Genetic identification of ricketsial isolates from fatal cases of Brazilian spotted fever, and comparison with *Rickettsia rickettsii* isolates from the American continent

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Running Head: Ricketsial isolates from fatal cases in Brazil

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Abstract
Fifteen bacterial isolates from spotted fever group ricketsiosis in Brazil were genetically identified as *Rickettsia rickettsii*. In a phylogenetic analysis with other *R. rickettsii* isolates from GenBank, the Central/South American isolates showed low polymorphism and formed a clade distinct from two North American clades; the later had greater in-branch polymorphism.

TEXT

The tick-borne bacterium *Rickettsia rickettsii* is the etiologic agent of Rocky Mountain spotted fever (RMSF), the most severe ricketsioses affecting humans in the western hemisphere (1). In Brazil, the disease has been referred to as Brazilian spotted fever (BSF). While RMSF fatality rates are usually 5 to 10% in the United States, general rates of 20 to 40% have been reported in Brazil (1).

In the state of São Paulo, southeastern Brazil, where BSF is a compulsory notifiable disease, various spotted fever group (SFG) rickettsial isolates have been obtained from human clinical cases during the last few years (2, 3). Whereas all these isolates were confirmed to be SFG rickettsiae through indirect immunofluorescence assay using anti-*R. rickettsii* human sera (4), they were not genetically identified through molecular analysis. Herein, we perform genetic identification and molecular characterization of these rickettsial isolates, and compared their genetic profile with isolates from ticks in Brazil, and with *R. rickettsii* isolates from other American countries.

Blood clot or skin lesion biopsy from BSF suspected cases were processed by the shell vial technique, as described (5). Once rickettsiae were visualized within Vero cells through immunofluorescence assay using anti-*R. rickettsii* human polyclonal serum (4, 6), 1st passage-infected cells were harvested and one aliquot used for DNA
extracted through the PureLink™ Genomic DNA Kit (Invitrogen, Carlsbad, CA).

Extracted DNA were assayed with PCR protocols using primers targeting the rickettsial genes gltA, ompA, ompB, and the intergenic regions RR0155-rpmB, RR1240-tlc5′, and cspA-ksgA, as shown in Table 1. In addition, DNA extracted from 3rd passage-infected cells of three R. rickettsii isolates, previously isolated from the ticks Amblyomma sculptum (reported as Amblyomma cajennense), Amblyomma aureolatum, and Rhipicephalus sanguineus in Brazil (12-14), were submitted to the intergenic region-PCR protocols. PCR products were sequenced in an ABI automated sequencer (Applied Biosystems/Thermo Fisher Scientific, model ABI 3500 Genetic Analyser, Foster City, California) with the same primers used for PCR. Generated sequences were compared to each other and submitted to BLAST analyses (www.ncbi.nlm.nih.gov/blast) to infer closest similarities available in GenBank.

Phylogenetic analyses were performed using the program PAUP version 4.0b10 (15) to maximum parsimony (MP); confidence values for individual branches of the resulting tree were determined by bootstrap analysis with 1,000 replicates. Bayesian analysis (BA) was performed by Mrs. Bayes version 3.1.2 (16) software with 1,000,000 generations and GTR+I+G substitution model was used. Partial DNA sequences obtained from the amplified PCR products (gltA, RR0155-rpmB, RR1240-tlc5′, cspA-ksgA, and ompB) were concatenated and aligned with corresponding sequences of different strains of R. rickettsii available in GenBank using the CLUSTAL X (17) and adjusted manually using GeneDoc (18). Partial sequences of the ompA gene were not included because the region of the gene that was amplified in the present study showed no polymorphism among the R. rickettsii isolates. Corresponding sequences of R. rickettsii strain Hlp#2 (CP003311), and Rickettsia philipii strain 364D (CP003308) were used as outgroups.

Fifteen rickettsial isolates (designated as IAL 1 to 15) from BSF patients (13 fatal cases) in the state of São Paulo, southeastern Brazil, were identified as R. rickettsii.
since their *gltA* (737 nt), *ompA* (491 nt), and *ompB* (787 nt) DNA sequences were 100% identical to each other and to corresponding sequences on the genome of *R. rickettsii* strain Brazil (GenBank no. CP003305). While the *ompA* partial sequences were also 100% identical to corresponding sequences of *R. rickettsii* strains from North America (e.g., CP000848, CP000766), the *ompB* partial sequences were 100% identical to the North American Sheila Smith strain (CP000848), and at the same time, differed by one single nucleotide polymorphism from other North American strains (e.g., CP000766, CP003307). The *gltA* sequence of the 15 Brazilian human isolates differed by an extra codon (CGG) when compared to several North American strains, such as Sheila Smith (CP000848) and Bitterroot (U59729). This extra codon was also present in the *R. rickettsii* tick isolates (Taiaçu, Itu, and Rs1) from Brazil (12-14). The sequence of three intergenic regions (249 nt for RR0155-rpmB, 315 nt for RR1240-tlc5$, and 362 nt for *cspA-kgA*) were determined for the 15 human isolates, and for the three tick isolates (Taiaçu, Itu, and Rs1) from Brazil. For each intergenic region, the sequences were 100% identical to each other (no polymorphism was detected), and when submitted to BLAST analysis, they were 100% identical to corresponding sequences of *R. rickettsii* strain Brazil (CP003305). For the concatenated phylogenetic analysis, which included a total of 2,392 nt, the sequences of the 15 human isolates (IAL 1-15) and 3 tick isolates (Taiaçu, Itu, and Rs1) from Brazil were aligned with corresponding sequences from 10 other *R. rickettsii* isolates available in GenBank, 6 from the United States, 1 from Costa Rica, 2 from Colombia, and 1 from Brazil (Table 2). The 15 human and 3 tick isolates of *R. rickettsii* from Brazil formed a clade under high bootstrap support (99 to 100%) with all three South American isolates available in GenBank (one from Brazil, two from Colombia) and with the Central American isolate from Costa Rica (Fig. 1). This Central/South American clade, ecologically associated with 4 different tick species, namely *A. aureolatum*, *Amblyomma patinoi*, *A. sculptum*, and *R. sanguineus* (Table 2), had a sister
group formed by the North American isolates Sheila Smith and Bitterroot, ecologically
associated with the tick vector *Dermacentor andersoni*. A more divergent clade was
composed by North American isolates that have been ecologically associated with the
ticks *Dermacentor variabilis* and *R. sanguineus*.

GenBank nucleotide sequence accession numbers of the partial sequences of *R. rickettsii*
generated in this study are KJ994337-KJ994339 for the *gltA*, *ompA*, and *ompB*
genes, respectively, and KJ994340-KJ994342 for the RR0155-*rpmB*, RR1240-*tlc5b*, and
*cspA-ksgA* intergenic regions, respectively.

As reported in previous studies (10, 19-21), the North American isolates of *R. rickettsii*
presented relatively high polymorphism when compared to Central/South American isolates. Our concatenated analysis showed the formation of 3 North American haplotypes (A, B, C), each associated with a different tick species (Fig. 1, Table 2). On the other hand, and excluding strain Colombia (haplotype E), there was a single haplotype (D) in Central/South America, even though they were associated with 4 different tick species. Geographic distances cannot be inferred for this discrepancy because the distance between Costa Rica and southeastern Brazil is much higher than the distances between distinct North American isolates (Fig. 1). Interestingly, while low, mild, and high virulent strains of *R. rickettsii* have been reported in both the eastern and western parts of the United States (20, 22), only high virulent strains, responsible for high fatality rates, have been reported in Central/South America, regardless of the tick vector (2, 23, 24-29). While our results of no polymorphism among Central/South American isolates could be biased to the fact that most of these isolates were derived from fatal cases, a recent study reported the same clade distribution of our Fig. 1, when analyzing intergenic regions of *R. rickettsii* derived from fatal cases from the United States, Mexico, and Central/South America (21).

The relatively high polymorphism among North American isolates of *R. rickettsii*, and the contrasting low level of polymorphism in Central/South America.
suggest that *R. rickettsii* radiated in North America, and was introduced into South America during more recent periods. This scenario would also explain why there are a mixture of high and less virulent *R. rickettsii* strains in North America (due to longer coevolving periods with vertebrates), and at the same time, only highly virulent strains have been found in South America.

It has been suggested that the higher fatality rates of BSF, when compared to RMSF in the United States, are related to delayed treatment, use of less effective antibiotics, and more virulent *R. rickettsii* strains occurring in Brazil (2, 30-31). The present study corroborates previous studies that provided genetic evidence for very low polymorphism occurring among *R. rickettsii* isolates from South America. This fact should be a significant reason for the much higher fatality rates of BSF, although the other above factors could also be contributing factors.

ACKNOWLEDGMENTS

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Fig. 1. Molecular phylogenetic analysis of *Rickettsia rickettsii* isolates from the United States, Costa Rica, Colombia, and Brazil. A total of 2,392 unambiguously aligned nucleotide sites of two rickettsial genes (*gltA* and *ompA*) and three intergenic regions (RR0155-*rpmB*, RR1240-*tld5*, and *cspA-ksgA*) were concatenated and subjected to analysis by Maximum Parsimony and Bayesian methods. Corresponding sequences of *Rickettsia philipii* strain 364D and *R. rickettsii* strain Hlp#2 were used as an outgroup. Numbers at nodes are support values derived from bootstrap and posterior probability for MP and BA analyses (MP/BA). Sequence codes A–E, each with a different color, represent the five haplotypes generated from the 28 isolates of *R. rickettsii* described in Table 2. Grey braces or arrows at the clades indicate the tick species that has been ecologically associated with the *R. rickettsii* isolates. The geographical region of origin of the 28 isolates and their corresponding haplotypes (A to E) are indicated on the map of the American continent.
Table 1: Primer pairs used for amplification of rickettsial genes (gltA, ompA, ompB) or intergenic regions (RR0155-rpmB, RR1240-tlcS5, and cspA-ksgA).

<table>
<thead>
<tr>
<th>Primer pairs</th>
<th>Target and primers</th>
<th>Primer sequences (5’ – 3’)</th>
<th>Amplicon size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CS-239 g</td>
<td>GCTTTCTCATCATATGCTATTAT</td>
<td>834</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>CS-1069</td>
<td>CAGGGTTTCGTGATTTTTT</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Rs190.70p</td>
<td>ATGGCGAATATTTTCTCAAAA</td>
<td>530</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Rs190.602n</td>
<td>AGTGCAGCATTGCTCCCCT</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>120-M59</td>
<td>CCGAGGTGGTGGTAACTG</td>
<td>862</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>120-807</td>
<td>CCTTAGATTAACGCCCTAA</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RR0155-rpmB</td>
<td>TTTCTAGCAGCGGTGTATCC</td>
<td>290</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Reverse RR1240-tlcS5</td>
<td>TTAGCCCATGTTGAGGTTTACT</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Forward</td>
<td>CGGATAACGCCGAGCTTAATA</td>
<td>357</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Reverse cspA-ksgA</td>
<td>ATGCCGCTCTGAATTTGTTT</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Forward</td>
<td>CATCAGTCTCGCTTATT</td>
<td>405</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TTTCTCTCCCTCCTCATCAA</td>
<td>10</td>
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</table>
TABLE 2 Isolates of *Rickettsia rickettsii* used in the phylogenetic analysis of the present study.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Isolation source</th>
<th>Clinical outcome</th>
<th>Source of DNA sequences for the following rickettsial genes (GenBank accession numbers or reference number)</th>
<th>Haplotype</th>
<th>Tick</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iowa</td>
<td>Tick</td>
<td>-</td>
<td>gltA CP000766 RR0155-rpmB CP000766 RPMB CP000766 tlc5b CP000766</td>
<td>A</td>
<td>D.v.</td>
</tr>
<tr>
<td>Hino</td>
<td>Human Fatal</td>
<td>CP003309</td>
<td>gltA CP000766 RR0155-rpmB CP000766 RPMB CP000766 tlc5b CP000766</td>
<td>A</td>
<td>D.v.</td>
</tr>
<tr>
<td>Hauke</td>
<td>Human Fatal</td>
<td>CP003318</td>
<td>gltA CP000766 RR0155-rpmB CP000766 RPMB CP000766 tlc5b CP000766</td>
<td>A</td>
<td>D.v.</td>
</tr>
<tr>
<td>Arizona</td>
<td>Human Fatal</td>
<td>CP003307</td>
<td>gltA CP000766 RR0155-rpmB CP000766 RPMB CP000766 tlc5b CP000766</td>
<td>B</td>
<td>R.s.</td>
</tr>
<tr>
<td>Sheila Smith</td>
<td>Human Fatal</td>
<td>CP000848</td>
<td>gltA CP000766 RR0155-rpmB CP000766 RPMB CP000766 tlc5b CP000766</td>
<td>C</td>
<td>D.a.</td>
</tr>
<tr>
<td>Bitterroot</td>
<td>Tick</td>
<td>-</td>
<td>gltA CP000766 RR0159729 RPMB EF216032 RPMB EF215893 RPMB EF215860 RPMB X16353</td>
<td>C</td>
<td>D.a.</td>
</tr>
<tr>
<td>Costa Rica</td>
<td>Human Fatal</td>
<td>19, 27</td>
<td>gltA CP000766 RR0155-rpmB CP000766 RPMB CP000766 tlc5b CP000766</td>
<td>D</td>
<td>?</td>
</tr>
<tr>
<td>Rs1</td>
<td>Tick</td>
<td>13</td>
<td>gltA CP000766 RR0155-rpmB CP000766 RPMB CP000766 tlc5b CP000766</td>
<td>D</td>
<td>A.s.</td>
</tr>
<tr>
<td>Itu</td>
<td>Tick</td>
<td>-</td>
<td>gltA CP000766 RR0155-rpmB CP000766 RPMB CP000766 tlc5b CP000766</td>
<td>D</td>
<td>A.a.</td>
</tr>
<tr>
<td>Taiaçu</td>
<td>Tick</td>
<td>-</td>
<td>gltA CP000766 RR0155-rpmB CP000766 RPMB CP000766 tlc5b CP000766</td>
<td>D</td>
<td>A.s.</td>
</tr>
<tr>
<td>IAL 1-2</td>
<td>Human Fatal</td>
<td>This study</td>
<td>gltA CP000766 RR0155-rpmB CP000766 RPMB CP000766 tlc5b CP000766</td>
<td>D</td>
<td>A.s.</td>
</tr>
<tr>
<td>IAL 4, 9</td>
<td>Human Cure</td>
<td>This study</td>
<td>gltA CP000766 RR0155-rpmB CP000766 RPMB CP000766 tlc5b CP000766</td>
<td>D</td>
<td>A.s.</td>
</tr>
<tr>
<td>IAL 3,5,8,10,15</td>
<td>Human Fatal</td>
<td>This study</td>
<td>gltA CP000766 RR0155-rpmB CP000766 RPMB CP000766 tlc5b CP000766</td>
<td>D</td>
<td>A.s.</td>
</tr>
<tr>
<td>Brazil</td>
<td>Human ?</td>
<td>This study</td>
<td>gltA CP000766 RR0155-rpmB CP000766 RPMB CP000766 tlc5b CP000766</td>
<td>D</td>
<td>A.s.</td>
</tr>
<tr>
<td>Colombia</td>
<td>Human Fatal</td>
<td>This study</td>
<td>gltA CP000766 RR0155-rpmB CP000766 RPMB CP000766 tlc5b CP000766</td>
<td>E</td>
<td>A.p.</td>
</tr>
</tbody>
</table>

* refers to the concatenated haplotypes shown in the phylogenetic tree (Fig. 1).  

1 for tick isolates, refers to the tick species from which the isolate was obtained; for human isolates, refers to the incriminated vector of *R. rickettsii* to humans in the area of origin of the isolate, according to Ogrzewalska et al. (32) for *Amblyomma aureolatum* (A.a.), Faccini-Martínez et al. (33), Nava et al. (34), and Pacheco et al. (13) for *Amblyomma patinoi* (A.s.) and *Amblyomma sculptum* (A.s.), Karpathy et al. (10) for *Dermacentor variabilis* (D.v.), *Dermacentor andersoni* (D.a.), and *Rhipicephalus sanguineus sensu lato* (R.s.); ?: unknown vector, according to Hun et al. (27).  

1 Geographic origin (municipality in the state of São Paulo, Brazil) of these isolates: São Paulo (IAL 1), São Bernardo do Campo (IAL 2).  

1 Geographic origin (municipality in the state of São Paulo) of these isolates: Piracicaba (IAL 4), Valinhos (IAL 9).  

1 Geographic origin (municipality in the state of São Paulo) of these isolates: Valinhos (IAL 3,5,8,11,12,15), Campinas (IAL 6,13), Jaguariúna (IAL 7), Piracicaba (IAL 10), Limeira (IAL 14).