***Rickettsia honei* sp. nov., the aetiological agent of Flinders Island spotted fever in Australia**

John Stenos,¹ Véronique Roux,² David Walker¹ and Didier Raoult²

Author for correspondence: David Walker. Tel: +1 409 772 2856. Fax: +1 409 772 2500.
e-mail: dwalker@marlin.utmb.edu

¹ Department of Pathology, University of Texas Medical Branch, 301 University Blvd, 1.116 Keiller Bldg, Galveston, TX 77550-0609, USA

² Unité des Rickettsies, CNRS UPRESA 6020, Faculté de médecine, 27 Bd Jean Moulin, 13385 Marseille, France

The name *Rickettsia honei*, strain RB^T, has been proposed for a unique spotted fever group (SFG) agent which is pathogenic for humans. This agent has previously been compared to the other SFG agents and was shown to be distinct in protein structure by SDS-PAGE and by immunoblotting. Genetic comparisons of the 16S rRNA, *rompA*, *gltA* and the 17 kDa antigen genes with the other SFG rickettsiae confirmed the phylogenetic distance between *R. honei* and the previously described species. Genetically, *Rickettsia honei* is more closely related to the Thai tick typhus (TT-118) rickettsia than to any other member of the SFG. Indeed, it is proposed that TT-118 is a strain of *R. honei* which was previously isolated in Thailand. These results elucidate the presence of a unique SFG rickettsial species in Australasia.

Keywords: *Rickettsia honei* sp. nov., spotted fever group

INTRODUCTION

The human disease, Queensland tick typhus (QTT), which is tick transmitted and is caused by *Rickettsia australis*, was believed to be limited to the Australian states of Queensland and New South Wales. However, recent reports have described spotted fever group (SFG) rickettsial infections occurring in the south-eastern region of Victoria (6), Flinders Island (28) and Tasmania (5).

An SFG rickettsiosis-like ailment was identified on Flinders Island in 1991 by the local medical practitioner, Dr Robert Stewart, who suspected the clinical symptoms of the patients to be rickettsial in origin (28). Signs and symptoms of the disease included fever, headache, myalgia, slight cough and a maculopapular rash. Serological analysis, including the Weil–Felix agglutination and rickettsial-specific immunofluorescence (IF) tests, suggested that the causative agent was a member of the SFG (10).

Blood was collected from patients presenting with Flinders Island spotted fever (FISF) prior to antibiotic therapy. Concentrated buffy coat from the blood was inoculated onto Vero cell monolayers, which were subsequently centrifuged (26). Weekly cell culture

scrapings of the monolayer were examined by IF, and on the fourth week a microbe of typical rickettsial morphology was observed (11).

There was no apparent cytopathic effect in the primary isolation. On subsequent passages, cytopathic effect was observed and was characterized by scattered irregularly shaped lesions with cells detaching from the monolayer (1). Electron microscopy revealed a Gram-negative bacillary bacterium measuring 0.4 µm in diameter and 1.0 µm in length, and having a low-density cytosol and encompassed by a multilayered outer membrane which was loosely adherent to the inner membrane (1).

At the genetic level, the 17 kDa antigen and the 16S rRNA genes of *Rickettsia honei* have been sequenced and compared with other members of the SFG. The analysis revealed a unique set of sequences for this agent, grouping it with *Rickettsia rickettsii* and *Rickettsia slovaca* (2). Subsequently, there have been several more sequence additions to the 17 kDa antigen and 16S rRNA gene database, and hence the phylogenetic position of *R. honei* needs to be re-evaluated. We further characterize the genetic position of the FISF agent by citrate synthase (*gltA*) and rickettsial outer-membrane protein A (*rompA*) gene sequence analyses. These two genes have been sequenced for most of the other members of the SFG rickettsiae and were shown to be useful tools to study the phylogenetic organization of these obligately intracellular bacteria (9, 23). On the basis of our results together with the previous publications on the geographic isolation,

Abbreviations: FISF, Flinders Island spotted fever; IF, immunofluorescence; SFG, spotted fever group.

The GenBank accession numbers of the *R. honei* gene sequences in this study are AF018074 (*gltA*), AF018075 (*rompA* 590 bp fragment) and AF018076 (*rompA* 3176 bp fragment).

genetic sequencing, phenotypic and protein analysis of this agent, we formally propose the name *Rickettsia honei*, the type strain of which is RB^T.

METHODS

Cell culture. *R. honei* was seeded into confluent Vero cell monolayers in RPMI 1640 medium as described previously (11). The infected cell culture was harvested, and the rickettsiae purified using renografin density-gradient centrifugation (27).

Rickettsial chromosomal DNA extraction. Purified *R. honei*

were incubated with 1 mg proteinase K ml⁻¹ and 1 % SDS at 56 °C for 1 h. This digestion was followed by two phenol/chloroform (50:50) extractions and ethanol precipitation. The resultant DNA pellet was washed with 70 % ethanol, air-dried and reconstituted with nuclease-free water.

PCR amplification of the *R. honei* *gltA* and *ompA*. Oligonucleotides were designed from the sequence of the *R. rickettsii* *gltA* gene with the exception of RpCS1258, which has been described previously (Table 1) (20). The *R. honei* *gltA* was amplified using 2 µmol primer pair CS-162-F and RpCS1258, 200 µmol dNTPs and 10 ng *R. honei* DNA in Advantage Tth Polymerase mix (Clontech). The amplifi-

Table 1. Primer sequences of the rickettsial *gltA* gene

Primer	Primer sequence
CS-162-F	5' GCA AGT ATC GGT GAG GAT GTA ATC 3'
CS-398-SF	5' ATT ATG CTT GCG GCT GTC GG 3'
CS-764-SF	5' ATT GCC TCA CTT TGG GGA CC 3'
RpCS1258	5' ATT GCA AAA AGT ACA GTG AAC A 3'
CS-731-SR	5' AAG CAA AAG GGT TAG CTC C 3'
CS-411-SR	5' AAT GCC GCA AGA GAA CCG ACA G 3'

Table 2. GenBank accession numbers of rickettsial sequences used in this study

Organism	Strain	Accession no. for:			
		17 kDa antigen	16S rDNA	<i>gltA</i>	<i>ompA</i>
<i>Rickettsia parkeri</i>	Maculatum 20	U17008	L36673	U59732	U43802, U83449
<i>Rickettsia sibirica</i>	246, ATCC VR-151 ^T		L36218	U59734	U43807, U83455
<i>Rickettsia mongolotimonae</i>	HA-91		L36219	U59731	U43796, U83439
Strain S	Strain S		U25042	U59735	U43805, U83452
<i>Rickettsia africae</i>	ESF-5		L36098	U59733	U43790, U83436
<i>Rickettsia honei</i>	RB ^T	M99391	U17645	AF018074	AF018075, AF018076
Thai tick typhus rickettsia	TT-118, ATCC VR-599 ^T	AF027124	L36220	U59726	U43809, U83456
Astrakhan fever rickettsia	A-167		L36100	U59728	U43791, U83437
Israeli tick typhus rickettsia	ISTT CDC1		L36223	U59727	U43797, U83441
<i>Rickettsia conorii</i>	Moroccan, ATCC VR-141	M28480	L36105	U59730	U43244, U83448
<i>Rickettsia rickettsii</i>	R (Bitterroot), ATCC VR-89 ^T	M16486	L36219	U59729	U43804, U83451
<i>Rickettsia slovaca</i>	13-B		L36224	U59725	U43808, U83454
<i>Rickettsia japonica</i>	YM	D16515	L36213	U59724	U43795, U83442
<i>Rickettsia massiliae</i>	Mtu1		L36214	U59719	U43799, U83445
<i>Rickettsia rhipicephali</i>	3-6-7	U11020	L36216	U59721	U43803, U83450
<i>Rickettsia aeschlimannii</i>	MC16		U74757	U59722	U43800, U83446
<i>Rickettsia montanensis</i>	M/5-6	U11017	L36215	U74756	U43801, U83447
<i>Rickettsia helvetica</i>	C9P9		L36212	U59723	
<i>Rickettsia akari</i>	MK (Kaplan), ATCC VR-148 ^T		L36099	U59717	
<i>Rickettsia australis</i>	Phillips	M74042	L36101	U59718	
<i>Rickettsia typhi</i>	Wilmington, ATCC VR-144 ^T	M28481	L36221	U59714	
<i>Rickettsia prowazekii</i>	Breinv, ATCC VR-142 ^T	M28482	M21789	M17149	
AB bacterium		U04162	U04163	U59712	
<i>Rickettsia canadensis</i>	2678, ATCC VR-610 ^T	M82879	L36104	U59713	
<i>Rickettsia amblyommii</i>	MO 85-1084	U11013			
<i>Rickettsia felis</i>		M82878			
<i>Rickettsia bellii</i>	369L42-1	U11013	L36103	U59716	
<i>Bartonella quintana</i>	Fuller, ATCC VR-358 ^T	M82878	M73228	L38987	

cation was performed on a thermal cycler (Perkin Elmer Cetus) with 35 cycles of denaturation at 95 °C, annealing at 48 °C and extension at 70 °C for 1 min intervals. The resultant PCR product was purified from unincorporated primers and nucleotides using the QIAquick PCR purification kit (Qiagen). Amplification of *rompA* was performed as described previously (9).

DNA sequencing of PCR products. The purified *gltA* and *rompA* PCR products were sequenced using the ABI PRISM 377 Sequencer (Perkin Elmer) and ALF DNA sequencer (Pharmacia LKB), respectively.

DNA analysis. The gene sequences of the 16S rRNA, 17 kDa, *gltA* and *rompA* for the rickettsial species were extracted from GenBank (Table 2). The homologous rickettsial gene segments were aligned with those of other rickettsial sequences using the multisequence alignment program CLUSTAL. Phylogenetic relationships were inferred using the PHYLIP version 3.4 software package (J. Felsenstein, University of Washington, Seattle, USA). Evolutionary distance matrices, generated by DNADIST, were determined by the methods of Kimura (16). These matrices were used to infer dendrograms using the neighbour-joining method (24). The data were also examined using the parsimony analysis (DNAPARS). The stability of predicted branching orders was assessed by generation of a consensus tree from 100 trees derived from bootstrap samples of the original alignment using SEQBOOT and CONSENSE in the same package.

RESULTS AND DISCUSSION

Genetic analysis

Phylogenetic trees derived from distance matrix analysis generated from 16S rRNA, *gltA*, *rompA* and 17 kDa antigen gene sequence alignments are presented in Figs

Fig. 1. Phylogenetic tree obtained by a neighbour-joining analysis of the 16S rRNA gene sequences, showing the position of *R. honei* in the α -1 subclass of the *Proteobacteria*. Bootstrap values from 100 analyses are shown at the branch points of the tree. The accession numbers of the sequences of the organisms used in the analysis are listed in Table 2.

Fig. 2. Phylogenetic tree obtained by a neighbour-joining analysis of the *gltA* sequences.

1, 2, 3 and 4, respectively. The general organization of the four dendrograms was the same within the trees derived from parsimony analysis as that found in evolutionary distance matrix analysis. However, branching order for each gene was slightly different especially when bootstrap values were low. In all four phylogenetic trees, *R. honei* and the Thai tick typhus rickettsia (TT-118) are grouped together in a unique branch which is closely associated to the core SFG branch but far from *R. australis*. In fact these two agents had identical gene sequences except for the 16S rDNA gene where they differed by 1 nucleotide. Trees generated using the 17 kDa antigen gene by parsimony and distance matrix analysis revealed low bootstrap values. This situation indicated that this particular gene is not a suitable tool for phylogenetic analysis. However, this phylogenetic tree, like the others, does display a similar relationship between *R. honei* and the other SFG members. As previously determined, *rompA* sequence comparison is the best tool to infer phylogenetic relationships among the SFG rickettsiae which diverged late from the common ancestor of this group of bacteria (9). Our data support this opinion since the bootstrap values are the greatest for this phylogenetic tree, in most cases equalling 100.

The plethora of rickettsial genetic information currently available in GenBank, including 16S rRNA, 17 kDa, *gltA* and *rompA* genes, has helped with the speciation of several human and tick rickettsial isolates including *Rickettsia massiliae* and *Rickettsia aeschlimannii* (3, 4).

Justification of a new species

Traditionally, microimmunofluorescence using a particular protocol for developing mouse antisera against the SFG rickettsiae has been the reference method for defining the relationships between taxa of the SFG

Fig. 3. Phylogenetic tree obtained by a neighbour-joining analysis of the *romA* sequences.

Fig. 4. Phylogenetic tree obtained by a neighbour-joining analysis of the 17 kDa antigen gene sequences.

rickettsiae (18, 29). However other assay systems including SDS-PAGE, immunoblotting and gene sequencing are more objective methods that can be utilized to determine the phylogenetic identity of a particular agent (19).

SDS-PAGE and immunoblot analysis of *R. australis* and *R. honei* protein lysates revealed differences in protein profiles and structure. There were distinct differences between these rickettsiae in terms of protein electrophoretic mobility and antigenicity. Further

analysis with species-specific monoclonal antibodies derived from other members of the SFG showed no reactivity with *R. honei* (27).

Although the 16S rRNA gene has been used for the speciation of bacterial isolates, it has been proposed that sequence homologies greater than 97% should rely on DNA–DNA reassociation for taxonomic classification (25). The SFG rickettsiae share more than 97.8% homology in the 16S rRNA gene, and with the exceptions of *Rickettsia akari* and *R. australis* DNA–DNA homology analysis groups the rest of the spotted fever group members into a single species (22, 30). However, it has been proposed that the evolutionary selective pressures of the rickettsiae may differ from the enterobacteria due to the differences in genome size and strict intracellular growth and thus the criteria for classification should be different (15).

Based on the calibration of rRNA gene evolution by Ochman & Wilson (17), the divergence of *R. honei* and *R. australis* occurred 65 million years ago whereas the divergence of *R. honei* and TT-118 has occurred recently. It seems plausible that the divergence of the common ancestors of *R. australis* and *R. honei* can be explained by continental drift. The close relationship of *R. honei* and TT-118 suggests that they are strains of the same species. Although the vector ecology of *R. honei* and TT-118 is not clearly defined and both strains are found in Australasia, they may have been introduced to one site or the other by migratory birds. For example, the Curlew sandpiper (*Calidris ferruginea*), Sanderling (*Calidris alba*) and black-bellied plover (*Pluvialis squatarola*) migrate south from the Arctic tundras through Asia to coastal regions of Australia (7). The arthropod that harbours *R. honei* has not been identified, but a reptile-associated tick namely, *Aponoma hydrosauri*, has been suspected based on temporal association of bite with onset of illness in a patient (11). TT-118 was isolated from a mixed pool thought to represent larval *Ixodes* sp. and *Rhipicephalus* sp. (21). This situation would suggest that the rickettsial species may have been introduced by a rickettsemic bird or a tick carried upon a bird. We propose that *R. honei* (strain RB^T) be designated as the reference strain since it is an isolate from a patient who displayed the clinical symptoms of SFG rickettsial disease (11). The likeness of these two strains will be investigated further by microimmunofluorescence and monoclonal antibody typing.

Recent publications have shown the distinctiveness of *R. honei*, both at the protein and genetic levels (1, 2, 27). We have pooled the previously available genetic information together with the *gltA* and *rompA* sequences to show this agent's relationship to the other SFG members. Characteristically the SFG members are all genetically closely related to one another, but have been assigned species names due to their geographic isolation and diverse arthropod host life cycles. Both rickettsial *gltA* and *rompA* genes have been proposed as phylogenetic tools for determining the relationships among the rickettsiae (8, 23). We have

used these genes together with the 17 kDa antigen and 16S rRNA genes to demonstrate that *R. honei* is a unique species of the SFG rickettsiae.

Description of *Rickettsia honei* sp. nov.

Rickettsia honei [ho.ne.i. L. gen. n. *honei* of Hone, named after Frank Sandland Hone, an Australian physician who was an early pioneer in Australian rickettsiology (8, 12–14)].

This agent is an obligately intracellular bacterium which can be grown at 32 °C in Vero cells with RPMI 1640 supplemented with 10% foetal calf serum and 2 mM L-glutamine (11). Its morphology is characteristically rickettsial in nature as determined by electron microscopy (1). SDS-PAGE, immunoblotting and species-specific monoclonal antibody analyses demonstrated the uniqueness of *R. honei* (27). We have further characterized this agent by sequence analysis and have shown that *R. honei* is truly a new member of the SFG. The type strain of *R. honei* is strain RB^T, isolated from a person with the clinical manifestations of Flinders Island spotted fever. This strain has been deposited in the American Type Culture Collection as ATCC VR-1472^T.

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REFERENCES

1. Baird, R. W., Lloyd, M., Stenos, J., Ross, B. C., Stewart, R. S. & Dwyer, B. (1992). Characterization and comparison of Australian human spotted fever group rickettsiae. *J Clin Microbiol* **30**, 2896–2902.
2. Baird, R. W., Stenos, J., Stewart, R., Hudson, B., Lloyd, M., Aiuto, S. & Dwyer, B. (1996). Genetic variation in Australian spotted fever group rickettsiae. *J Clin Microbiol* **34**, 1526–1530.
3. Beati, L., Meskini, M., Thiers, B. & Raoult, D. (1997). *Rickettsia aeschlimannii* sp. nov., a new spotted fever group rickettsia associated with *Hyalomma marginatum* ticks. *Int J Syst Bacteriol* **47**, 548–554.
4. Beati, L. & Raoult, D. (1993). *Rickettsia massiliae* sp. nov., a new spotted fever group rickettsia. *Int J Syst Bacteriol* **43**, 839–840.
5. Chin, R. H. & Jennens, I. D. (1995). Rickettsial spotted fever in Tasmania [letter]. *Med J Aust* **162**, 669.
6. Dwyer, B. W., Graves, S. R., McDonald, M. I., Yung, A. P., Doherty, R. R. & McDonald, J. K. (1991). Spotted fever in East Gippsland, Victoria: a previously unrecognised focus of rickettsial infection. *Med J Aust* **154**, 121–125.
7. Elphick, J. (1995). The atlas of bird migration. Tracing the great journeys of the world's birds, pp. 128–129. New York: Random House.
8. Fenner, F. (1990). History of microbiology in Australia. In *Rickettsioses*, pp. 265–266. Maryborough, Victoria: Australian Print Group.

9. Fournier, P. E., Roux, V. & Raoult, D. (1998). Phylogenetic analysis of spotted fever group rickettsiae by study of the outer surface protein rOmpA. *Int J Syst Bacteriol* **48**, 839–849.
10. Graves, S. R., Dwyer, B. W., McColl, D. & McDade, J. E. (1991). Flinders Island spotted fever: a newly recognised endemic focus of tick typhus in Bass Strait. 2. Serological investigations. *Med J Aust* **154**, 99–104.
11. Graves, S. R., Stewart, L., Stenos, J., Stewart, R. S., Schmidt, E., Hudson, S., Banks, J., Huang, Z. & Dwyer, B. (1993). Spotted fever group rickettsial infection in south-eastern Australia: isolation of rickettsiae. *Comp Immunol Microbiol Infect Dis* **16**, 223–233.
12. Hone, F. S. (1922). A series of cases closely resembling typhus fever. *Med J Aust* **1**, 1–13.
13. Hone, F. S. (1923). A further series of cases closely resembling typhus fever. *Med J Aust* **1**, 435–443.
14. Hone, F. S. (1927). Endemic typhus fever in Australia. *Med J Aust* **2**, 213–226.
15. Kelly, P. J., Beati, L., Mason, P. R., Matthewman, L. A., Roux, V. & Raoult, D. (1996). *Rickettsia africae* sp. nov., the etiological agent of African tick bite fever. *Int J Syst Bacteriol* **46**, 611–614.
16. Kimura, M. (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.
17. Ochman, H. & Wilson, A. C. (1987). Evolution in bacteria: evidence for a universal substitution rate in cellular genomes. *J Mol Evol* **26**, 74–86.
18. Phillip, R. N., Casper, E. A., Burgdorfer, W., Gerloff, R. K., Hughes, L. E. & Bell, E. J. (1978). Serologic typing of rickettsiae of the spotted fever group by indirect immunofluorescence. *J Immunol* **121**, 1961–1968.
19. Raoult, D. & Roux, V. (1997). Rickettsioses as paradigms of new or emerging infectious diseases. *Clin Microbiol Rev* **10**, 694–719.
20. Regnery, R. L., Spruill, C. L. & Plikaytis, B. D. (1991). Genotypic identification of rickettsiae and estimation of intraspecies sequence divergence for portions of two rickettsial genes. *J Bacteriol* **173**, 1576–1589.
21. Robertson, R. G. & Wisseman, C. L., Jr (1973). Tick-borne rickettsiae of the spotted fever group in west Pakistan. *Am J Epidemiol* **97**, 55–64.
22. Roux, V. & Raoult, D. (1995). Phylogenetic analysis of the genus *Rickettsia* by 16S rDNA sequencing. *Res Microbiol* **146**, 385–396.
23. Roux, V., Rydkina, E., Ereemeeva, M. & Raoult, D. (1997). Citrate synthase gene comparison, a new tool for phylogenetic analysis, and its application for the rickettsiae. *Int J Syst Bacteriol* **47**, 252–261.
24. Saitou, N. & Nei, M. (1997). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
25. Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.
26. Stenos, J., Graves, S. & Dwyer, B. (1992). Quantification of *Rickettsia australis*. *Am J Trop Med Hyg* **47**, 141–146.
27. Stenos, J., Ross, B., Feng, H. M., Crocquet-Valdes, P. & Walker, D. (1997). Protein characterization of Australian spotted fever group rickettsiae and monoclonal typing of *Rickettsia honei*. *J Clin Microbiol* **35**, 261–263.
28. Stewart, R. S. (1991). Flinders Island spotted fever: a newly recognised endemic focus of tick typhus in Bass Strait. 1. Clinical and epidemiological features. *Med J Aust* **154**, 94–99.
29. Uchida, T., Yu, X. J., Uchiyama, T. & Walker, D. H. (1989). Identification of a unique spotted fever group rickettsia from humans in Japan. *J Infect Dis* **159**, 1122–1126.
30. Walker, D. H. (1989). Rocky Mountain spotted fever: a disease in need of microbiological concern. *Clin Microbiol Rev* **2**, 227–240.