Genetic Differentiation of Chinese Isolates of *Rickettsia sibirica* by Partial *ompA* Gene Sequencing and Multispace Typing

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Current data on rickettsiae and rickettsial diseases in China remain limited. Using partial *ompA* gene sequencing and multispace typing, we identified 15 rickettsial isolates from China. All isolates were found to belong to *Rickettsia sibirica* subsp. *sibirica*. Four isolates from *Dermacentor sinicus* collected in Beijing, China, were fully identical to strain BJ-90, previously demonstrated to belong to *R. sibirica* subsp. *sibirica* despite antigenic and genotypic specificities. All 11 remaining isolates were similar to the *R. sibirica* subsp. *sibirica* type strain, 246. These were widely distributed in China in humans and different tick species. We emphasize the importance of surveying the distribution of *R. sibirica* in China.

Spotted fever rickettsioses are a group of tick-, flea-, or mite-borne infections caused by spotted fever group (SFG) rickettsiae. The distribution of each rickettsiosis is directly dependent on that of its arthropod vector or vectors (16). A large-scale epidemiological investigation of rickettsioses has been performed in China over the past 50 years. This survey has demonstrated a modification of the geographic distribution of SFG rickettsiae, from a limited focus in northern China at the beginning of the survey to a wide area expanding from the Xinjiang province in the west to the Heilongjiang province in the east and from Inner Mongolia in the north to Hainan island in the south and finally to virtually all areas investigated to date (3, 4). Currently, there are three SFG rickettsiae present in China: *Rickettsia sibirica* subsp. *sibirica*, *R. sibirica* subsp. *mongolotimonae*, and *R. heilongiangensis* (7). The majority of Chinese SFG isolates belong to the *R. sibirica* species.

*R. sibirica* contains two subspecies (12), i.e., *R. sibirica* subsp. *sibirica*, the agent of North Asian tick typhus, and *R. sibirica* subsp. *mongolotimonae*, the agent of “lymphangitis-associated rickettsiosis.” The former subspecies was first isolated in Russia but it has subsequently been found in northern China (19). In contrast, *R. sibirica* subsp. *mongolotimonae* was first isolated in Inner Mongolia and then found in southern Europe and Africa (9, 12). *R. heilongiangensis*, first isolated from *Dermacentor silvarum* ticks in the Heilongjiang province of China (22), was subsequently demonstrated to cause a human spotted fever in China and the Russian Far East (15). *R. hulinii* was first isolated from *Haemaphysalis concinna* ticks in the Heilongjiang province of China, and its pathogenic role for humans is suspected but has not been demonstrated as yet (22).

From 1974 to 2000, 16 rickettsial strains identified as *R. sibirica* were isolated in the Chinese Center for Rickettsial Diseases, Beijing, China. Herein, in order to precisely identify these 16 strains, we used partial *ompA* sequencing and multispace typing (11), both tools being described as highly discriminant among *Rickettsia* isolates (10, 17).

**MATERIALS AND METHODS**

*R. sibirica* strain cultivation and DNA extraction. The 16 studied isolates were obtained from patients or ticks from different locations at different times (Fig. 1; Table 1). Rickettsiae were inoculated in L929 cell monolayers in Eagle’s minimal essential medium (Seromed, Berlin, Germany) supplemented with 4% fetal bovine serum and 2 mM glutamine. They were then cultivated at 32°C in a 5% CO2-enriched atmosphere. Bacteria were harvested after Gimenez stain was heavily positive, and the total genomic DNA was extracted from culture using a QIAamp tissue kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions. Two hundred microliters of elution buffer was used to resuspend each extracted DNA. Genomic DNAs were then stored at −20°C until further processing.

**PCR amplification and sequencing.** The primers used to amplify the *ompA* gene (17) and the three variable intergenic spacers are listed in Table 2. PCR amplifications were performed with a PE 9600 thermal cycler (Applied Biosystems). For each specimen, the 25-μl reaction mixture contained the following: 1 μl of the appropriate DNA template, 10 pM of each primer, 0.2 mM of deoxynucleoside triphosphate mixture (dATP, dCTP, dGTP, and dTTP), 0.5 U of Hot Star Taq DNA polymerase (QIAGEN), 2.5 μl of 10× Taq buffer, and 1 μl of 25 mM MgCl2. The following conditions were used for amplification:

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Strain Origin</th>
<th>Location</th>
<th>Yr of isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>JH-74</td>
<td>D. nuttalli</td>
<td>Jinghe, Xinjiang</td>
</tr>
<tr>
<td>2</td>
<td>SFH-053</td>
<td>D. silvarum</td>
<td>Suifenhe, Heilongjiang</td>
</tr>
<tr>
<td>3</td>
<td>An-84</td>
<td>Patient</td>
<td>Jinghe, Xinjiang</td>
</tr>
<tr>
<td>4</td>
<td>MT-84</td>
<td>D. nuttalli (male)</td>
<td>Jinghe, Xinjiang</td>
</tr>
<tr>
<td>5</td>
<td>FT-84</td>
<td>D. nuttalli (female)</td>
<td>Jinghe, Xinjiang</td>
</tr>
<tr>
<td>6</td>
<td>Se-85</td>
<td>Patient</td>
<td>Humeng, Inner Mongolia</td>
</tr>
<tr>
<td>7</td>
<td>To-85</td>
<td>D. nuttalli (tick ova)</td>
<td>Humeng, Inner Mongolia</td>
</tr>
<tr>
<td>8</td>
<td>W-88</td>
<td>Patient</td>
<td>Zhemeng, Inner Mongolia</td>
</tr>
<tr>
<td>9</td>
<td>BJ-90</td>
<td>D. sinicus</td>
<td>Changping, Beijing</td>
</tr>
<tr>
<td>10</td>
<td>BJ-91</td>
<td>D. sinicus</td>
<td>Changping, Beijing</td>
</tr>
<tr>
<td>11</td>
<td>BJ-93</td>
<td>D. sinicus</td>
<td>Changping, Beijing</td>
</tr>
<tr>
<td>12</td>
<td>BJ-95</td>
<td>D. sinicus</td>
<td>Changping, Beijing</td>
</tr>
<tr>
<td>13</td>
<td>NH-95</td>
<td>Haemaphysalis concinna</td>
<td>Ninghua, Fujian</td>
</tr>
<tr>
<td>14</td>
<td>NH-98</td>
<td>Patient</td>
<td>Ninghua, Fujian</td>
</tr>
<tr>
<td>15</td>
<td>GDFK-58</td>
<td>Unidentified tick</td>
<td>Fengkai, Guangdong</td>
</tr>
<tr>
<td>16</td>
<td>GDFK-59</td>
<td>Unidentified tick</td>
<td>Fengkai, Guangdong</td>
</tr>
</tbody>
</table>
predenaturation for 15 min at 94°C, followed by 39 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 54°C for amplifying intergenic spacer sequences and 53°C for the ompA gene, and extension for 1 min at 72°C. Amplification was completed by holding the reaction mixture for 5 min at 68°C for final extension of the PCR products. Positive and negative controls were used to confirm the reagent quality and avoid manipulation errors and contamination (DNA extracted from R. sibirica strain 246 was used as the positive control and sterile water as the negative control). PCR products were purified using a QIAquick Spin PCR purification kit (QIAGEN) as described by the manufacturer. PCR products were sequenced in both directions by using PCR primers and a d-rhodamine Terminator cycle sequencing ready reaction kit (AppliedBiosystems) as described by the manufacturer. Sequencing products were resolved using an ABI 3100 automated sequencer (Perkin-Elmer). Sequence assembly was performed using ABI Prism DNA sequencing analysis software, version 3.4 (Perkin-Elmer), and multisequence alignment was done using CLUSTAL W software, version 1.81 (18).

Nucleotide sequence accession numbers. The sequences for DQ008262, DQ008247, and DQ008283 have been submitted to GenBank.

RESULTS

Partial ompA gene sequencing. A 602-bp fragment of ompA was obtained for all strains except strain NH-98, which could not be cultivated. Nucleotide sequence alignment showed that all 15 isolates were divided into two types (Fig. 2). Four strains, i.e., BJ-90, BJ-91, BJ-93, and BJ-95, isolated from Dermacentor sinicus ticks collected in Beijing in 1990, 1991, 1993, and 1995, respectively, had an identical ompA nucleotide sequence (GenBank accession number AF179365). All 11 remaining strains had a nucleotide sequence identical to that of R. sibirica strain 246 (GenBank accession number U43807). The degrees of nucleotide and derived amino acid sequence similarity between BJ-type isolates and all other isolates described in this paper were 99.8 and 100%, respectively.

Multispace typing. PCR amplification of the three intergenic spacers in the 15 strains we tested yielded products of the expected sizes. For each spacer, all 15 strains exhibited identical sequences. The nucleotide sequences of the three intergenic spacer sequences were found to be identical to those of R. sibirica subsp. sibirica (GenBank accession numbers DQ008262 for dksA-xerC, DQ008247 for rpmE-tRNA^{Met}, and DQ008283 for mppA-purC).

DISCUSSION

Using partial ompA gene sequencing and multispace typing, we demonstrated that 15 rickettsial isolates obtained from human or ticks over a 26-year period in various areas of China belong to R. sibirica subsp. sibirica. In 1993, Yu et al., by using PCR-restriction fragment length polymorphism, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and Western blotting, demonstrated that Chinese rickettsial isolates could be classified within three types (19). Type 1 was antigenically and genotypically identical to R. sibirica and was represented by isolate To-85, previously isolated from tick ova of Dermacentor nuttallii in Humeng county, Inner Mongolia, in 1985 (5). Type 2 was genotypically identical to but antigenically slightly different from R. sibirica. The representative isolate was BJ-90, isolated from D. sinicus ticks in the Changping district of Beijing in 1990 (7). Type 3 exhibited unique genotypic and antigenic characteristics. Its representative strain, HA-91, was first isolated from Hyalomma asiaticum kozlovi olenew ticks collected from Inner Mongolia in 1991 (14). Since that work, strains BJ-90 and HA-91 have been demonstrated to belong to the R. sibirica species (8) and the creation of two subspecies

![FIG. 1. Detailed geographic distribution of studied isolates.](http://jcm.asm.org/)
within *R. sibirica* has been proposed (12). Strain HA-91 is the type strain of *R. sibirica* subsp. *mongolotimonae*, whereas strain BJ-90 belongs to *R. sibirica* subsp. *sibirica*. Herein, we found a great genetic homogeneity among BJ-type isolates from the Beijing area (BJ-90, BJ-91, BJ-93, and BJ-95). These differed from the other *R. sibirica* isolates, which themselves were highly homogeneous. It is important to note that BJ-type isolates have been obtained from only one tick species, that is, *D. sinicus*, in a single location over different years (2, 20, 21). However, it is not clear whether the Changping area of Beijing is the only area of distribution of this rickettsia, as few other rickettsia isolates from *D. sinicus* ticks from other locations are available. In addition, the pathogenic status of BJ-type strains is currently unknown.

Another significant outcome of this report is that *R. sibirica* subsp. *sibirica* is distributed nationwide in China, from the Xinjiang province in the west to the Heilongjiang province in the north to the Guangdong province in southern China (13). This rickettsia is vectored by at least four tick species. We emphasize the fact that four of the studied isolates were obtained from patients, thus demonstrating the potential risk of North Asian tick typhus in many areas of China (1, 6). Further surveillance of spotted fever group rickettsiae and rickettsioses in China will be conducted to estimate the prevalence of the disease.

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**REFERENCES**