un aislado de *A. ovale*.

Palabras clave: *Rickettsia bellii*, *Amblyomma ovale*, *Amblyomma incisum*, Brasil.

INTRODUCTION

Rickettsiae are pathogenic bacteria that require eukaryotic cells in order to grow. They are gram-negative, small, rod-shaped, α -proteobacteria causing mild to severe diseases in humans and animals. The genus subdivides into the conventionally well defined typhus group (TG) and spotted fever group (SFG) based on phylogenetic analyses. The TG includes the louse-borne *Rickettsia prowazekii* (the agent of epidemic typhus) and the flea-borne *Rickettsia typhi* (the agent of endemic typhus). The SFG includes tick-borne *Rickettsia rickettsii* (the agent of Rocky Mountain spotted fever), *Rickettsia conorii* (the agent of Mediterranean spotted fever), *Rickettsia africae* (the agent of African tick bite fever), *Rickettsia sibirica* (the agent of North Asian tick-borne fever), mite-borne *Rickettsia akari* (the agent of rickettsialpox), and flea-borne *Rickettsia felis* (the agent of flea-borne spotted fever) (1,2). *Rickettsia bellii* is the most divergent species, being representative of a distinct group within the genus *Rickettsia*, together

with *Rickettsia canadensis* (3). *R. bellii*, the most common rickettsia found in ticks in America, was isolated for the first time in embryonated chicken eggs from a triturated pool of unfed adult *Dermacentor variabilis* in the United State, one of the most important vectors of *R. rickettsii* in that country, but there is no evidence that *R. bellii* can infect humans (4). It has been found in various other hard ticks, including species of *Amblyomma*, *Ixodes*, *Haemaphysalis*, and in soft ticks, including species of *Argas* and *Ornithodoros* (4-12).

In Brazil, despite *R. bellii* was also the most common rickettsial species identified in ticks from the state of Rondônia, Western Amazon, it was only successfully isolated and established in continuous cell passages from *Amblyomma scalpturatum* (7) and in *Amblyomma aureolatum*, *Amblyomma dubitatum*, and *Haemaphysalis juxtakochi* in the state of São Paulo (10-12). In the present study, we tested samples of

Amblyomma ovale and *Amblyomma incisum* ticks collected in the state of São Paulo (southeastern Brazil), attempting to isolate rickettsiae in cell culture and establishment in continuous cell passages.

MATERIALS AND METHODS

During 2004-2006, adult free-living *A. ovale* and *A. incisum* ticks were collected on the vegetation in an Atlantic rainforest area in the Intervales State Park, Ribeirão Grande Municipality, state of São Paulo (24°18'S, 48°24'W) by visual inspection of vegetation during another study on ecology of free-living ticks in the Intervales State Park (13). Ticks were brought alive to the laboratory and before testing live ticks by the hemolymph test, ticks were held in an incubator at 32°C and 95% RH for 3 to 5 days, in order to stimulate rickettsial growth (14).

Thereafter, each tick was subjected to the hemolymph test for detection of *Rickettsia*-like structures, as previously described (14). Briefly, the distal portion of one leg of each tick was cut with scissors and a drop of hemolymph was fixed by air in a glass slide and stained by Gimenez staining (15). Right after the collection of hemolymph, ticks were frozen in a -80°C freezer.

Attempts to isolate *Rickettsia* in Vero cell culture were performed with ticks shown to contain *Rickettsia*-like structures by the hemolymph test. For this purpose, frozen ticks were thawed and subjected to the shell vial technique as previously described (16), with some modifications (8). Briefly, individual ticks were thawed in water bath at 37°C and disinfected for 10 minutes in iodine alcohol followed by several washes in sterile water. Then each tick was triturated in 500 µl of brain heart infusion broth (BHI) and the resultant tick homogenate inoculated into shell vials containing a monolayer of confluent Vero cells. After inoculation, the shell vials were centrifuged for one hour at 700 g at 22°C. Thereafter, the monolayer was washed once with minimal essential medium containing 5% bovine calf

serum, and then incubated at 28°C with medium containing antibiotics (1% penicillin, streptomycin). After three days, the medium was switched to antibiotic-free medium, and the aspirated medium was checked by Gimenez staining for the presence of *Rickettsia* like-organisms. If the result was positive, the monolayer of the shell vial was harvested and inoculated into a 25 cm² flask containing a monolayer of confluent uninfected Vero cells. Cells of the 25 cm² flask were checked by Gimenez staining until more than 90% of the cells were infected, when they were harvested and inoculated into 150 cm² flasks of Vero cells. At all instances, inoculated Vero cells were incubated at 28°C. The level of infection of cells was monitored by Gimenez staining (15), of scrapped cells from inoculated monolayer. A rickettsia isolate was considered established in the laboratory after at least three passages through 150 cm² Vero cell flasks, each reaching more than 90% of infected cells (8).

Cell passages of isolates were genotypically identified by polymerase chain reaction (PCR) amplification and sequencing the product of the resultant infected cells. For this purpose, DNA was extracted using the Dneasy Tissue Kit (Qiagen®), following manufacturer's protocol for isolation of DNA from culture cells samples and DNA from infected cell passages were tested by a battery of PCR using all primer pairs listed in table 1, targeting fragments of three rickettsial genes: citrate synthase (*gltA*), 17-kDa outer membrane protein (*htrA*), and 190-kDa outer membrane protein (*ompA*). For each set of reactions, a negative control (5 µl of water) and positive control (5 µl of DNA extracted from *A. cajennense* ticks experimentally infected with *R. parkeri*) was included (17).

All PCR products of the expected size obtained in the present study were purified using ExoSap (USB) and sequenced in an automatic sequencer (Applied Biosystems/Perkin Elmer, model ABI Prism 310 Genetic, California, USA) according to the manufacturer's protocol. Partial sequences obtained were submitted to BLAST analysis, (18), to determine similarities to other *Rickettsia* species.

Table 1. Primer pairs used for amplification of rickettsial genes.

Primer pairs	Target genes	Nucleotide sequences (5' – 3')	Product size	Reference
<i>gltA</i>				
1	CS-78	GCAAGTATCGGTGAGGATGTAAT	401	-8
	CS-323	GCTTCCTTAAAATTCAATAAATCAGGAT		-8
2	CS-239	GCTCTTCTCATCCTATGGCTATTAT	834	-8
	CS-1069	CAGGGTCTTCGTGCATTCTT		-8
<i>htrA</i>				
3	17k-5	GCTTTACAAAATTCTAAAAACCATATA	549	-8
	17k-3	TGTCTATCAATTCACAACCTGCC		-8
<i>ompA</i>				
4	Rr190.70p	ATGGCGAATATTTCTCCAAAA	530	-19
	Rr190.602n	AGTGCAGCATTCGCTCCCCCT		-19

RESULTS

A total of 388 ticks *A. incisum* were collected (180 males, 208 females) and 50 ticks *A. ovale* (17 males, 33 females). By the hemolymph test, one *A. incisum* male and one *A. ovale* female that showed to contain rickettsia-like organisms by the hemolymph test were processed by the shell vial technique (8,16). *Rickettsia* was successfully isolated and established in Vero cell culture from tick *A. ovale*. Although the establishment of *Rickettsia* in Vero cell culture from the *A. incisum* tick was unsuccessful, the initial cultivation of rickettsia was possible due to the presence of numerous Giménez-stained intracellular rickettsia-like organisms from the inoculated shell vials, which could not be cultivated further than the first passage owing to contamination of the cell cultured by extracellular bacteria.

Even though, DNA of contaminated cell culture at first passage was tested by PCR, as described below. The isolate from *A. ovale* generated in the present study has been deposited in the Rickettsial Collection of the Laboratory of Parasitic Diseases of the Faculty of Veterinary Medicine, in the University of São Paulo, where it is available upon request.

DNA of infected cells at fourth passage of *A. ovale* and first passage of *A. incisum* were subjected to PCR targeting the rickettsial genes *gltA*, *ompA* and *htrA*. PCR products of the expected size were obtained with the *gltA* and *htrA* primers for two isolates, but no product was obtained with the *ompA* primers (these primers have been specific for SFG rickettsiae). We sequenced 1060 and 500 nucleotides of the *gltA* and *htrA* genes, respectively, from the *A. ovale* isolate, and 1096 and 499 nucleotides of the *gltA* and *htrA* genes, respectively, from the *A. incisum* isolate. By BLAST analysis, partial *gltA* sequence of the *A. ovale* rickettsia in the present study was 100% (1060/1060) similar to the corresponding sequence of *R. bellii* strain HJ7 (DQ865204), and partial *gltA* sequence of the *A. incisum* rickettsia was 99.9% (1095/1096) similar to the corresponding sequence of *Rickettsia bellii* RML369-C (CP000087). Partial *htrA* sequence of the *A. ovale* rickettsia was 99.6% (497/499) similar to the corresponding sequence of *R. bellii* (AY362702), whereas partial *htrA* sequence of the *A. incisum* rickettsia was 100% (499/499) similar to *R. bellii* (AY362702). Thus, both rickettsial agents from *A. ovale* and *A. incisum* were genotypically identified as *R. bellii*.

DISCUSSION

R. bellii has been reported in 18 tick species belonging to the genera *Amblyomma* (8 species), *Dermacentor* (5 species), *Haemaphysalis* (2 species), *Ixodes* (1 species), *Argas* (1 species), and *Ornithodoros* (1 species) from different parts of Brazil, Argentina and United States, (4-12). Previously in Brazil, this bacterium was reported infecting *A. ovale* from Rondônia (northern Brazil), besides the ticks *A. dubitatum*, *A. aureolatum*, *Amblyomma oblongoguttatum*, *A. scalpturatum*, *Amblyomma humerale*, *Amblyomma rotundatum*, *Amblyomma nodosum*, *Ixodes loricatus*, and *H. juxtakochi*, (6-12). Among the genus *Rickettsia*, *R. bellii* is indeed the species with the greatest number of tick records. The present study reports for the first time *R. bellii* infecting the tick *A. incisum*, adding one more species for the list of ticks that are naturally found infected with this rickettsia in the New World. We also provide the first successful *in vitro* culture of *R. bellii* from *A. ovale*.

Although infection rates by *R. bellii* in tick populations are high variable (3.6 to 100%), but usually high (5,7,8), there has been no direct or indirect evidence of human infection by *R. bellii*. Among animals, there is only one study showing serological evidence of *R. bellii* infection in capybaras from several parts of the state of São Paulo, possibly transmitted by *A. dubitatum* ticks, (20).

A. incisum and *A. ovale* are ticks of Neotropical origin, (21) and while conducting projects on ticks from deer and on tick ecology in animal trails in this same Atlantic rainforest reserve of the present study, researchers were bitten by ticks numerous times, with most of the reports caused by *A. incisum*, (22), which was the most abundant tick species along the trails (23). The occurrence of human infestation by *A. ovale* was also reported in the study area, (22), beyond being the most frequent human-biting tick in the state of Rondônia, western Brazilian Amazon, (24). Thus, it is very important to know the *Rickettsia* species that are infecting these important human-biting ticks in South America. In addition, less pathogenic rickettsiae could have important ecological implications if one considers that the presence of less pathogenic rickettsiae within a tick population can minimize the transmission of a greater pathogenic rickettsia. (25)

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