

Detection of spotted fever group *Rickettsiae* in ticks from Zhejiang Province, China

Jimin Sun · Junfen Lin · Zhenyu Gong · Yue Chang · Xiaodong Ye ·
Shiping Gu · Weilong Pang · Chengwei Wang · Xiaohua Zheng ·
Juan Hou · Feng Ling · Xuguang Shi · Jianmin Jiang ·
Zhiping Chen · Huakun Lv · Chengliang Chai

Received: 2 July 2014 / Accepted: 30 December 2014 / Published online: 30 January 2015
© The Author(s) 2015. This article is published with open access at Springerlink.com

Abstract Tick species distribution and prevalence of spotted fever group *Rickettsiae* (SFGR) in ticks were investigated in Zhejiang Province, China in 2010 and 2011. PCR was used to detect SFGR and positive amplicons were sequenced, compared to published sequences and phylogenetic analysis was performed using MEGA 4.0. A total of 292 adult ticks of ten species were captured and 7.5 % (22/292) of the ticks were PCR-positive for SFG *Rickettsia*. The PCR-positive rates were 5.5 % (6/110) for *Haemaphysalis longicornis*, 3.6 % (1/28) for *Amblyomma testudinarium* and 16 % (15/94) for *Ixodes sinensis*, respectively. Phylogenetic analyses of *gltA* genes detected in ticks indicated that there are two dominating groups of SFGR. Sequences of group one were closely related to *Rickettsia monacensis*, whereas sequences of group two were closest related to *Rickettsia*

Jimin Sun, Junfen Lin and Zhenyu Gong have contributed equally to this work.

J. Sun · J. Lin · Z. Gong · J. Hou · F. Ling · X. Shi · J. Jiang · Z. Chen · H. Lv · C. Chai (✉)
Zhejiang Provincial Center for Disease Control and Prevention, Hangzhou, China
e-mail: zjdccl@126.com

Y. Chang
Taizhou Municipal Center for Disease Control and Prevention, Taizhou, China

X. Ye
Jindong Center for Disease Control and Prevention, Jindong, China

S. Gu
Anji Center for Disease Control and Prevention, Anji, China

W. Pang
Tiantai Center for Disease Control and Prevention, Taizhou, China

C. Wang
Daishan Center for Disease Control and Prevention, Daishan, China

X. Zheng
Xianju Center for Disease Control and Prevention, Xianju, China

heilongjiangensis and *Rickettsia japonica*, which are human pathogens. Our findings underline the importance of these ticks in public health surveillance in Zhejiang Province, China.

Keywords Spotted fever group *Rickettsiae* · Public health · *Rickettsia monacensis* · *Rickettsia heilongjiangensis* · *Rickettsia japonica* · Ticks

Introduction

Rickettsia is a genus of non-motile, gram-negative, intracellular bacteria transmitted by ticks, fleas, lice and mites and cause diseases in humans such as typhus, Rickettsial pox, Boutonneuse fever, African tick bite fever, Rocky Mountain spotted fever, Flinders Island spotted fever and Queensland tick typhus (Raoult and Roux 1997; Nathan et al. 2007). The genus *Rickettsia* is traditionally classified into the conventionally well-defined typhus group (TG), the spotted fever group (SFG), ancestral group and transitional group, based mainly on phenotypic and serological features (Gillespie et al. 2008). SFG *Rickettsiae* (SFGR) are widely distributed throughout the world in foci of endemicity and cause sporadic outbreaks in areas such as Japan, southern China and eastern Russia (Choi et al. 2005). From about 30 SFGR described so far, at least 15 are known to be pathogenic for humans (Parola et al. 2005).

In China, many SFGR belong to *Rickettsia sibirica* group, including two subspecies, i.e., *R. sibirica sibirica*, the agent of North Asian tick typhus detected in *Dermacentor silvarum* and *Dermacentor sinicus* in northern China, and *Rickettsia sibirica mongolitimonae*, the agent of lymphangitis-associated rickettsiosis isolated from *Hyalomma asiaticum* in Inner Mongolia (Yu et al. 1993; Zhang et al. 2006). *Rickettsia heilongjiangensis*, firstly isolated from *D. silvarum* ticks in Heilongjiang Province, can cause spotted fever in humans (Fournier et al. 2003; Jiao et al. 2005). Additionally, ticks are widely distributed in Zhejiang Province where humans are frequently bitten by ticks. This stimulated us to explore the tick species distribution in different areas of Zhejiang Province and the prevalence of SFGR species in these ticks.

Materials and methods

Tick sampling

The investigated sites included Daishan, Xinchang, Jindong, Tiantai, Xianju and Anji which were randomly chosen based on their geographical and administrative locations (Fig. 1). Ticks were collected from sheep, cattle, hedgehogs, domestic dogs, wild boar and small mammals including *Apodemus agrarius*, *Rattus niviventer*, and *Suneus murinus* during January 2010 to December 2011. All ticks were identified to the species level by standard guides (Deng and Jiang 1991) according to morphology and were stored at -80°C prior to DNA extraction.

DNA extraction

Each adult tick was subjected individually to DNA extraction. Ticks were washed using 70 % ethanol once; then they were washed three times with sterile deionized water to



Fig. 1 Geographical distribution of investigated sites in Zhejiang Province

decontaminate the surface. Individual ticks were placed into different sterilized mortars and crushed with corresponding sterile pestles with liquid nitrogen. DNA was prepared from the crushed ticks using the QIAamp Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

Polymerase chain reaction amplification

All tick samples were screened for SFGR infection through testing them individually by polymerase chain reaction (PCR) amplification with the use of primer (5'-GGGGCCTGCTCACGGCGG-3'; 5'-ATTGCAAAAAGTACAGTGAACA-3') designed to amplify a fragment of the citrate synthase gene (*gltA*) of SFGR as described previously (Regnery et al. 1991; Roux et al. 1997).

The reaction mixture contained 10 mM Tris-HCl, 1.5 mM MgCl₂, 50 mM KCl (pH 8.3), 200 mM each dNTP, 1.25 U Taq polymerase, and 0.5 mM each respective primer. PCR products were electrophoresed in a 1.5 % agarose gel, stained with gold view, and visualized using ultraviolet light. To avoid cross contamination, all steps were performed in separate rooms; mastermix was prepared under a laminar air flow bench. In each PCR, at least two negative controls contained mastermix and sterile water instead of DNA template were used.

Cloning and sequencing of PCR products

After electrophoresis, all positive DNA amplicons were purified using the Promega Wizard PCR Preps Kit (Promega, Madison, WI, USA) and then cloned into the PGEM-T Easy vector system (Promega) following the manufacturer's protocol. The white recombinant clones were selected for sequencing. Bidirectional sequencing of positive PCR products were commercially conducted by Shanghai Sangon Biotechnology (Shanghai, China).

Database DNA comparisons

Our sequences were submitted to GenBank and compared to published sequences using the BLAST program from the National Center for Biotechnology Information Website (<http://www.ncbi.nlm.nih.gov/BLAST/>), and phylogenetic analysis was performed using MEGA 4.0. For each gene analyzed, a phylogram was constructed by the neighbor-joining method. Confidence values for individual branches of the resulting tree were determined by bootstrap analysis with 1,000 replicates.

Data analysis

A Chi-square test or Fisher's exact test, as appropriate, was used to compare SFGR prevalence among different species of ticks and different sampling sites. The difference was considered statistically significant when $P < 0.05$. Statistical analysis was performed with 16.0 (SPSS, Chicago, IL, USA).

Results

Tick samples

A total of 292 adult ticks of ten species were captured (Table 1). The species of ticks from different areas varied significantly ($\chi^2 = 408.915$, $P < 0.001$). The dominant tick species differed at various study areas. *Haemaphysalis longicornis* was dominant in Daishan (91.38 %) and Xinchang (80.00 %); *H. longicornis* (21.43 %), *Rhipicephalus haemaphysaloides* (21.43 %), and *Ixodes sinensis* (22.86 %) in Jindong; *I. sinensis* (100 %) in Tiantai; *H. longicornis* (58.14 %) and *Rhipicephalus microplus* (41.86 %) in Xianju; *Amblyomma testudinarium* (23.96 %) and *I. sinensis* (65.63 %) in Anji.

Prevalence of SFGR infection

Overall, 7.53 % (22/292) of the ticks were PCR-positive to SFGR. The PCR-positive rates were 5.5 % (6/110) for *H. longicornis*, 3.8 % (1/28) for *A. testudinarium*, and 16 % (15/94) for *I. sinensis*, respectively (Table 1). No *Rickettsia* DNA was detected in *R. haemaphysaloides*, *R. microplus*, *I. granulatus*, *Haemaphysalis yeni*, *D. taiwanensis*, *Haemaphysalis hystrix*, and *Haemaphysalis asiaticum*. The prevalence of *Rickettsia* was not significantly different among species ($\chi^2 = 15.776$, $P = 0.072$).

Prevalences in Daishan, Xinchang, Jindong, Tiantai, Xianju, and Anji were 1.7, 10, 10, 0, 9.3, 9.4, and 7.5 %, respectively. The prevalence was similar among different areas ($\chi^2 = 5.391$, $P = 0.37$).

Table 1 Prevalence of spotted fever group *Rickettsiae* (SFGR) infection among tick species from different areas in Zhejiang Province, China

	Daishan	Xinchang	Jindong	Tiantai	Xianju	Anji	Total (n)	SFGR positive (n)	Positive rate (%)
<i>H. longicornis</i>	53	8	15	0	25	9	110	6	5.45
<i>R. haemaphysaloides</i>	3	2	15	0	0	0	20	0	0
<i>A. testudinarium</i>	0	0	5	0	0	23	28	1	3.57
<i>I. sinensis</i>	0	0	16	1.5	0	63	94	15	15.96
<i>R. microplus</i>	1	0	0	0	18	0	19	0	0
<i>I. granulatus</i>	1	0	0	0	0	0	1	0	0
<i>H. yeni</i>	0	0	5	0	0	0	5	0	0
<i>D. taiwanensis</i>	0	0	9	0	0	0	9	0	0
<i>H. hystrix</i>	0	0	5	0	0	0	5	0	0
<i>H. asiaticum</i>	0	0	0	0	0	1	1	0	0
Total (n)	58	10	70	1.5	43	96	292	22	7.53
SFGR positive (n)	1	1	7	0	4	9	22		
Positive rate (%)	1.72	10.00	10.00	0	9.30	9.38	7.53		

Phylogenetic analysis

All sequences were submitted to GenBank, and the accession numbers were from KM886876 to KM886897. Phylogenetic analyses of *gltA* genes (340 bp) detected in ticks indicated that there were two dominating groups of SFGR (Fig. 2).

Group one consisted of 16 detected sequences, which were from Anji ($n = 9$) and Jindong ($n = 7$). Moreover, all sequences were detected in *I. sinensis* except that one sequence was detected in *A. testudinarium*. The majority of sequences of this group were closely related to *R. monacensis* (JX040639), which was detected in *I. ricinus* ticks from Romania. One sequence was most similar to *R. tamurae* (AF394896) that came from *A. testudinarium* in Japan.

Group two included six detected sequences, which were from three areas (Daishan, Xianju and Xinchang). Of note, these sequences were all from *H. longicornis* and were most similar to *R. heilongjiangensis* (AF178034) and *R. japonica* (DQ909073).

Discussion

In our study, ten species of ticks were found in six areas and the tick species varied significantly at different areas. This may be due to the different animal hosts from which they were removed, geographic sites, unbalanced number of ticks in different areas. The positive rate of SFGR was found to be 7.5 % among ticks, a percentage similar to the recently reported percentage (6.9 %) recorded in the Hebei Province, China (Zou et al. 2011). However, ticks were pooled prior to DNA extraction and the pools consisted of 2–10 ticks collected from the same site in their study.

Although no SFGR DNA was detected in Tiantai and infection rates varied at different survey areas, we could not determine the geographic diversity of SFGR in ticks. The number of ticks examined was limited and the species of ticks varied significantly; therefore, infection rates in the current study might be biased. In addition, because intensity of circulation of any vector-borne agent fluctuates dramatically throughout the year and from year to year, even at the same location (Bown et al. 2003; Wielinga et al. 2006), we could not justify comparing infection rates between different areas on the basis of unsynchronized single collections over a 2-year period. A randomized sampling scheme and further collection of ticks are required to clarify this issue.

Usually, for SFGR, ticks are both the reservoir and the vector of the bacteria and the geographical distribution of the disease is superimposed upon that of the tick (Raoult and Roux 1997). Among ten tick species collected in this study, only three were infected with SFGR. This demonstrated that these three tick species were the main carriers of SFGR. But it is not excluded that other ticks might act as vectors for SFGR because of small sample sizes.

Phylogenetic analyses indicated that two groups of SFGR were detected in ticks, one identified in Jindong and Anji, another identified in Xinchang, Daishan and Xianju. Interestingly, sequences of group 1 were all from *I. sinensis* and *A. testudinarium*, sequences of group two were all from *H. longicornis*. Additionally, one sequence which was detected in *A. testudinarium* was most similar to one sequence, which was also detected in *A. testudinarium* in Japan. These indicate that SFGR species might be related to tick species.

Some sequences were most similar to *R. monacensis* which had been isolated from *I. ricinus* in Germany (Simser et al. 2002) and Hungary (Sreter et al. 2005). *Rickettsia*

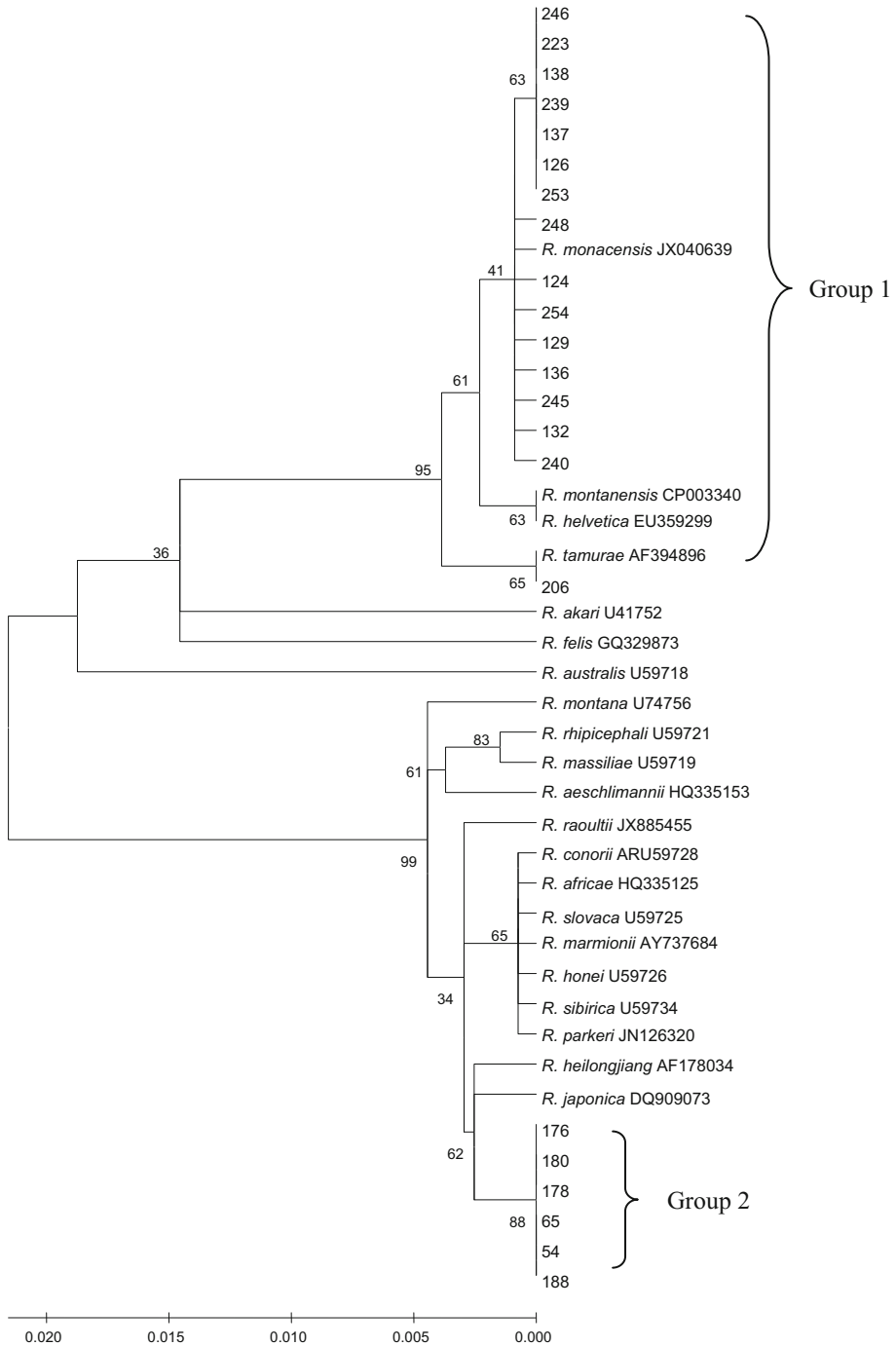


Fig. 2 Phylogenetic analyses of partial *gltA* genes of *Rickettsia* species identified in ticks from Zhejiang Province, China

monacensis can cause spotted fever in humans, as the disease attributed to this agent has not received a specific name. The other sequences were most related to *R. heilongjiangensis* and *R. japonica* which were also human rickettsial pathogens. *Rickettsia heilongjiangensis* was first isolated in 1982 from *D. silvarum* in Heilongjiang Province in the northeast of China and subsequently demonstrated to cause human spotted fever in China and in the Russian Far East (Mediannikov et al. 2004). *Rickettsia japonica* was first isolated in 1984 in Japan (Mahara 1997) and is the agent of Japanese spotted fever (Uchida et al. 1992).

In comparable studies throughout Zhejiang Province, SFGR were detected in *H. longicornis*, *R. haemaphysaloides* (Cheng et al. 2010; Meng et al. 2008; Jiang et al. 2010). However, sequences detected in their study were most similar to *R. rhipicephali* and *R. massiliae*. This is the first identification of *R. monacensis* in ticks from Zhejiang Province which demonstrate the importance of epidemiological survey of *R. monacensis* infection in Zhejiang Province.

There were several limitations to our study. Firstly, the low numbers of ticks of each species in each region collected in different times and from different animals reduced the probabilities of getting useful information from this study. Secondly, the examined ticks were collected from animals rather than as questing specimens which brings into question whether SFGR would survive moulting of the ticks, especially for non-*Ixodes* ticks. Finally, tick species from different sites varied significantly, which may also influence infection rates of different sites.

In summary, we detected SFGR in diverse species of ticks from different areas suggesting that SFGR is widely prevalent in ticks in Zhejiang Province. SFGR detected were similar to *R. monacensis*, *R. heilongjiangensis* or *R. japonica* which can cause human infection. SFGR infections are largely unrecognized but may become more frequently diagnosed in Zhejiang Province.

Acknowledgments We thank the physicians and staff at Daishan, Xinchang, Jindong, Tiantai, Xianju and Anji Center for Disease Control and Prevention for their support and assistance with this investigation. No conflict of interest exists in the submission of this manuscript, and manuscript is approved by all authors for publication. This research was supported by a grant from Zhejiang Province major science and technology program (2012C13016-2), the Project of the State Scientific and Technological Development of the 12th Five Year Plan grant (2012ZX10004219), the medical research program of Zhejiang Province (2012KYA045, 2014RCA002) and national Science Foundation of China (81201319).

Open Access This article is distributed under the terms of the Creative Commons Attribution License which permits any use, distribution, and reproduction in any medium, provided the original author(s) and the source are credited.

References

- Bown KJ, Begon M, Bennett M, Woldehiwet Z, Ogden NH (2003) Seasonal dynamics of *Anaplasma phagocytophilum* in a rodent-tick (*Ixodes trianguliceps*) system, United Kingdom. *Emerg Infect Dis* 9:63–70
- Cheng SY, Zheng SQ, Meng Z, Ye XD, Jiang LP, Wang ZQLuQY (2010) Analysis of DNA sequences of spotted fever group *Rickettsia* detected from ticks in Zhejiang Province. *Dis Surveill* 25:466–468
- Choi YJ, Lee SH, Park KH, Koh YS, Lee KH, Baik HS, Choi MS, Kim IS, Jang WJ (2005) Evaluation of PCR-based assay for diagnosis of spotted fever group rickettsiosis in human serum samples. *Clin Diagn Lab Immunol* 12:759–763
- Deng GF, Jiang ZJ (1991) Economic insect fauna of Chinese, 39, Acari Leach Science Press

- Fournier PE, Dumler JS, Greub Q, Zhang J, Wu Y, Raoult D (2003) Gene sequence-based criteria for identification of new *Rickettsia* isolates and description of *Rickettsia heilongjiangensis* sp. nov. *J Clin Microbiol* 41:5456–5465
- Gillespie JJ, Williams K, Shukla M, Snyder EE, Nordberg EK, Ceraul SM, Dharmanolla C, Rainey D, Soneja J, Shallom JM, Vishnubhat ND, Wattam R, Purkayastha A, Czar M, Crasta O, Setubal JC, Azad AF, Sobral BS (2008) *Rickettsia* phylogenomics: unwinding the intricacies of obligate intracellular life. *PLoS One* 3:e2008. doi:10.1371/journal.pone.0002018
- Jiang LP, Meng Z, Cui QR, Tong WS, Ling F, Wang Z (2010) Detection of rOmpA and gltA genes of spotted fever group *Rickettsiae* from tick specimens in Zhejiang Province. *Chin J Vector Biol Control* 21:350–352
- Jiao YM, Wen BH, Chen ML, Niu DS, Zhang J, Qiu L (2005) Analysis of immunoprotectivity of the recombinant OmpA of *Rickettsia heilongjiangensis*. *Ann N Y Acad Sci* 1063:261–265
- Mahara F (1997) Japanese spotted fever: report of 31 cases and review of the literature. *Emerg Infect Dis* 3:105–111
- Mediannikov OY, Sideonikov Y, Ivanov L, Mokressoova E, Fournier PE, Tarasevich I, Raoult D (2004) Acute tick borne rickettsiosis, caused by *Rickettsia heilongjiangensis* variant in the Russian Far East. *Emerg Infect Dis* 10:810–817
- Meng Z, Jiang LP, Lu QY, Cheng SY, Ye JL, Zhan L (2008) Detection of co-infection with Lyme spirochetes and spotted fever group *Rickettsiae* in a group of *Haemaphysalis longicornis*. *Chin J Epidemiol* 29:1217–1220
- Nathan BU, Stenos J, Graves SR, Faa AG, Cox GE, Dyer JR, Boutlis CS, Lane AM, Shaw MD, Robson J, Nissen MD (2007) Flinders Island spotted fever rickettsioses caused by “marmionii” strain of *Rickettsia honei*, Eastern Australia. *Emerg Infect Dis* 13:566–573
- Parola P, Paddock CD, Raoult D (2005) Tick-borne rickettsioses around the world: emerging diseases challenging old concepts. *Clin Microbiol Rev* 18:719–756
- Raoult D, Roux V (1997) Rickettsioses as paradigms of new or emerging infectious diseases. *Clin Microbiol Rev* 10:694–719
- Regnery RL, Spruill CL, Plikaytis BD (1991) Genotypic identification of *Rickettsiae* and estimation of intraspecies sequence divergence for portions of two rickettsial genes. *J Bacteriol* 173:1576–1589
- Roux V, Rydkina E, Ereemeeva M, Raoult D (1997) Citrate synthase gene comparison, a new tool for phylogenetic analysis, and its application for the *Rickettsiae*. *Int J Syst Bacteriol* 47:252–261
- Simser JA, Palmer AT, Fingerle V, Wilske B, Kurtti TJ, Munderloh UG (2002) *Rickettsia monacensis* sp. nov., a spotted fever group *Rickettsia*, from ticks (*Ixodes ricinus*) collected in a European city park. *Appl Environ Microb* 68:4559–4566
- Sreter L, Sreter Z, Szelli T, Egyed ZL (2005) Molecular evidence of *Rickettsia helvetica* and *R. monacensis* infections in *Ixodes ricinus* from Hungary. *Ann Trop Med Parasitol* 99:325–330
- Uchida T, Uchiyama T, Kumano K, Walker DH (1992) *Rickettsia japonica* sp. nov., the etiological agent of spotted fever group rickettsiosis in Japan. *Int J Syst Bacteriol* 42:303–305
- Wielinga PR, Gaasenbeek C, Fonville M, Deboer A, Vries AD, Dimmers W, Jagers GAO, Schouls LM, Borgsteede F, van der Giessen JWB (2006) Longitudinal analysis of tick densities and *Borrelia*, *Anaplasma*, and *Ehrlichia* infections of *Ixodes ricinus* ticks in different habitat areas in The Netherlands. *Appl Environ Microb* 72:7594–7601
- Yu XJ, Jin Y, Fan MY, Xu GM, Liu QH, Raoult D (1993) Genotypic and antigenic identification of two new strains of spotted fever group *Rickettsiae* isolated from China. *J Clin Microbiol* 31:83–88
- Zhang L, Jin J, Fu X, Raoult D, Fournier PE (2006) Genetic differentiation of Chinese isolates of *Rickettsia sibirica* by partial ompA gene sequencing and multispacer typing. *J Clin Microbiol* 44:2465–2467
- Zou YX, Wang QY, Fu ZX, Liu PP, Jin HT, Yang HH, Gao HW, Xi Z, Liu Q, Chen LF (2011) Detection of spotted fever group *Rickettsia* in *Haemaphysalis longicornis* from Hebei Province. *J Parasitol* 97:960–962