Genetic Classification of “Rickettsia heilongjiangii” and “Rickettsia hulinii,” Two Chinese Spotted Fever Group Rickettsiae

J. Z. ZHANG,1,2 M. Y. FAN,2 Y. M. WU,3 P. E. Fournier,1 V. ROUX,1 AND D. RAOULT1*

Unité des Rickettsies, Faculté de Médecine, CNRS UPRÉS-A6020, 13385 Marseille Cedex 05, France,1 and Department of Rickettsiology, Institute of Epidemiology & Microbiology, Chinese Academy of Preventive Medicine, Beijing 102206,2 and Department of Microbiology, Institute of Military Medicine, Shenyang Military Command, Shengyang 110034,3 Peoples Republic of China

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To determine the phylogenetic position of two new rickettsial strains isolated from ticks in China, 16S ribosomal DNA, gltA, and ompA (apart from the tandem repeat units) genes were amplified by PCR and sequenced. The phylogenetic relationships between these strains and other rickettsiae were inferred from the comparison of sequences of the three genes by the parsimony, neighbor-joining, and maximum-likelihood methods. The results demonstrated that the 054 strain, a rickettsia pathogenic in humans, and the HL-93 strain were related and clustered together with Rickettsia japonica. Significant statistical bootstrap values (100 and 92%) supported the nodes in this cluster. Based on previous genotypic and antigenic data and the phylogenetic analysis presented here, the 054 and HL-93 strains should be considered as new species, and we formally propose that they be named “Rickettsia heilongjiangii” and “Rickettsia hulinii,” respectively.

The advent of new culture techniques, such as the shell vial-centrifugation technique (16), and the detection of rickettsial DNA have dramatically increased the number of rickettsial species detected (20). Before 1984, only six spotted fever group (SFG) rickettsioses were recognized in the world (20): Rocky Mountain spotted fever caused by Rickettsia rickettsii, Mediterranean spotted fever caused by Rickettsia conorii, Siberian tick typhus caused by Rickettsia sibirica, Queensland tick typhus caused by Rickettsia australis, rickettsialpox caused by Rickettsia akari, and Israeli spotted fever caused by Israeli tick typhus rickettsia. Over the last 12 years, eight new rickettsioses have been reported, including Japanese spotted fever caused by Rickettsia japonica, Flinders Island spotted fever caused by Rickettsia honei, Astrakhan fever caused by Astrakhan fever rickettsia, African tick bite fever caused by Rickettsia africana, California flea typhus caused by the ELB agent, and three unnamed spotted fevers caused by “Rickettsia slovaca,” Rickettsia schwartzii, and Rickettsia helvetica. In China, 20 strains of tick-associated rickettsiae, belonging to at least five serotypes, have been isolated over the last few years, including R. sibirica; the BJ-90 isolate (42); the Heilongjiang isolate (strain 054, also called “Rickettsia heilongjiangii”); the Inner Mongolian isolate HA-91, or “R. mongolotimonae,” also found in France; and the Hulin isolate (strain HL-93, also called “Rickettsia hulinii”) (9, 19, 33). Three of these rickettsiae, R. sibirica, “R. mongolotimonae,” and “R. heilongjiangii,” are human pathogens. “R. heilongjiangii” was first isolated in 1982 from Dermacentor silvarum ticks collected in Suifenhe, a city of Heilongjiang Province in China (Fig. 1). Between May and July 1988, 12 patients in Chunhua, Huichun County, Jilin Province, were reported to be infected by this strain on the basis of serological results. All patients had a history of fever, headache, rash, eschar, regional lymphadenopathy, and conjunctivitis following a tick bite, with fourfold rises in complement fixation antibodies in convalescent-phase sera (14). Between May and June 1996, seven patients with clinical manifestations of SFG rickettsial infections were found in Suifenhe and Dongning, in Heilongjiang Province. Isolates from these patients were found to be identical to the 054 strain by immunofluorescence and PCR plus restriction fragment length polymorphism (30). The “R. hulinii” strain was first isolated in 1993 from Haemaphysalis concinna ticks collected in Hulin County, Heilongjiang Province (Fig. 1), and was considered to be a unique SFG rickettsia (38). To date, the pathogenicity of this strain has been experimentally demonstrated in animals (38), but not in humans. In order to estimate the phylogenetic classification of strains 054 and HL-93, we sequenced their 16S rRNA (16S ribosomal DNA [rDNA]–, citrate synthase [gltA]–, and rOmpA [ompA]–encoding genes and compared these sequences with those of other SFG rickettsiae.

Strains 054 and HL-93 were cultivated and purified as previously described (12), and their DNA was extracted from purified organisms with the QIAmp tissue kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. PCR amplification and sequencing reactions were performed with the oligonucleotide primers mentioned below. All primers were purchased from GIBCO BRL (Custom Primers, Life Technologies Sarl. B.P., Cergy Pontoise Cedex, France), and amplifications were carried out in a PTC-200 DNA thermal cycler (MJ Research, Inc., San Francisco, Calif.) under conditions previously described (11, 22–24). R. japonica and distilled water were used as a positive and negative control, respectively. Each amplicon obtained was purified for DNA by using the QIAquick Spin PCR Purification kit; Perkin-Elmer) as described by the manufacturer. Sequencing reactions were carried out with the Dye Terminator kit (d-rhodamine terminator cycle DNA sequencing kit; Perkin-Elmer) as described by the manufacturer. Sequencing reaction products were resolved by electrophoresis with an ABI Prism 377 Sequencer (Perkin-Elmer). The results obtained were processed into sequence data by using the Se-
The primers fD1 and rP2 amplified 1,463- and 1,461-bp fragments of the 16S rDNA gene from strains 054 and HL-93, respectively. The gltA gene was amplified in two fragments. Overall, sequences of 1,238 and 1,237 bp were obtained from the O54 and HL-93 strains, respectively. PCR amplification and sequencing of the ompA gene excluded the central tandemly repeat region. The 5′-end fragment of the gene was 611 bp for both the O54 and HL-93 strains. The 3′ part of the gene was amplified in four fragments. Overall, sequences of 3,187 and 3,181 bp were obtained from strains 054 and HL-93, respectively. The sequenced fragments of the rOmpA gene were analyzed from base positions 91 to 680 and 3608 to 6789 with respect to the sequence published for *R. rickettsii* (2) and were therefore submitted to GenBank as two separate fragments. Strains 054 and HL-93 clustered together with *R. japonica* into a well-defined, strongly supported monophyletic group within the SFG rickettsiae, with bootstrap values of 91 and 100% for the node where strains and HL-93 branched together in the gltA and ompA trees, respectively, and values of 92 and 100% for the node where both strains clustered with *R. japonica*. Similar phylogenetic organizations were inferred from the analysis of all three genes by using the three phylogenetic analysis methods (Fig. 2).

Historically, the classification and identification of rickettsiae have been based on differences in their epidemiology, serology, and intracellular growth characteristics. Because different species may have common ecological features (e.g., geographic distribution, arthropod vectors, etc.) and due to serological cross-reactivity, it is difficult to distinguish between rickettsiae. Moreover, DNA-DNA hybridization, a criterion used for the definition of species in the family *Enterobacteriaceae*, is not applicable to SFG rickettsiae (8). Other recognized tools for the taxonomic classification of bacteria, such as the study of protein profiles by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, monoclonal antibodies, Western blotting, and hybridization of labeled cloned DNA probes (1, 3, 17, 21, 31, 32, 34), are not widely applicable and lack interlaboratory reproducibility. Therefore, for over 10 years, the reference method for the identification of members of SFG rickettsiae has been the indirect microimmunofluorescence serologic typing test with mouse sera (18). Recently, phylogenetic analyses and the determination of taxonomic relationships based on comparisons of sequences of a combination of various genes have greatly facilitated the study of rickettsial taxonomy. The 16S rDNA (23, 26, 28), gltA (24), and ompA (11, 22) genes appear to be the most useful sequences. In our study, the 054 and HL-93 strains demonstrated ompA sequence homologies of 98.1% with each other and 96.6 and 95.6% with *R. japonica*, respectively, which are higher than
those observed for strain HL-93 with \textit{R. rickettsii} or \textit{R. conorii} (94 and 90\%, respectively). Although closely related, the data
on serotyping (11), and, to date, there is no consensus on
differentiation of epidemic typhus rickettsiae (\textit{Rickettsia prowazekii})
until the mid-twentieth century (12, 21, 36). Some characteristics of heavy and light bands of \textit{Rickettsia prowazekii}
in concordance with previous phenotypic and genotypic studies
of epidemic typhus rickettsiae by sequencing and analysis of restriction fragment length polymorphism of PCR-amplified DNA of the gene encoding the protective protein antigen of \textit{Rickettsia rickettsii} has tandemly repeated, near-

**REFERENCES**


