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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.[1] 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.[2, 3]

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.[4] In the first trials, the identification of *Rickettsia* harbored by individual ticks was made using indirect microimmunofluorescent serologic typing[5, 6] and microagglutination[7] 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,[8–11] and secondarily for the detection of such rickettsiae in different vectors (ticks,[12–14] fleas,[15, 16] or mites[17]) and humans.[3, 18, 19]

Analysis of ribosomal gene sequences has proven to be useful for identifying genotypic relationships between major groups of rickettsia-like organisms,[20] differentiating at a specific level between recognized and unrecognized species of the genus *Rickettsia*,[21–23] 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.[24, 25]

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.[26–31] Recently, the presence of *Ehrlichia phagocytophila* was reported in *I. ricinus* in Switzerland,[32] and *Coxiella burnetii* and an undefined rickettsial organism were isolated from *I. ricinus* in Austria.[33]

MATERIALS AND METHODS

Collection of ticks. Adult *I. ricinus* were obtained from red deer (*Cervus elaphus*) hunted in woodland areas of Ubrique, Alcala 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 μl of lysis buffer (100 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM EDTA), added to 40 μl of 10% sodium dodecyl sulfate and 20 μl 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.[34] 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,[8] and the primer pair derived from the 120-kD outer membrane protein (*rOmpB*) gene of *R. rickettsii* (BG 1-21 and BG 2-20).[10] The sequences and orientations of these primers (Boehringer, Mannheim, Germany) are described in Table 1.

A 100-μl reaction mixture, which contained 1 μl of sample, 56 μl of distilled water, 10 μl of 10× *Taq* polymerase

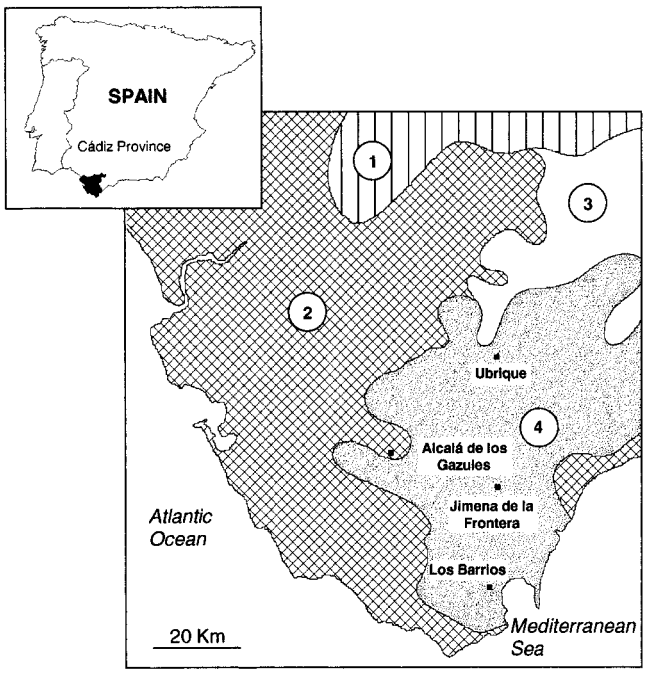

FIGURE 1. Geographic localization of the sites in the province of Cadiz, Spain where *Ixodes ricinus* were collected. Numbered areas correspond to very dry (1), dry (2), subhumid (3), and humid (4) climate areas.

buffer (Promega), 8 μl of 25 mM $MgCl_2$, 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.[8,10] All amplification reactions were conducted in a DNA Thermocycler 9600 (Perkin Elmer, Norwalk, CT), using thin-wall microamp® tubes (Perkin Elmer). After amplification, 7 μl of the PCR mixture was electrophoresed for 1 hr at 7 V/cm in a 4% Nusieve® 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)[8] 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[20] 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
R. prowazekii	CS	RpCS.877p	GGGGGCCTGCTCACGGCGG	381	Regnery and others[8]
		RpCS1258n	ATTGCAAAAAGTACAGTGAACA		
R. rickettsii	120-kD genus common antigen (rompB)	RrBG.1-21	GGCAATTAATATCGCTGACGG	650	Anderson and others[39]
		RrBG.2-20	GCATCTGCACTAGCACTTTC		
	190-kD protein antigen (rompA)	Rr190.70p	ATGGCGAATATTTCTCCAAAA	532	Regnery and others[8]
		Rr190.602n	AGTGCAGCATTCGCTCCCCCT		
Protobacteria	16S rRNA	fD1	AGAGTTTGATCCTGGCTCAG	426	Weisburg and others[20]
R. canada		Rc16S.452n	AACGTCATTATCTTCCTTGC		This report

* bp = basepairs; CS = citrate synthase (*glta*).

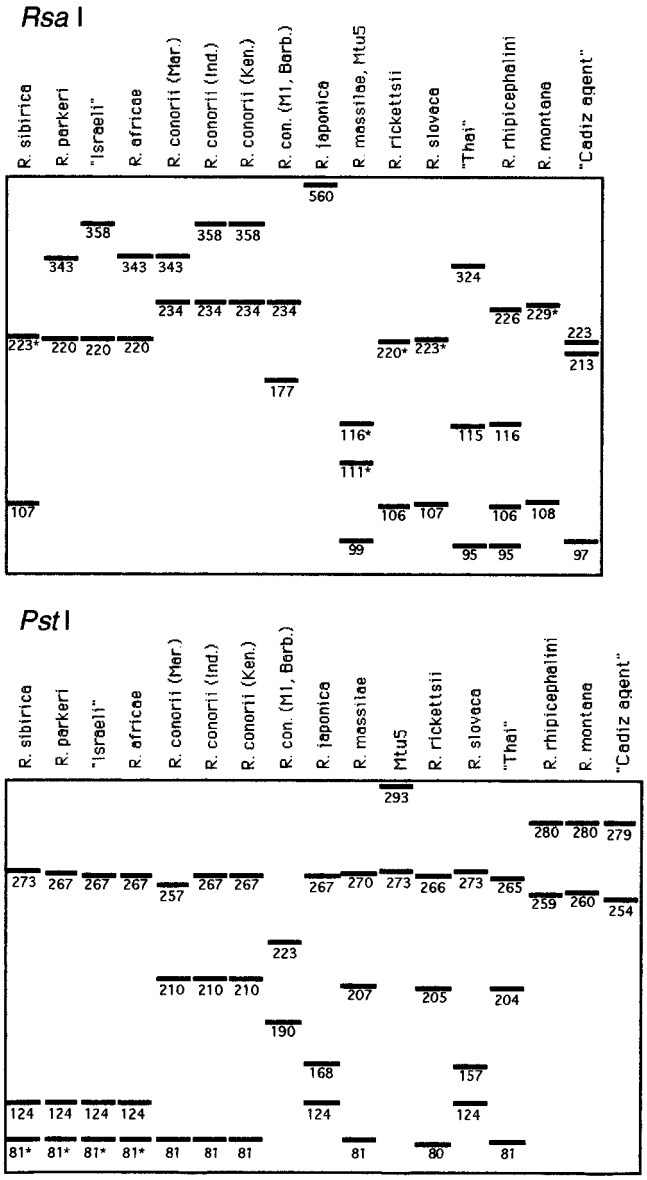

FIGURE 2. Electrophoretic migration patterns of polymerase chain reaction–amplified rickettsial DNA with the Rr 190.70p and Rr 190.602n primer pair digested with the restriction endonucleases *Rsa* I and *Pst* I. The schematic profiles of the other species of *Rickettsia* were adopted from Regnery and others[8] and Eremeeva and others.[10] Mar. = Morocco strain; Ind. = India strain; Ken. = Kenya strain; con = *conorii;* Barb. = Barbados strain. * = double band.

formed for 30 cycles in a reaction mixture at 95°C for 20 sec, 59°C for 30 sec, and 72°C for 45 sec.

The specific primers assayed were used to sequence the amplified fragments of *glta, rOmpA,* and a 5' portion of the 16S rRNA genes of the rickettsiae detected in the infected ticks. Thermal cycling conditions and product observation were as described in the above protocols.

After amplification, primers and nucleotides were removed from 300 μl of PCR products by purification on the Wizard™ PCR preps DNA purification system (Promega), according to the manufacturer's protocol. The remaining DNA was eluted in 50 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.6). In this case, the amount of DNA obtained was quantified in a 4% Nusieve® GTG agarose gel in 1 × TAE buffer by comparing fluorescence emission with 1 μg of MWM VI. Approximately 100 fmol of the purified PCR product (4–5 μl) was used directly in the sequencing reaction.

The PCR cycle sequencing was performed (Silver sequence™ DNA Sequencing System; Promega) for each amplicon using the correct forward or reverse primers. Four reactions of 9 μl containing 3 μl of the appropriate termination mixture and 6 μl of the sample reaction mixture were made (100 fmol of purified product of the PCR, 1.8 μl of 5× DNA sequencing buffer [75 mM Tris-HCl, pH 9.0, 3 mM $MgCl_2$], 6.75 pmol of respective primer and 0.25 U of *Taq* DNA polymerase sequencing grade). Tubes were preheated to 95°C for 2 min and subjected to linear amplification for 55 cycles at 95°C for 30 sec, 42°C or 59°C depending on the calculated melting temperature of the primer concerned for 30 sec, and 72°C for 1 min. A volume of 4.5 μl of sequencing stop solution (10 mM NaOH, 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol) was added to each sequencing tube and heated at 65°C for 2 min.

Sequencing reaction products were loaded twice on 40-cm, 6% polyacrylamide, 7 M urea gels by electrophoresis in the Sequi-Gen Nucleic Acid Sequencing System (Bio-Rad, Hercules, CA) at 55 W of constant electrophoresis (55°C) and separated for 4.5 and 2.5 hr, respectively. Gels were fixed in 2 liters of fix/stop solution (10% glacial acetic acid) for 20 min (until diffusion of the tracking marks in the gel occurred), rinsed three times with ultrapure water for 2 min, stained with 2 liters of 0.1 % $AgNO_3$ and 0.055% formaldehyde for 30 min, rinsed for 5 sec with ultrapure water, developed with 2 liters of 0.14 M sodium carbonate, 0.055% formaldehyde, and 4 mg of sodium thiosulfate, and chilled to 8–10°C (8–12 min) until the bands appeared. To terminate the developing reaction, an equal volume of fix/stop solution was added. Finally, the gel was rinsed twice with ultrapure water and completely dried in an oven at 45°C. A permanent record was made using APC film (Promega) exposed to overhead fluorescent lighting. Development of APC film was carried out according to the manufacturer's protocol.

For electron microscope studies, infected *I. ricinus* females were dissected in a mixture of 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M cacodylate buffer, postfixed in a 2% osmium tetroxide solution, dehydrated in increasing concentrations of ethanol, and embedded in the resin Epon 812. Ultrathin sections were cut by ultramicrotome (model Ultracut S; Reichert-Jung, Vienna, Austria), mounted on grids, stained with uranyl acetate and lead citrate, and examined by transmission electron microscope (model EM10C; Carl Zeiss, Oberkochen, Germany) at 80 kV.

RESULTS

Preliminary detection and identification of SFG rickettsia in *I. ricinus* adults was performed using PCR/RFLP with specific rickettsial molecular biology tools. We attempted to amplify the *glta* gene with rickettsial genus-specific primers, and the *rOmpA* and *rOmpB* genes with SFG-specific primers.

The RpCS.877p-RpCs1258n (CS) and Rr190.70p-

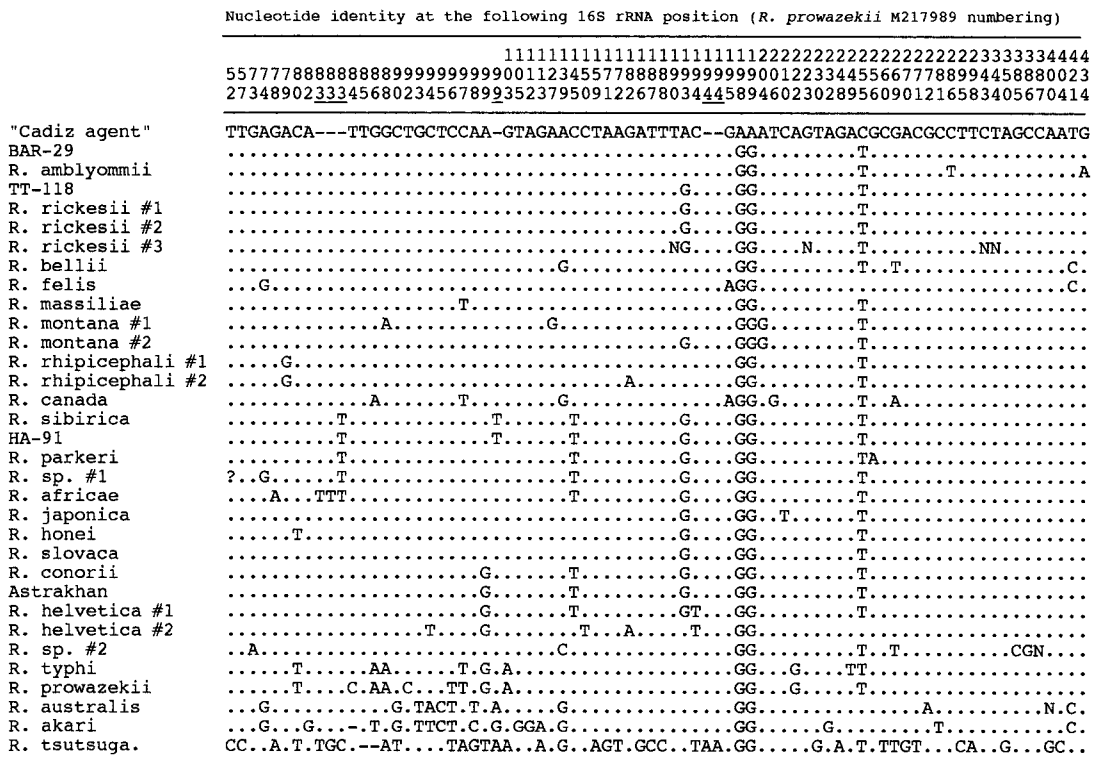

FIGURE 3. Nucleotide identities of 16S rRNA variable and signature positions in *Rickettsia* species in the sequenced window. Nucleotides are conserved between sequences (.) except where indicated. Deletion of nucleotides (-) are indicated. Accession numbers of GenBank deposited sequences are L36102 (*Rickettsia* BAR- 29), U11012 (*R. amblyommii*), L36220 (*Rickettsia* TT-118), L36217 (*R. rickettsii* #1), U11021 (*R. rickettsii* #2), M21293 (*R. rickettsii* #3), U11014 (*R. bellii*), L28944 (*R. felis*), L36214 (*R. massiliae*), L36215 (*R. montana* #1), U11016 (*R. montana* #2), L36216 (*R. rhipicephali* #1), U11019 (*R. rhipicephali* #2), L36104 (*R. canada*), L36218 (*R. sibirica*), L36219 (*Rickettsia* HA-91), L36673 (*R. parkeri*), U25042 (*Rickettsia* sp. #1, strain S), L36098 (*R. africae*), L36213 (*R. japonica*), U17645 (*R. honei*), L36224 (*R. slovaca*), U12460 (*R. conorii*), L36100 (Astrakhan fever rickettsiae), L36223 (*R. helvetica* #1), L36212 (*R. helvetica* #2), U04163 (*Rickettsia* sp. #2, from *Adalia bipunctata*), L36221 (*R. typhi*), M21789 (*R. prowazekii*), U12459 (*R. australis*), U12458 (*R. akari*), and L36222 (*R. tsutsugamushi* [*tsutsuga.*]).

Rr190.602n (*rOmpA*) DNA fragments were amplified. The *rOmpB* was not amplified with the RrBG.1-21-RrBG2-20 primer pair. The impossibility of amplifying this fragment in *R. helvetica, R. akari, R. bellii,* and *R. massiliae* was demonstrated by Eremeeva and others[10] using the same primer sequences and thermocycling conditions.

Restriction of the *glta* fragment with *Alu* I results in five migrating bands with sizes ranging from 43 (double band), 84, 91, and 124 basepairs (bp), corresponding to four *Alu* I sites, as previously described by Eremeeva and others[10] for the species relating to SFG. The amplified DNA fragment from *rOmpA* has two *Rsa* I sites and one *Pst* I site, and was cleaved by these two enzymes into fragments of 223, 213 and 97 bp, and 279 and 254 bp, respectively. A comparison with the schematic electrophoretic migration patterns on the same PCR amplified and restricted fragments as stated previously[8, 10] shows a characteristic pattern in the Cadiz agent, the new rickettsial agent detected in *I. ricinus* from the Cadiz hills (Figure 2).

Sequence data from the 5' end of a 16S rRNA gene partially sequenced over a total of 392 nucleotide positions (sites 51 to 442, numbered according to the *R. prowazekii* GenBank accession number M21789) shows a high nucleotide identity between the Cadiz agent and other recognized species and strains of *Rickettsia.* Because of the extreme conservation of 16S rRNA among members of the genus (less than 3% sequence divergence), which does not permit a phylogenetic inference based on this gene alone,[4] several differences were observed when this fragment was aligned with homologous rickettsiae fragments deposited in the GenBank database (Figure 3).

Sequencing of the *glta* gene fragment. The *glta* fragment was sequenced (Figure 4A) and compared with equivalents in other known *Rickettsia.*[35] The new rickettsial agent has a sequence that resembles those of *R. helvetica* and *R. canada* (Figure 4B), differing in only five and 12 nucleotide mutations respectively. This results in one amino acid change in the translation of the sequenced fragment.

Protein translation of the *rOmpA* sequence data (Figure 5) compared with sequence information available in GenBank[36] demonstrates an inordinate genetic distance in this gene between the Cadiz agent and other reported rickettsiae species, with a minimum of 20 exclusive changes over 149 amino acid positions considered.

The nucleotide sequence data reported in this paper will appear in the European Molecular Biology Laboratory, Genbank, and nucleotide sequence databases under the accession numbers Y08783, Y087784, and Y08785 for 16S rRNA, *glta,* and *rOmpA* genes, respectively. The alignments of 16S rRNA,

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[GGGGGCCTGC TCACGGCGG]G GCTAATGAAG CGGTAATAAA TATGCTTAAA GAAATTGGTA
GTTCTGAGAA TATCCCTAAA TATATAGCTA AAGCTAAGGA TAAAAATGAT CCGTTTAGGT
TAATGGGCTT CGGTCATCGT GTATATAAAA ACTATGACCC ACGTGCCGCA GTACTTAAAG
AGACGTGTAA GGAAGTATTA AAGGAACTCG GACAGCTAGA AAACAATCCG CTCTTACAAA
TAGCAATAGA ACTTGAAGCT ATCGCTCTTA AAGATGAATA TTTTATTGAG AGAAAATTAT
ATCCAAATGT TGATTTTTAT TCGGGTATTA TCTATAAAGC TATGGGTATA CCGTCGCAAA
[TGTTCACTGT ACTTTTTGCA AT]
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A

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                 Nucleotide identity at the following CS position (R. prowazekii U59715 numbering)
                 ____________________________________________________________________________________________
                                               11111111111111111111111111111111111111111111111111111111111111
                 89999999999999999999999999999900000000000000000000000000000000001111111111111111112222222222
                 90011233334444556677778888889900011122333344444566666778888999990000001223345689990011223333
                 56706214890349794936790156784503656867035623478703689280247167892456891066843531381736891345
                 ____________________________________________________________________________________________
"Cadiz agent"    GCGATTTTTCTGACAATCGTAAAATCCGGTGCCTGAACTCCACGCTAGGTGAAAGACCACAAACTCGCCTAAAACTAGTTTGTCATGTGAAA
R.helvetica      ..............................A.......CT.......AT...........................................
R.canada         ..................A...G.C..A.......C.......T...AT.C...............T.......T.............A...
R.massiliae      .T....C...A.TT.............A...TT.A......G.....A.CA.......GTC...............................
Bar 29           .T....C...A.TT.............A...TT.A......G.....A.CA.......GTC...............................
R.rhipicephali   .T....C...A.TT.............A...TT.A......G.....A.CA.......G.C...............................
R.sp. #2         .T....C.....TT.............A...TT........G.....A.CA.......G.C...............................
R.africae        ......C.....TT.............AA..TT........G.....A.CA.......G.C...............................
R.conorii        ......C.....TT.............AA..TT........G.....A.CA.......G.C......T........................
R.parkeri        ......A.....TT.............AA..TT........G.....A.CA.......G.C...............................
R.sibirica       ......C.....TT.............AA..TT........G.....A.CA.......G.C...............................
R.sp. #3         ......C.....TT.............AA..TT........G.....A.CA.......G.C...............................
R.sp. #4         ......C.....TT.............AA..TT........G.....A.CA.......G.C...............................
Astrakhan        ......C.....TT.............AA..TT........G.....A.CA....C..G.C...............................
R.slovaca        ......C.....TT.............AA..TT........G.....A.CA.......G.C.............T.................
Thai             ......C.....TT.............AA..TT........G.....A.CA.......G.C...............................
Israeli          ......C.....TT.............AA..TT........G.....A.CA....C.TG.C...............................
R.japonica       ......C.....TT.............A...TT........G.....A.CA.......G.C.......................G.......
R.ricketsii      ......C.....TT.............AA..TT........G..T..A.CA.......G.C...---A........................
R.australis      ....C.C......AT..T.............T.............C.A.CAC......G....................CCA...G......
R. akari         A...C.C......A.........G.......T...........T...A.CA......TG....T..A.T............A..........
R. felis         A.AG..C......T.................T...T...........ATCA.................T.G..........A.......XXX
R.sp. #1         ...G..C....A.T..................T......CTTT....AT........G.TGGTC..TA...G..A...........A.....
R.prowazekii     ..AG...C.....T.G..A..G.....A...TTA..G....G.....AT.A...T.TATTC..T....G....C...A...AC.........
R. typhi         ..AG.........T.G..AC.......A...TTA..GT...GT....AT.A...T..AGT...T...TG....CT......AC.........
R.bellii         ..AG.GACGTA..A..G.A......AAT.C..T......T.GG...G.CC..GGA.TAG.T...C.ATAC.GG...G.C....T.GCC..G.
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B

FIGURE 4. **A,** partial nucleotide sequence of the Cadiz agent citrate synthase (*glta*) gene fragment. Boxed letters represent the 5' and complementary 3' primer sequences described in Table 1. **B,** nucleotides identities of *glta* gene fragment variable and signature positions in *Rickettsia* species in the sequenced window. Nucleotides are conserved between sequences (.) except where indicated. Deletion of nucleotides (-) is indicated. Accession numbers of GenBank deposited sequences are U59723 (*R. helvetica*), U20241 (*R. canada*), U59719 (*R. massiliae*), U59720 (BAR-29), U59721 (*R. rhipicephali*), U59722 (*Rickettsia* sp. #2), U59733 (*R. africae*), U20243 (*R. conorii*), U59732 (*R. parkeri*), U59734 (*R. sibirica*), U59731 (*Rickettsia* sp. #3), U59735 (*Rickettsia* sp. #4), U59728 (Astrakhan rickettsiae), U59725 (*R. slovaca*), U59726 (Thai tick typhus), U59727 (Israeli tick typhus), U59724 (*R. japonica*), U59729 (*R. rickettsii*), U33923 (*R. australis*), U41752 (*R. akari*), U33922 (*R. felis*), U59712 (*Rickettsia* sp. #1), U59715 (*R. prowazekii*), U20245 (*R. typhi*), and U59716 (*R. bellii*).

glta, and *rOmpA* genes considered in this study should be requested on the e-mail address fmarquez@cica.es.

Ultrastructure. *Rickettsia* were surrounded by two membranes, an inner cytoplasmic membrane and an outer cell wall membrane, with the latter having a dense, rippling appearance (Figure 6).

DISCUSSION

The PCR-RFLP analysis has been shown to be a rapid and adequate method for the direct detection of the presence of SFG rickettsiae in tick vectors and for the identification and classification of such rickettsiae.[12–14, 30, 37, 38] These investigators successfully used specific pairs of primers derived from *R. prowazekii* and *R. rickettsii* that amplified a part of the rickettsial genome that has a degree of species specificity among the rickettsiae (*rOmpA* and *rOmpB*) or which provides group specificity (*glta*). The 16S rRNA gene partial sequence data indicates that this new rickettsiae is a member of the SFG (Figure 3). The similarities in the *Alu* I restriction fragments pattern observed in the *glta* gene of different SFG species can be explained by the sequence conservation of this gene in relation to the main metabolic role of this rickettsial enzyme.

The fact that *rOmpA* was not amplified in *R. helvetica, R. akari, R. australis,* and *R. bellii* with the Rr 190 pair primers[10] might correlate with the antigen pattern obtained in Western blotting of these species.[29] Differences observed in the *rOmpA* fragment sequence in the Cadiz agent may be explained as a consequence of the genetic drift of this gene, rather than as due to external contamination of our samples, as is corroborated by the unsuccessful amplification of this

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"Cadiz agent" GIKTDLFTTSTAALMLSSSGALGVAA-GDLFTINDAAFSDLAAAGNWNKITAGGVANGTSVDGPQDNKAFTYGGA
R. montana    .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
R. africae    .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
R.conorii #1  .L.AA.....I.....I.VS.VIA.N.N.....NVGNN...E...A.....PAH...N.W.....D
R.conorii #2  .L.AA.....I.....I.VS.VIA.N.N.....NVGNN-..E...A.....PAR...N.W.....D
R.conorii #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
R.japonica    .L.AA.....I.....VIA.N.....ND...N...Q...R.E.D.NPAG...N.W.....D
R.massiliae   .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
R.parkeri     .L.AA.....I.....VIA.N.N.....DVNNN..SE...A.....VPAGS..N.W.....D
R.rhipicep.   .L.AA.....I.....V..VIS.N.N.....V.N...D...K.....P.....NGM.....D
R.ricketsii   .L.AA.....I.....T..VIA.N.N....NNVGNN...E...A.....PAG...N.W.....D
Rick. S       .L.AA.....I.....VIA.N.N.T...NVGNN...E...A.....NPAG...N.W.....D
R. sibirica   .L.AA.....I.....VIA.N.N.I...NVGNN...E...A.....PAG...N.W.....D
R. slovaca    .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" HIITADEVGRIITAINVAATNPIGIKIAGNTSVGSIVTDRNLLPVNITAGKSLTLTGTAAFVPRHGVGAFADTY
R. montana    .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.
R. africae    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.
R.conorii #1  YT....VADH.....D.T...LN..Q..V.....GG.....T.....N.NN.DAAN..F..P..N.
R.conorii #2  YT....VADH.....D.T...LN..Q..V.....GG.....T.....N.NN.DAAN..F..P..N.
R.conorii #3  YT....VADH.....D.T...LN..Q..V....--------.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.
R.japonica    YT....V.NC..K.....N...V.LN..Q..V....I.GG.....T.....N.NN.VAAN..FN.P..N.
R.massiliae   .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.
R.parkeri     YT....AAD.....G.T.V.LN..Q..V....I.GG.....T.....N.NN.VAAN..FD.P..N.
R.rhipicep.   .T.....A.C.....G.T.V.LN.TQ..V.....GG.....T.....N.TN.VAAN..FD.P..N.
R.ricketsii   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.
R. sibirica   YT....AAD.....G.T.V.LN..Q..V....I.GG.....T..V.....N.NN.VAAN..FD.P..N.
R. slovaca    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.
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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* [*rhipicep.*]), U59722 (*Rickettsia* sp. #2), U43794 (*R. conorii*), U59732 (*R. parkeri*), U43804 (*R. rickettsii*), U43805 (*Rickettsia* S), U43807 (*R. sibirica*), U43808 (*R. slovaca*), and U43809 (Thai tick typhus).

gene in *I. ricinus* infected with *R. helvetica* collected in Switzerland.[30]

Ereemeva and others[10] 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.[21, 39, 40]

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,[21–23] 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.[8, 10] Extending the discussion initiated by Beati and others,[30] 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,*[29] 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-

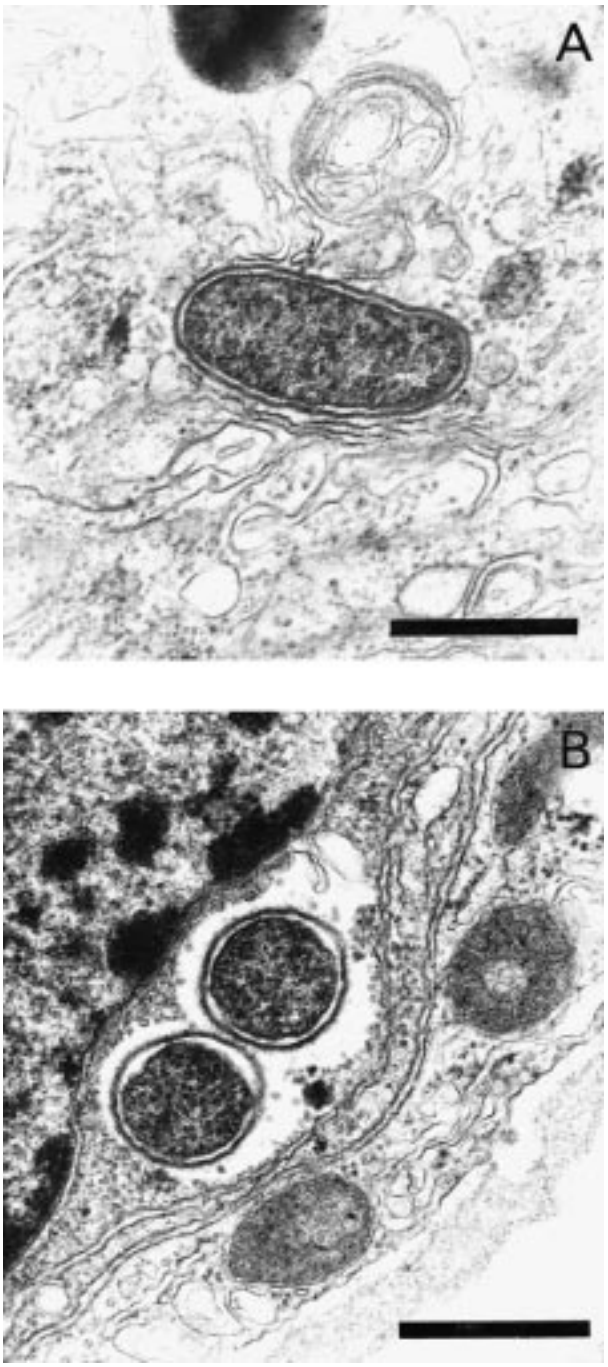

FIGURE 6. Electron micrographs of rickettsiae-infected *Ixodes ricinus* tissues. **A,** longitudinal section of a rickettsiae living free in the cytoplasm of a *Ixodes ricinus* cell. **B,** transversal sections of two rickettsiae associated with the endoplasmic reticulum. Bars = 0.5 μm.

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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