Experimental infection of the opossum Didelphis aurita by Rickettsia felis, Rickettsia bellii, and Rickettsia parkeri and evaluation of the transmission of the infection to ticks Amblyomma cajennense and Amblyomma dubitatum
Experimental Infection of the Opossum *Didelphis aurita* by *Rickettsia felis*, *Rickettsia bellii*, and *Rickettsia parkeri* and Evaluation of the Transmission of the Infection to Ticks *Amblyomma cajennense* and *Amblyomma dubitatum*

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Abstract

This work evaluated the infection of opossums (*Didelphis aurita*) by *Rickettsia felis*, *Rickettsia bellii*, and *Rickettsia parkeri* and their role as amplifier hosts for horizontal transmission to *Amblyomma cajennense* and/or *Amblyomma dubitatum* ticks. Infection in *D. aurita* was induced by intraperitoneal inoculation with *R. felis* (*n* = 4 opossums), *R. bellii* (*n* = 4), and *R. parkeri* (*n* = 2). Another group of six opossums were inoculated intraperitoneally with Leibovitz-15 sterile culture medium, representing the uninfected groups (*n* = 2 opossums simultaneously to each infected group). Opossum blood samples collected during the study were used for DNA extraction, followed by real-time polymerase chain reaction targeting the rickettsial gene *gltA*, hematology, and detection of *Rickettsia* spp.-reactive antibodies by indirect immunofluorescence assay. Opossums were infected with uninfected *A. cajennense* and/or *A. dubitatum* for 30 days postinoculation (DPI). Flat ticks molted from ticks fed on opossums were allowed to feed on uninfected rabbits, which were tested for seroconversion by immunofluorescence assay. Samples of flat ticks were also tested by real-time polymerase chain reaction. Inoculated opossums showed no clinical abnormalities. Antibodies to *Rickettsia* spp. were first detected at the second to fourth DPI, with detectable titers until the 150th DPI. Rickettsemia was detected only in one opossum inoculated with *R. parkeri*, at the eighth DPI. Only one *A. cajennense* tick (2.0%) previously fed on a *R. parkeri*-inoculated opossum became infected. None of the rabbits infested with opossum-derived ticks seroconverted. The study demonstrated that *R. felis*, *R. bellii*, and *R. parkeri* were capable to produce antibody response in opossums, however, with undetectable rickettsemia for *R. felis* and *R. bellii*, and very low rickettsemia for *R. parkeri*. Further studies must be done with different strains of these rickettsiae, most importantly the strains that have never gone through *in vitro* passages.

Key Words: *Rickettsia parkeri*—*Rickettsia Felis*—*Rickettsia bellii*—Opposum—*Amblyomma*.

Introduction

The genus *Rickettsia* comprises obligate intracellular bacteria of the order Rickettsiales. A large number of *Rickettsia* species are agents to human diseases in different parts of the world, most of them vectored by ticks (Raoult and Roux 1997, Walker 2007). In Brazil, *Rickettsia rickettsii* is the etiological agent of Brazilian spotted fever (BSF), an acute febrile disease that has been reported in the northeastern region of the country (Silva and Galvão 2004). Other two pathogenic species were reported in Brazil: *Rickettsia felis* (Raoult et al. 2001, Horta et al. 2006a) and *Rickettsia parkeri* (Labruna et al. 2004a, Silveira et al. 2007).

The vectors implicated in the transmission of BSF belong to the genus *Amblyomma*, namely *Amblyomma cajennense* and *Amblyomma aureolatum* ticks, which bite humans and have been reported to be infected by *R. rickettsii* (Guedes et al. 2005, Pinter and Labruna 2006). *Amblyomma dubitatum*, a tick that infests chiefly capybaras (*Hydrochaeris hydrochaeris*), also infests opossums (*Didelphis spp.*) and humans and has been...
reported to be infected by \textit{R. parkeri} and \textit{Rickettsia bellii} (Labruna et al. 2004a, 2007a, Horta et al. 2007). \textit{R. felis} was first identified as a human pathogen in 1994 (Schriever et al. 1994). The organism has been detected infecting fleas in various countries among all continents of the world, except Antarctica (Pérez-Osorio et al. 2008). Although \textit{R. felis} is highly prevalent among fleas, the presence of the bacteria in ticks has also been reported (Cardosa et al. 2003, Ishikura et al. 2003, Oliveira et al. 2008).

\textit{R. parkeri} was considered nonpathogenic for humans until the past 60 years, since its first isolation from \textit{Amblyomma maculatum} ticks in the United States (Parker 1939). During the last few years, human cases of infection by \textit{R. parkeri} were reported in the United States, with patients presenting mild febrile illness and multiple eschars (Paddock et al. 2004, Whitman et al. 2007). Labruna et al. (2004a) isolated a \textit{R. parkeri}-like strain from \textit{A. duibatum} (= \textit{Amblyomma cooperi}) in a BSF endemic area in São Paulo state. Horta et al. (2004, 2007) and Pacheco et al. (2007) reported serological evidence of canine, equine, capybara, and opossum infections by \textit{R. parkeri} in areas where these animals were exposed to \textit{A. duibatum} ticks.

\textit{R. bellii} has been reported in various tick species, including \textit{A. duibatum}, representing the greatest number of tick records among the genus \textit{Rickettsia} (Philip et al. 1983, Horta et al. 2006b, Labruna 2009). Serological evidence for \textit{R. bellii} infection was recently reported in capybaras in Southeastern Brazil (Pacheco et al. 2007). No evidence for human infection by \textit{R. bellii} has been recorded.

In Brazil, only opossums and capybaras have been reported to act as efficient infection source of \textit{R. rickettsii} to ticks, that is, these vertebrates act as amplifier hosts (Horta et al. 2009, Souza et al. 2009). Moreover, opossums have been the only wild animal in Brazil, from which \textit{R. rickettsii} was isolated under natural conditions (Moreira and Magalhães 1935, Travassos 1937). Opossums are abundant in all BSF endemic areas, where they are usually infested by larvae and nymphs of \textit{A. cajennense}, and they also present high antibody titers to \textit{R. rickettsii} (Horta et al. 2007). In a recent study, opossums \textit{Didelphis aurita} were susceptible to experimental infection by \textit{R. rickettsii}, developing long rickettsemia periods (\textless 4 weeks), when they were infested with 5–20% of \textit{A. cajennense} ticks to feed on them, indicating that opossums can act as amplifier hosts for \textit{A. cajennense} larvae and nymphs (Horta et al. 2009).

Based on these statements, our study performed experimental infection of opossums by \textit{R. felis}, \textit{R. bellii}, and \textit{R. parkeri} and evaluated the role of these animals as amplifier hosts for \textit{A. cajennense} and/or \textit{A. duibatum} ticks, under laboratory conditions.

Materials and Methods

Opossums

Capture of free-living adult opossums was performed within the São Paulo metropolitan area, from October 2007 to February 2008. Opossums were identified as \textit{D. aurita} according to Nowaki (1991) and were brought to the laboratory where they were maintained in individual cages (50×50×50 cm) and fed with canine commercial pellets, fruits, vegetables, and water \textit{ad libitum}. A total of 16 adult opossums, either males or females, weighing 0.95–2.88 kg (mean: 1.7 kg) were used in our study. Before starting the experiments, opossums were clinically healthy, and their paired serum samples (14-day interval) were shown to contain no reactive antibodies (serum dilution: 1/32) to \textit{R. rickettsii}, \textit{R. parkeri}, \textit{R. felis}, and \textit{R. bellii} (serologic reaction performed as described below). Our study was approved by the Ethical Committee of the Faculty of Veterinary Medicine of the University of São Paulo (protocol no. 12.285-1).

\textit{R. felis}, \textit{R. bellii}, and \textit{R. parkeri inocula}

The following strains of rickettsiae were used to inoculate opossums: \textit{R. felis} strain Pedreira, originally isolated from a \textit{Ctenocephalides felis felis} flea from Pedreira Municipality, São Paulo (Horta et al. 2006a); \textit{R. bellii} strain Mogi, originally isolated from an \textit{A. aureolatum} tick from the district of Taiaçupeba, Mogi das Cruzes Municipality, São Paulo (Pinter and Labruna 2006); \textit{R. parkeri} strain At24, originally isolated from an \textit{Amblyomma triste} tick from Paulicéia Municipality, São Paulo (Silveira et al. 2007). These rickettsial strains have been maintained in our laboratory through \textit{in vitro} passages in \textit{C6/36} cells (for \textit{R. felis}) and Vero cells (for \textit{R. bellii} and \textit{R. parkeri}), always at 28 \textdegree C. For our study, frozen stocks of each rickettsial strain were thawed and inoculated in a monolayer of \textit{C6/36} or Vero cells. When the monolayer was close to 100\% infected, it was harvested and used to prepare a fresh homogenate in Leibovitz-15 (L-15) sterile culture medium.

Ticks

The \textit{A. cajennense} ticks used in our study were derived from uninfected engorged females collected from naturally infested horses in Pirassununga Municipality, São Paulo. The \textit{A. duibatum} ticks were derived from uninfected engorged females collected from naturally infested capybaras in Itatiba Municipality, São Paulo. We certify that both \textit{A. cajennense} and \textit{A. duibatum} larvae were not infected by any \textit{Rickettsia} species by testing the engorged females at the end of oviposition by polymerase chain reaction (PCR) targeting the rickettsial gene \textit{gltA}, which resulted in no amplified product. In addition, laboratory rabbits infested with these larvae remained seronegative for \textit{Rickettsia} spp. (techniques described below).

Experimental infection of opossums with \textit{Rickettsia} spp.

The 16 opossums were randomly assigned into six experimental groups for three independent experiments (two groups per experiment). Experiment 1—\textit{R. felis} infection: group 1 (G1) opossums (\textit{n} = 4 animals) were each inoculated intraperitoneally with 2 mL of \textit{R. felis}-infected \textit{C6/36} cells, prepared in \textit{L-15} sterile culture medium; group 2 (G2) opossums (\textit{n} = 2) were the uninfected control group, in which each opossum was inoculated intraperitoneally with only 2 mL of \textit{L-15} sterile culture medium. Experiment 2—\textit{R. bellii} infection: group 3 (G3) opossums (\textit{n} = 4 animals) were each inoculated intraperitoneally with 2 mL of a \textit{R. bellii}-infected Vero cells, prepared in \textit{L-15} sterile culture medium; group 4 (G4) opossums (\textit{n} = 2) were the uninfected control group, in which each opossum was inoculated intraperitoneally with only 2 mL of
L-15 sterile culture medium. Experiment 3—R. parkeri infection: group 5 (G2) opossums (n = 2 animals) were each inoculated intraperitoneally with 2 mL of a R. parkeri-infected Vero cells, prepared in L-15 sterile culture medium; group 6 (G6) opossums (n = 2) were the uninfected control group, in which each opossum was inoculated intraperitoneally with only 2 mL of L-15 sterile culture medium.

In each experiment, aliquots of the same inoculum utilized to infect opossums were inoculated intraperitoneally into two guinea pigs. Rectal temperature was examined daily for 20 days. At the 21st days postinoculation (DPI), the guinea pigs were euthanized and the blood was obtained to verify the infection by analysis of anti-Rickettsia antibodies by indirect immunofluorescence assay (IFA). Guinea pig rectal temperatures were considered normal when they were between 37.5 °C and 39.5 °C, as determined in an early extensive study on R. rickettsii infection in guinea pigs (Monteiro 1931).

Procedures with infected and uninfected opossums

Since the day of inoculation on the opossums (designated as day 0), each opossum was examined daily for clinical abnormalities and rectal temperature until 30 DPI. Blood samples (1.5 mL per sample) were collected in ethylene diamine tetraacetic acid (EDTA) from each opossum through the caudal vein, every 2 days from 0 to 30 DPI, and every 15 days from 31 to 150 DPI, when opossums were euthanized and submitted to necropsy and collection of spleen, liver, and brain, which were then submitted to DNA extraction followed by real-time PCR assay for detection of rickettsial DNA, as described below. From 0 to 30 DPI, each blood sample was divided into three subaliquots to be used for hematology, DNA extraction, and plasma separation to be tested for serology (techniques described below). From 31 to 150 DPI, blood was collected without EDTA and the serum was separated for serological analysis.

At the first DPI, all opossums were infested by ticks as follows: each of the G1 and G2 opossums (experiment 1) were infested with ∼300 larvae and 200 nymphs of uninfected A. cajennense; each of the G3 and G4 opossums was infested with ∼500 larvae and 100 nymphs of uninfected A. dubitatum; each of G5 and G6 opossums was infested with ∼400 larvae and 200 nymphs of uninfected A. cajennense and A. dubitatum ticks. These infestations were repeated on 8 and 15 DPI, comprising a total of three infestations at 7-day interval per opossum (Table 1). All infestations were conducted inside cotton sleeves glued to the shaved dorsum of opossums, as previously described (Horta et al. 2009). In each opossum of experiments 1 and 2, a single sleeve served for infestations with uninfected larvae and nymphs. In experiment 3, two sleeves per opossum were utilized, each one for larvae and nymphs of the same tick species. Each sleeve was opened daily, and the engorged ticks that had naturally detached were collected and taken to an incubator at 25 °C and with 90% relative humidity (RH) to molt to the next developmental stage.

Procedures with ticks recovered from opossums

Samples of unfed (flat) nymphs and adult ticks that molted from engorged larvae and nymphs, respectively, collected from opossums were individually submitted to DNA extraction and tested for the presence of rickettsial DNA by real-time PCR (technique described below). Additional samples of nymphs and adults were used to infect tick-naive rabbits, two rabbits per each group of opossum-derived ticks: one rabbit infected with nymphs and another one infected with adult ticks. Blood samples collected from each rabbit through the ear marginal vein at 0 and 30 DPI were tested for the presence of Rickettsia spp-reactive antibodies, as described below. Rabbit rectal temperature was measured daily from 0 to 20 DPI.

Hematological tests

The following hematological parameters were determined for opossums: hematocrit, total proteins, hemoglobin concentration, total number of erythrocytes, and leukocytes (the latter one discriminated for lymphocytes, neutrophils, eosinophils, monocytes, and basophils). Values were compared with reference values previously reported for D. aurita (Malta and Luppi 2006, Casagrande et al. 2009).

Indirect IFA

Opossum, rabbit, and guinea pig blood serum samples were tested by IFA using R. felis, R. bellii, and R. parkeri crude antigen, and a fluorescein isothiocyanate-labeled sheep anti-opossum immunoglobulin G (IgG) (Centro de Controle de Zoonoses, São Paulo/SP, Brazil), goat anti-rabbit IgG (Sigma, St. Louis, MO), or rabbit anti-guinea pig IgG (Sigma) as previously described (Horta et al. 2007). Animal sera were

<table>
<thead>
<tr>
<th>Experiment-group (no. of opossums)</th>
<th>Intraperitoneal inoculation</th>
<th>Tick infestations at 1, 8, and 15 DPI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-G1 (4)</td>
<td>Rickettsia felis + L-15 sterile medium</td>
<td>Amblyomma cajennense (≈300 larvae and 200 nymphs)</td>
</tr>
<tr>
<td>1-G2 (2)</td>
<td>L-15 sterile medium</td>
<td>A. cajennense (≈300 larvae and 200 nymphs)</td>
</tr>
<tr>
<td>2-G3 (4)</td>
<td>Rickettsia bellii + L-15 sterile medium</td>
<td>A. dubitatum (≈500 larvae and 100 nymphs)</td>
</tr>
<tr>
<td>2-G4 (2)</td>
<td>L-15 sterile medium</td>
<td>A. dubitatum (≈500 larvae and 100 nymphs)</td>
</tr>
<tr>
<td>3-G5 (2)</td>
<td>Rickettsia parkeri + L-15 sterile medium</td>
<td>A. cajennense + A. dubitatum (≈400 larvae and 200 nymphs of each species)</td>
</tr>
<tr>
<td>3-G6 (2)</td>
<td>L-15 sterile medium</td>
<td>A. cajennense + A. dubitatum (≈400 larvae and 200 nymphs of each species)</td>
</tr>
</tbody>
</table>
diluted in twofold increments with phosphate-buffered saline starting from 1:64 dilution. For each serum sample, the endpoint titer reacting with *R. rickettsii* was determined. In each slide, a serum previously shown to be nonreactive (negative control) and a known reactive serum (positive control) were tested at the 1:64 dilution. Opossum, rabbit, and guinea pig positive control sera were obtained from previous studies (Horta et al. 2009, Souza et al. 2009). Geometric mean antibody titer for each of the six experimental groups of opossums was obtained by taking the base-2 logarithm of each titer. The arithmetic mean of the result was calculated for each group for each date. The base-2 antilogarithm was taken on each resulting mean. Sera nonreactive at the 1:64 dilution were considered negative and were calculated as 1:1 for calculation purposes (Breitschwerdt et al. 1988).

Besides the antigens aforementioned, three other antigens, *R. rickettsii* strain Taiaçu (Pinter and Labruna 2006), *Rickettsia amblyommi* strain Ac37 (Labruna et al. 2004b), and *Rickettsia rhipicephali* strain HJ#5 (Labruna et al. 2007b) were tested by IFA with sera from two opossums from each of the *Rickettsia*-inoculated groups (G1, G3, and G5) to determine homologous and heterologous endpoint titers among the six rickettsial antigens.

**DNA extraction and real-time PCR**

DNA extraction of blood and organ samples was performed using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI), starting with 50 μL of blood from each individual sample, according to the manufacturer’s protocol for isolation of DNA from animal blood or tissues. Ticks were always processed individually. For this purpose, DNA extraction was performed on individual nymph or a group of eight legs extirpated from a single adult tick in 50 μL of TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0) in sterile microtubes. The final suspension was boiled at 100°C for 20 min and held at –20°C until tested by real-time PCR. The efficacy of this boiling protocol was previously shown with ticks and fleas naturally infected by *Rickettsia* spp. (Horta et al. 2007). For every 10 individual samples, a blank tube was included in the DNA extraction.

Tick and blood DNA samples were individually processed by real-time PCR assay using primers CS-5 (5’-GAGAGGAATTATATCAAAGGTAATG-3’) and CS-6 (AGGTCTCTCGTGCAATTCTC), which amplify a 147-bp fragment of the rickettsial gltA gene. These primers have shown sensitivity down to a single copy of *R. rickettsii* (Labruna et al. 2004a). A fluorogenic probe [5’ 6-FAM d(CATTGTGCCATCCAGCTACGGT) BHQ-1 3’] (Integrated DNA Technologies, San Diego, CA) positioned 76 bp downstream of the forward primer and 3 bp upstream of the reverse primer was used in the reactions (Labruna et al. 2004a). The reactions were performed in a 7500 Real-Time PCR System apparatus (Applied Biosystems, Foster City, CA). For each reaction, 1 or 2.5 μL of tick or blood DNA templates, respectively, were added to 2.5 μL of PCR buffer (10×), 4 μL of deoxynucleotide triphosphate mixture (1.25 mM), 1.0 MgCl2 (50 mM), 10 pmol of each primer, 0.1 μL of Taq polymerase (5000 U/mL), 0.25 μL of probe, and bidistilled water to a final volume of 25 μL. For each set of reactions, DNA extracted from *R. felis* strain Pedreira was used as positive control (Horta et al. 2006a), and at least three aliquots of bidistilled water were used as negative controls.

**Results**

**Experimental infection of opossums with *Rickettsia* spp.**

None of the 16 opossums showed marked clinical changes such as fever or hematological values outside the reference range reported elsewhere for *Delphips* spp. (Malta and Luppi 2006, Casagrande et al. 2009, Horta et al. 2009). Attempts to detect rickettsial DNA by real-time PCR was successful in only one G5-opossum (inoculated with *R. parkeri*) at the eighth DPI. No rickettsial DNA was detected in G1 (inoculated with *R. felis*), G3 (inoculated with *R. bellii*), G2, G4, and G6 (control groups) opossums. Also, no rickettsial DNA was detected in any opossum tissue at the 150th DPI.

**Experimental infection of guinea pigs with *Rickettsia* spp.**

All guinea pigs infected with the same inoculum of *R. felis*, *R. bellii*, and *R. parkeri* utilized in opossums at day 0 showed antibody titers at 21st DPI, ranging from 64 to 4,096. Only one guinea pig inoculated with *R. parkeri* developed fever (>39.5°C) for 8 consecutive days (Table 2).

**Table 2. Rectal Temperature, Fever Duration, and Antibody Titer to the Corresponding *Rickettsia* Species in Guinea Pigs Inoculated with *Rickettsia felis*, *Rickettsia bellii*, or *Rickettsia parkeri***

<table>
<thead>
<tr>
<th>Guinea pig no.</th>
<th>Inoculum</th>
<th>Days of fever</th>
<th>Rectal temperature range</th>
<th>Antibody titer at 21 DPI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>R. felis</em></td>
<td>0</td>
<td>38.4–39.1°C</td>
<td>4,096</td>
</tr>
<tr>
<td>2</td>
<td><em>R. felis</em></td>
<td>0</td>
<td>38.5–39.4°C</td>
<td>4,096</td>
</tr>
<tr>
<td>3</td>
<td><em>R. bellii</em></td>
<td>0</td>
<td>38.6–39.5°C</td>
<td>2,048</td>
</tr>
<tr>
<td>4</td>
<td><em>R. bellii</em></td>
<td>0</td>
<td>38.8–39.4°C</td>
<td>4,096</td>
</tr>
<tr>
<td>5</td>
<td><em>R. parkeri</em></td>
<td>0</td>
<td>38.1–39.4°C</td>
<td>4,096</td>
</tr>
<tr>
<td>6</td>
<td><em>R. parkeri</em></td>
<td>8</td>
<td>38.2–40.9°C</td>
<td>64</td>
</tr>
</tbody>
</table>
**TABLE 3. ENDPOINT ANTIBODY TITERS BY INDIRECT IMMUNOFLUORESCENCE ASSAY FOR SIX Rickettsia SPECIES IN OPPOSUMS EXPERIMENTALLY INFECTED WITH Rickettsia felis (G1 GROUP), Rickettsia bellii (G2 GROUP), Rickettsia parkeri (G3 GROUP), AND Rickettsia rickettsii (HORTA ET AL. 2008)**

<table>
<thead>
<tr>
<th>Opossum group-no.</th>
<th>Inoculum</th>
<th>DPI with Rickettsia</th>
<th>IFA endpoint titer to the following antigens</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>R. rickettsii</td>
</tr>
<tr>
<td>G1-1</td>
<td>R. felis</td>
<td>20</td>
<td>128</td>
</tr>
<tr>
<td>G1-1</td>
<td>R. felis</td>
<td>15</td>
<td>64</td>
</tr>
<tr>
<td>G1-3</td>
<td>R. felis</td>
<td>20</td>
<td>—</td>
</tr>
<tr>
<td>G1-3</td>
<td>R. felis</td>
<td>15</td>
<td>—</td>
</tr>
<tr>
<td>G3-1</td>
<td>R. bellii</td>
<td>8</td>
<td>1,024</td>
</tr>
<tr>
<td>G3-1</td>
<td>R. bellii</td>
<td>15</td>
<td>64</td>
</tr>
<tr>
<td>G3-3</td>
<td>R. bellii</td>
<td>10</td>
<td>256</td>
</tr>
<tr>
<td>G3-3</td>
<td>R. bellii</td>
<td>75</td>
<td>64</td>
</tr>
<tr>
<td>G5-1</td>
<td>R. parkeri</td>
<td>20</td>
<td>1,024</td>
</tr>
<tr>
<td>G5-1</td>
<td>R. parkeri</td>
<td>15</td>
<td>128</td>
</tr>
<tr>
<td>G5-2</td>
<td>R. parkeri</td>
<td>20</td>
<td>1,024</td>
</tr>
<tr>
<td>G5-2</td>
<td>R. parkeri</td>
<td>15</td>
<td>128</td>
</tr>
<tr>
<td>G1-S²</td>
<td>R. rickettsii</td>
<td>20</td>
<td>65,356</td>
</tr>
<tr>
<td>G1-S²</td>
<td>R. rickettsii</td>
<td>180</td>
<td>4,096</td>
</tr>
<tr>
<td>G2-1 a</td>
<td>R. rickettsii</td>
<td>18</td>
<td>16,384</td>
</tr>
<tr>
<td>G2-1 a</td>
<td>R. rickettsii</td>
<td>180</td>
<td>1,024</td>
</tr>
</tbody>
</table>

Homologous titers are in bold.

aGroup and opossum numbers refer to opossums inoculated by Horta et al. (2009).

IFA, immunofluorescence assay; —, nonreactive at 1:64 serum dilution.

**IFA of opossums**

Antibodies anti-R. felis, anti-R. bellii, and anti-R. parkeri were first detected in opossums on the fourth, fourth, and second DPI, respectively, and remained with detectable titers until 150 DPI. Control group opossums showed no reactivity against *Rickettsia* spp. antigens during the study. Antibodies in G1 (inoculated with *R. felis*), G3 (inoculated with *R. bellii*), and G5 (inoculated with *R. parkeri*) opossums peaked at 20, 6, and 8 DPI, respectively, and then declined slowly toward the end of the experiment, when opossums still had titers ranging from 64 to 128. The antibody curves were similar among inoculated opossums (Fig. 1). Table 3 shows homologous and heterologous antibody titers against six *Rickettsia* species from two opossums of each inoculated group (G1, G3, G5) at two time points, and from two opossums that were inoculated with *R. rickettsii* in a previous study (Horta et al. 2009). In all cases, homologous titers were higher or equal to heterologous titers. In some cases, homologous titers were fourfold higher than heterologous titers.

**Tick infestations**

Engorged larvae and nymphs were recovered from 5 to 24 DPI in opossums of the six experimental groups. After ecdisis inside the incubator, a total of 480 flat nymphs and adults were tested by real-time PCR assay, which detected rickettsial DNA in only one G5-*A. cajennense* nymph that had fed as larva on a *R. parkeri*-infected opossum (Table 4).

A total of 40 rabbits were infested with flat nymphs or adults derived from ticks that fed previously on *R. parkeri*-infected opossum (Table 4). However, this parameter was not valuable because other

**TABLE 4. REAL-TIME POLYMERASE CHAIN REACTION FOR DETECTION OF RICKETTSIAL DNA IN TICKS THAT FED ON OPOSSUMS OF SIX EXPERIMENTAL GROUPS (G1–G6) OF THREE EXPERIMENTS (1–3)**

<table>
<thead>
<tr>
<th>Experiment-group (no. of opossums)</th>
<th>Inoculum</th>
<th>Nymphs</th>
<th>Adults</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Amblyomma Cajennense</td>
<td>Amblyomma Dubitatum</td>
</tr>
<tr>
<td>1-G1 (4)</td>
<td>R. felis</td>
<td>0/48 (0)</td>
<td>—</td>
</tr>
<tr>
<td>1-G2 (2)</td>
<td>Sterile medium</td>
<td>0/24 (0)</td>
<td>—</td>
</tr>
<tr>
<td>2-G3 (4)</td>
<td>R. bellii</td>
<td>—</td>
<td>0/48 (0)</td>
</tr>
<tr>
<td>2-G4 (2)</td>
<td>Sterile medium</td>
<td>0/24 (0)</td>
<td>—</td>
</tr>
<tr>
<td>3-G5 (2)</td>
<td>R. parkeri</td>
<td>1/24 (4.2)</td>
<td>0/24 (0)</td>
</tr>
<tr>
<td>3-G6 (2)</td>
<td>Sterile medium</td>
<td>0/24 (0)</td>
<td>0/24 (0)</td>
</tr>
<tr>
<td>Total</td>
<td>—</td>
<td>1/120 (0.8)</td>
<td>0/120 (0)</td>
</tr>
</tbody>
</table>
seven rabbits infested with ticks that had fed on uninfected opossums also developed fever for few days (Table 5).

Discussion

Our study showed that opossums (D. aurita) are susceptible to the infection by R. felis, R. bellii, and R. parkeri, as verified by seroconversion of all inoculated opossums, sometimes with high homologous antibody titers to R. felis, R. bellii, or R. parkeri. Similarly to R. rickettsii-inoculated D. aurita (Horta et al. 2009), Rickettsia-infected opossums of our study showed no signs of fever or hematological abnormalities. All guinea pigs inoculated with aliquots of the same Rickettsia inoculum utilized in opossums seroconverted, most of them developing high anti-Rickettsia homologous titers. At least one guinea pig inoculated with R. parkeri presented high fever (>40°C), but no other clinical abnormality was observed. This result contrasts to previous studies, in which guinea pigs inoculated with R. parkeri showed temperature never above 39.5°C (Goddard 2003). As our single febrile guinea pig developed a
weak antibody response to R. parkeri (titer 64) in contrast to the other inoculated guinea pigs (titer ≥2,048), it is possible that the high fever was caused by an unknown factor that could have precluded a normal antibody response. Absence of fever in guinea pigs inoculated with R. bellii corroborates previous studies that demonstrated that R. bellii is not pathogenic for guinea pigs (Philip et al. 1983). Our results also indicate that R. felis is not pathogenic to guinea pigs.

Antibody titers of opossums infected by R. felis, R. bellii, and R. parkeri showed similar standard curves, with increasing titers during the first 15–30 DPI, when highest mean antibody titers were between 256 and 1,024, followed by gradual decrease to minimum mean titers (64–128) at 150 DPI. Although similar antibody standard curves were also observed in D. aurita experimentally infected with R. rickettsii (Horta et al. 2009), much higher titers (mean between 32,768 and 65,536) were observed in R. rickettsii-inoculated opossums (Horta et al. 2009).

Opossums inoculated with one Rickettsia species showed cross-reacting antibodies to other Rickettsia species, as expected for organisms in this bacterial genus (La Scola and Raoult 1997). It is noteworthy that opossum homologous titers were always higher than heterologous titers, or at least equal between closely related species. This finding is consistent with previous studies in mouse and dogs inoculated with Rickettsia (Philip et al. 1978, La Scola and Raoult 1997, Pirandia et al. 2008). In some cases (opossums G1-1 at 20 DPI, G1-3 at 20 DPI, and G2-1 at 18 and 180 DPI), endpoint titer of homologous serum was at least fourfold higher than heterologous titers (Table 3), a condition that has been applied to epidemiological studies for incriminating a possible Rickettsia species involved in a homologous reaction under natural conditions (Horta et al. 2007, Labruna et al. 2007c, Pacheco et al. 2007).

There was no detectable rickettsial DNA in opossum blood after being inoculated with R. felis, R. bellii, or R. parkeri, except for one opossum at 8 DPI with R. parkeri. Although we used a highly sensitive real-time PCR protocol, capable of detecting down to 1–100 copies of Rickettsia spp. (Labruna et al. 2004a), our results indicate that if opossums developed rickettsemia, its level was very low, a condition that is not surprising during active infection by Rickettsia spp., because these bacteria do not multiply in any blood cell (they multiply within endothelial cells). Recent studies in our laboratory showed that direct detection of rickettsial DNA in the blood samples of dogs, opossums, guinea pigs, and capybaras experimentally infected with R. rickettsii were highly variable; some animals had no detectable DNA by real-time PCR despite active rickettsiae in their blood demonstrated by guinea pig inoculation (Pirandia et al. 2008, Horta et al. 2009, Souza et al. 2009). Similarly, all flat ticks that had fed on inoculated opossums in the previous parasitic stage were also negative by PCR, except for a single A. cajennense tick that had fed on a R. parkeri-infected opossum.

As rickettsemia was not efficiently detected in opossums by PCR, if rickettsemia occurred, it was below the minimal required concentration of R. felis, R. bellii, or R. parkeri to infect a significant number of feeding ticks. Alternatively, the ticks used in our study could be refractory to acquisition of rickettsial infection via feeding on a rickettsemic host. In fact, ticks of the same A. cajennense colony used in our study were previously shown to be partially refractory to infection by R. rickettsii, as only 35% of them became infected by R. rickettsii after feeding on infected guinea pigs, in contrast to 95–100% infection in A. aureolatum and Rhizophus sanguineus ticks that fed on the same guinea pigs (Labruna et al. 2008). On the other hand, ticks from the same A. cajennense colony used in our study have been shown to be susceptible to the infection by R. parkeri (Sangioni et al. 2005) and R. felis (Horta et al. 2008); however, ticks were needle-inoculated directly into the hemocele, a condition very different from acquisition via feeding on a rickettsemic host. In fact, A. cajennense is rarely found infected with rickettsiae under natural conditions in São Paulo (Sangioni et al. 2005, Pacheco et al. 2009). Regarding A. dubitatum, it is normally found infected by R. bellii under natural conditions, usually with 10–40% infection rates (Labruna et al. 2004a, Pacheco et al. 2009). There is also a report of a natural infection of A. dubitatum by a different strain of R. parkeri (strain COOPERI) (Labruna et al. 2004a).

Previous studies have shown that R. rickettsii-infected opossums (both D. aurita and D. virginiana) develop long rickettsemia lasting for up to 3–4 weeks (Bozeman et al. 1967, Horta et al. 2009), during which they can act as amplifier hosts of R. rickettsii infection to A. cajennense ticks (Horta et al. 2009). Contrastingly, results of our study indicate that opossums do not act as amplifier hosts of R. felis, R. bellii, and R. parkeri to A. cajennense and/or A. dubitatum ticks, suggesting that opossums play no role on the ecology of these rickettsiae under natural conditions in Brazil. It must be stated that in our study, we used rickettsial strains with several in vitro passages in cell culture. Although never reported for the genus Rickettsia, it has been reported that a strain of Ehrlichia chaffeensis with a history of various in vitro passages lost its ability to infect dogs via infected ticks (Long et al. 2003). Also, our rickettsial strains were cultivated at 28°C before being inoculated into opossums. Lower temperatures like 28°C have been associated with less expressed proteins and decreased pathogenicity of R. rickettsii to vertebrate hosts (Spencer and Parker 1923, Gilford and Price 1955, Policastro et al. 1997). These statements are even more interesting when we compare our unsuccessful results with those successful ones of Horta et al. (2009), who worked with a strain of R. rickettsii that had never gone into in vitro passage. Thus, before we can definitely conclude that opossums are not important in the ecology of R. felis, R. bellii, and R. parkeri, further studies must be done with different strains of these rickettsiae, most importantly the strains that have never gone through in vitro passages. Finally, a R. felis natural cycle between opossums and fleas has been suggested (Pérez-Osorio et al. 2008). This possibility also deserves further investigations, because opossums are commonly found infected by R. felis-infected fleas in Brazil (Horta et al. 2007).

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Disclosure Statement

No competing financial interests exist.

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