Genotypic and Antigenic Identification of Two New Strains of Spotted Fever Group Rickettsiae Isolated from China

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Four isolates of spotted fever group rickettsiae isolated from ticks in China were compared with all known species and strains of spotted fever group rickettsiae by immunofluorescence assay, DNA polymerase chain reaction followed by restriction endonuclease fragment length polymorphism analysis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and Western immunoblot. The Chinese isolates belonged to three types, including a novel serotype which has not been described before. One isolate obtained from tick ova of Dermacentor nutalli in Inner Mongolia was antigenically and genotypically identical to Rickettsia sibirica. Two isolates obtained from Dermacentor sinicus collected from Beijing were identical, different from other members of spotted fever group rickettsiae but apparently closely related to R. sibirica. HA-91, a strain isolated from Hyalomma asiaticum bv. kozlovi olenew, was antigenically and genotypically unique among spotted fever group rickettsiae, and we feel that data presented here should prompt consideration of it as a new species on the basis of current rickettsial taxonomy.

MATERIALS AND METHODS

Isolation of rickettsiae from ticks. Ticks were collected from domestic or wild animals and classified by local entomologists. Dermacentor sinicus ticks were collected from Changping County of Beijing in June 1990 and May 1991, and Hyalomma asiaticum kozlovi olenew ticks were collected from the Alashan region of the Inner Mongolia autonomous region in May 1991 (Fig. 1). The ticks were surface sterilized by immersion in 70% alcohol for 10 min and then rinsed with distilled water for 10 min. Each tick was initially tested by the hemolymph test (4). One leg of the tick was cut off and a drop of hemolymph was applied to a slide and stained by the method of Gimenez (12). Ticks positive by hemolymph test were homogenized and suspended in SPG (218 mM sucrose, 3.8 mM KH₂PO₄, 7.2 mM K₂HPO₄, and 4.9 mM monosodium L-glutamic acid; pH 7.0) buffer (2). The suspension from each group of ticks was injected intraperitoneally into two guinea pigs. The rectal temperature of the guinea pigs was measured twice a day. The blood of guinea pigs that had fever was sampled after 3 days of fever and inoculated into embryonated hens’ eggs for further isolation of rickettsiae.

Rickettsia strains. BJ-90 and BJ-91, two isolates from D. sinicus, were named after Beijing, where the ticks were collected and numbered according to the year that the rickettsiae were isolated (1990 and 1991, respectively). HA-91 was isolated from H. asiaticum kozlovi olenew collected from Inner Mongolia in 1991. A Chinese reference strain, IMTO-85, was previously isolated from tick ova of Dermacentor nutalli (9). Barbash strain, originally from the World Health Organization, was provided by Rickettsial Laboratory, Institute of Epidemiology and Microbiology, Chinese Academy of Preventive Medicine (Beijing, China). R. japonica, R. montana, and R. parkeri were kindly provided by David H. Walker (University of Texas Medical Branch at Galveston, Galveston, Tex.), and R. sibirica 232, Ethiopia tick typhus rickettsia, Kenya tick typhus rickettsia, Israel tick typhus rickettsia, R. australis, R. belli, R. rhipicephali, and R. slovaca were obtained from G. A. Dasch (Naval Medical Research Institute, Bethesda, Md.). The following reference rickettsiae were obtained from the American Type Culture Collection: R. conori Indian strain (ATCC VR-591), R. conori Moroccan strain (ATCC VR-141), R. akari strain Kaplan (ATCC VR-148), R. rickettsii strain Sheila Smith (ATCC VR-149), and Thai tick typhus rickettsia (ATCC VR-599).

Cultivation of rickettsiae. Except for the initial isolation of Chinese strains by using embryonated hens’ eggs, all rickettsiae were cultivated in L929 cells in preparation for PCR and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Cell culture medium (Eagle’s minimal essential medium) was supplemented with 4% fetal calf serum. The infected cells were incubated at 32°C and were harvested with glass beads when rickettsial infection in the cells was heavy.

Electron microscopy. After 3 days of rickettsial infection, the L929 cell monolayer was fixed in 3.15% glutaraldehyde...
for 30 min and then washed in sucrose-cacodylate buffer for 30 min and fixed again in 1% osmium-1.5% potassium ferricyanide for 1 h. Dehydration was performed by using increasing concentrations of acetone (15 to 100%). The layer was embedded in Epon, sectioned, stained with uranyl acetate and lead citrate, and examined with a JEOL JEM 1200 EX electron microscope (3).

**Antisera.** Mouse antisera were prepared by the method of Philip et al. (15); female Swiss Webster mice were injected intravenously via the tail vein with 0.5 ml of rickettsia-infected L929 cells on day 0 and 7 and were exsanguinated on day 10. The sera from each group were pooled and stored at −70°C. Rickettsia-infected L929 cells were used as antigens for immunofluorescence assay IFA. Fluorescein (DTAF)-labeled goat anti-mouse immunoglobulin G and immunoglobulin M (heavy plus light chains) conjugate was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, Pa.).

The specificity difference (SPD) between each pair of strains was calculated according to the formula SPD = (Aa + Bb) − (Ab + Ba) (15), where Aa (or Bb) is the antibody titer of serum A (or B) reacted with heterologous antigen b (or a). In this formula, antibody titers were expressed as the log_{10} of the endpoint titer. If the SPD between two strains was ≥3 (P < 0.01), they were considered two distinct species (15).

**Purification of rickettsiae.** The rickettsia-infected cells were centrifuged at 7,000 × g for 10 min. The pellet was resuspended in K36 buffer (0.1 M KCl, 0.05 M phosphate buffer, 0.015 M NaCl; pH 7.0) (20) and sonicated in an ice bath. The suspension was loaded onto a cushion of 30% sucrose in K36 buffer and centrifuged at 7,000 × g for 30 min. The resulting pellet was purified by Renografin density gradient centrifugation (20). The light and heavy bands were harvested and washed with K36 buffer at 7,000 × g for 10 min. The purified rickettsiae were resuspended in distilled water. The rickettsial-protein concentrations were quantified by the method of Lowry et al. (14).

**SDS-PAGE and Western blot.** SDS-PAGE was performed as described previously (13); 4% stacking and 7.5% separating gels with 2.6% cross-linking were used. The purified rickettsiae were solubilized in Laemmli (13) solubilizer (4% SDS, 0.125 M Tris hydrochloride [pH 6.8], 25% glycerol, 10% 2-mercaptoethanol, 0.5% bromophenol blue) at room temperature, and 10 µg of rickettsial protein was loaded into each well of the gels. The gel (6 by 8 by 0.1 cm) (Bio-Rad, Richmond, Calif.) was run at 10 mA in an ice bath. Western blot (immunoblot) was performed as described previously (19) in an ice bath. The nitrocellulose paper was blocked with 5% nonfat milk in TBS (20 mM Tris-HCl, 500 mM NaCl; pH 7.5). The mouse antisera were diluted 1/100 in TBS containing 1% nonfat milk. Peroxidase-conjugated goat anti-mouse immunoglobulin G (heavy plus light chains) (Jackson ImmunoResearch Laboratories) was diluted 1/500 in TBS. The nitrocellulose paper was incubated with 4-chloro-1-naphthol (Sigma, St. Louis, Mo.) to develop the color. Low-range and high-range prestained molecular weight standards (Bio-Rad) were used to estimate the molecular weights of proteins.

**PCR amplification, DNA digestion, and electrophoresis.** The samples for PCR amplification were prepared as described previously (1, 7). A 1.5-ml sample of rickettsia-infected L929 cells was washed three times with distilled water by centrifugation at 17,500 × g for 5 min. The pellet was resuspended in 100 µl of distilled water and boiled for 10 min; 10 µl of this material was used as DNA templates. DNA amplification, digestion, and electrophoresis were carried out as described previously (16). DNA amplification was done in a thermal cycler (PREM III; Lep Scientific, Fllobio, Courbevoie, France) with 35 cycles of denaturation (20 s at 95°C), annealing (30 s at 48°C), and extending (2 min at 60°C). Noninfecting L929 cells and distilled water were used as negative controls. PCR amplification of DNA was verified by 1% agarose (Sigma Chimie, La Verpillière, France) gel electrophoresis of 10 µl of PCR product. A 23.5-µl volume of PCR product was digested with restriction endonuclease at 37°C for 2 h. The digested products were separated on 8% polyacrylamide vertical gels (Bio-Rad Laboratories) by standard procedures (18). Gels were run at 100 V for 4 h and then stained with ethidium bromide. The gels were photographed with type 667 P/N film (Polaroid Corp., Cambridge, Mass.) and a 365-nm UV light source (Bioblock Scientific, Illkirch, France). DNA molecular marker V (Boehringer GmbH, Mannheim, Germany) was used to determine the sizes of the DNA fragments. The sizes of DNA fragments were calculated by entering digitized migration data directly into a desktop computer (The Imager; Appli gen, Heidelberg, Germany). PCR-RFLP was performed by using the following oligonucleotide primer pairs (Bioprobe Systems, Montreuil-sous-Bois, France) and restriction endonucleases (New England Biolabs, Beverly, Mass.): Rp CS.877p and Rp CS.1258n (16) (encoding a 381-bp sequence) with Ali1, Rr 190.70p and Rr 190.602n (532 bp) (16) with RsaI and PstI, and Rr 120.BG3 and Rr 120.BG4 (615 bp) (7, 11) with Ali1 and RsaI.

**Comparison with other rickettsiae.** Our laboratory is equipped with an image system (Appli gen) connected with a QGL program retaining pictures and molecular weights from previous works. R. akari, R. australis, R. conori, R. rhizophilus, R. parkeri, R. slovaca, R. helvetica, R. bellii, R. rickettsi, Thai tick typhus rickettsia, and R. massiliae have been tested by both SDS-PAGE (1) and PCR-RFLP (1, 7, 8), and the results constitute a data bank allowing comparison of new strains with previously tested species.
RESULTS

Isolation of the new strains. Two strains of rickettsiae were isolated from D. sinicus and one strain was isolated from H. asiaticum kozlovi olenew. All of these rickettsiae were pathogenic to guinea pigs. All caused fever (40 to 42°C) and scrotal swelling. No deaths among guinea pigs were observed. The incubation time for the guinea pigs was between 2 and 5 days, depending on the initial numbers of rickettsiae inoculated. The rickettsiae were observed intracellularly on the smear of guinea pig scrotal tissue after staining by the method of Gimenez (12) (data not shown). All isolates grew well in embryonated hens' eggs and cell culture.

PCR-RFLP. Results of the PCR-RFLP analysis are shown in Fig. 2 to 7. Amplification by using the Rp CS.877p and Rp CS.1258n primer pair and digestion with restriction endonuclease Aul\textsubscript{i} demonstrated that all Chinese isolates (Fig. 2) had the typical profiles of the SFG rickettsiae (16) and that the DNA migration patterns of R. japonica were unique. When the Rr 190.70p and Rr 190.602n primer pair was used, the PCR products of all rickettsiae had the same patterns on agarose gels except for that of Barbash strain, whose molecular weight was much less than those for other SFG rickettsiae (data not shown). The amplification products of all rickettsiae with these primer pairs were digested by restriction endonucleases PstI and RsaI except for the PCR product of R. japonica, which could not be digested by RsaI (Fig. 3). Restriction of these products with RsaI demonstrated that all Chinese strains were divided into two distinct genotypic groups: one group including BJ-90, BJ-91, and IMTO-85 had PCR-RFLP patterns identical to those of R. sibirica; HA-91 belonged to another group whose PCR-RFLP patterns were different from those of R. sibirica but identical to those of R. parkeri and similar to those of strains of R. conorii (Fig. 3 and 6). PstI digestion of PCR-amplified rickettsial DNA with the Rr 190.70p and Rr 190.602n primer pair showed that all Chinese strains, R. sibirica, R. parkeri,
Ethiopia tick typhus rickettsia, and Israel tick typhus rickettsia had identical profiles, but HA-91 had patterns different from those of *R. conorii* Moroccan strain and Indian strain and Kenya tick typhus rickettsia (Fig. 4).

The *Rr* 120.BG 3 and *Rr* 120.BG 4 primer pair was further used for PCR-RFLP analysis of strain HA-91 and *R. parkeri*, since they were not differentiated by digestions with *RsaI* and *Pst*I, respectively, of PCR-amplified DNA derived from the *Rr* 190.70p and *Rr* 190.602n primer pair. Rickettsial DNA amplified with the *Rr* 120.BG3 and *Rr* 120.BG4 primer pair digested with *RsaI* demonstrated that the profiles of strain HA-91 and *R. parkeri* were different from those of Ethiopia tick typhus rickettsia and Israel tick typhus rickettsia (Fig. 5 and 7). *AluI* digestion of these PCR products did not disclose additional differences between strain HA-91 and these rickettsiae (data not shown).

**FIG. 6.** Schematic electrophoresis migration patterns of PCR-amplified DNA (*Rr* 190.70p and *Rr* 190.602n) of rickettsiae *R. sibirica* (Sib), *R. parkeri* (Pak), Israel tick typhus rickettsia (Isr), Ethiopia tick typhus rickettsia (Eth), *R. conorii* Moroccan strain (Mor), *R. conorii* Indian strain (Ind), Kenya tick typhus rickettsia (Ken), Barbash strain (Bar), and *R. japonica* (Jap) digested with *RsaI*. Doublet bands are indicated with an asterisk. Comigrating fragments are connected by dashed lines. Numbers are molecular sizes in base pairs.

**FIG. 8.** Profiles of Coomassie brilliant blue-stained, SDS-PAGE-separated rickettsial whole proteins with low (A) and high (B) molecular masses. HA, HA-91; TO, IMTO-85; sib, *R. sibirica*; 90, BJ-90; 91, BJ-91. Molecular mass markers (in kilodaltons) are indicated. Distinctive rickettsial protein bands are indicated by arrows.

**SDS-PAGE and Western blot.** The purity of rickettsiae was tested by SDS-PAGE by comparison with host cell lysates. The results demonstrated that only one protein with a molecular mass of 60 kDa was common among rickettsiae and L929 cells (data not shown). The 60-kDa protein of rickettsiae was probably not contaminated with host protein. It may be a heat shock protein and was shared by rickettsiae and host cells (6). The protein profiles of the rickettsiae showed that all strains shared many common proteins with low molecular masses (<106 kDa) (Fig. 8A), but the major proteins in the high-molecular-mass range (>106 kDa) were distinctive among these strains of rickettsiae (Fig. 8). IMTO-85 and *R. sibirica* had protein profiles identical to those of the major outer membrane proteins of 106, 118, and 155 kDa. The protein profiles of BJ-90 and BJ-91 were identical to each other and slightly different from that of *R. sibirica*, with a distinct protein band of 162 instead of 155 kDa. HA-91 strain had one distinct major protein band with a molecular mass of 180 kDa. Western blot with typing sera demonstrated that the major antigenic proteins among Chinese isolates were different from each other (Fig. 9).
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HA-91 rickettsiae of proteins for distinctive R. ali, R. conorii, R. massiliae, Halo-like species such as R. typhus, R. rickettsiae, R. sibirica, R. rhipicephali, R. sotticata, and Thai tick typhus rickettsia. The size of this species is 7,000 kDa for HA-91. The derived patterns for each rickettsia in the cytoplasm and surrounded the strain HA-91. Our previous study demonstrated that there are three types of SFG rickettsiae distributed in northern China. Type 1 was antigenically and genotypically identical to R. sibirica, which was represented by IMTO-85, the rickettsia previously isolated from tick ova (9). Type 2 was antigenically identical to and antigenically related to R. sibirica, but its profile was different and the representative strain for this type is BJ-90. Type 3 was both antigenically and antigenically unique among SFG rickettsiae, and the representative strain was HA-91. This was confirmed by IFA because the SPDs between HA-91 and other SFG rickettsiae were ≥3. SFG rickettsia serotypes are considered species according to current criteria (21). One of our new isolates, HA-91, should be considered a new species of SFG rickettsiae. The new isolates BJ-90 and BJ-91 could probably be considered variant strains of R. sibirica.

Previous studies demonstrated that all SFG rickettsiae isolated from the broad area of China from Xinjiang in the west to Heilongjiang in the east were antigenically identical to R. sibirica (5, 10). Our study not only identified two new types of SFG rickettsiae but also expanded the documented distribution of SFG rickettsiae in China. The south boundary of SFG rickettsiae distribution in China was moved to latitude 40°N. Probably the geographic distribution of SFG rickettsiae is not limited to this area. D. sinicus is distributed broadly in China, including Hebei and Shandong provinces in the south. Any area where D. sinicus is found could be a natural focus of SFG rickettsiae.

These new isolates were pathogenic to guinea pigs; however, their pathogenicity for humans remained to be investigated.

FIG. 10. Transmission electron micrograph of L929 cell infected with HA-91 rickettsiae (indicated by an arrow). Magnification, ca. x7,000.

FIG. 11. Transmission electron micrograph of L929 cell infected with BJ-90 rickettsiae (indicated by an arrow). Magnification, ca. x7,000.
TABLE 1. IFA titers of reciprocal reaction of mouse antiseras with rickettsial antigens and SPDs between strains of rickettsiae

<table>
<thead>
<tr>
<th>Mouse antiserum type</th>
<th>Titer of rickettsial antigen (SPD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA-91</td>
<td>1,024 (0)</td>
</tr>
<tr>
<td>BJ-90</td>
<td>128 (4)</td>
</tr>
<tr>
<td>R. sibirica</td>
<td>128 (5)</td>
</tr>
<tr>
<td>R. slovaca</td>
<td>256 (4)</td>
</tr>
<tr>
<td>R. rickettsii</td>
<td>16 (6)</td>
</tr>
<tr>
<td>R. parkeri</td>
<td>256 (4)</td>
</tr>
<tr>
<td>R. conorii</td>
<td>128 (5)</td>
</tr>
<tr>
<td>EthTT</td>
<td>256 (4)</td>
</tr>
</tbody>
</table>

*a* Moroccan strain.

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REFERENCES


