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Characterization and Comparison of Australian Human Spotted Fever Group Rickettsiae

ROBERT W. BAIRD,[1]* MEGAN LLOYD,[1] JOHN STENOS,[1] BRUCE C. ROSS,[1] ROBERT S. STEWART,[2] AND BRIAN DWYER[1]

Clinical Pathology Laboratory, Fairfield Hospital, Yarra Bend Road, Fairfield, Victoria, 3078,[1] and Whitemark, Flinders Island, 7225,[2] Australia

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The microbiological and molecular characteristics of the rickettsiae isolated from humans with Queensland tick typhus (QTT) caused by *Rickettsia australis* and the recently described Flinders Island spotted fever (FISF) were compared. Clinically and serologically, the diseases are similar. Cell culture reveals differences in the plaque-forming abilities of the isolates. Characterization of the gene encoding the genus-specific 17-kDa antigen of *R. australis* revealed a unique nucleotide sequence unlike those of the FISF isolate and *Rickettsia rickettsii*. Southern blot analysis of rickettsial DNA from the isolates with a 17-kDa-antigen gene probe revealed the presence of this gene in all isolates but no difference in banding patterns. When a probe for the rRNA genes was used, clear differences in banding patterns of isolates from patients with QTT and FISF were revealed. Thus, the rickettsiae isolated from patients with FISF differ from those from patients with QTT and may represent a new rickettsial species.

Tick-borne rickettsiae of the spotted fever group (SFG) cause disease throughout the world (15). Typical SFG infections include Rocky Mountain spotted fever, caused by *Rickettsia rickettsii*, in the United States and Mediterranean spotted fever, caused by *Rickettsia conorii*, in southern Europe, Africa, and Asia. Australia has the unique spotted fever illness Queensland tick typhus (QTT), caused by *Rickettsia australis* (3). Two strains of *R. australis* currently exist from the three original human isolations (3, 13). One of these, the PHS strain, is from the original outbreak described by Andrew et al. in 1946 (3), and the second strain, the JC or Cutlac strain, was isolated by Pope 11 years later (13). More recently, a spotted fever-like illness, Flinders Island spotted fever (FISF), in southeastern Australia has been described (6, 17, 18); it shares many clinical features with QTT. The isolation of two rickettsial organisms from the buffy coat fractions of blood samples from two patients with FISF (9) has allowed us to confirm the rickettsial nature of this disease by serology and electron microscopy. The rickettsia genus-specific 17-kDa-antigen gene was cloned and sequenced from the original *R. australis* isolates and was used as a probe to confirm the rickettsial nature of the two new isolates. In this paper, the microbiological and molecular characteristics of the FISF rickettsia are compared with those of previous Australian human rickettsial SFG isolates.

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MATERIALS AND METHODS

Patients. The patients with FISF from whom the rickettsiae were isolated have been described previously (9). Briefly, the rickettsiae were isolated from samples of the leukocyte-enriched fraction of blood (buffy coat) taken early in the illness (on 28 November 1990 for patient 1 and on 11 December 1990 for patient 2) and centrifuged onto monolayers of buffalo green monkey kidney (BGMK) cells. The clinical illnesses and the timing of samples from these patients are summarized in Fig. 1.

Cell culture. Rickettsial cultivation was performed under containment in a geographically isolated C3 laboratory in class 2 biosafety cabinets. Rickettsial strains were seeded into BGMK cells in antibiotic-free RPMI 1640 medium (Flow Laboratories, Irvine, Scotland) as previously described (7, 9). Ron Grice and Neville Stallman of the Queensland State Health Department kindly provided *R. australis* PHS. David Walker (University of Texas) kindly provided *R. australis* JC. The rickettsiae from patients 1 and 2, who both had FISF, were similarly but separately cultured. Infected monolayers were harvested 10 days postinoculation. Cells were pelleted from cell culture medium at 6,000 × *g* for 10 min before heat inactivation at 56°C for 30 min. The rickettsial preparations were stored at −70°C prior to DNA preparation. Cultivation and purification of the *R. australis* strains were separated both temporally and physically to avoid possible cross-contamination. All manipulations were performed in class 2 biosafety cabinets with positive-displacement pipettes.

Preparation of rickettsial DNA. Differential centrifugation followed by isopycnic sedimentation through a renografin density gradient (11) was used to obtain purified rickettsial preparations from the infected cell cultures. Purified DNA was obtained by cell lysis in 1 mg of proteinase K per ml–1% sodium dodecyl sulfate followed by two extractions with phenol-chloroform (50:50) and ethanol precipitation. The DNA pellets were washed with 70% ethanol, dried, and stored at 4°C until reconstitution with sterile water (4).

PCR amplification of the 17-kDa-antigen rickettsial gene. Oligonucleotides were synthesized from published conserved sequences (2) to the amino- and carboxy-terminal ends of the rickettsial 17-kDa common antigen gene. The amino-terminal oligonucleotide 5′-d(CCG GAA TTC TAA AAA CCA TA TAC TATT)-3′ and the complementary carboxy-terminal oligonucleotide 5′-d(CGG GAA TTC CAA TTC ACA ACT TGC CAT)-3′ each contained an introduced *Eco*RI restriction site (underlined) to facilitate cloning of the polymerase chain reaction (PCR) product. The 17-kDa-

* Corresponding author.

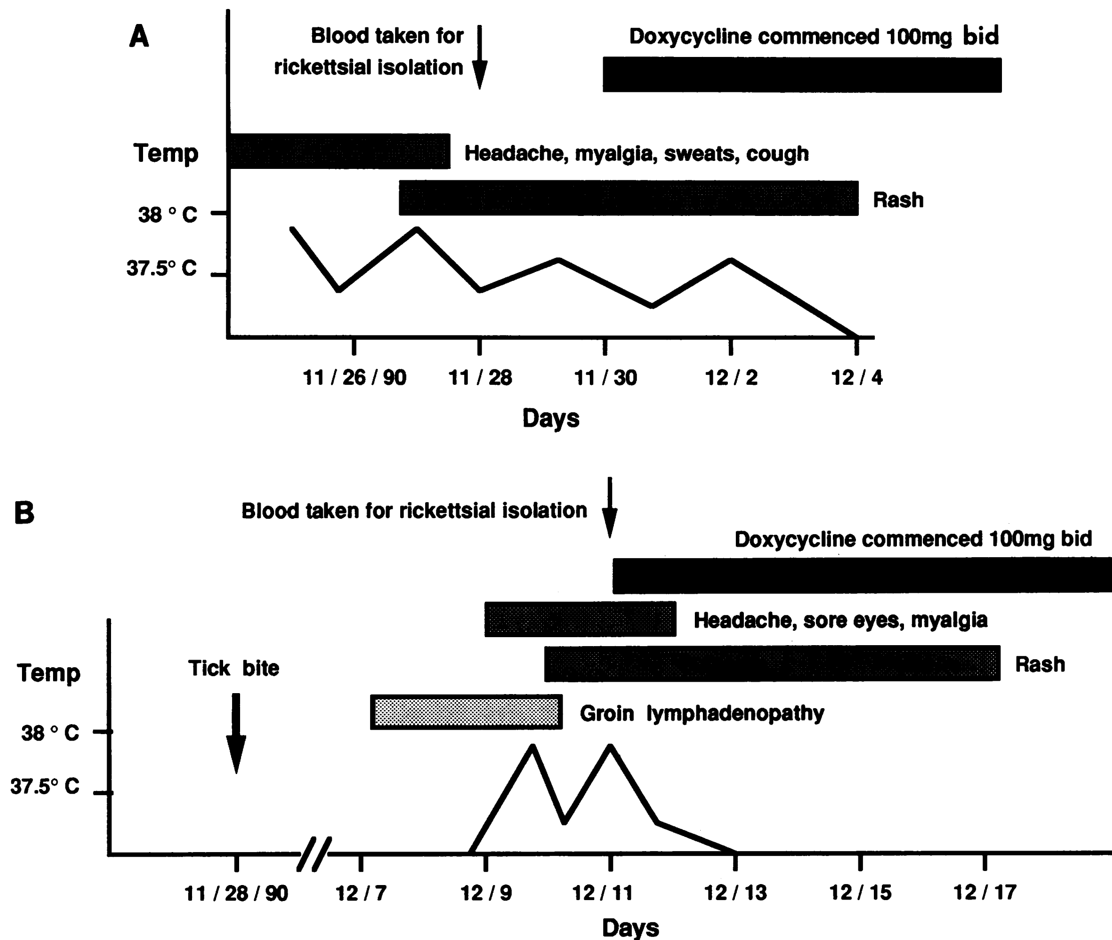

FIG. 1. Clinical courses of the two FISF patients from whom rickettsial isolations were made. (A) Patient 1 (FISF isolate 1) presented with typical headaches, myalgias, sweats, and a slight cough followed by a rash initially comprising a few scattered red macules over her body and limbs. The rash continued appearing until day 5. She responded to doxycycline. (B) Patient 2 (FISF isolate 2), a 50-year-old nurse, developed her illness on 8 December 1990. The infection was characterized initially by regional groin lymphadenopathy following a tick bite on 28 November 1990. This was followed by the development of a fever, headache, myalgia, and sweats. The rash, described as macular and slightly papular, was present over the trunk, thighs, calves, arms, and forearms. The illness responded to doxycycline. bd, bid, twice daily.

antigen gene was amplified (16) in a 100-μl reaction mixture containing 10 ng of rickettsial DNA, reaction buffer (Perkin-Elmer Cetus, Melbourne, Australia), 1.0 μM primer, 200 μM deoxynucleoside triphosphate mix, and 3 U of *Taq* polymerase (Perkin-Elmer Cetus). The amplification reaction was conducted on a thermal cycler (Perkin-Elmer Cetus) for 32 repeated cycles of denaturation at 95°C for 1 min, annealing at 52°C for 1 min, and primer extension at 70°C for 2 min. Products of PCR amplification were analyzed by electrophoresis through 1.5% agarose gels stained with ethidium bromide.

Cloning of amplified fragment. The products of PCR amplification were purified by phenol-chloroform extraction and ethanol precipitation before being suspended in 10 mM Tris–1 mM EDTA (TE buffer) and digested with *Eco*RI. Purification of the cleaved products was achieved with GeneClean (BIO 101, LaJolla, Calif.) or high-pressure liquid chromatography. The digestion products were ligated into the *Eco*RI-digested dephosphorylated plasmid vector pT7T3 (Pharmacia, Sweden) and transformed into *Escherichia coli* NM522 by electroporation (Gene Pulser; BioRad, Richmond, Calif.). Transformants were selected on agar plates containing ampicillin, and *E. coli* harboring recombinant plasmids was identified by subculture onto agar plates containing isopropyl β-D-thiogalactoside (IPTG) and 5-bromo-4-chloro-3-indolyl β-D-thiogalactoside (X-Gal). Recombinant plasmids containing the 520-bp insert were confirmed by *Eco*RI digestion.

Sequencing of the PCR product. Plasmid templates for double-stranded DNA sequencing were purified from *E. coli* by using an alkaline lysis procedure (4) followed by gel filtration chromatography through a Superose 6 fast protein liquid chromatography column as described by the manufacturer (Pharmacia). Double-stranded-DNA sequencing was performed by the dideoxy method (4) with [α-^{35}S]dATP,T7 DNA polymerase (Sequenase, USB, Cleveland, Ohio), and primers for either T3 or T7 promoters (Pharmacia). Two clones of each *R. australis* strain (pRB2 and pRB3 for the PHS strain and pRAJC9 and pRAJC10 for the JC strain) were sequenced in parallel to exclude the possibility of *Taq* polymerase-induced errors. One clone containing the 17-kDa-antigen gene from patient 1 (FISF isolate 1) was bidirectionally sequenced to obtain the FISF nucleotide sequence.

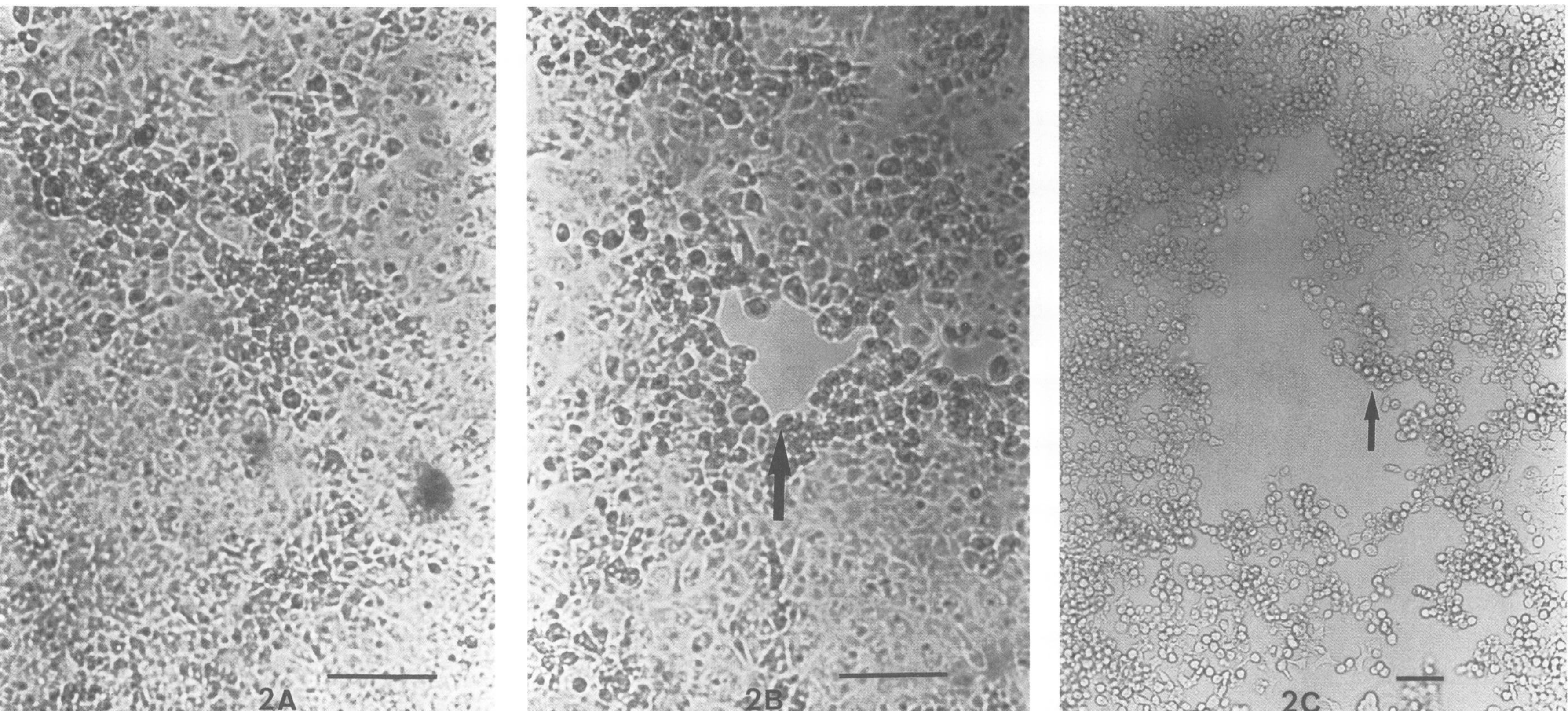

FIG. 2. (A) Photomicrograph of an uninfected BGMK cell monolayer. Bar = 50 μm. (B) Single characteristic demarcated plaque of *R. australis* PHS, with its well circumscribed, circular, circumferential, and complete heaped-up edge (arrow). Bar = 50 μm. (C) Irregular nondiscrete rounding up and dislodging of cells infected with rickettsiae from patient 1 (FISF isolate 1; arrow). Bar = 50 μm.

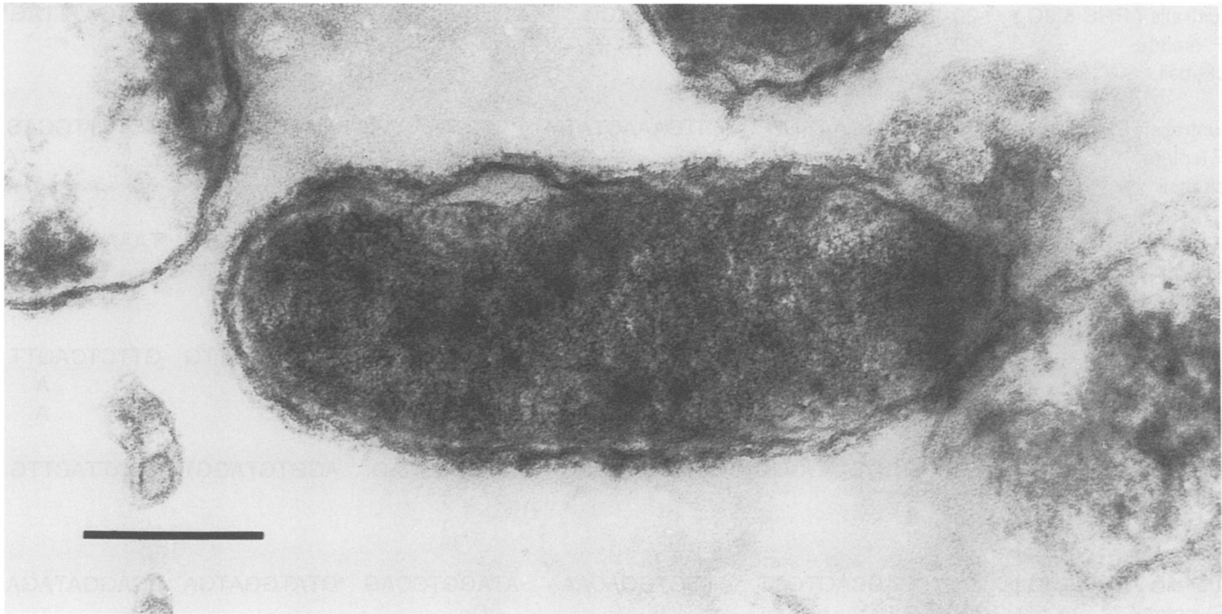

FIG. 3. Ultrathin-section electron micrograph of the rickettsia isolated from infected cell culture medium of FISF isolate 1 (patient 1). The specimen was centrifuged, pelleted, and suspended in fixative, revealing in the sections an organism with a typical multilayered outer membrane loosely adherent to the cell membranes and covering a relatively low-density cytosol. Bar = 0.25 μm.

Probes and hybridizations. All DNA probes used in this study were labeled with digoxigenin by using a nonradioactive-DNA labeling and detection kit (Boehringer, Mannheim, Germany). The first probe to the 17-kDa-antigen gene was prepared by PCR amplification of the 17-kDa-antigen gene in the presence of digoxigenin-labeled dUTP. The second probe used to investigate the rRNA gene restriction patterns was prepared by random primer labeling of plasmid pKK3535 (19) with Klenow polymerase (Boehringer Mannheim). This plasmid contains the rRNA genes of *E. coli* representing the 5S RNA, 16S RNA, 23S RNA, and $tRNA^{Glu_2}$ (5) and has been used previously to distinguish between a variety of bacteria (1, 10).

Southern blot hybridization. Samples containing approximately 1 μg of rickettsial DNA were digested with 10 U of the restriction enzyme *Eco*RI at 37°C for 2 h. DNA fragments were separated by electrophoresis through 0.7% agarose gels at 60 V for 16 h (20-cm gel) in 40 mM Tris-borate–2 mM EDTA. Fragments were transferred to positively charged nylon filters (Boehringer Mannheim) by using a vacuum transfer apparatus (Hybaid, United Kingdom). Nonradioactive detection was done as described by the manufacturer (Boehringer Mannheim) except that 1% casein was used in the blocking step and as a conjugate diluent.

Nucleotide sequence accession number. The GenBank accession number for the nucleotide sequence of *R. australis* is M74042. The FISF 17-kDa antigen gene accession number is M99391.

RESULTS

Microbiology. The rickettsial isolates from patients with FISF were separated temporally by at least 36 years and physically by over 3,000 km from the previous QTT isolates. The rickettsiae from the two patients with FISF exhibited cytopathic effects different from those of the previously multiply passaged *R. australis* isolates. The appearances of *R. australis* PHS and JC in actively growing culture were characterized by clusters of infected BGMK cells forming single discrete plaques, whereas the FISF isolates displayed visible single-host-cell infections and nonuniform lesions, with cells detaching from the monolayer in a random fashion (Fig. 2). These differences may have resulted from the repeated passage of the *R. australis* PHS and JC isolates over many years compared with the relatively few cell culture passages of the FISF isolates. All isolates exhibited strong microimmunofluorescence to rickettsia-specific polyclonal antisera. Briefly, cell scrapings were fixed to glass slides with acetone and assayed with polyclonal guinea pig antisera raised against *R. australis* PHS in a dilution of 1:64. After incubation with a fluoroscein-labeled conjugate, the scrapings were observed for the presence of cell-associated and free rickettsiae.

Electron microscopy (Fig. 3) of the FISF isolate purified from tissue culture of patient 1 (FISF isolate 1) revealed an organism 250 nm in diameter and 1 μm in length. By Giemsa stain and electron microscopy, this organism had the appearance typical of rickettsiae, being a short intracellular rod with a multilayered outer membrane. The outer membrane of the isolate was convoluted and loosely apposed to the cytoplasmic membranes and outlined a relatively low-density cytosol. Some damaged organisms were present in most of the sections.

Analysis of the 17-kDa-antigen gene. The 17-kDa-antigen gene has been previously well characterized in other rickettsial species and is thought to be genus specific for the rickettsial group of organisms (2). The PCR was used with primers to common regions of the gene to amplify, clone, and sequence this gene from *R. australis* PHS and JC and FISF isolate 1 (patient 1). The *R. australis* PHS and JC genes had identical nucleotide sequences. Sequence analysis revealed unique differences in *R. australis* PHS and JC genes compared with those of the FISF isolate and *R. rickettsii* Sheila Smith (Fig. 4). Comparison with the FISF isolate gene

Organism	Start						End
R. australis (PHS & JC)	-30	GAGCTCGAAT	TCTAAAAACC	ATATACTTAT	TAAATAATAT	ATTGATTTAG	20
FISF isolate	-29				- T	A	20
R.rickettsii	-29				- T	A	20
R. australis (PHS & JC)	21	TGAGAATTAT	ATGAAACTAT	TATCTAAAAT	TATGATTATA	GCTCTTGCAG	70
FISF isolate	21	A				A	70
R.rickettsii	21	A				A	70
R. australis (PHS & JC)	71	CTTCTATGTT	ACAAGCCTGT	AACAGTCCGG	GCGGTATGAA	TAAACAAGGT	120
FISF isolate	71			G			120
R.rickettsii	71			G			120
R. australis (PHS & JC)	121	ACAGGAACAC	TTCTTGGCGG	TGCCGGCGGT	GCATTACTTG	GTTCTCAGTT	170
FISF isolate	121			T C		A	170
R.rickettsii	121			T C		A	170
R. australis (PHS & JC)	171	CGGTAAGGGC	AAAGGACAGC	TTGTCGGAG	AGGTGTAGGT	GCATTACTTG	220
FISF isolate	171			T			220
R.rickettsii	171			T			220
R. australis (PHS & JC)	221	GAGCAGTGCT	TGGTGGACAA	ATAGGTGCAG	GTATGGATGA	GCAGGATAGA	270
FISF isolate	221	T		C			270
R.rickettsii	221	T		C		A	270
R. australis (PHS & JC)	271	AGACTTGCAG	AGCTCACCTC	ACAAAGAGCT	TTAGAAACAG	CTCCTAGCGG	320
FISF isolate	271		T	G		T	320
R.rickettsii	271		T	G		T	320
R. australis (PHS & JC)	321	TAGTAACGTA	GAATGGCGTA	ATCCGGATAA	CGGCAATTAT	GGTTACGTAA	370
FISF isolate	321				G C		370
R.rickettsii	321				C		370
R. australis (PHS & JC)	371	CACCTAATAA	AACTTATAGA	AATAGCAATG	GTCAATATTG	CCGTGAGTAC	420
FISF isolate	371		C	C	G	T	420
R.rickettsii	371			C			420
R. australis (PHS & JC)	421	ACTCAAACAG	TTGTAATAGG	CGGAAAACAA	CAAAAAGCAT	ACGGTAACGC	470
FISF isolate	421					T	470
R.rickettsii	421					T	470
R. australis (PHS & JC)	471	ATGCCGCCAA	CCTGACGGACAA	TGGCAAGTTG	TGAATTGG		511
FISF isolate	471					95.50%	511
R.rickettsii	471					96.10%	511

FIG. 4. Nucleotide sequence of the gene coding for the 17-kDa antigen of *R. australis* PHS and JC compared with the nucleotide sequence of *R. rickettsii*. Nucleotides are conserved between the two sequences except where indicated. Deletion of nucleotides (−) is indicated. Boxed letters represent the 5′ and complementary 3′ primer sequences described in the text. The presumptive initiating methionine residue coded for by nucleotides 31 to 33 is indicated by underlining. The percent sequence homology is indicated at the end of the sequence.

revealed 22 nucleotide differences. Eighteen of these occur in the coding region of the gene; of these, 12 are conservative differences, but the six base substitutions at nucleotide positions 70, 94, 356, 398, 406, and 416 all lead to changes in the putative amino acid structure. Comparison with *R. rickettsii* revealed 19 base differences over the length of the gene, with 4 base substitutions in the 5′ noncoding region and 15 base substitutions within the predicted coding region of the gene. Twelve of these substitutions are conservative, leading to no change in the amino acid composition of the 17-kDa protein. The base substitutions at nucleotide positions 70, 94, and 398 lead to amino acid changes of threonine for alanine, glycine for serine, and threonine for asparagine, respectively, in the putative protein structure.

A comparison of the FISF rickettsial isolate with *R. rickettsii* reveals much closer homology (98.9%), with only five base differences over the coding regions of the genes. These differences, at base positions 261, 356, 384, 406, and 416, lead to three amino acid differences between their putative protein structures. This gene remains highly conserved within SFG rickettsiae (2), but the nucleotide substitutions described for *R. australis* and the FISF isolate are far more varied than those observed between the geographically separate but genetically similar *R. rickettsii* and *R. conorii*, which share 99.8% homology within this particular gene.

Southern blot analysis. After purification of rickettsial DNAs from the four human isolates, the DNAs were compared by Southern blot analysis. The first probe was to the

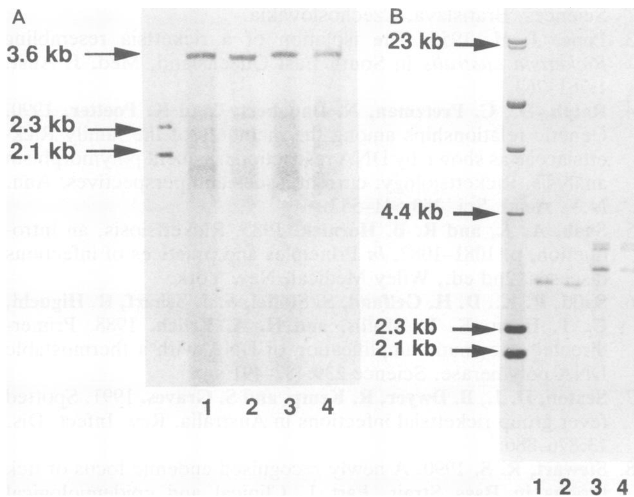

FIG. 5. Southern blot analysis of two *R. australis* strains and two isolates of FISF rickettsia. Genomic DNA was digested with *Eco*RI, electrophoresed through 0.7% agarose gels, and transferred to nylon membranes before being probed with either a 17-kDa-antigen gene probe (A) or a probe for the rRNA genes (B). Lanes: 1, *R. australis* JC; 2, *R. australis* PHS; 3, FISF isolate 1 (patient 1); 4, FISF isolate 2 (patient 2). Molecular weight indicators are on the left-hand side of the gel.

17-kDa-antigen gene. This probe revealed an identical band in all four rickettsiae at a molecular size of 3.6 kb, confirming the FISF isolates as members of the SFG of rickettsiae and indicating conservation of the *Eco*RI sites flanking the 17-kDa-antigen gene between all isolates (Fig. 5A). A probe to the rRNA genes (Fig. 5B) revealed marked differences between the isolates from Queensland and Flinders Island. The two *R. australis* PHS and JC isolates shared a similar ribosomal-gene banding pattern, with bands at 2.8, 4.6, 5.2, and 8 kb. The two Flinders Island isolates also had similar banding patterns, with fragments of 3, 3.8, and 3.9 kb which were different from those of the QTT isolates. This obvious genetic difference suggests that the Flinders Island isolates are distinct from the *R. australis* isolates and may represent a new subspecies.

DISCUSSION

This isolation of an SFG rickettsia is one of a number of new rickettsial isolations that have occurred recently, including isolations of *Rickettsia japonica* (20) and *Rickettsia helvetica* (12). Our studies show that the rickettsial organisms isolated from patients with FISF differ from the isolates obtained previously from QTT patients. The confirmation of the rickettsial nature of FISF extends the range of SFG rickettsial infections along the entire eastern seaboard of Australia.

Rickettsiae are genetically closely related, and comparison of 16S rRNA sequences in *R. rickettsii*, *Rickettsia typhi*, and *Rickettsia prowazeki* indicates greater than 98% homology between these species (14). *R. australis* has been previously compared with other members of the family *Rickettsiaceae* by DNA probing and restriction fragment length polymorphisms (14, 21). These comparisons reveal marked differences in DNA homology compared with that between the other SFG rickettsiae *R. rickettsii* and *R. conorii*, which are more closely related.

This finding is substantiated by sequence comparison of the 17-kDa-antigen genes of *R. australis* with *R. rickettsii*, which reveals 19 nucleotide differences over the length of this highly conserved gene (2). Interestingly, the FISF isolate also is markedly different with respect to the 17-kDa nucleotide sequence of *R. australis*, this gene having 22 nucleotide differences over the coding area of the gene. The FISF isolate appears to be more closely related to *R. rickettsii*, as only five nucleotide differences occur with respect to the 17-kDa-antigen gene structures of these two isolates. This gene is highly conserved among all spotted fever isolates studied to date, and a previous sequence comparison of *R. rickettsii* and *R. conorii* revealed only one conservative nucleotide difference over the entire length of the 17-kDa-antigen gene. Though the Australian rickettsial isolates from patients with QTT and FISF were separated by 3,000 km geographically and 35 years temporally, this does not seem to account for the marked genetic differences between the strains. Therefore, the marked number of nucleotide differences between the two Australian human rickettsial isolates is unique among SFG rickettsiae studied to date. The function of this highly conserved gene has yet to be determined, though its antigen is expressed on the surfaces of rickettsiae. The use of rRNA genes as a probe to distinguish between bacterial species and subspecies has received wide use (1, 5, 19). The demonstration of banding differences of the rRNA genes between the isolates from patients with FISF is strong evidence for more-general genetic differences between the FISF isolates and those from patients with QTT. Profound differences in the restriction patterns may reflect a number of differences in the sequences of the 16S rRNAs. Currently, efforts to determine the 16S rRNA gene sequences of the FISF and *R. australis* isolates are under way.

The isolation of the FISF rickettsia is the first isolation of a new rickettsia from humans in Australia for 35 years. This agent differs from the agent of QTT on the basis of cytopathology in cell culture and rRNA gene restriction patterns. Is the FISF rickettsia a new species of rickettsia, or is it a subspecies of *R. australis*? Before these questions can be answered, questions relating to its immunogenic relationship to other SFG rickettsiae must be answered, and further genetic characterization, such as of 16S ribosomal sequences, remains to be done. Other questions involve transmission vectors, host reservoirs of endemic infection, and distribution of disease (8).

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