

## Molecular Detection of Spotted Fever Group *Rickettsia* in *Dermacentor silvarum* from a Forest Area of Northeastern China

WU-CHUN CAO,<sup>1,2</sup> LIN ZHAN,<sup>1</sup> SAKE J. DE VLAS,<sup>3</sup> BO-HAI WEN,<sup>1</sup> HONG YANG,<sup>1</sup>  
JAN H. RICHARDUS,<sup>3</sup> AND J. DIK F. HABBEMA<sup>3</sup>

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**ABSTRACT** In total, 676 *Dermacentor silvarum* Olenov (Acari: Ixodidae) from a forest area of Jilin Province in northeastern China were examined by polymerase chain reaction for the presence of spotted fever group (SFG) *Rickettsia*. The overall positive rate was 10.7%, with a 95% confidence interval from 8.3 to 13.0%. The SFG *Rickettsia* infection was more prevalent in adults than in nymphs, and in fed ticks obtained from domestic animals than in those collected on vegetation. Sequence analysis of the partial outer membrane protein A gene confirmed the existence of *R. sibirica* and discovered a novel rickettsial agent in this area, the sequence of which was identical to that of DnS14 genotype *Rickettsia* previously reported in the former Soviet Union.

**KEY WORDS** *Rickettsia*, *Dermacentor silvarum*, China

Spotted fever group (SFG) rickettsiae are obligately intracellular gram-negative bacteria associated with arthropods. Ticks, which maintain the microorganisms in a natural cycle through transovarial and transstadial passage, act as both reservoirs and vectors. While feeding, they can transmit SFG infections to humans and animals (Parola and Raoult 2001). In China, five species of tick-transmitted SFG rickettsiae were reported previously, three of which have been known to cause human diseases (reviewed in Fan et al. 1999). *Rickettsia sibirica*, the pathogen of Siberian tick typhus, transmitted by *Dermacentor* spp., is frequently encountered, especially in the northern China. Another SFG *Rickettsia* was first identified from *Hyalomma asiaticum* in Inner Mongolia, and it was isolated from the blood and skin of a patient in Marseille, France. Therefore, the agent was named *Rickettsia mongolotimona* (Raoult et al. 1996). "*Rickettsia heilongjiangensis*" initially isolated from *Dermacentor silvarum* Olenov (Acari: Ixodidae) in Heilongjiang Province was then found in patients from Jilin and Heilongjiang provinces (Lou et al. 1989, Zhang et al. 2000a). In addition to the three human pathogens, some rickettsiae of unknown pathogenicity were recognized from ticks, including "*Rickettsia hulunii*" from *Hemaphysalis concinna* (Zhang et al. 2000a) and BJ-90 strain from *Dipentodon sinicus* (Zhang et al. 2000b).

Detection and identification of the tick-borne SFG rickettsiae before the 1990s mainly depended on culture and epitope recognition techniques, such as im-

munofluorescence and agglutination tests as well as serotyping with monoclonal antibodies. Recently, new molecular techniques have enabled the development of useful, sensitive, and rapid tools to detect agents in ticks (Sparagano et al. 1999). Sequence comparison of the gene encoding outer membrane protein A, *ompA* (Fournier et al. 1998), is a currently accepted method for recognizing rickettsiae (Billings et al. 1998, Marquez et al. 1998, Weller et al. 1998, Rydkina et al. 1999, Ammerman et al. 2004). The molecular sequence-based identification techniques facilitate genetic characterization without need for bacterial culture. In the current study, polymerase chain reaction (PCR) and sequence analysis of amplified products were carried out to identify rickettsiae in *D. silvarum* from a forest area of Jilin Province in northeastern China.

### Materials and Methods

**Tick Collection.** *D. silvarum* were collected in 2005 in the hinterland of Changbai Mountains situated at 42–43° N, 126–128° E within Jingyu County, Jilin Province. The terrain consists of forested rolling hills, with an average elevation of 775 m. The annual precipitation is ≈800 mm, and the relative humidity is ≈70%. The temperature ranges from –41.4 to 33.6°C, with the average of 4.0°C. Questing ticks were collected by dragging a standard 1-m<sup>2</sup> flannel flag over vegetation. Fed ticks were collected from domestic animals including cattle, sheep, and dogs. Ticks were identified by an entomologist to the species level, and the development stages were kept alive in a refrigerator until DNA extraction was performed.

**DNA Extraction.** The ticks to be tested were soaked in 70% ethanol for 15 min, and then they were rinsed

<sup>1</sup> Beijing Institute of Microbiology and Epidemiology, State Key Laboratory of Pathogen and Biosecurity, 20 Dong-Da St., Fengtai District, Beijing 100071, People's Republic of China.

<sup>2</sup> Corresponding author, e-mail: caowe@nic.bmi.ac.cn.

<sup>3</sup> Department of Public Health, Erasmus MC, University Medical Center, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands.

three times in sterile water. DNA was extracted by a method described previously (Cao et al. 2000). Briefly, the ticks were individually placed into micro-tubes and mechanically crushed with sterile scissors in 50  $\mu$ l of DNA extraction buffer (10 mM Tris, pH 8.0, 2 mM EDTA, 0.1% SDS, and 500  $\mu$ g/ml proteinase K). The samples were incubated for 2 h at 56°C, and then they were boiled at 100°C for 10 min to inactivate proteinase K. After centrifugation, the supernatant was transferred to fresh sterile micro-tubes and purified by extracting twice with an equal volume of phenol-chloroform. The DNA was precipitated by adding three volume of ice-cold absolute ethanol and 100  $\mu$ l of 3 M sodium acetate to the samples, and then they were placed at -20°C for 24 h. The DNA was pelleted at 10,000g for 15 min at 4°C in a microcentrifuge tube and washed twice with ice-cold 70% ethanol. After drying, the DNA was resuspended in 50  $\mu$ l of DNase-free water and used as template for PCR amplification. The blank micro-tubes with only 50  $\mu$ l of DNA extraction buffer were assessed in parallel with the tick samples during DNA extraction.

**PCR Amplification.** PCR was performed using primers Rr190.70p (Regnery et al. 1991) and 190-701 (Roux et al. 1996), which amplifies a 629- or 632-bp fragment of the gene encoding a 190-kDa *ompA* specific for the SFG rickettsiae. The PCR amplifications were performed in a 30- $\mu$ l reaction mixture containing of 3.0  $\mu$ l of purified DNA, 200  $\mu$ M each deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP), 0.8  $\mu$ M *Taq* polymerase, and forward and reverse primers (0.5  $\mu$ M each). In parallel with each amplification of tick specimens, negative (sterile water) and positive controls (DNA from *Rickettsia conorii*) were included. The reaction was carried out in a PCR thermal cycler (AB Geneamp, PCR System 2700, Applied Biosystems, Foster City, CA), with the following stepwise procedure: initial denaturation at 94°C for 6 min, followed by 35 cycles of 94°C for 15 s, 54°C for 15 s, 70°C for 30 s, and a final extension at 70°C for 5 min. The PCR products were visualized under UV light after electrophoresis on a 1.2% agarose gel stained with ethidium bromide. To avoid contamination, DNA extraction, the reagent setup, amplification, and electrophoresis were conducted in separate rooms.

**DNA Sequencing and Data Analysis.** The amplicons of positive ticks were then sequenced directly by a dideoxynucleotide cycle sequencing method with an automated DNA sequencer (ABI PRISM 377, PerkinElmer Life and Analytical Sciences, Boston, MA). Two sequencing reactions of each PCR product were performed to limit errors in sequencing. If different sequences were obtained, the further sequencing reactions were carried out to generate a consensus sequence and to determine the potential presence of another co-infected *Rickettsia*. The sequences of either 629- or 632-bp fragments obtained at current study were compared with the corresponding sequences of other rickettsiae deposited in GenBank by means of BLAST.

SFG *Rickettsia* infection rate with its 95% confidence interval (CI) of the ticks was estimated on the

**Table 1.** Prevalences of SFG *Rickettsia* infection in *D. silvarum* ticks from Jilin province of northeastern China

Characteristics	No. ticks tested	Prevalence (95% CI)	P value
Sex/stage			0.002
Male	239	14.6 (10.2-19.1)	
Female	352	10.2 (7.1-13.4)	
Nymph	85	1.2 (0-3.5)	
Origin			<0.001
Questing ticks from vegetation	518	8.3 (5.9-10.7)	
Fed ticks from animals	158	18.4 (12.3-24.4)	
Mo of collection			0.265
May	213	8.9 (5.1-12.7)	
June	346	11.0 (7.7-14.3)	
July	117	12.8 (6.8-18.9)	

basis of binomial distribution. A chi-square test was used to compare infection rates across the strata of selected characteristics. A *P* value <0.05 was considered statistically significant.

## Results

In total, 676 *D. silvarum* were examined by PCR for presence of SFG *Rickettsia*. The distribution and infection rates of ticks in terms of sex and stage, origin, and month of collection are shown in Table 1. The overall positive rate for SFG *Rickettsia* in *D. silvarum* was 10.7%, with a 95% CI from 8.3 to 13.0%. The variation in prevalence of rickettsial infection among male, female, and nymphal ticks was statistically significant ( $\chi^2 = 12.09$ , *df* = 2, *P* = 0.002). Although no difference in infection rate was observed between male and female ticks ( $\chi^2 = 2.63$ , *df* = 1, *P* = 0.105), infection with *Rickettsia* was more frequent in adults than that in nymphs ( $\chi^2 = 9.17$ , *df* = 1, *P* = 0.002). The infection rate in fed ticks obtained from domestic animals was significantly higher than that in questing ticks collected on vegetation ( $\chi^2 = 12.86$ , *df* = 1, *P* < 0.001). There was no significant diversification in positive rates among ticks in different months ( $\chi^2 = 1.29$ , *df* = 2, *P* = 0.525).

All the amplicons of the 72 positive ticks were directly sequenced. The partial nucleotide sequences of *ompA* gene obtained using primers Rr190.70p/190.701 from 47 ticks (accounting for 65.3% of the total positive samples) were identical to those of *R. sibirica* genotype (GenBank accession U43807). Although an unreported agent in China (named JL-02, GenBank accession AY093696) was discovered from the other 25 ticks, the sequences of which were 100% homologous with that of DnS14 genotype previously recognized in the former Soviet Union (Rydikina et al. 1999). The sequence was closely related to DnS28 and RpA4 genotypes in the former Soviet Union and *Rickettsia montanensis* in the United States, but they were different from *R. sibirica*, *R. mongolotimonae*, *R. heilongjiangii*, and *R. hulinii* reported previously in China.

### Discussion

The prevalence of SGF *Rickettsia* infection in *D. silvarum* was investigated in a forest area of Jilin Province in northeastern China. As a result, 10.7% ticks were found to be infected, which is comparable with the infection rate in *Dermacentor nuttallii* from the former Soviet Union (Rydkina et al. 1999), *Ixodes ricinus* from Italy (Beninati et al. 2002), but it is lower than that in *Amblyomma variegatum* from Caribbean (Kelly et al. 2003). The phenomena of wide range of *Rickettsia* infection rate in various ticks has been observed in an annual survey in United States (Azad and Beard 1998). The finding that the infection rates of male and female ticks were not significantly different is consistent with that of a previous study (Ammerman et al. 2004). However, adult ticks seem more likely to be infected with *Rickettsia* than nymphs. This may be because the ticks at adult stage have fed on more animal hosts with rickettsemias. *Rickettsia* infection was found more common in fed ticks from animals than that in ticks on vegetation. The possibility is that the feeding activation of *Rickettsia* spp., in which the agent infecting a tick can multiply in response to feeding, increases the probability of detecting the *Rickettsia* by using PCR techniques. *D. silvarum* is one of the most abundant tick species in northeastern China, and it often parasitizes large domestic and wild animals such as cattle, horse, sheep, and deer, and it also readily feeds on humans as alternate hosts. The significance of the tick species in both veterinary medicine and public health deserves further investigation.

The findings of sequence analysis confirmed the existence of *R. sibirica* in Jilin Province, where Siberian tick typhus is endemic. In addition, a *Rickettsia* variant (JL-02) was found in *D. silvarum* from this region. The partial nucleotide sequences of *ompA* gene of the agent is identical to the corresponding part of the *Rickettsia* recognized in *D. nuttallii* from former Soviet Union (Rydkina et al. 1999), and it is genetically distinct from all the rickettsiae previously reported in China. Unfortunately, we did not sequence two separate PCR products or two clones from one PCR reaction, dual infection of the *Rickettsia* variant and *R. sibirica* in a single tick remains to be investigated. In the United States, various tickborne rickettsiae exist in areas endemic for *R. rickettsii*, the agent of Rocky Mountain spotted fever (Niebylski et al. 1997). Similarly, several recently described rickettsiae were found in ticks of the *Rhipicephalus sanguineus* complex in the regions endemic for Mediterranean spotted fever caused by *R. conorii* (Marquez et al. 1998). Although coinfections of *Rickettsia* could be detected in ticks, the transmission to the egg was for only one species. It is believed that only a single species of *Rickettsia* is transmitted transovarially. The clinical importance of the phenomenon is that "nonpathogenic" *Rickettsia* can exclude tick infection with "pathogenic" strains, altering the incidence and prevalence of rickettsial disease in animals and humans in one area (Burgdorfer et al. 1981).

The sequence analysis of *ompA* gene fragment alone is not entirely sufficient to identify the agent as a new *Rickettsia*. However, it is a useful tool for preliminary classification, and it has been widely used to recognize tickborne *Rickettsia* in field surveys (Billings et al. 1998, Marquez et al. 1998, Weller et al. 1998, Rydkina et al. 1999, Ammerman et al. 2004). A recently published article discussed an approach to genetic classification of *Rickettsia* species, in which four to five gene targets were used for sequencing. As an example, the approach was applied for confirming the classification of *R. heilongjiangensis* (Fournier et al. 2003). Further studies are required to classify the JL-02 strain and to understand its public health significance.

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