Molecular Characterization of *Rickettsia rickettsii* Isolated from Human Clinical Samples and from the Rabbit Tick *Haemaphysalis leporispalustris* Collected at Different Geographic Zones in Costa Rica

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**Abstract.** Five strains of spotted fever group (SFG) rickettsiae previously isolated from human clinical cases and from the tick *Haemaphysalis leporispalustris* were used for molecular characterization in this study to establish their genetic relationship compared with the prototype *Rickettsia rickettsii* strain Sheila Smith. Samples were tested by polymerase chain reaction (PCR) targeting the rickettsial genes *gltA*, *ompA*, and *ompB*. PCR products of the latter two genes were DNA sequenced and compared with available sequences in GenBank. The *ompA* partial sequences of the five Costa Rican isolates showed 100% identity to several *R. rickettsii* sequences available in GenBank, including the sequence of the virulent reference strain Sheila Smith, whereas the *ompB* partial sequences of the five Costa Rican isolates showed 99.8–100% identity to *R. rickettsii* sequences from GenBank. This study showed the first molecular detection of *R. rickettsii* isolates from Rocky Mountain Spotted Fever patients and from the rabbit tick *H. leporispalustris* in different geographical zones in Costa Rica.

**INTRODUCTION**

Rocky Mountain Spotted Fever (RMSF) is a severe, tick-borne febrile disease caused by *Rickettsia rickettsii*, a *Rickettsia* belonging to the spotted fever group (SFG). The disease has been reported in the United States, Mexico, Panama, Colombia, Brazil, Argentina, and Costa Rica, where it represents a major public health concern.1–3 According to Dumler and Walker,4 “although the causative agent is still reluctant to reveal its secrets and the virulence mechanisms have not been identified yet, infections are sporadic but persistent.”

The first human cases of RMSF reported in Costa Rica date to 1977; thereafter, confirmed cases were detected in 1980, 1982, 1987, and 1994 in wet lands located along the Caribbean coast.5 Five additional cases were detected in 2003 in a location close to the most populated cities of the country (Figure 1). Human clinical samples from these RMSF cases were cultured in our laboratory and were identified as SFG rickettsiae by immunofluorescence.7

In 1985, we isolated two strains of *R. rickettsii* from rabbit ticks of the species *Haemaphysalis leporispalustris* collected from wild rabbits (*Sylvilagus brasiliensis*) living in RMSF-endemic areas of Costa Rica. Both isolates exhibited high virulence (high mortality and intense scrotal lesions) to guinea pigs and could not be differentiated from human isolates by immunofluorescence.8–10 These results contrasted to previous studies in the United States, where the *R. rickettsii* isolates obtained from *H. leporispalustris* were shown to be only milder pathogenic to guinea pigs.9 More recently, genetic studies have shown that the Hlp82 strain of *R. rickettsii*, isolated from *H. leporispalustris* from the United States, is distinct from the other isolates, suggesting that it could represent a different species or subspecies.11 In 1986, field studies were conducted to evaluate the distribution of RMSF in Costa Rica, and although the population of ticks parasitizing animals and vegetation was scarce, rickettsial infection was indirectly shown by the detection of SFG-reactive antibodies in humans, dogs, and wild rabbits.12

Traditionally, cultivation and identification of rickettsiae from clinical samples are laborious and time consuming but do not allow the genetic differentiation of isolates. The purpose of this study was to characterize five *R. rickettsii* isolates obtained from naturally infected persons from different geographic regions in Costa Rica, as well as from ticks (*H. leporispalustris*) collected on rabbits *S. brasiliensis*. This was done by PCR amplification of portions of the rickettsial genes *gltA*, *ompA*, and *ompB* and DNA sequencing of PCR amplified *ompA* and *ompB* products.

**MATERIALS AND METHODS**

**Samples.** Fifteen rickettsial isolates were previously obtained from 10 clinically ill patients and 2 *H. leporispalustris* ticks through the inoculation of eggs yolk, guinea pigs, and/or Vero cells with patient samples or tick homogenate. All samples were identified as SFG rickettsiae by immunofluorescence as previously described,7 and the prototype Sheila Smith strain (SS; ATCC-VR-149) was used as a positive control. These isolates have been cryopreserved in our laboratory, and aliquots of them were thawed to be processed in this study.

**DNA extraction and PCR.** Samples were processed by DNA extraction using a silica gel system QIAamp Viral DNA mini kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. Polymerase chain reaction (PCR) for the citrate synthase gene (*gltA*) of *Rickettsia spp.* was performed in all samples. A 147-nucleotide region was amplified using the primers CS-5 (forward) and CS-6 (reverse) under the following conditions: 12.5 μL 2× Master Mix Taq polymerase (Fermentas Inc, Hanover, MD), 0.75 μL of 15 μmol/L of each primer, 1.25 μL DMSO, and 5 μL of extracted DNA in a final volume of 25 μL.13 The reaction was performed in a cycler (2700; Perkin Elmer, Waltham, MA): 95°C for 3 minutes, 50 cycles at 95°C for 15 seconds, 58°C for 30 seconds, and 72°C for 30 seconds, with one extension of 7 minutes at 72°C. Positive samples were confirmed with a PCR that amplifies the other region of 401 bp in the *gltA* gene using the primers CS-78 (forward) and CS-323 (reverse).14,15 This was done under the same conditions described above with 1 μL of each primer (20 μM) and 40 cycles.

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Molecular characterization was done by amplification of a 532-bp fragment of the rickettsial ompA gene (that codes to a major outer membrane protein) using the primers Rr190.70p (forward) and Rr190.602n with the same conditions described above. It was carried out in a cycler (2700; Perkin Elmer) under 95°C for 5 minutes, 35 cycles of 95°C for 40 seconds, 58°C for 30 seconds, and 65°C for 45 seconds, and a final extension of 10 minutes at 72°C.14,15 Also, an 855-bp fragment of the ompB gene (codes to another major outer membrane protein) region using primers 120-M59 (forward) and 120-807 (reverse)15 was attempted by 95°C for 3 minutes, 40 cycles at 95°C for 30 seconds, 50°C for 30 seconds, and 68°C for 90 seconds, with a final extension of 1 minute at 68°C. These genes have been used in different studies to characterize the rickettsial species.15,16

Sequencing, PCR products from five rickettsial isolates were selected to be sequenced: one isolate from San Carlos, Strain CR, 1977; one isolate from Sarapiqui, Strain Srp, 1987; one isolate from Limón, Strain NC, 1987; one isolate from San Ramón, Strain SR, 2003; and one isolate from the rabbit tick H. leporispalustris, Strain J-79, 1985. PCR products of ompA and ompB were gel purified using QIAquick gel extraction Kit (Qiagen) and quantified in an agarose gel by visual comparison with DNA ladder Mass Ruler (Fermentas). Ten nanograms of the product was sequenced with the BigDye terminator V3.1 Cycle Sequencing Kit according to the manufacturer's instructions in a 20-μL total reaction volume using GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA). The products were precipitated with ethanol/EDTA/sodium acetate and sequenced in forward and reverse directions two to three times with an ABI PRISM 3130 DNA Analyzer. Sequences were edited using the SeqManII software package (DNASTar, Madison, WI), and the basic local alignment search tool (BLAST, National Center for Biotechnology Information [NCBI]) was used to find similarity with reference strains.

RESULTS

DNA from all 15 rickettsial isolates yielded expected PCR products using both primers sets for the rickettsial gtlA gene. From these, 13 yielded expected PCR products for the ompA gene and 11 for the ompB gene. PCR products derived from ompA and ompB genes of five rickettsial isolates (each one from a different region of Costa Rica) were selected to be processed by DNA sequencing. For the ompA amplicons, we were able to obtain fragments between 300 and 400 bp, and for the ompB amplicons, fragments of > 600 bp were obtained except for Strain Srp (Table 1). DNA sequences from the five isolates perfectly matched (100% identity) to corresponding sequences of R. rickettsii in GenBank. Regarding the ompA gene, partial sequences of the five isolates were 100% identical to R. rickettsii Sheila Smith, a highly virulent reference strain, and other R. rickettsii isolates available in the database (Table 1). Regarding the ompA gene, partial sequences of the five isolates were 100% identical to R. rickettsii Sheila Smith-a highly virulent reference strain, and to other R. rickettsii isolates available in the database (Table 1).

DISCUSSION

This study reports the first genetic identification of R. rickettsii isolates from RMSF patients from different geographical
zones in Costa Rica and the first from the rabbit tick *Haemaphysalis leporispalustris* outside the United States.

Analysis based on the partial *ompA* and *ompB* sequences showed that all samples share a high degree of sequence identity with *R. rickettsii* reference strain Sheila Smith and also with the avirulent strain *Rickettsia rickettsii* Iowa from the United States. As recently reported, these two North American strains are closely related, with genomic comparisons showing deletions and single nucleotide polymorphisms; however, their role in bacterial pathogenesis remains to be elucidated.

The American *R. rickettsii* strain Hpl#2 from the rabbit tick, *H. leporispalustris*, has a lower virulence in animal models, and very recently it was suggested that it could be a novel subspecies or rickettsial species. In contrast, our Strain J-79, also isolated from *H. leporispalustris*, shows high virulence to guinea pigs. This information supports the importance of further molecular studies to characterize national isolates and to elucidate our J79 classification.

Although there is not sufficient information to show genetic differences between the Costa Rican isolates, their geographic region, and the years of isolation, this preliminary work may suggest a wide distribution of *Rickettsia* in the country, and more ecological studies should be undertaken to establish the tick responsible for transmitting the *Rickettsia* to humans to better understand the natural cycle of *R. rickettsii* in Costa Rica.

The first documented case of RMSF in Costa Rica in 1977 was reported from our laboratory, which is the only one in the country that developed all the currently used methods for isolation and characterization of this bacterium. Since then, we have isolated several *R. rickettsii* from human and tick samples and have done most of the serologic studies in the country. We have shown positive serology in human sera from residents of Cedral, Jimenez, and Puerto Limon in a field study in 1986. During the last 10 years, we have analyzed 109 serum samples that were sent to our laboratory from patients hospitalized in endemic regions; 31 were reactive to *R. rickettsii* by immunofluorescence. We have also reported seropositivity in rabbits, and we have evidence of seropositive blood obtained from dogs belonging to families where clinical cases were reported (unpublished data from the Virology Laboratory, University of Costa Rica).

There is not enough awareness of RMSF as an important cause of morbidity and mortality in humans in Costa Rica. For example, patients are already on antibiotic therapy when samples are sent to laboratory, and only a few laboratories send these samples for etiologic agent diagnosis.

Costa Rica is known for its conservation programs; 33% of the country is made up of National Parks. The hundreds of thousands of tourists that visit these conserved areas every year, along with the continuous growth of urban towns in former forest zones, increase the transmission risk. It is important to educate and to inform health authorities for better epidemiologic surveillance so that local residents and travelers to endemic regions take preventive measures.

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**Table 1**

<table>
<thead>
<tr>
<th>Isolate code</th>
<th>Locality year</th>
<th>Origin</th>
<th>Source for DNA extraction</th>
<th>Amplification by PCR</th>
<th>Highest similarity (%) of partial <em>ompA</em> sequence</th>
<th>Highest similarity (%) of partial <em>ompB</em> sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR</td>
<td>San Ramón 2003</td>
<td>Human</td>
<td>Yes</td>
<td>Yes</td>
<td>100% (376/376) to <em>R. rickettsii</em> Sheila Smith (CP000848) and Panama-2004 (DQ164838)</td>
<td>99.8% (680/681) to <em>R. rickettsii</em> Sheila Smith (CP000848) and Panama-2004 (DQ164838)</td>
</tr>
<tr>
<td>Srp</td>
<td>Sarapiquí 1987</td>
<td>Human</td>
<td>Yes</td>
<td>Yes</td>
<td>100% (319/319) to <em>R. rickettsii</em> Sheila Smith (CP000848) and Iowa (CP000766)</td>
<td>99.7% (679/681) to <em>R. rickettsii</em> Sheila Smith (CP000848) and Panama-2004 (DQ164838)</td>
</tr>
<tr>
<td>CR</td>
<td>San Carlos 1977</td>
<td>Human</td>
<td>Yes</td>
<td>Yes</td>
<td>100% (407/407) to <em>R. rickettsii</em> Sheila Smith (CP000848) and Iowa (CP000766)</td>
<td>99.8% (482/482) to <em>R. rickettsii</em> Sheila Smith (CP000848) and Iowa (CP000766)</td>
</tr>
<tr>
<td>NC</td>
<td>Limón 1987</td>
<td>Human</td>
<td>Yes</td>
<td>Yes</td>
<td>100% (325/325) to <em>R. rickettsii</em> Sheila Smith (CP000848) and Iowa (CP000766)</td>
<td>99.8% (753/754) to <em>R. rickettsii</em> Sheila Smith (CP000848) and Iowa (CP000766)</td>
</tr>
<tr>
<td>J-79</td>
<td>Limón 1985</td>
<td>Tick*</td>
<td>Yes</td>
<td>Yes</td>
<td>100% (358/358) to <em>R. rickettsii</em> Sheila Smith (CP000848) and Iowa (CP000766)</td>
<td>99.8% (717/717) to <em>R. rickettsii</em> Sheila Smith (CP000848) and Iowa (CP000766)</td>
</tr>
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* *Haemaphysalis leporispalustris* (Fuentes et al. 1985).

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Acknowledgments: The authors thank and recognize Dr. Luis G. Fuentes who pioneered the research on rickettsial diseases in Costa Rica and Francisco Vega, Reynaldo Pereira, and Carlos Vargas for providing technical support and assistance.

Financial support: This study was supported by the Microbiology Faculty of the University of Costa Rica.

Disclosure: The authors declare that they have no competing interest.
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