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Genetic Variation in Australian Spotted Fever Group Rickettsiae

ROBERT W. BAIRD,[1]* JOHN STENOS,[2] ROB STEWART,[3] BERNIE HUDSON,[4] MEGAN LLOYD,[2] SANDRA AIUTO,[2] AND BRIAN DWYER[5]

Microbiology Department, Melbourne Pathology, Collingwood, Victoria 3066,[1] Clinical Pathology Laboratory, Fairfield Hospital, Fairfield, Victoria 3078,[2] Whitemark, Flinders Island 7255,[3] Microbiology Department, Royal North Shore Hospital, St. Leonards, New South Wales 2065,[4] and Microbiology Department, Royal Darwin Hospital, Casuarina, Northern Territory 5792,[5] Australia

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Rickettsiae were isolated by cell culture of buffy coat blood from six patients with spotted fever from southeastern Australia and Flinders Island in Bass Strait. The isolates were genetically compared with two previous *Rickettsia australis* patient isolates. The genus-specific 17-kDa genes from the isolates were compared after DNA amplification and restriction fragment analysis of the amplified DNA. This comparison revealed that mainland rickettsial isolates from southeastern Australia were identical to two previous isolates of *R. australis* from northeastern Australia. Rickettsial isolates from Flinders Island were distinct from the mainland isolates. The 16S rRNA gene sequences from the isolates were determined and compared. The Flinders Island rickettsial agent was most closely related (0.3% structural divergence) to *Rickettsia rickettsii*, *Rickettsia conorii*, and *Rickettsia slovaca*. The Flinders Island rickettsial agent was 1.3 and 2.1% structurally divergent from *R. australis* and *Rickettsia akari*, respectively. The 16S rRNA gene sequence from the Flinders Island agent shows that this rickettsia is more closely related to the rickettsial spotted fever group than is *R. australis*. We conclude that there are two populations of spotted fever group rickettsiae in Australia and propose that the genetically distinct causative organism of Flinders Island spotted fever be designated *Rickettsia honei*. The extent of distribution and animal host reservoirs remain to be elucidated.

Tick-borne rickettsiae of the spotted fever group (SFG) cause disease worldwide (17). Australia has two rickettsial spotted fever illnesses: Queensland tick typhus, which is caused by *Rickettsia australis* (2, 14) and occurs eastern Australia, and Flinders Island spotted fever, which is caused by a unique SFG rickettsia (4). The clinical illnesses have been previously reviewed (4, 6, 8, 18, 20); they have similar symptoms and signs, and both respond well to therapy with doxycycline. To localize the geographical ranges and distributions of these two illnesses, we compared eight rickettsial isolates obtained from patients with spotted fever on the mainland and Flinders Island. The comparison of these isolates was based on previously published differences in the genus-specific 17-kDa antigen gene (4) and 16S rDNA sequence analysis. We have compared six recent patient rickettsial isolates with the two remaining *R. australis* isolates in existence. These two strains of *R. australis*, designated PHS and JC, were isolated from humans in 1946 and 1955, respectively (2, 14).

MATERIALS AND METHODS

Patients. *R. australis* PHS and JC were kindly supplied by Ron Grice of the Queensland Health Department, Queensland, Australia, and David Walker, Galveston, Tex. The four rickettsial isolates from Flinders Island are isolates from two previously described patients (4) (designated here as patients 1 and 2) and two other patients (designated here as patients 3 and 4). Patients 3 and 4 suffered the typical spotted fever illness of fever and headache followed by rash. The rickettsia causing their illness was isolated in December 1991 by culture of buffy coat blood placed directly into cell culture flasks. The two rickettsial isolates from the mainland were from two sites. Patient 5, a 71-year-old man, sustained a bite from an adult *Ixodes holocyclus* tick in his garden in Sydney on 18 November 1991. Five days later he developed fever, myalgia, lethargy, and a rash. On 28 November 1991 he reported to his local doctor, who detected an eschar at the bite site. The rash was papulovesicular in nature and involved the trunk, neck, and proximal aspects of all limbs. The second mainland isolate was from patient 6, who suffered a rickettsial illness in March 1992, 10 days after bush walking at Malacoota, East Gippsland, Victoria.

PCR typing. Rickettsial cultivation was performed under containment in a geographically isolated C3 laboratory in class 2 biosafety cabinets. Australian rickettsial strains (PHS and JC) were seeded into Vero cells in antibiotic-free RPMI 1640 medium (Flow Laboratories, Irvine, Scotland) as previously described (19). The rickettsiae from the patients were isolated by culture of buffy coat-enriched blood centrifuged onto cell monolayers, and the cells were observed for plaque formation (19). The rickettsia isolated from patient BP required a subsequent passage through mice and reisolation to remove contaminating bacteria from the cell culture. The rickettsiae were subsequently stored at −70°C and recultivated for these studies. The infected cell monolayers were harvested 10 days postinoculation and purified by Renografin centrifugation (19). The rickettsiae were heat inactivated at 56°C for 30 min and stored at −70°C prior to DNA preparation. Both the cultivation and purification of the known *R. australis* strains were done separately, both temporally and physically, to avoid possible cross-contamination. Purified DNA was obtained by cell lysis in a solution containing 1 mg of proteinase K per ml and 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 (3). The 17-kDa antigen gene was amplified (1, 4) in 50 μl of a reaction mixture containing 100 ng of crude rickettsial DNA, reaction buffer (Promega, Madison, Wis.), 1.0 μM each primer, 200 μM each deoxynucleoside triphosphate, and 1 U of *Taq* polymerase (Promega). The amplification reaction, conducted in a thermal cycler (Hybaid, Middlesex, United Kingdom), consisted of 35 repeated cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and primer extension at 70°C for 2 min. The products of PCR amplification were purified by phenol-chloroform extraction and ethanol precipitation before resuspension in 10 mM Tris–1 mM EDTA and digestion with *Cfo*I or *Dde*I (Boehringer GmbH, Mannheim, Germany). The products of PCR amplification and restriction enzyme digestion were analyzed by electrophoresis through 8% acrylamide gels and either ethidium bromide or silver staining. Analysis of previously published genetic differences (4) in the base pair structures of these genes had revealed a unique *Dde*I cleavage site at base 184 in the 17-kDa gene of *R. australis* and a unique *Cfo*I site at base 170 in the gene from the Flinders Island rickettsial isolates.

16S rRNA cloning. Oligonucleotide primers rRNA1 and rRNA2 (Table 1)

* Corresponding author. Mailing address: Microbiology Department, Melbourne Pathology, 32 Smith St., Collingwood, Victoria 3066, Australia. Phone: 011-61-3-92877700. Fax: 011-61-3-92877710.

TABLE 1. Primers used in amplification and sequencing of *R. australis* and *R. honei* 16S rRNA genes

Gene	Primer sequence	Positions[a]
rRNA1	5′ CGG AAT TCA GAG TTT GAT CCT GGC TCA G 3′	10–29
rRNA2	5′ CCG AAT TCA AGG AGG TGA TCC AGC CGC A 3′	1501–1482
SFG-16S-1	5′ CGT CAT TAT CTT CCT TGC 3′	444–427
SFG-16S-2	5′ GAG ATG CTT TTC TTC AGC 3′	973–990
SFG-16S-3	5′ TGT AGC CCA ACC CGT AAG GG 3′	1190–1171

[a] Homologous positions in the *R. bellii* 16S rRNA gene (GenBank accession number U11014).

were used to amplify the 16S rRNA gene of *R. australis* and the Flinders Island agent's chromosomal DNA by PCR. *Eco*RI (Boehringer) restriction enzyme sites were incorporated into primers rRNA1 and rRNA2 to facilitate the cloning of the PCR-amplified 16S rRNA gene product (13, 15). The DNA was amplified by PCR in 50 μl of the reaction mixture described above. After an initial denaturation at 93°C for 4 min, the reaction mixtures were exposed to 35 cycles of denaturation at 93°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min. The resulting PCR product of approximately 1,500 bp was purified by using a DNA purification kit, Wizard PCR preps (Promega), according to the manufacturer's instructions. The purified product was then digested with *Eco*RI for 3 h. After digestion, two fragments of approximately 600 and 900 bp were liberated because of an internal *Eco*RI site in the PCR product. Following a second DNA purification, the products were ligated with an *Eco*RI-digested, dephosphorylated plasmid vector pT7T3 (Pharmacia Biotech, Sullentuna, Sweden). The ligated products were then transformed into *Escherichia coli* NM522 by electroporation with a Gene Pulser (Bio-Rad, Richmond, Calif.). Transformants were randomly selected from Luria-Bertani (LB) agar plates (15 g of agar, 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter) containing 100 μg of ampicillin per ml and grown in LB medium to saturation. Minipreps were performed, and clones harboring the appropriate inserts were identified.

16S rRNA sequencing. The *fmol* Sequencing System (Promega) was used to sequence the four clones, taking advantage of the T3 and T7 promoters on either end of the multiple cloning site of the vector. The T3 and T7 promoter primers (Promega) were used to sequence in from either end of the 16S rRNA gene fragment, followed by primer design (Table 1) and further sequencing to complete the procedure.

Phylogenetic analysis. A phylogenetic analysis and sequence distance table, in which 16S ribosomal DNA (rDNA) sequences were compared, was calculated by importation of 16S sequences into MegAlign-DNAstar-Lasergene (Madison, Wis.).

Nucleotide sequence accession numbers. The 16S rRNA nucleotide sequences used in this study were obtained from the GenBank database. The following organisms (with accession numbers in parentheses) were compared: *Rickettsia africae* (RIRRGDA), *Rickettsia akari* (RAU12458), *Rickettsia amblyommii* (RAU 11012), Astrakhan fever agent (RIRRGDC), *Rickettsia bellii* (RBU11014), *R. australis* (REU17644), *Rickettsia conorii* (RIRRGDH), *Rickettsia helvetica* (RIR RGDK), *Rickettsia honei* (U17645), *Rickettsia japonica* (RIRRGDL), *Rickettsia massiliae* (RIRRGDI), *Rickettsia montana* (RMU11016), *Rickettsia parkeri* (RPU 12461), *Rickettsia rhipicephali* (RRU11019), *Rickettsia rickettsii* (RRU11021), *Rickettsia sibirica* (RSU12462), and *Rickettsia slovaca* (RIRRGDX).

RESULTS

Patient characteristics are detailed in Table 2. The clinical illnesses produced by *R. australis* and the Flinders Island rickettsia have been compared previously (18, 20), the major differences being a seasonal summer-autumn occurrence of disease on Flinders Island and the maculopapular nature of the rash in the described cases from Flinders Island without vesiculation.

The rickettsial isolations complement previous serological and clinical studies that extend the range of *R. australis* to southeastern Australia. A postulated geographic range for SFG rickettsiae in eastern Australia is presented in Fig. 1. The serological representation is based on serological data collected at the Fairfield Laboratories and previously published reports of Australian rickettsial illness.

TABLE 2. Human SFG rickettsial isolates: patient details

Strain or patient no.	Sex[a]	Age (yr)	Date of exposure	Location of exposure	Rickettsial titer[b]
R. australis PHS	M	27	1944	Atherton Tablelands	WF 1:320
R. australis JC	M	19	1957	Brisbane	WF positive
1	F	67	Dec. 1990	Flinders Island	1/512
2	F	50	Dec. 1990	Flinders Island	1/512
3	M	72	Dec. 1991	Flinders Island	1/1,024
4	M	79	Dec. 1991	Flinders Island	1/256
5	M	71	Nov. 1991	Sydney, New South Wales	1/512
6	F	37	Mar. 1992	Malacoota, Victoria	ND

[a] M, male; F, female.
[b] Measured by immunofluorescence using an antibody to immunoglobulin G. WF, Weil-Felix; ND, not determined.

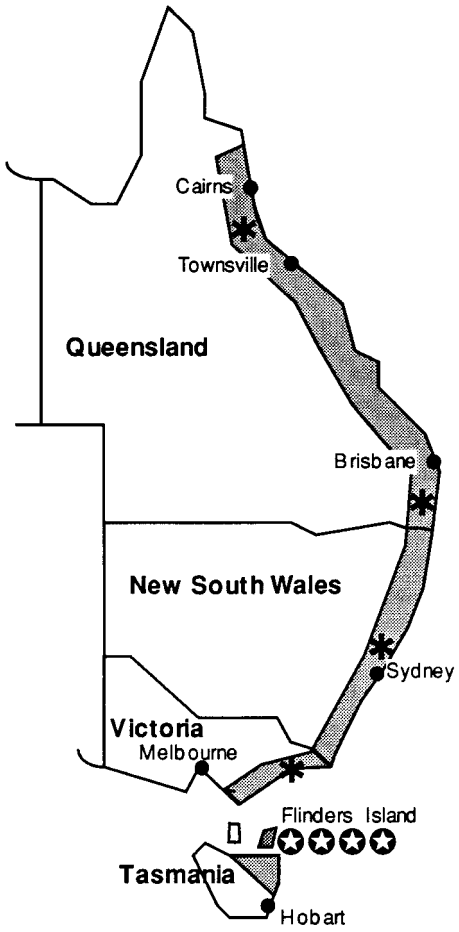

FIG. 1. Diagram of eastern Australia showing the sites of isolation of SFG rickettsiae from patients. The shaded areas represent the range of SFG infections in eastern Australia as demonstrated by serological studies. ✶, site of isolation of *R. australis* from infected patients. ✪, site of isolation of rickettsia from infected patients on Flinders Island.

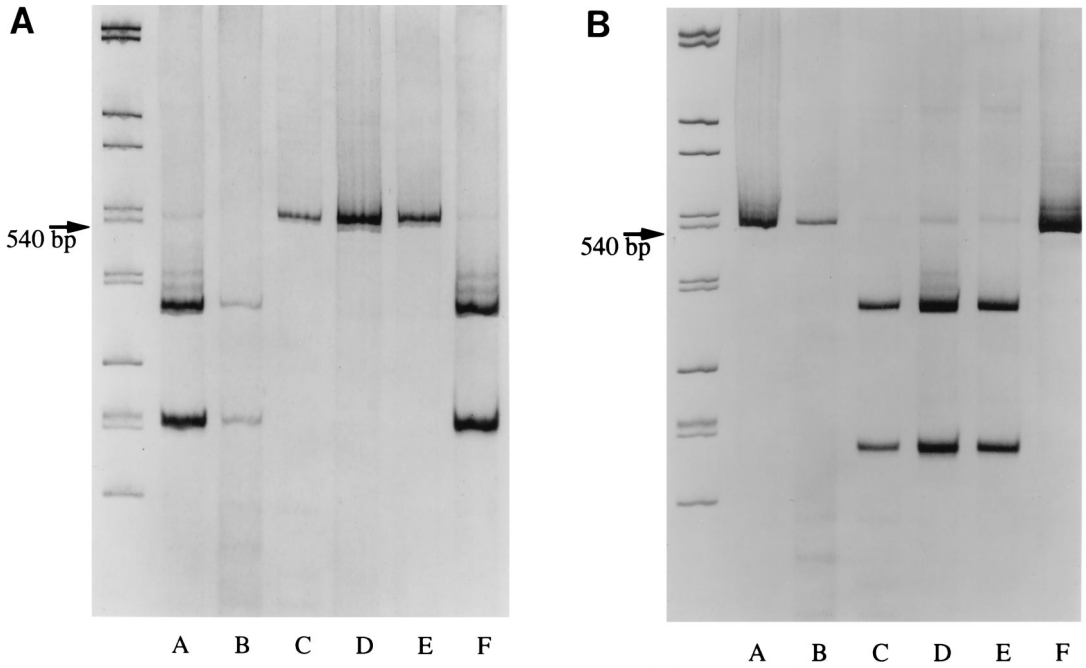

FIG. 2. (A) Restriction pattern of the amplified 17-kDa gene cleaved with *Dde*I. Lanes: A, *R. australis* PHS; B, *R. australis* JC; C, isolate from patient 1; D, isolate from patient 2; E, isolate from patient 4; F, isolate from patient 5. (B) Complementary restriction pattern of the amplified 17-kDa gene cleaved with *Cfo*I. The lanes represent the same rickettsial isolates as in panel A. Molecular weight markers are on the left.

The results of the PCR typing are shown in Fig. 2 and reveal that the amplified 17-kDa genes from the mainland rickettsial isolates (lanes A, B, and F), including Queensland and New South Wales rickettsiae, had identical restriction fragment patterns, being cleaved by *Dde*I but not by *Cfo*I. The PCR-amplified 17-kDa gene from patient 6, from Malacoota, Victoria, had an identical pattern. Fig. 2 reveals that the rickettsial isolates from the three patients on Flinders Island (lanes C, D, and E) are cleaved by *Cfo*I but not by *Dde*I. The PCR-amplified 17-kDa gene of the rickettsial isolate from patient 3, from Flinders Island, had a cleavage pattern identical to those of the other Flinders Island isolates. These results reveal that the Australian mainland SFG isolates are *R. australis* and that the Flinders Island isolates are different from *R. australis.*

Phylogenetic analysis (presented in Fig. 3) and structural similarity and divergence values (Fig. 4) confirm that *R. australis* is distinct from the other SFG rickettsiae. Analysis of the 16S rRNA sequences reveals that the Flinders Island agent, *R. honei*, occurs as a typical but distinct SFG rickettsial species and is most closely homologous to *R. rickettsii* (0.3% divergent), *R. conorii* (0.3% divergent), and *R. slovaca* (0.3% divergent) and most divergent from *R. australis* (1.3% divergent) and *R. akari* (2.1% divergent). *R. australis* and the Flinders Island agent, *R. honei*, were 1.1 and 0.3% divergent from *R. rickettsii*, respectively. In summary, phylogenetically the Flinders Island agent is a true SFG rickettsia while *R. australis* appears to be distinct from the SFG.

DISCUSSION

Earlier studies of the Flinders Island rickettsial agent revealed its unique geographical location, spotted-fever-like clinical characteristics, and variances in cell culture characteristics, 17-kDa antigen gene sequences, and immunogenic profiles (4, 9) compared with *R. australis.*

The studies described here on *R. australis* isolates from Victoria and New South Wales confirmed previous serological studies showing that the entire eastern seaboard of Australia is

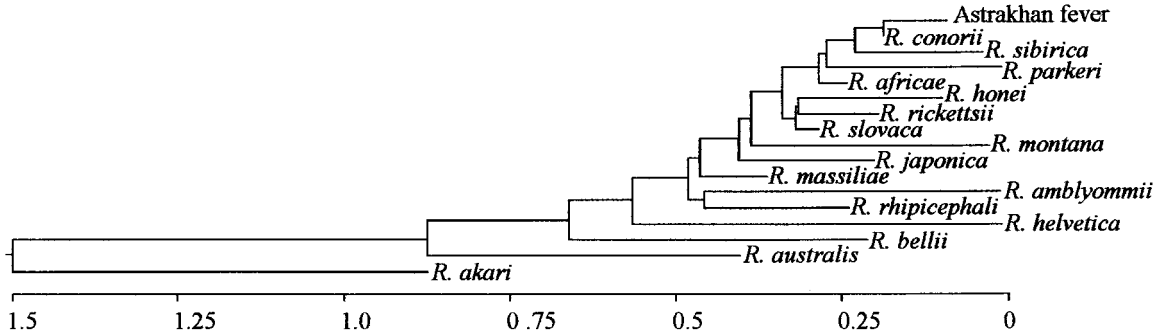

FIG. 3. Phylogenetic relationships of *R. honei* and *R. australis* and other members of the rickettsiae, defined on the basis of 16S rRNA analysis. The percentages of sequence divergence are indicated by the markers.

Percent Similarity

Percent Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	
1		97.1	98.5	99.2	98.0	98.5	99.2	98.4	99.0	99.0	99.0	99.0	98.7	98.6	99.0	99.2	99.2	1
2	2.1		97.5	96.8	97.9	98.0	98.0	97.2	97.7	97.2	97.2	97.6	96.7	97.9	97.9	97.4	97.7	2
3	0.8	2.1		97.7	98.5	98.8	99.1	98.0	99.0	98.4	98.5	98.9	97.9	99.0	99.0	98.5	99.0	3
4	0.3	1.8	0.8		97.9	98.4	99.5	99.1	99.0	99.5	99.4	98.1	97.7	97.9	99.1	99.0	99.7	4
5	1.3	1.8	1.4	1.4		98.9	98.6	98.5	98.7	98.7	99.0	98.4	97.5	98.6	98.3	98.1	98.7	5
6	1.0	1.8	1.0	1.2	1.1		99.1	98.8	99.1	99.1	99.2	98.9	97.9	98.9	99.1	98.5	99.2	6
7	0.2	1.9	0.8	0.1	1.3	0.9		98.7	99.7	99.2	99.1	99.4	99.0	99.2	99.7	99.4	99.7	7
8	1.0	2.0	1.2	0.9	1.4	1.2	0.8		99.0	99.2	99.1	98.1	97.5	98.3	99.0	98.2	99.1	8
9	0.4	2.1	0.8	0.5	1.3	0.9	0.3	1.0		99.5	99.5	99.3	98.3	99.2	99.1	99.1	99.7	9
10	0.5	2.0	0.8	0.5	1.2	0.9	0.4	0.8	0.5		99.6	98.7	98.0	98.5	99.6	98.7	99.7	10
11	0.5	1.9	0.6	0.5	1.0	0.8	0.4	0.8	0.5	0.4		98.7	98.0	98.7	99.6	98.6	99.7	11
12	0.5	2.1	0.9	0.6	1.4	1.0	0/6	1.1	0.6	0.6	0.6		98.1	99.0	99.1	98.8	99.3	12
13	0.4	2.1	0.9	0.4	1.4	1.0	0.4	1.1	0.6	0.6	0.6	0.8		98.1	98.3	99.2	98.7	13
14	0.6	1.9	0.7	0.6	1.2	0.9	0.6	0.8	0.6	0.6	0.4	0.7	0.8		99.1	98.7	99.1	14
15	0.3	1.8	0.8	0.6	1.1	0.9	0.3	1.0	0.3	0.4	0.4	0.6	0.5	0.6		99.2	99.8	15
16	0.3	2.1	0.9	0.3	1.4	1.0	0.2	1.1	0.5	0.6	0.6	0.7	0.5	0.7	0.4		99.2	16
17	0.3	2.0	0.7	0.3	1.2	0.8	0.2	0.9	0.3	0.3	0.3	0.5	0.4	0.5	0.2	0.4		17
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	

R. africae
R. akari
R. amblyommii
Astrakhan fever
R. australis
R. bellii
R. conorii
R. helvetica
R. honei
R. japonica
R. massiliae
R. montana
R. parkeri
R. rhipicephali
R. rickettsii
R. sibirica
R. slovaca

FIG. 4. Matrix showing structural divergence and similarity of selected rickettsial strains.

affected by SFG has rickettsial disease. In addition, genetic differences in the rickettsial isolates from Flinders Island and the mainland *R. australis* isolates were demonstrated. Restriction fragment length polymorphism analysis of PCR-amplified genes has been previously used to compare SFG rickettsiae (5). We have exploited differences in the previously published genetic structure of the genus-specific 17-kDa common antigen gene to rapidly compare the isolates and show their differences. Primers rRNA1 and rRNA2 have been used routinely to amplify the 16S rRNA gene from mycobacterial species (13, 15), and we were able to amplify this gene from both *R. australis* and the Flinders Island isolates. The results of 16S rRNA gene sequencing confirm the genetic differences between the two distinct rickettsial isolates. Recent phylogenetic analysis of rickettsial species (7, 16, 21, 22) has suggested that *R. australis* should be classified separately from other SFG rickettsiae.

The closest relative of the Flinders Island agent, *R. honei*, appears to be the geographically distinct *R. rickettsii*. In this phylogenetic analysis, the other geographically close SFG rickettsia *R. japonica*, isolated in Japan, appears to be more structurally divergent than the more distantly geographically spaced isolates of *R. rickettsii* and *R. conorii*. Future studies based on analysis of multiple isolates to reveal the degree of intraspecies genetic variation may provide insight into this paradox.

The presence of two distinct isolates of SFG rickettsiae in Australia, in two distinct locations, from patients with clinical spotted fever raises a number of intriguing questions. Previous serological studies (18) have extended the range of SFG rickettsial disease down the eastern Australian seaboard to northern Tasmania. To date, rickettsial isolations have not been achieved in northern Tasmania to determine the SFG rickettsial species present. Interestingly, the unique focus of rickettsial infection on Flinders Island does not appear to occur on the other Bass Strait island, including King Island, an island which has a temperate climate similar to that of Flinders Island.

In view of the geographic, phenotypic, and genetic differences between the rickettsial isolates from Flinders Island and *R. australis*, we propose that the rickettsia from patients with Flinders Island spotted fever be designated *R. honei* after the distinguished Australian Frank Sandland Hone (8, 10, 11, 12). While chief quarantine officer for South Australia, Hone, a pioneer of Australian rickettsiology, described in 1922 and 1923 a large epidemic of an illness resembling typhus fever which had occurred in South Australia.

We conclude that there are two populations of SFG rickettsiae in Australia. The clinical illnesses produced by these rickettsiae are similar, but their distributions and animal host reservoirs remain to be elucidated.

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