

## GENOTYPIC IDENTIFICATION OF AN UNDESCRIBED SPOTTED FEVER GROUP *RICKETTSIA* IN *IXODES RICINUS* FROM SOUTHWESTERN SPAIN

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**Abstract.** An undescribed rickettsia was directly analyzed with specific rickettsial molecular biology tools on *Ixodes ricinus* L. collected in different localities of the province of Cadiz (southwestern Spain). On the basis of the results of the citrate synthase (*glta*) gene, 190 kD-outer membrane protein (*rOmpA*) gene, and 16S ribosomal RNA (16S rRNA) gene partial sequence data, it was found that this rickettsia is sufficiently genetically distinct from other *Rickettsia* to be considered a distinct taxonomic entity. The isolation and culture of this organism, as well as comparative antigenic analysis, are required to ensure its conclusive taxonomic placement among spotted fever rickettsiae. The epidemiologic role of this new rickettsial agent and its possible pathogenicity to wild and domestic animals or humans is still unknown and needs to be investigated.

Rickettsiae are gram-negative bacteria that multiply only inside host cells and require arthropods either as reservoirs or as vectors.<sup>1</sup> The bacterial genus *Rickettsia* is traditionally divided into three biotypes: the spotted fever group (SFG), the typhus group, and the scrub typhus group, based on vector host and antigenic cross-reactivity.

Some *Rickettsia* infect lice, fleas, and mites, but most are associated with various species of Ixodid ticks. As a first approach, field studies oriented to the detection of rickettsial and rickettsial-like organisms in ticks and other arthropods, and the subsequent precise identification of these bacteria are of critical importance in understanding the epidemiology, natural history, and potential threat to human health, along with a reconsideration of established components of the vector-reservoir cycle.<sup>2,3</sup>

Until the mid-1980s, the identification of new species of rickettsia was a tedious and time-consuming process that required successful propagation of the organism in a cell culture system to characterize such parameters as growth and cytopathology.<sup>4</sup> In the first trials, the identification of *Rickettsia* harbored by individual ticks was made using indirect microimmunofluorescent serologic typing<sup>5,6</sup> and microagglutination<sup>7</sup> after isolation of the strain, and comparing the isolate with prototype strains belonging to known rickettsia species. Recent advances in molecular biology, and the availability of amplification, restriction, and sequencing techniques have determined the development of new detection and characterization tests concerning rickettsiae. In this regard, the analysis of restriction endonuclease digest of DNA from purified rickettsiae has been used primarily for its genotypic identification and estimation of intraspecies divergence,<sup>8–11</sup> and secondarily for the detection of such rickettsiae in different vectors (ticks,<sup>12–14</sup> fleas,<sup>15,16</sup> or mites<sup>17</sup>) and humans.<sup>3,18,19</sup>

Analysis of ribosomal gene sequences has proven to be useful for identifying genotypic relationships between major groups of rickettsia-like organisms,<sup>20</sup> differentiating at a specific level between recognized and unrecognized species of the genus *Rickettsia*,<sup>21–23</sup> despite the extreme sequence conservation observed among these microorganisms. In this way, a consensus sequence polymerase chain reaction (PCR) relies on the use of highly conserved and specific DNA sequences, such as ribosomal 16S rRNA gene sequences, and

has been demonstrably useful in amplifying DNA from as yet undiscovered though related organisms.<sup>24,25</sup>

Rickettsiosis are zoonoses limited geographically by the distribution area of their infected vector. *Ixodes ricinus* is an exophilic, three-host tick widely distributed in European countries. To date, only one species of *Rickettsia* (*R. helvetica*, formerly called Swiss agent), has been isolated from *I. ricinus* ticks in Switzerland and Sweden.<sup>26–31</sup> Recently, the presence of *Ehrlichia phagocytophila* was reported in *I. ricinus* in Switzerland,<sup>32</sup> and *Coxiella burnetii* and an undefined rickettsial organism were isolated from *I. ricinus* in Austria.<sup>33</sup>

### MATERIALS AND METHODS

**Collection of ticks.** Adult *I. ricinus* were obtained from red deer (*Cervus elaphus*) hunted in woodland areas of Ubrique, Alcalá de los Gazules, Jimena de la Frontera and Los Barrios (Cadiz Province, Spain) (Figure 1) during the winters of 1995–1996 and 1996–1997, and classified by an entomologist (FJM).

**Extraction of DNA.** Individual *I. ricinus* adults were crushed in 340  $\mu$ l of lysis buffer (100 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM EDTA), added to 40  $\mu$ l of 10% sodium dodecyl sulfate and 20  $\mu$ l of proteinase K (Promega, Madison, WI) (50 mg/ml), and maintained in a bath preset at 55°C for 1 hr. After incubation, samples were extracted with phenol-chloroform as described elsewhere.<sup>34</sup> Nucleic acids were precipitated adding 0.1 volumes of potassium acetate solution (5 M based on acetate), 2.5 volumes of absolute ethanol, and chilling to -20°C.

**Polymerase chain reaction–restriction fragment length polymorphism (RFLP) method.** Amplification was assayed using three sets of primers: RpCS primer set (RpCS.877p and RpCS.1258n) and Rr190 primer set (Rr190.70p and Rr190.602n) derived from the sequences of *R. prowazekii* and *R. rickettsii* respectively,<sup>8</sup> and the primer pair derived from the 120-kD outer membrane protein (*rOmpB*) gene of *R. rickettsii* (BG 1-21 and BG 2-20).<sup>10</sup> The sequences and orientations of these primers (Boehringer, Mannheim, Germany) are described in Table 1.

A 100- $\mu$ l reaction mixture, which contained 1  $\mu$ l of sample, 56  $\mu$ l of distilled water, 10  $\mu$ l of 10 $\times$  *Taq* polymerase

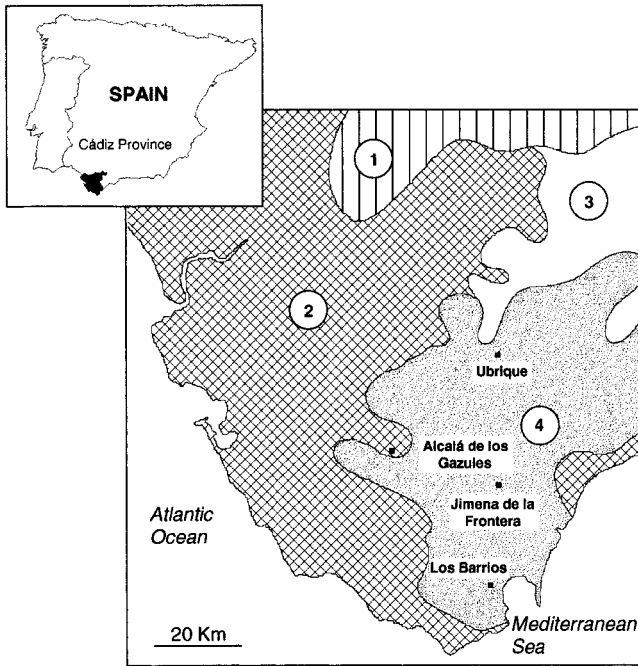


FIGURE 1. Geographic localization of the sites in the province of Cádiz, Spain where *Ixodes ricinus* were collected. Numbered areas correspond to very dry (1), dry (2), subhumid (3), and humid (4) climate areas.

buffer (Promega), 8 µl of 25 mM MgCl<sub>2</sub>, 20 µl of 1 mM dNTPs, and 0.5 U of *Taq* DNA polymerase (Promega), was prepared. Each of the 35 cycles of amplification consisted of denaturation at 95°C for 20 sec, annealing at 48°C for 30 sec, and sequence extension at 72°C for 2 min.<sup>8,10</sup> All amplification reactions were conducted in a DNA Thermocycler 9600 (Perkin Elmer, Norwalk, CT), using thin-wall microamp<sup>®</sup> tubes (Perkin Elmer). After amplification, 7 µl of the PCR mixture was electrophoresed for 1 hr at 7 V/cm in a 4% Nusieve<sup>®</sup> GTG agarose (FMC Bioproducts, Rockland, ME) gel in 1 × TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA), using the molecular weight marker VI (MWM VI) (Boehringer Mannheim) as a control, stained with ethidium bromide, and observed under ultraviolet light illumination.

Samples for RFLP analysis were prepared by digesting 15 µl of the PCR mixture containing the amplified fragment with the appropriate restriction endonuclease (Promega)<sup>8</sup> for the citrate synthase (*gltA*) and the outer membrane protein (*rOmpA*) genes.

**Sequencing.** Amplification of 5' region of the 16S rRNA gene was made using the primers fD1<sup>20</sup> and Rc16S.452n. To ensure the uniqueness of the Rc16S.452 primer, the 16S rRNA sequence of *R. canada* (GenBank accession numbers L36104 and U15162) was compared by homology with other GenBank related sequences using the FASTA algorithm of the Genetic Computer Group (GCG) (University of Wisconsin, Madison, WI) program, allowing for three or less mismatches. The *Rickettsia*-exclusive sequences were selected from the FASTA alignments file and the candidate primers were tested for exclusivity to SFG rickettsiae using the FINDPATTERNS algorithm from GCG. The PCR was per-

TABLE 1  
Oligonucleotide primers used for genotypic identification of rickettsial species\*

Species	Gene	Primer	Nucleotide sequence (5' → 3')	Amplified fragment size (bp)	Authors and reference
<i>R. prowazekii</i>	CS	RpCS.877p	GGGGCCTGCTCAGCGGG	381	Regnery and others <sup>8</sup>
		RpCS1258n	ATTGC AAAAGTACAGTGAACA		
<i>R. rickettsii</i>	120-kD genus common antigen (rompB)	RrBG.1-21	GGCAATTAATCGCTGACGG	650	Anderson and others <sup>39</sup>
		RrBG.2-20	GCACTGCACTAGCACATTC		
	190-kD protein antigen (rompA)	Rr190.70p	ATGGCGAATATTTCCAAAA	532	Regnery and others <sup>8</sup>
		Rr190.602n	AGTGCAGCATTCCGCCCT		
Protobacteria	16S rRNA	fD1	AGAGTTTGATCCTGGCTCAG	426	Weisburg and others <sup>20</sup>
<i>R. canada</i>		Rc16S.452n	AACGTCATTATCTTCCCTGC		This report

\* bp = basepairs; CS = citrate synthase (*gltA*).







"Cadiz agent"	GIKTDLFTTSTAALMLSSSGALGVAA-GDLFTINDAASFSDLAAAGNWNKITAGGVANGTSDVDPQDNKAFYYGGA
<i>R. montana</i>	.L.AA.....I.....F..V...VIS-N.....N..VN...E..V...T...PA...R..V.....D
Astrakhan	.L.AA.....I.....VIA.N.N.....NVGNN...E..A..T...PAGS..N.W.....D
Israeli	.L.AA.....I.....VIA.N.N.....NVGNN...E..A.....VAG..N.W.....D
<i>R. africae</i>	.L.AA.....I.....VIA.N.N.....NVGNN...E..A.....NPAGS..N.W.....D
BAR-29	.L.AA.....I.....V...V...VIS.N.N.....V.N...D...Q...P.....NGM.....D
<i>R. conorii</i> #1	.L.AA.....I.....I.VS.VIA.N.N.....NVGNN...E..A.....PAH..N.W.....D
<i>R. conorii</i> #2	.L.AA.....I.....I.VS.VIA.N.N.....NVGNN...E..A.....PAR..N.W.....D
<i>R. conorii</i> #3	.L.AA.....I.....I.VS.VIA.N.N.....NVGNN...E..A.....PAR..N.W.....D
HA-91	.L.AA.....I.....VIA.N.N.T...NVGNN...E..A.....APAG..N.W.....D
<i>R. japonica</i>	.L.AA.....I.....VIA.N.....ND...N...Q...R.E.D.NPAG..N.W.....D
<i>R. massiliae</i>	.L.AA.....I.....V...V...VIS.N.N.....V.N...D...Q...P.....NGM.....D
MC16	.L.AA.....I.....VVSNN.N.....N...D...E..I...P.....NGM.....D
<i>R. parkeri</i>	.L.AA.....I.....VIA.N.N.....DVNNN...SE..A.....VPAGS..N.W.....D
<i>R. rhipicep.</i>	.L.AA.....I.....V...VIS.N.N.....V.N...D...K...P.....NGM.....D
<i>R. rickettsii</i>	.L.AA.....I.....T.VIA.N.N.....NVGNN...E..A.....PAG..N.W.....D
Rick. S	.L.AA.....I.....VIA.N.N.T...NVGNN...E..A.....NPAG..N.W.....D
<i>R. sibirica</i>	.L.AA.....I.....VIA.N.N.I...NVGNN...E..A.....PAG..N.W.....D
<i>R. slovacica</i>	.L.AA.....I.....VIA.N.N.....NVGNN...E.....PAGS..N.W.....D
Thai	.L.AA.....I.....T.VIA.N.N.....NVGNN...E..A.....NPAGS..N.W.....D
"Cadiz agent"	HIITADEVGRITAINVAATNPIGIKIAGNTSVGSIVTDRNLLPVNITAGKSLTLTGTAAFVPRHGVGAFADTY
<i>R. montana</i>	.T...K.....G.T.V.LD.TQ..V.....GG.....T.I.....N.AN.I.AN..FD.P..N.
Astrakhan	YT...AAD.....D.T.V.LN..Q..V.....RG.....T.....N.NN.VAAN..F..P..N.
Israeli	YT...VAD.....D.T.V.LN.VQ..V.....RG.....T.....N.NN.VAAN..F..P..N.
<i>R. africae</i>	YT...AAD.....G.T.V.LN..Q..V...I.GG.....T.....N.NN.VAAN..FD.P..N.
BAR-29	.T...A.....G.T.V.LN.TQ..V.....GG.....T.....N.TN.VAAN..FD.P..N.
<i>R. conorii</i> #1	YT...VADH.....D.T...LN..Q..V.....GG.....T.....N.NN.DAAN..F..P..N.
<i>R. conorii</i> #2	YT...VADH.....D.T...LN..Q..V.....GG.....T.....N.NN.DAAN..F..P..N.
<i>R. conorii</i> #3	YT...VADH.....D.T...LN..Q..V.....GG.....T.....N.NN.DAAN..F..P..N.
HA-91	YT...AAD.....G.T.V.LD..Q..V...I.GG.....T.....N.NN.VAAN..FD.P..N.
<i>R. japonica</i>	YT...V.NC.K...N...V.LN..Q..V...I.GG.....T.....N.NN.VAAN..FN.P..N.
<i>R. massiliae</i>	.T...A.....G.T.V.LN.TQ..V.....GG.....T.....N.TN.VAAN..FD.P..N.
MC16	.T...A.....G.T.V.LN.TQ..V.....GG.....T.....N.TN.VAAN..FD.P..N.
<i>R. parkeri</i>	YT...AAD.....G.T.V.LN..Q..V...I.GG.....T.....N.NN.VAAN..FD.P..N.
<i>R. rhipicep.</i>	.T...A.C.....G.T.V.LN.TQ..V.....GG.....T.....N.TN.VAAN..FD.P..N.
<i>R. rickettsii</i>	YTV...AADR..K...G.T.V.LN.TQ..V...I.KG.....TLN.....N.NN.VAAN..FD.P..N.
Rick. S	YT...AAD.....G.T.V.LN..Q..V...I.GG.....T.....N.NN.VAAN..FD.P..N.
<i>R. sibirica</i>	YT...AAD.....G.T.V.LN..Q..V...I.GG.....T..V.....N.NN.VAAN..FD.P..N.
<i>R. slovacica</i>	YT...AAD.....G.T.V.LN..Q..V...I.RG.....T.N.....N.NN.VAAN..FDSP..N.
Thai	YT...AAD.....G.T.V.LNV.Q..V...I.RG.....T.....N.TN.VAAN..FDAP.NS.

FIGURE 5. Alignment of the deduced amino acid sequence of the 190-kD outer membrane protein (*rOmpA*) gene fragment between positions 25 and 468 (*Rickettsia rickettsii* numbering in GenBank accession number U43804). Amino acids are conserved between sequences (.) except where indicated. Amino acid deletions are indicated (-). Accession numbers of GenBank deposited sequences are U43801 (*R. montana*), U43791 (Astrakhan rickettsiae), U43797 (Israeli tick typhus), U43790 (*R. africae*), U43792 (BAR-29), U43794 (*R. conorii* #1), U43798 (*R. conorii* #2), U45244 (*R. conorii* #3), U43796 (*Rickettsia* HA-91), U43795 (*R. japonica*), U43793 (*R. massiliae*), U43800 (*Rickettsia* MC16), U43802 (*R. parkeri*), U43803 (*R. rhipicephali* [*rhhipicep.*]), U59722 (*Rickettsia* sp. #2), U43794 (*R. conorii*), U59732 (*R. parkeri*), U43804 (*R. rickettsii*), U43805 (*Rickettsia* S), U43807 (*R. sibirica*), U43808 (*R. slovacica*), and U43809 (Thai tick typhus).

gene in *I. ricinus* infected with *R. helvetica* collected in Switzerland.<sup>30</sup>

Ereemeva and others<sup>10</sup> pointed out that different methods of identification of rickettsiae (analysis of antigenic diversity, restriction profiles in pulsed-field electrophoresis, or PCR/RFLP) gave the same results, mainly because these identification methods are based on the analysis of the major surface proteins of the rickettsiae, *rOmpA* and *rOmpB*, which are extensively implicated in the antigenic properties of different species and strains, and thus in the protective immune response of the vertebrate host.<sup>21, 39, 40</sup>

Throughout the study, this new *Rickettsia* has differed considerably from other known rickettsiae. We believe that it represents a new genotype, with notable differences in the 16S rRNA sequence,<sup>21-23</sup> in the *rOmpA* PCR/RFLP pattern, in partial sequence data, and in the deduced protein sequences. Nonetheless, it must be included in the SFG by virtue of similarities inferred from the partial sequence of 16S rRNA and *gltA* genes and the electrophoretic band pattern obtained from the restriction of the PCR-amplified *gltA* fragment, according to criteria previously proposed.<sup>8, 10</sup> Extending the discussion initiated by Beati and others,<sup>30</sup> the failure to amplify the 190-kD (*R. akari*, *R. australis*, *R. bellii*, and *R.*

*helvetica*) and 120-kD (*R. akari*, *R. bellii*, *R. helvetica*, and *R. massiliae*) outer membrane proteins may imply that the major differences among the SFG *Rickettsia* are located in the outer membrane surface proteins. The *rOmpA* sequences indicate that this new species is different from other known SFG rickettsiae.

The Cadiz mountains are the wettest areas of the Iberian Peninsula, with annual rainfall reaching 1,200 mm per year at the Ubrique Meteorological Station, and they have a temperate climate (with an average maximum temperature of 15.7°C and an average minimum temperature of 4.3°C) that is very propitious to the successful growth of an isolated *I. ricinus* population.

The epidemiologic role of the Cadiz agent and its possible pathogenicity requires confirmation. As for *R. helvetica*,<sup>29</sup> low heterologous cross-reactive titers to sera derived from natural and experimental infection caused by this new *Rickettsia* could be expected. Responses to these rickettsiae may confuse the interpretation of serologic tests in patients from the province of Cadiz and contiguous areas who are suspected of having Mediterranean spotted fever, particularly if the high number of bites due to *I. ricinus* in humans and the main role played by this tick as the vector of Lyme borre-

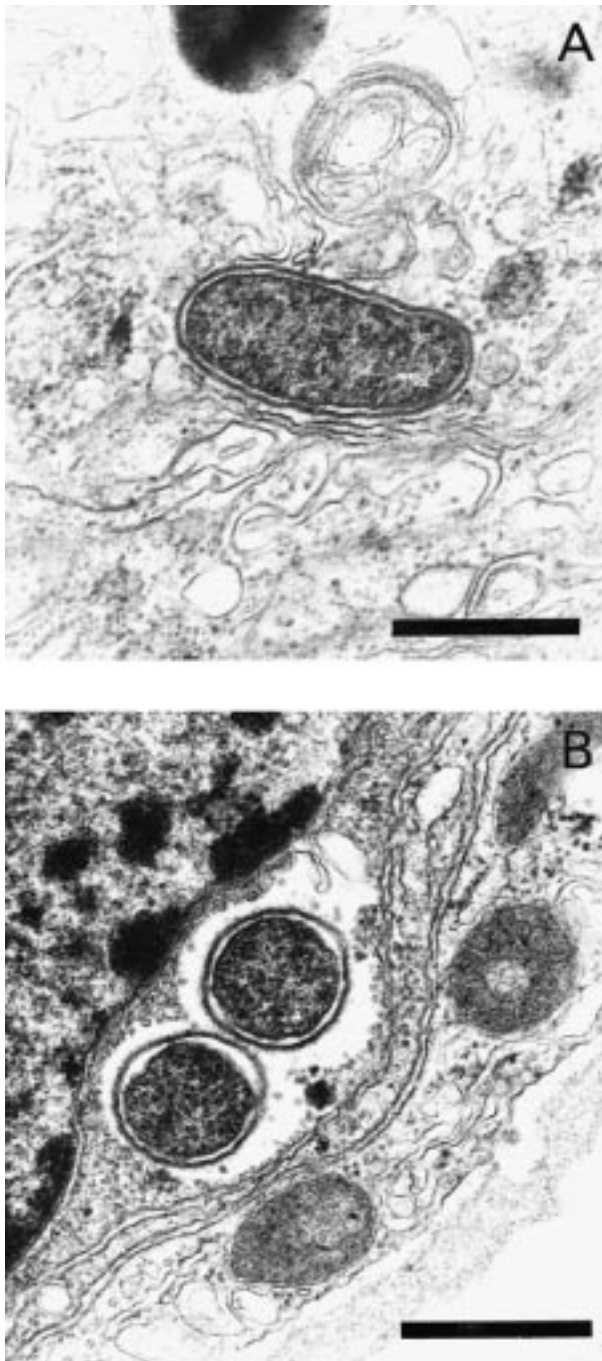


FIGURE 6. Electron micrographs of rickettsiae-infected *Ixodes ricinus* tissues. **A**, longitudinal section of a rickettsiae living free in the cytoplasm of a *Ixodes ricinus* cell. **B**, transversal sections of two rickettsiae associated with the endoplasmic reticulum. Bars = 0.5  $\mu$ m.

liosis in Europe are considered. In addition, immunologic characterization must be carried to conclude that this rickettsia is a new species.

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