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Investigation of *Borrelia* spp. in ticks (Acari: Ixodidae) at the border crossings between China and Russia in Heilongjiang Province, China

Shi Liu[1], Chao Yuan[2], Yun-Fu Cui[1*], Bai-Xiang Li[3], Li-Jie Wu[3], Ying Liu[4]

[1]*The 2nd Affiliated Hospital of Harbin Medical University, Harbin 150001, People's Republic of China*

[2]*Daqing Oilfield General Hospital Group Rangbei Hospital, Daqing, 163114, People's Republic of China*

[3]*Harbin Medical University School of Public Health, Harbin 150001, People's Republic of China*

[4]*The 3rd Affiliated Hospital of Qiqihar Medical College, QiqiHar 161002, People's Republic of China*

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ABSTRACT

Objective: To investigate the precise species of tick vector and the *Borrelia* spirochete pathogen at the Heilongjiang Province international border with Russia. **Methods:** In this study, ticks were collected from 12 Heilongjiang border crossings (including grasslands, shrublands, forests, and plantantions) to determine the rate and species type of spirochete-infected ticks and the most prevalent spirochete genotypes. **Results:** The ticks represented three genera and four species of the Ixodidae family [*Ixodes persulcatus*, *Dermacentor silvarum*, *Haemaphysalis concinna* and *Haemaphysalis japonica*]. *Ixodes persulcatus* had the highest amount of *Borrelia burgdorferi sensu lato* infection of 25.6% and the most common species of *Borrelia* isolated from *Ixodes persulcatus* was *Borrelia garinii*, strain PD91. **Conclusions:** Our results suggest that *Borrelia garinii* PD91-infected *Ixodes persulcatus* may be the principal cause of Lyme disease in the border crossing areas of Heilongjiang Province.

1. Introduction

Lyme disease is a tick-borne zoonotic infection caused by spirochetal bacteria of the *Borrelia* genus[1]. Three species of *Borrelia* account for the majority of cases in North America and Asia (*Borrelia burgdorferi sensu stricto*) and Europe [*Borrelia afzelii* (*B. afzelii*) and *Borrelia garinii* (*B. garinii*)][2]. The annual global incidence was estimated at more than 0.3 million cases in 2009[3], with a projected increase in case number due to continued urban sprawl and increased deforestation and contact between humans and animals.

In China, the first reported case of Lyme disease came from the forest region of Hailin County in Heilongjiang Province in 1986[4]. Since then, Lyme disease has been clinically reported by at least 29 provinces, and more than 130 strains of *Borrelia burgdorferi* sensu lato have been isolated from infected patients, ticks, and animals[5,6].

The prevalence of Lyme disease is known to correlate with the geographic distributions and activities of vector ticks[7]. In North America, the *Ixodes scapularis* (Acari: Ixodidae) and *Ixodidae dentatus* (Acari: Ixodidae) are the most common vectors in the Eastern regions[8], while *Ixodidae pacificus* (Acari: Ixodidae) is the most common in the western regions[9]. In Europe, *Ixodidae ricinus* (Acari: Ixodidae) is the most common vector[10]. In China, *Borrelia burgdorferi* sensu lato has been isolated from *Ixodidae persulcatus* (*I. persulcatus*)(Acari: Ixodidae), *Ixodidae granulatus* (*I. granulatus*) (Acari: Ixodidae), *Ixodidae acutitarsus* (Acari: Ixodidae), *Haemaphysalis concinna* (*H. concinna*) (Acari: Ixodidae), *Haemaphysalis japonica* (*H. japonica*) (Acari: Ixodidae), *Haemaphysalis longicornis* (*H. longicornis*) (Acari: Ixodidae), *Haemaphysalis bispinosa* (*H. bispinosa*) (Acari: Ixodidae), *Haemaphysalis cornigera* (Acari: Ixodidae), *Rhipicephalus microplus* (Acari: Ixodidae), *Dermacentor nuttalli* (Acari: Ixodidae), and *Dermacentor silvarum* (*D. silvarum*) (Acari: Ixodidae)[11,12].

*: Corresponding author: Prof. Yun-Fu Cui, Department of General Surgery, The 2nd Affiliated Hospital of Harbin Medical University, No.148 Baojian Road, Nangang District, Harbin 150001, People's Republic of China.

Tel: +86-451-86605113
E-mail: yfcui666@yahoo.cn

Each of these species occupies different geographic niches. The *I. persulcatus* species is the most prevalent tick in the Inner Monglia, Heilongjiang, Jilin, Liaoning, Xinjiang, Ningxia and Hebei Provinces. *H. longicornis* dominates the Henan and Shandong Provinces. *I. granulatus* dominates the Fujian, Guangdong, Yunnan and Guizhou Provinces. *H. bispinosa* is most prevalent in Sichuan, Hunan, Hubei, Jiangxi, Jiangsu and Anhui Provinces[13].

The Heilongjiang Province in Northeast China is not only an endemic region of Lyme disease but represents a unique envirionment that supports transmission since it is situated on the Russian border and the site of international trade. However, little is known about the distinctive epidemiologic factors of the 12 border crossings encompassed within this region. Thus, this study was designed to investigate the rates and species of spirochete-infected ticks and *Borellia* pathogens in the 12 border crossings of Heilongjiang.

2. Materials and methods

2.1. Survey area and tick collection

The 12 Heilongjiang border crossings include Dongning, Suifenhe, Mishan, Hulin, Raohe, Fuyuan, Tongjiang, Luobei, Jiayin, Xunke, Heihe, and Mohe. These crossings join China to the far-Eastern region of Russia, located at 43°25′-50°47′ N and 125°03′-135°05′ E (Figure 1). The international border region ranges in altitude from 40 m to 1 100 m above sea level, and has a subarctic climate (3.6 ℃ annual mean temperature, ~120 day frost-free season, and 500-600 mm annual mean rainfall) and geographic regions ranging from swamps and wetlands to plains and mountains.

For this study, ticks were collected by flagging from plantations, forests, shrublands and grasslands of each of the 12 border crossing areas during their peak activity period (early May to mid June) in 2009. All specimens were stored at 4 ℃ until analyses.

2.2. Spirochete isolation and cultivation

An entomologist (Lin Shi) identified the species of the collected ticks. Isolation and cultivation of spirochetes from infected ticks were carried out according to the previously described methods[14]. The spirochetes from inidividual ticks were cultivated for eight weeks, with weekly monitoring by dark-field microscopy, after which the cultures split and propagated as subcultures. The infectivity rate of each spirochete culture was determined according to the previously described procedure[15]. Once identified, the strains were deposited in the repository at the Heilongjiang Province Center for Disease Control.

Figure 1. Survey areas.
1, Dongning (43°25′-44°35′N and 129°53′-131°18′E); 2, Suifenhe (43°20′-44°40′N and 130°00′-130°40′E); 3, Mishan (45°01′-45°55′N and 131°14′-133°08′E); 4, Hulin (45°23′-46°36′N and 132°11′-133°56′E); 5, Raohe (46°30′-47°34′N and 133°07′-134°20′E); 6, Tongjiang (47° 25′-48° 17′N and 132° 18′-134° 07′E); 7, Luobei (47°12′-48°21′N and 130°01′-131°34′E); 8, Fuyuan (47°25′-48°27′N and 133°40′-135°05′E); 9, Jiayin (48° 83′-49° 26′N and 129° 09′-130° 50′E); 10, Xunke (47°58′-49°36′N and 127°24′-129°17′E); 11, Heihe (47°42′-51°03′N and 124° 45′-129° 18′E); and 12, Mohe (52°10′-53°33′N and 121°07′-124°20′E).

2.3. DNA extraction

Total DNA was extracted from the cultured spirochetes, a panel of international standard strains (B31, 20047 and VS461), and a panel of Chinese representative strains (PD91, FP1, GS1 and CS4; supplied by the Epidemiology Institute, Chinese Center for Disease Control, Beijing, China) using the previously described method[16]. DNA concentration was determined by spectrophotometry.

2.4. PCR and sequencing

PCR amplification of the 5S-23S rRNA gene from each subculture of spriochetes was carried out by the previously described method[17]. PCR products were separated using 1.5% agarose gel electrophoresis, stained with ethidium bromide, visualized under UV, and gel purified. An aliquot of the purified sample was confirmed by sequencing (Boya Biotechnology Corp., Shanghai, China).

2.5. Restriction fragment length polymorphism (RFLP) analysis

For RFLP anlaysis, 5S-23S rRNA amplicons were digested with *Mse* Ⅰ and *Dra* Ⅰ, as previously described[18]. The

digested DNA was then electrophoresed through 16% polyacrylamide gels for 3 h at 100 V. The gels were silver stained and the banding pattern was visualized under white light.

2.6. Statistical analysis

The *Chi*-square test was used to evaluate the preferred habitats among the various tick species. One-way ANOVA and *Chi*-square tests were used to examine the regional differences for the various tick species. The spirochete prevalence ratios were calculated as binomial error estimates and analyzed among the locations by the *Chi*-square test. A *P*-value less than 0.05 indicated statistical significance.

3. Results

3.1. Tick species distribution among the different habitats and locations

A total of 10 126 unfed, host-seeking adult ticks were collected in this survey. All were from the Ixodidae family and represented three genera and four species, including *I. persulcatus*, *D. silvarum*, *H. concinna* and *H. japonica*. Each of the four species had a significant preference among the different habitats (χ^2=2 352.332, *P*<0.005) (Table 1). Specifically *I. persulcatus* was more abundant in forests (35.1%) and plantations (41.6%). There were also significant preferences among the tick species for particular locations (χ^2=4 170.241, *P*<0.005) (Table 2). These locations were divided into northern and southern regions by using the 48°N coordinate. The southern regions included Dongning, Suifenhe, Mishan, Hulin, Raohe, Tongjiang, and Luobei, while the northern regions included Fuyuan, Jiayin, Xunke, Heihe, and Mohe. However, the composition of tick species among the southern and northern regions was not different.

3.2. Prevalence and strains of spirochetes

The spirochete prevalence among *I. persulcatus* was similar among all the locations examined (by *Chi*-square test, *P*=0.065) (Figure 2). The overall average spirochete prevalence ratio was 25.6%. Three strains of spirochetes were isolated from *I. persulcatus*, including HS1, HS2 and HS3. The HS1 strain came from Dongning and the others came from Tongjiang.

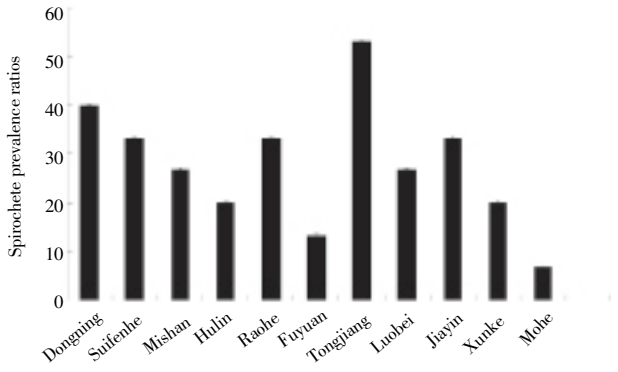

Figure 2. The spirochete prevalence ratios of *I. persulcatus* at different locations.
From left to right, the ratios (%) are: 40.00±0.49, 33.33±0.47, 26.67±0.44, 20.00±0.40, 33.33±0.47, 13.33±0.34, 53.33±0.50, 26.67±0.44, 33.33±0.47, 20.00±0.40, 6.67±0.25, and 0.00±0.00.

3.3. Strain identificaton by sequencing

PCR amplifications were carried out on three international standard strains, four representative strains from China, and the three strains isolated in our study. As shown in Figure 3, all strains produced a 240 bp amplicon. Sequence analysis revealed that the 5S-23S intergenic spacer was identical in HS1, HS2 and HS3 strains (*i.e.* 5′-TAGAATAATATATATCTTT GTTTAATCCATGTCAATATATATTTTA TTTTTTATATTATTTAA ATAAAACATTCAAAAACATGAACATCTAAAAATATAAAAAA TAAAATCAATGTTTAAAGCATAAAATAAAAACCCTGGCAATA ACCTACTCTCCCGCA-3′). In addition, the three strains were also found to be highly homologous to the PD91 strain of *B. garinii* (100% similarity).

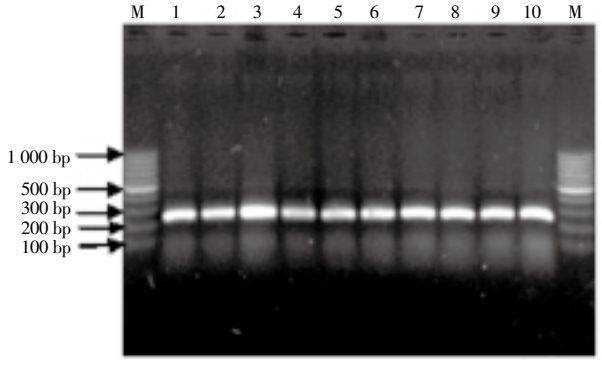

Figure 3. PCR amplicon banding patterns of each strains′ 5S-23S rRNA inergenic spacer.
DNA were electrophoresed through a 1.5% agarose gel, stained with ethidium bromide, and UV illuminated. Lanes are: amplicons produced from 1, B31; 2, 20047; 3, VS461; 4, PD91; 5, FP1; 6, GS1; 7, CS4; 8, HS1; 9, HS2; 10, HS3; and M, markers.

3.4. RFLP analysis of the strains′ 5S-23S rRNA intergenic spacer amplicons

As shown in Table 3, the 5S-23S rRNA RFLP digestion

patterns of strains HS1, HS2 and HS3 were consistent with the Chinese standard *B. garinii* strain PD91 (Figures 4 and 5). For HS1, HS2 and HS3, the *Mse* I restriction fragment sizes were 96, 54, 41 and 38 bp, and those produced by *Dra* I were 128, 52 and 41 bp. Both of these digestion patterns match those of the PD91 strain. These results suggest that the HS1, HS2 and HS3 strains are genospecies *B. garinii* of the PD91 subgroup.

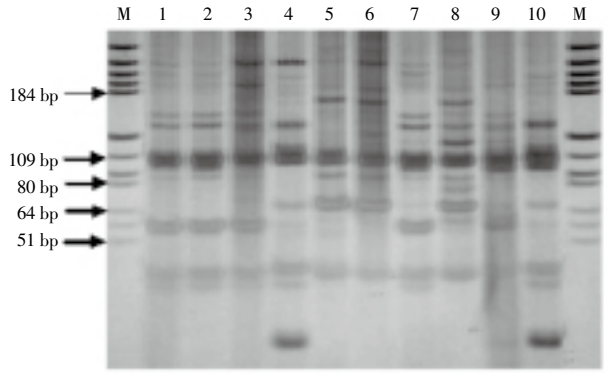

Figure 4. *MseI* digestion patterns of stains' 5S-23S rRNA spacer amplicons.

DNA were electrophoresed through a 16% polyacrylamide gel, stained with silver, and visualized under white light. Lanes are: amplicons produced from 1, HS3; 2, HS2; 3, HS1; 4, CS4; 5, GS1; 6, FP1; 7, PD91; 8, VS461; 9, 20047; 10, B31; and M, markers.

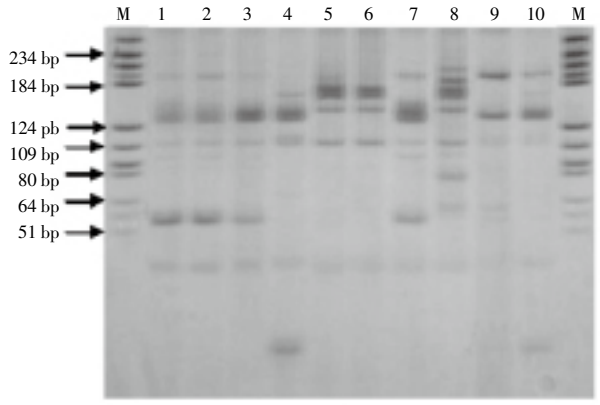

Figure 5. DraI digestion patterns of strains' 5S-23S rRNA spacer amplicons.

DNA were electrophoresed through a 16% polyacrylamide gel, stained with silver, and visualized under white light. Lanes are: amplicons produced from 1, HS3; 2, HS2; 3, HS1; 4, CS4; 5, GS1; 6, FP1; 7, PD91; 8, VS461; 9, 20047; 10, B31; and M, markers.

4. Discussion

Our study showed that *I. persulcatus* was the most abundant tick species in the forest and plantation habitats of the Heilongjiang Province. Thus, border crossings located

Table 1

Tick species distributions among different habitats.

Habitats	Ticks No.	Labor hours	Densites, individuals/labor hour	*I. persulcatus* [*n*(%)]	*D. silvarum* [*n*(%)]	*H. concinna* [*n*(%)]	*H. japonicap* [*n*(%)]
Shrublands	1 908	40	47.7	106(5.6)	1 620(84.9)	155(8.1)	27(1.4)
Forests	6 595	128	51.5	2 316(35.1)	2 037(30.9)	2 040(30.9)	202(3.1)
Grasslands	966	40	21.2	69(7.1)	574(59.4)	293(30.3)	30(3.1)
Plantations	657	62	10.6	273(41.6)	63(9.6)	259(39.4)	62(9.4)
Total	10 126	270	37.5	2 764(27.3)	4 294(42.4)	2 747(27.1)	321(3.2)

Table 2

Tick species distributions among different regions.

Locations	Ticks No.	Labor hours	Densites, individuals/labor hour	*I. persulcatus* [*n*(%)]	*D. silvarum* [*n*(%)]	*H. concinna* [*n*(%)]	*H. japonicap* [*n*(%)]
Dongning	1 301	46	28.3	145(11.1)	980(75.3)	157(12.1)	19(1.5)
Suifenhe	1 514	43	35.2	827(54.6)	467(30.8)	176(11.6)	44(2.9)
Mishan	530	28	18.9	147(27.7)	82(15.5)	255(48.1)	46(8.7)
Hulin	1 352	36	37.6	53(3.9)	867(64.1)	400(29.6)	32(2.4)
Raohe	661	13	50.8	445(67.3)	23(3.5)	170(25.7)	23(3.5)
Tongjiang	684	9	76.0	248(36.3)	–	397(58.0)	39(5.7)
Luobei	916	14	65.4	112(12.2)	292(31.9)	483(52.7)	29(3.2)
Fuyuan	512	12	42.7	203(39.6)	19(3.7)	274(53.5)	16(3.1)
Jiayin	479	12	39.9	81(16.9)	308(64.3)	72(15.0)	18(3.8)
Xunke	893	18	49.6	292(32.7)	487(54.5)	114(12.8)	–
Heihe	1 158	35	33.1	153(13.2)	724(62.5)	226(19.5)	55(4.7)
Mohe	126	4	31.5	58(46.0)	45(35.7)	23(18.3)	–
Total	10 126	270	37.5	2 764(27.3)	4 294(42.4)	2 747(27.1)	321(3.2)

Table 3
5S–23S rRNA RFLP patterns of strains in this study.

Isolate	Origin (Tissue)	Country (Province)	Genotype	*Mse* Ⅰ digestion fragments sizes (bp)	*Dra* Ⅰ digestion fragments sizes (bp)	RFLP patterns	
						Mse Ⅰ	*Dra* Ⅰ
B31	*Ixodes scapularis*	United States	*Borrelia burgdorferi* sensu stricto	96, 41, 36, 25, 24	128, 41, 30, 28	A	A′
20047	*Ixodes ricinus*	France	*B. garinii*	96, 85, 41	204, 41	B	B′
VS461	*Ixodes ricinus*	Swizerland	*B. afzelii*	96, 62, 41, 18	142, 41, 22	D	D′
PD91	Human (blood)	China (Inner Mongolia)	*B. garinii*	96, 54, 41, 38	128, 52, 41	C	C′
FP1	Human (blood)	China (Sichuan)	*B. afzelii*	96, 62, 41, 18	142, 41, 22	D	D′
GS1	*I. granulatus*	China (Guizhou)	*Borrelia valaisiana*	130, 48, 20	144, 64	G	G′
CS4	Rabbit (bladder)	China (Hunan)	*Borrelia burgdorferi* sensu stricto	96, 41, 36, 25, 24	128, 41, 30, 28	A	A′
HS1	*I. persulcatus*	China (Heilongjiang)	*B. garinii*	96, 54, 41, 38	128, 52, 41	C	C′
HS2	*I. persulcatus*	China (Heilongjiang)	*B. garinii*	96, 54, 41, 38	128, 52, 41	C	C′
HS3	*I. persulcatus*	China (Heilongjiang)	*B. garinii*	96, 54, 41, 38	128, 52, 41	C	C′

in these regions may be characterized by higher risk of contracting or transmitting Lyme disease. In addition, we noted that the presence of ticks among the various habitats followed the local environmental and climatic variations (data not shown). Even though the border crossing cities evaluated in our study are all located along with Heilongjiang River in the Heilongjiang Province, Mohe has the highest latitude, Hulin has the highest humidity, and Dongning has the highest accumulated temperature. Therefore, we designed our tick collection efforts with respect to the distinctive features of each city, using unequal labor hours and different dates/times to eliminate potential bias introduced by the sample collection itself.

Our study determined that the average prevalence ratio of *Borrelia burgdorferi sensu lato* in *I. persulcatus* from Heilongjiang Province was 25.6%. This was higher than that reported in previous investigations focused on the Xinjiang Uygur Autonomous Region of China[19] and the Inner Mongolia Autonomous Region of China[20], but was much lower than that in the previous studies of the Heilongjiang Province[21,22]. The discrepancy among these ratios may reflect the differences in sampling approaches and geographic/seasonal variations of infected ticks. Nonetheless, all the research supports the conclusion that *I. persulcatus* is the primary vector of Lyme disease in China. It is possible, however, that other tick species may act as important vectors by maintaining the *Borrelia* foci.

The *D. silvarum* and *H. concinna* tick species have been previously investigated to determine their ability to transmit *Borrelia burgdorferi* sensu lato under laboratory conditions, and were found to be very ineffective vectors[23,24]. Therefore, our current study focused on the *I. persulcatus* species of ticks collected throughout the Heilongjiang Province. PCR and RFLP analysis of the 5S–23S rRNA intergenic spacer amplicons showed that the isolates (HS1, HS2 and HS3) had a similar genotype to *B. garinii*, as seen in previous studies by others[11,25–28]. A previous RFLP-based study found that isolates (n=45) from Northeast China were comprised of 64.4% *B. garinii* and 35.6% *B. afzelii*[29]. Unfortunately, only three strains of spirochetes were isolated in our study, and we cannot make any speculations on their existence of the *B. afzelii* species in our study population.

In our current study, HS1 was isolated from Dongning city, while HS2 and HS3 were from Tongjiang city of Heilongjiang Province. Both of these cities also had the highest spirochete-infected *I. persulcatus* prevalence ratios (53.3% and 40.0%, respectively). Therefore, it is possible that the high spirochete prevalence may have influenced the presence of HS1, HS2 and HS3 strains.

In conclusion, our study determined that the tick populations in the China–Russia border crossing regions of Heilongjiang Province, Northeast China are principally composed of the Ixodidae family member *I. persulcatus*, *D. silvarum*, *H. concinna* and *H. japonica*. The *Borellia* species infecting these ticks were of the *B. garinii* genotype and PD91 subgroup. The average *Borellia* prevalence ratio in *I. persulcatus* was 25.6%. Thus, *B. garinii* was identified as the primary causative agent of Lyme disease in Heilongjiang Province. Owing to limitions in the number of survey sites, ticks, and spirochetes evaluated in our study, other common *Borellia* species, such as the Lyme disease-associated *B. afzelii*, were not examined. Thus, further study of the high risk China–Russia border region of Heilongjiang Province will likely provide important insights into the disease epidemiology and its transmission features.

Conflict of interest statement

We declare that we have no conflict of interest.

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