Presence of pathogenic *Borrelia burgdorferi sensu lato* in ticks and rodents in Zhejiang, south-east China

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A molecular epidemiological survey was conducted to investigate the presence of pathogenic *Borrelia burgdorferi sensu lato* (s.l.) species in the forest areas of Zhejiang province, south-east China. A total of 182 ticks of 6 species and 200 rodents of 8 species were collected and individually examined for the presence of *B. burgdorferi* s.l. DNA by nested PCR targeting the 5S–23S rRNA intergenic spacer. Forty-one ticks of four species, *Haemaphysalis concinna*, *Haemaphysalis longicornis*, *Rhipicephalus microplus* and *Haemaphysalis warburtoni*, were infected with *B. burgdorferi* s.l., with an overall infection rate of 23%. Sixteen rodents of four species, *Nivivener confucianus*, *Nivivener coxingi*, *Apodemus sylvaticus* and *Rattus losea*, were positive for *B. burgdorferi* s.l., with an overall prevalence of 8%. MseI RFLP analysis and sequence analysis of the positive PCR products showed that *Borrelia* spirochaetes in specimens consisted of *Borrelia garinii*, *Borrelia afzelii* and *Borrelia valaisiana*-related group. Forty (98%) of the *B. burgdorferi* s.l.-positive ticks were infected with *B. garinii* and one (2%) was infected with *B. afzelii*. Twelve (75%) of the positive rodents were infected with *B. garinii* and four (25%) were infected with the *Borrelia* spirochaete belonging to *B. valaisiana*-related group.

Received 29 September 2007

Accepted 26 March 2008

INTRODUCTION

Lyme disease (LD) is a multi-organ infection caused by certain spirochaetes in the *Borrelia burgdorferi sensu lato* (s.l.) species complex (Steere *et al.*, 1983; Hengge *et al.*, 2003). The complex is considered to comprise at least 12 different genospecies to date (Masuzawa *et al.*, 2001; Richter *et al.*, 2006; Wang *et al.*, 1999). Among them, *Borrelia burgdorferi sensu stricto* (s.s.), *Borrelia garinii* and *Borrelia afzelii* are the major aetiological agents of human LD (Baranton *et al.*, 1992; Wang *et al.*, 1999). In addition, *Borrelia bissettii* (Picken *et al.*, 1996), *Borrelia valaisiana* (Rijpkema *et al.*, 1997), *Borrelia lusitaniae* (Collares-Pereira *et al.*, 2004) and *Borrelia spielmannii* (Foldvari *et al.*, 2005) are considered to be potentially pathogenic to humans. *B. burgdorferi* s.l. is mainly maintained in natural foci through the transmission cycles of competent tick vectors and vertebrate reservoirs (Anderson, 1991; Burgdorfer *et al.*, 1991; Hengge *et al.*, 2003). Humans accidentally enter this cycle and represent dead-end hosts for the agent (Hengge *et al.*, 2003).

LD was first documented in north-east China in 1986 (Ai *et al.*, 1987). One typical case of human lyme borreliosis

was reported in Zhejiang province of south-east China (Cheng *et al.*, 1992), where a good many of the popular tourist destinations and the well-known tea gardens are located. Antibody to *B. burgdorferi* s.l. was also detected in serum samples from some residents living in the forest areas of this province (Zhang *et al.*, 1997, 2001). However, only *Borrelia sinica* and *B. valaisiana*-related strains, which are not known to be pathogenic to humans, have been isolated in this area to date (Masuzawa *et al.*, 2001). Furthermore, no information is available concerning the prevalence and distribution of *B. burgdorferi* s.l. in tick and rodent vectors in this area. Lack of such knowledge has hampered our understanding of the ecology, epidemiology, and potential threats of the pathogens to human health. The objectives of this study were to investigate the prevalence of *B. burgdorferi* s.l. in ticks and rodents, and to confirm the presence of pathogenic *B. burgdorferi* s.l. in the Zhejiang province of south-east China.

METHODS

Study area. Surveys were performed in three areas of Zhejiang province in south-east China: Jinhua county (28°51' N, 119°57' E) located in the central part, Tiantai county (29°08' N, 121°01' E) situated in the eastern part, and Qintian county (27°56' N, 120°26' E) located in the southern part. The terrain of the survey areas consists of

Abbreviation: LD, Lyme disease.

The GenBank accession numbers for 5S–23S rRNA intergenic spacers reported in this study are EU160458 and EU160459.

forested rolling hills with an average elevation of 900 m. It has a humid subtropical monsoon climate with annual precipitation of about 1500 mm. The temperature ranges from 4.8 to 29 °C, with an average of 18.3 °C.

Specimen collection. This survey was conducted from October to December, 2004. Actively questing ticks were collected by flagging the vegetation in areas where rodents were sampled. Ticks were identified to the species, and were stored alive until use. Rodents were captured using box traps with peanuts as bait. After identification of species and sex, the spleen was removed from each rodent and stored at -20 °C until tested.

DNA extraction. DNA extraction from ticks was performed as previously described (Cao *et al.*, 2000). Briefly, each tick was placed into a microtube and mechanically disrupted with sterile scissors in 50 µl DNA extraction buffer (10 mM Tris/HCl pH 8.0, 2 mM EDTA, 0.1 % SDS, and 500 µg proteinase K ml⁻¹). The sample was incubated for 2 h at 56 °C, and then boiled at 100 °C for 10 min to inactivate proteinase K. After centrifugation, the supernatant was transferred to a fresh sterile microtube and purified by extracting twice with an equal volume of phenol/chloroform before use.

A small piece of spleen (about 500 mg) from an individual rodent was used for DNA extraction. Briefly, each spleen specimen was crushed with Trizol (Invitrogen) to separate DNA from RNA after centrifugation. The precipitated DNA was obtained after washing twice in a solution containing 0.1 M sodium citrate in 10 % ethanol, then the DNA pellet was suspended in 75 % ethanol and kept at room temperature for 10–20 min. After centrifuging at 2000 g at 2–8 °C for 5 min, the DNA was dissolved in weak base by adding 300 µl 8 mM NaOH and centrifuged to remove insoluble material. The supernatant containing the DNA was transferred to a new tube, adjusted with HEPES to pH 7–pH 8, and stored at 4 °C for use in PCR.

PCR. A nested PCR was performed with primers designed to amplify the variable spacer between two conserved structures: the 3' end of the 5S rRNA and the 5' end of the 23S rRNA. Primers 23S3 (5'-CGACCTTCTTCGCTTAAAGC-3') and 23Sa (5'-TAAGCTGACT-AATACTAATTACCC-3') were designed on the basis of a previously published rDNA sequence (Schwartz *et al.*, 1992), and applied for the primary amplification. In the nested PCR, a primer set [primer 1 (5'-CTGCGAGTTTCGCGGGAGA-3') and primer 2 (5'-TCCTAGGCA-TTCACCATA-3')], previously described by Postic *et al.* (1994), was used and expected to yield 226–266 bp fragment depending on the strain. Both primary and nested PCR amplification were performed in a volume of 20 µl containing 2 µl 10× PCR buffer (containing 100 mM Tris/HCl, 500 mM KCl, 15 mM MgCl₂), 0.2 µl Taq DNA polymerase (5 U µl⁻¹), 0.2 µl dNTP mix (10 mM) (all from TaKaRa, Shuzo Co. Ltd, Kyoto, Japan), 15.8 µl deionized water, 1 µl DNA template and 0.4 µl of each primer (12.5 µM). The cycling conditions for primary amplification involved 2 min denaturation at 96 °C, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 40 s, and a final extension at 72 °C for 5 min. The nested amplification was carried out with the following profile: 2 min initial denaturation at 96 °C, followed by 30 cycles of 94 °C for 30 s, 59 °C for 30 s and 72 °C for 40 s, and a final extension at 72 °C for 5 min. In parallel with each amplification, a positive control (previously extracted DNA of *B. garinii* strain JW1) and a negative control (distilled water) were included.

All the PCR products were separated by 1.5 % agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light. To minimize contamination, DNA extraction, the reagent setup, amplification, and agarose gel electrophoresis were performed in separate rooms.

RFLP analysis. The positive PCR products of the 5S–23S rRNA intergenic spacer were further analysed by RFLP. Previously extracted DNA of *B. garinii*, *B. afzelii* and *B. burgdorferi s.s* were used as positive controls. For each positive sample, 13 µl amplified DNA was digested at 37 °C overnight with endonuclease *MseI* (New England Biolabs) according to the manufacturer's recommendations. Electrophoresis was conducted in 16 % polyacrylamide gel at 100 V for 3 h. The gels were silver stained, and bands were subsequently visualized under white light. A 50 bp DNA Ladder Marker (TaKaRa, Shuzo) was used as a molecular mass marker. Genospecies of *B. burgdorferi s.l.* were identified according to RFLP profiles of each sample. RFLP profiles that differed from the known profiles of positive controls were further analysed by sequence analysis.

DNA sequencing of PCR products. PCR products that showed unique RFLP profiles were electrophoresed on 1 % agarose gels, and purified by using the Qiaquick Gel Extraction kit (Qiagen). The nucleotide sequences were determined by a dideoxynucleotide cycle sequencing method with an automated DNA sequencer (ABI Prism 377, Perkin-Elmer). The sequences obtained in the present study were compared with the previously published sequences deposited in GenBank using the BLAST program from the National Center for Biotechnology Information website. *MseI* RFLP analysis of the 5S–23S rRNA intergenic spacer was performed on the basis of the DNA sequences obtained using software Vector NTI 9.0 (Lu & Moriyama, 2004).

The accession numbers of the 5S–23S rRNA intergenic spacer sequences obtained in this study are EU160458 and EU160459.

Statistical analysis. The results were analysed with descriptive statistics (rate, proportion, and confidence intervals with 5 % error). The χ^2 test or Fisher's exact test (when necessary) were used to compare the proportions. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

B. burgdorferi s.l. prevalence in ticks

B. burgdorferi s.l. DNA was detected by a nested PCR specifically targeting the 5S–23S rRNA intergenic spacer. A total of 182 unfed, host-seeking adult ticks from six species, *Haemaphysalis concinna*, *Haemaphysalis longicornis*, *Rhipicephalus microplus*, *Haemaphysalis warburtoni*, *Ixodes vespertilionis* and *Ixodes kashmirius*, were collected on vegetation and individually examined. Forty-one ticks from four species, *H. concinna*, *H. longicornis*, *R. microplus* and *H. warburtoni*, were naturally infected, with an overall infection rate of 22.53 % [95 % confidence interval (CI): 16.68–29.30 %]. Infection rates of ticks with respect to each spirochaete species are shown in Table 1. The difference in infection rates among species was not statistically significant (Fisher's exact test, $P = 0.164$).

The infection rate of *B. burgdorferi s.l.* in *H. longicornis* ticks was 28.07 % (95 % CI: 20.06–37.26 %), which is higher than the prevalence of 14 % and 12 % in the same tick species that were previously collected in Shandong and Beijing in China, respectively (Wan *et al.*, 1998). The positive rate of *B. burgdorferi s.l.* in *R. microplus* (14.29 %, 95 % CI: 3.05–36.34 %) is also higher than that of the same tick species collected in Sichuan, south-west China

Table 1. Prevalence of *B. burgdorferi* s.l. in specimens collected in Zhejiang province, south-east China

Specimen	No. tested	No. positive (%)	No. positive for species (% of positive animals)		
			<i>B. garinii</i>	<i>B. afzelii</i>	<i>B. valaisiana</i> -related group
Ticks					
<i>Haemaphysalis longicornis</i>	114	32 (28.07)	31 (96.88)	1 (3.12)	0 (0)
<i>Rhipicephalus microplus</i>	21	3 (14.29)	3 (100)	0 (0)	0 (0)
<i>Haemaphysalis warburtoni</i>	40	5 (12.5)	5 (100)	0 (0)	0 (0)
<i>Haemaphysalis concinna</i>	2	1 (50)	1 (100)	0 (0)	0 (0)
<i>Ixodes vespertlionis</i>	4	0 (0)	0 (0)	0 (0)	0 (0)
<i>Ixodes hyatti</i>	1	0 (0)	0 (0)	0 (0)	0 (0)
Total	182	41 (22.53)	40 (97.56)	1 (2.44)	0 (0)
Rodents					
<i>Nivivener confucianus</i>	108	10 (9.26)	6 (60)	0 (0)	4 (40)
<i>Rattus losea</i>	25	2 (8)	2 (100)	0 (0)	0 (0)
<i>Apodemus sylvaticus</i>	21	3 (14.29)	3 (100)	0 (0)	0 (0)
<i>Nivivener coxingi</i>	4	1 (25)	1 (100)	0 (0)	0 (0)
<i>Cricetulus barabensis</i>	20	0 (0)	0 (0)	0 (0)	0 (0)
<i>Rattus norvegicus</i>	12	0 (0)	0 (0)	0 (0)	0 (0)
<i>Nivivener fulvescens</i>	2	0 (0)	0 (0)	0 (0)	0 (0)
<i>Apodemus agrarius</i>	8	0 (0)	0 (0)	0 (0)	0 (0)
Total	200	16 (8)	12 (75)	0 (0)	4 (25)

(6.38%) (Wan *et al.*, 1998). One of the two *H. concinna* ticks, which are mainly distributed in north-east China (Teng & Jiang, 1991) and have a *B. burgdorferi* s.l. prevalence of 14% in the Inner Mongolia Autonomous Region (Wan *et al.*, 1998), was infected with *B. burgdorferi* s.l. Moreover, *H. warburtoni*, which are mainly distributed in south-west China (Teng & Jiang, 1991), was found for the first time to be infected with *B. burgdorferi*. These diversities could be attributable to geographical and seasonal variations of infected ticks or to different sampling approaches and examination methods.

*Mse*I RFLP analysis of the amplified products from tick specimens resulted in three distinct profiles. On the basis of *Mse*I RFLP patterns of positive controls of *B. garinii*, *B. afzelii* and *B. burgdorferi* s.s., the profiles obtained in this study consist of two restriction patterns for *B. garinii* species and one restriction pattern for *B. afzelii* species (Fig. 1). DNA of pathogenic *B. burgdorferi* s.l., including *B. garinii* and *B. afzelii*, was initially detected in Zhejiang province. Of the 41 *B. burgdorferi* s.l.-positive ticks, 40 (98%) were infected with *B. garinii* and one (2%) was infected with *B. afzelii*. The numbers and proportions of ticks infected with different *Borrelia* species are listed in Table 1. Different species of pathogenic *B. burgdorferi* s.l. are associated with distinct clinical manifestations of human LD: *B. garinii* infection usually results in neuroborreliosis, and *B. afzelii* infection often causes acrodermatitis chronica atrophicans (ACA) (Balmelli & Piffaretti, 1995; van Dam *et al.*, 1993). Therefore, LD patients in Zhejiang province might mainly present neurological symptoms and the late cutaneous manifestation. It will be necessary to alert public health officials and clinicians

about the dominant *B. garinii* infection and the existence of *B. afzelii*-positive in ticks in forest areas of Zhejiang province.

In forest areas of Zhejiang province, free-living *H. longicornis* is the most abundant tick species on vegetation and is responsible for tick bites on domestic animals and

Fig. 1. *Mse*I RFLP patterns of the 5S–23S rRNA intergenic spacer of *B. burgdorferi* s.l. in specimens in Zhejiang province, south-east China. M, 50 bp DNA ladder marker; A, *Borrelia garinii* NT29 subtype; B and C, isolates from the *Borrelia valaisiana*-related group; D, *Borrelia garinii* 20047 subtype.

humans (Teng & Jiang, 1991). Moreover, *H. longicornis* plays an important role in maintenance of *B. burgdorferi* s.l. in enzootic cycles in China, and is considered to be a potential vector in southern China (Wan *et al.*, 1998). Pathogenic *B. burgdorferi* s.l. infection in *H. longicornis* ticks poses a potential health threat both to local residents and to tourists and animals in this area. *H. concinna*, *R. microplus* and *H. warburtoni* are three-host ticks, and infest a variety of domestic and wild animals such as cattle, horses, sheep, goats and deer in their respective habitats, and are also invasive to humans (Teng & Jiang, 1991). The results of *B. garinii* infection in *H. concinna*, *R. microplus*, and *H. warburtoni* obtained in the present study present the additional human and animal infection risks following the bites of the three tick species.

The association of *B. burgdorferi* s.l. with the *Ixodes* ticks that act as the principal vectors is well-documented. The *Ixodes persulcatus* tick is the major vector in northern China, with *Ixodes granulatus* and *H. longicornis* as potentially important vectors in southern China (Wan *et al.*, 1998). *Ixodes sinensis* was also considered to act as the competent vector which transmits the LD spirochaete in southern China on the basis of the experimental evidence of the tick–animal–tick transmission cycle (Sun *et al.*, 2003). *I. sinensis* is an abundant tick species in Zhejiang province with high infestation of habitats and cattle (Teng & Jiang, 1991). *I. sinensis* was absent in the present survey, because the method of flagging the vegetation was applied to collect ticks. Attempts to detect *B. burgdorferi* s.l. in *I. vespertilionis* and *I. kashmirius* were unsuccessful. This study was intended to investigate the presence of pathogenic *B. burgdorferi* s.l. in Zhejiang province. Because the number of survey sites and samples collected were limited, the infection rates obtained in this study could be biased. A randomized sampling scheme should be made and a more comprehensive survey and further collection of ticks should be done to obtain more accurate data about the prevalence and distribution of *B. burgdorferi* s.l. in ticks in Zhejiang province.

***B. burgdorferi* s.l. prevalence in rodents**

A total of 200 rodents from eight species, *Nivivener confucianus*, *Nivivener coxingi*, *Apodemus sylvaticus*, *Rattus losea*, *Cricetulus barabensis*, *Rattus norvegicus*, *Nivivener fulvescens* and *Apodemus agrarius*, were collected and examined by nested PCR for the presence of *B. burgdorferi* DNA. Sixteen rodents from four species, *N. confucianus*, *N. coxingi*, *A. sylvaticus* and *R. losea*, were PCR positive for *B. burgdorferi* DNA, with an overall positive rate of 8 % (95 % CI: 4.64–12.67 %). Infection rates of rodents with respect to spirochaete species are shown in Table 1. There was no significant difference in positive rate among the four species of rodents (Fisher's exact test, $P=0.462$).

The overall infection rate of the rodents captured (8%) is obviously lower than that of the ticks that were simultaneously collected in this survey (23 %), probably because they received few bites from the infected ticks.

Lower prevalence of *B. burgdorferi* s.l. in rodents (3 %) than that in questing ticks collected simultaneously (18 %) was also reported in the Inner Mongolia Autonomous Region of north-east China (Chu *et al.*, 2006), where LD is endemic. All of the four *B. burgdorferi* s.l.-positive rodent species are the most common rodent species in Zhejiang province (Wang, 1997). Among them, *A. sylvaticus* and *N. coxingi* are usually parasitized by host-seeking ticks in their habitats (Teng & Jiang, 1991; Wang, 1997). The results obtained in this study imply that these two species of rodents may have an important role in maintaining *B. burgdorferi* s.l. in the tick–rodent cycle in the forest areas of Zhejiang province. Since *N. confucianus* and *R. losea* are occasionally infested by ticks (Teng & Jiang, 1991; Wang, 1997), they also assist in maintenance and transmission of *B. burgdorferi* s.l. in this area. *A. agrarius* rodents are distributed over an extensive area in mainland China (Wang, 1997) and are known to be a major reservoir for *B. burgdorferi* s.l. in China (Zhang *et al.*, 1997). However, *B. burgdorferi* s.l. DNA was not detected in any of the eight rodent species collected, probably because of the limited number of specimens.

MseI RFLP analysis of the amplified products from rodent specimens generated four distinct profiles, including two patterns for *B. garinii* species, one pattern for *B. afzelii* species, and two unique patterns (Fig. 1). Representative samples of RFLP profiles that were different from the known profiles of positive controls were sequenced. One sequence of the 5S–23S rRNA intergenic spacer obtained from a *B. burgdorferi* s.l.-positive rodent's spleen (R48) was 100 % identical to these of OS66/01 (AB091454), OG45/01 (AB091455) and OM50 (AB091443), which belong to the *B. valaisiana*-related group. Another sequence obtained from another *B. burgdorferi* s.l.-positive rodent's spleen (R54) was 100 % identical to these of OR5/01 (AB091446), OM92/01 (AB091445) and KR3 (AB037119), which also belong to the *B. valaisiana*-related group. *MseI*-restricted fragments of the 5S–23S rRNA intergenic spacer obtained from *B. burgdorferi* s.l.-positive spleen from rodent R48 were 147, 60, 24 and 22 bp long, and those from rodent R54 were 105, 60, 43, 24 and 22 bp long based on RFLP analysis of two sequences using Vector NTI 9.0 software.

B. garinii, *B. afzelii* and the *Borrelia* spirochaetes belonging to the *B. valaisiana*-related group were found in rodents by *MseI* RFLP analysis and sequence analysis. Among the 16 infected rodents, 12 (75 %) from four rodent species were infected with *B. garinii*, and four (25 %) *N. confucianus* were infected with a *Borrelia* spirochaete belonging to the *B. valaisiana*-related group. The numbers and proportions of rodents infected with different *Borrelia* species are listed in Table 1 with respect to species. The occurrence of *B. garinii* species in the majority of positive rodents (75 %) suggested that *B. garinii* might be the dominant *Borrelia* species in the areas of Zhejiang province that were sampled.

Borrelia strains belonging to the *B. valaisiana*-related group have been isolated from *A. agrarius* rodents,

N. confucianus rodents and *I. granulatus* ticks in Zhejiang province of mainland China (Masuzawa *et al.*, 2001), from *Ixodes nipponensis* ticks in Korea (Masuzawa *et al.*, 1999), and from *I. granulatus* ticks and a variety of rodents in Japan (Masuzawa *et al.*, 2004). At first, these *Borrelia* spirochaetes were tentatively considered to belong to the *B. afzelii*-related group on the basis of 16S rDNA gene sequences (Kee *et al.*, 1996). Then they were suggested to be members of the *B. valaisiana* species, because these strains showed higher similarity to *B. valaisiana* than to *B. afzelii* in their 16S rDNA gene sequences and were distinguishable from *B. afzelii* by RFLP analysis of the 5S–23S rRNA intergenic spacer sequence (Masuzawa *et al.*, 1999). Masuzawa *et al.* (2004), suggested that these *Borrelia* spirochaetes found in East Asia should be classified as a new genospecies of *B. burgdorferi* s.l. (temporarily named *B. valaisiana*-related group) according to phylogenetic analyses of 16S rDNA gene sequences and flagellin gene sequences. The results obtained from previous and present studies suggested that these *B. valaisiana*-related spirochaetes most probably circulated between their rodent reservoir hosts and tick vectors in East Asia. This seems to be different from the transmission cycle maintaining *B. valaisiana* in Europe, where *B. valaisiana* has been found in various avian reservoirs but never in rodent hosts (Hanincová *et al.*, 2003; Humair *et al.*, 1998; Kurtenbach *et al.*, 1998a). Moreover, phenotypic differences might also exist between *B. valaisiana* isolates found in Europe and *B. valaisiana*-related strains isolated in East Asia, since the experiment on *Borrelia* sensitivities for the sera of their reservoir hosts showed that rodent serum lysed *B. valaisiana* while bird serum did not (Kurtenbach *et al.*, 1998b). It remains to be determined whether this *B. burgdorferi* s.l. species can cause disease in humans.

ACKNOWLEDGEMENTS

This study was supported by the National Science Fund for Distinguished Young Scholars (no. 30725032). We are grateful to Shu-Yun Xie and Li-Ping Jiang [the Centers for Disease Control and Prevention (CDC) of Zhejiang province] for assistance in specimen collection.

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