SHORT REPORT: ISOLATION AND IDENTIFICATION OF TWO SPOTTED FEVER GROUP RICKETTSIAL STRAINS FROM PATIENTS IN CATALONIA, SPAIN

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Abstract. Two rickettsial strains, 16B (previously isolated) and FB1, were isolated from blood from patients with Mediterranean spotted fever in Catalonia, Spain. These are the only 2 human rickettsial isolates of the spotted fever group obtained so far in Spain. These strains were identified by the polymerase chain reaction and sequence analysis of a fragment of the outer membrane protein A (ompA) gene. The partial ompA sequence was found to be 100% identical with that of Rickettsia conorii (Malish 7 strain) for both strains. These results confirm the presence of R. conorii in Catalonia, despite the fact that in a previous study, no R. conorii were isolated, but a new rickettsial strain of the spotted fever group (Bar29) was isolated from dog ticks (Rhipicephalus sanguineus) in Catalonia. Further studies are necessary to get a better knowledge of the epidemiology of rickettsiae in Catalonia.

Rickettsia conorii, a spotted fever group (SFG) rickettsia, is the causative agent of Mediterranean spotted fever (MSF) and is transmitted in the Mediterranean area by the brown dog tick Rhipicephalus sanguineus. In Spain, the epidemiology of rickettsiae and rickettsial diseases is still poorly described, although several MSF seroepidemiologic surveys1–3 and clinical studies of this disease4–5 have been reported. The first tick rickettsial isolate was obtained in Spain by Vero cell inoculation with eggs of Rhipicephalus sanguineus isolated from dogs near Madrid and identified by polymerase chain reaction (PCR)–restriction fragment length polymorphism as R. rhipicephali,7 an SFG rickettsia that seems to be nonpathogenic for humans. In Catalonia, Spain, the first 2 SFG rickettsial strains isolated from humans differed in cytopathogenicity from the reference R. conorii Moroccan strain,7 although 1 of them, the 16B strain, was identical to R. conorii by pulsed-field gel electrophoresis.8

In 1996, 6 SFG rickettsial strains were isolated on shell vials from ticks (Rhipicephalus sanguineus) collected in different Catalonian areas.9 The 6 strains were phenotypically and genotypically identical and seemed to be identical to the Mtu5 strain, an SFG rickettsial strain that was previously isolated from the tick Rhipicephalus turanicus in southern France.10 This new SFG rickettsial strain (called Bar29) is closely related to R. massiliiae,11 a recently described SFG rickettsia that has been detected in France, Portugal, Greece, and central Africa. The Bar29 strain is slightly different from R. massiliiae by genotypic and antigenic criteria and shows an unexpected resistance to rifampin. Rickettsia conorii was not isolated in this study.

In 1995, 15 blood samples from 15 patients clinically (presence of fever, maculopapular or purpuric rash, and tache noire or a history of contact with dog ticks) and serologically (a positive single serum sample or 2 serum samples with a 4-fold elevation in titer within 2–3 weeks detected by a specific microimmunofluorescence assay) diagnosed as having MSF were collected 2–7 days after the onset of disease before antibiotic therapy into tubes containing heparin and stored at −80°C. Samples were collected after informed consent was obtained. The study was reviewed and approved by the Ethical Committee of Sabadell Hospital and followed the rules of the Spanish Health Ministry.

Isolation was carried out by the centrifugation-shell vial technique as described previously,12 and each blood sample was assayed in triplicate. Rickettsiae were detected directly inside the shell vial by immunofluorescence staining and microscopic examination of coverslips at 6, 11, and 14 days after incubation. Only 1 rickettsial strain, designated FB1, was isolated from 1 of the 15 blood samples. Positivity was detected 14 days after inoculation. The supernatant of this shell vial was successfully subcultured onto confluent monolayers of MRC5 cells in culture flasks. Harvesting was done when the degree of infection estimated by Gimenez staining12 was high.

The DNA was extracted by using a QIAmp Tissue kit (Qiagen, Hilden, Germany). For the PCR amplification, we used the outer membrane protein A (ompA) gene primer pair Rr90.70p–602n described by Regnery and others14 (Table 1). Amplification was performed under the following conditions: 3 min of denaturation at 95°C, followed by 35 cycles of denaturation for 20 sec at 95°C, annealing for 30 sec at 46°C, and extension for 1 min at 72°C. A further 7-min extension at 72°C completed the polymerization. Primers Rr 190.70p–602n amplified a fragment of the ompA gene of the expected size (530 basepairs) for both strains (16B and FB1).

Sequence determination of the amplicon was done with the reagents of an ampicycle kit (Pharmacia, Uppsala, Sweden) incorporating previously described fluorescein-labeled primers15 (Table 1). Sequencing reactions were resolved on 6% polyacrylamide gels (ready mix gel, automated laser fluorescent grade, Pharmacia), and electrophoresis was performed in an A.L.F. automated sequencer (Pharmacia) and associated software. The partial ompA sequence of both strains was found to be 100% identical with that of R. conorii (Malish 7 strain) when compared with the ompA gene sequences from known SFG rickettsiae (Figure 1).

In this report, we have described the isolation by the shell vial technique of a new rickettsial strain from human origin in Catalonia (FB1) and its genotypic identification by sequencing of a fragment of the ompA gene of this strain and strain 16B, which was previously isolated from blood from 1 patient with MSF in the same area. Both rickettsial isolates have been identified as the R. conorii Malish 7 strain. This strain seems to be typical of the species R. conorii, at least in the Mediterranean area, since it has been identified from
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TABLE 1

<table>
<thead>
<tr>
<th>Primer Nucleotide sequence (5′—3′)</th>
<th>ompA positions</th>
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<tbody>
<tr>
<td><strong>Rr 90.70p</strong> ATG-GCG-AAT-ATT-TCT-CCA-AA 70-90</td>
<td></td>
</tr>
<tr>
<td><strong>190.264</strong> CGT-TAT-CTC-ATT-CCA-ATT-AT 264-245</td>
<td></td>
</tr>
<tr>
<td><strong>190.4852†</strong> GCA-AAA-GCT-TAA-CTT-TAA-A 485-503</td>
<td></td>
</tr>
<tr>
<td><strong>Rr 190.6021†</strong> AGT-GCA-GCA-TTC-GCT-CCC-CCT 602-580</td>
<td></td>
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</tbody>
</table>

* The primer was used for the polymerase chain reaction and the sequencing reaction.
† The primer was located on the complementary strand of DNA.

The 2 human strains tested in this study and from 5 strains from humans isolated in Marseille, France.15

These results confirm the presence of *R. conorii* in the area studied. In contrast, in a previous study, no *R. conorii* were isolated, but a new strain closely related to *R. massiliae* (Bar29) was isolated from Catalan Rhipicephalus sanguineus ticks, the tick species that is more likely to bite humans in the Mediterranean area. The differences between Bar29 and *R. conorii*, which include differences in the growth patterns of both strains, as well as the low number of isolates obtained, could explain the failure to isolate Bar29 from patients or *R. conorii* from ticks.

Similar findings were reported by Babalis and others16 in Greece. After testing 242 adult ticks, only 1 strain was isolated from *R. sanguineus*. This strain was identified as a new isolate of *R. massiliae*. No *R. conorii* was found in this study despite a high seroprevalence of MSF in humans (46%) in Greece.

Further attempts to isolate a larger number of strains from ticks and human patients are currently underway in our laboratory to get a better knowledge of the epidemiologic situation of rickettsiae in Catalonia.

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