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PREVALENCE OF *ANAPLASMA PHAGOCYTOPHILA* AND *BORRELIA BURGDORFERI* IN *IXODES PERSULCATUS* TICKS FROM NORTHEASTERN CHINA

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Abstract. A total of 1,345 *Ixodes persulcatus* ticks collected from northeastern China were investigated for the presence of *Anaplasma phagocytophila* and *Borrelia burgdorferi* by a nested polymerase chain reaction (PCR). Sixty-two (4.6%) ticks were positive for *A. phagocytophila* and 454 (33.8%) were positive for *B. burgdorferi*. Seven (0.5%) were coinfected with both agents. Sequence analysis of 919-basepair PCR amplicons revealed three types of *A. phagocytophila*. Type 1 was identical to the published sequences of *A. phagocytophila*s responsible for human granulocytic ehrlichiosis (HGE). The other two variants differed from the HGE agent sequence at one and four positions, respectively. These findings imply that infection with *A. phagocytophila* poses a potential health threat to both humans and animals in northeastern China, and that ehrlichiosis should be considered in the differential diagnosis of febrile patients with a history of tick bite, particularly when clinical manifestations are atypical for Lyme disease.

INTRODUCTION

Human granulocytic ehrlichiosis (HGE) is an emerging tick-borne disease first recognized in the upper midwestern United States in 1994.[1] Patients with HGE often present relatively nonspecific symptoms that include fever, myalgia, headache, chills, lethargy, arthralgia, leukopenia, thrombocytopenia, and a mild elevation in levels of transaminases.[2,3] The etiologic agent of HGE is closely related to *Ehrlichia phagocytophila* and *E. equi.*[4] However, these species are now considered one species, and they were renamed *Anaplasma phagocytophila* in a recent reclassification.[5]

Anaplasma phagocytophila is transmitted by genus *Ixodes* ticks, including *I. scapularis*,[6,7] and *I. pacificus*[8,9] in the United States, *I. ricinus* in Europe,[10,11] and *I. persulcatus* in Asia.[12] These ticks also serve as the vectors of *Borrelia burgdorferi*, the causative agent of Lyme disease. Coinfection of ticks with *A. phagocytophila* and *B. burgdorferi* has been increasingly reported in recent years.[13–16] Northeastern China is highly endemic for Lyme disease,[17] where the infection rate of *B. burgdorferi* in adult *I. persulcatus* ticks can be as high as 44.1%.[18] Recently, the presence of *A. phagocytophila* in ticks from the region has been reported.[12] Therefore, an extensive study was carried out to confirm the previous findings, and to investigate coinfection of *I. persulcatus* ticks with *A. phagocytophila* and *B. Burgdorferi*.

MATERIALS AND METHODS

Collection of ticks. *Ixodes persulcatus* ticks were collected by flagging vegetation from four areas in Inner Mongolia Autonomous Region and Heilongjiang Province in the summers of 1999 and 2001, respectively. All the collection sites are forested highlands belonging to Great Xing-An Mountains and Small Xing-An Mountains. Ticks were kept alive in a refrigerator until tested.

Extraction of DNA. After identification, ticks were soaked in 70% ethanol for a few minutes, and then rinsed three times in sterile water. Extraction of DNA was performed as previously described.[19] Briefly, the ticks were placed into microtubes and mechanically disrupted with sterile scissors in 50 μL of DNA extraction buffer (10 mM Tris, pH 8.0, 2 mM EDTA, 0.1% sodium dodecyl sulfate, 500 μg of proteinase K/mL). The samples were incubated for two hours at 56°C and then boiled at 100°C for 10 minutes to inactivate the proteinase K. After centrifugation, the supernatant was transferred to fresh sterile microtubes and purified by extracting twice with an equal volume of phenol-chloroform before use.

Amplification by a polymerase chain reaction (PCR). A nested PCR was performed with primers designed to amplify the 16S rRNA gene of *A. phagocytophila*.[12] Primers GE9f and GE10r, previously described by Chen and others,[4] were used for the primary amplification. The PCR amplifications were performed in a volume of 30 μL in a Perkin-Elmer (Norwalk, CT) model 2400 thermal cycler. An initial three-minute denaturation at 95°C was followed by 35 cycles at 94°C for 15 seconds, 55°C for 20 seconds, and 72°C for 15 seconds, and a final extension at 72°C for five minutes. In nested PCR, the components and conditions were similar to those for the primary amplification, except that primers GE9f and GE2 were used,[20] and 1 μL of the primary PCR product was used as the template. A positive control (a plasmid containing the 16S rRNA gene of the HGE agent (kindly provided by Dr. J. Stephen Dumler, Department of Pathology, The Johns Hopkins Medical Institutions, Baltimore, MD) and a negative control (distilled water) were included with each set of amplifications.

For amplification of *B. burgdorferi* DNA, a nested PCR was carried out with primers derived from the *B. burgdorferi* outer surface protein A (*ospA*) gene.[21] For the primary amplification, 3 μL of each template sample was amplified in a 30-μL reaction mixture containing the primers OA1 and OA4. One microliter of the primary PCR product was then used as the template in a second 30-μL reaction mixture with primers BSL and OA4. The PCR amplifications were performed in a Perkin-Elmer model 2400 thermal cycler using the following protocol: preheating at 95°C for three minutes, followed by 40 cycles at 94°C for 20 seconds, 50°C for 20 seconds, and 72°C for 30 seconds, and a final extension at 72°C for seven minutes. In parallel with each amplification of tick specimens, DNA from a *B. burgdorferi* isolate was used as a positive control and distilled water was used as a negative control.

Reaction products were separated by 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.

Cloning of PCR products and DNA sequencing. The products of nested amplification were collected, and the purified DNA fragments were then cloned into the plasmid vector pGEM-T (Promega Corp., Madison, WI) and transformed into competent cells (*Escherichia coli* XL1-Blue) according to the manufacturer's instructions. The recombinant plasmids were extracted and purified from overnight cultures using QIA prep Spin Miniprep Kit (Qiagen, Valencia, CA). The nucleotide sequences of the plasmid inserts were determined by a dideoxynucleotide cycle sequencing method with an automated DNA sequencer (ABI PRISM 377; Perkin-Elmer).

RESULTS

A total of 1,345 *I. persulcatus* ticks were examined for the presence of *A. phagocytophila* and *B. burgdorferi* sensu lato. The distribution of ticks according to origin, sex and stage, and infection status is shown in Table 1. *Anaplasma phagocytophila* were detected in 4.6% of the ticks. Of 643 *I. persulcatus* ticks collected from the Wuerqihan and Moerdaoga forestry farms in the Great Xingan Mountains of Inner Mongolia, 40 (6.2%) contained *A. phagocytophila.* The difference in infection rates among male, female, and nymphal ticks was not significant ($\chi^2 = 1.69$, degrees of freedom [df] = 2, $P = 0.43$). The frequency of positive ticks from the Weihe forestry farm in the Small Xingan Mountains of Heilongjiang was 3.1% (22 of 702 ticks), which was significantly lower than that at Inner Mongolia ($\chi^2 = 6.59$, df = 1, $P = 0.01$). Although *A. phagocytophila* DNA was not detected in nymphal ticks in this area, the frequency of positive ticks was not statistically different regardless of sex or stage ($\chi^2 = 3.71$, df = 2, $P = 0.16$).

The prevalence of *B. burgdorferi* in ticks from the Great Xingan Mountains was 27.7% (178 of 643 ticks). The infection rate in adults (47.9%) was significantly higher than that in nymphs (8.0%) ($\chi^2 = 29.28$, df = 1, $P < 0.001$). In contrast to infection with *A. phagocytophila*, infection with *B. burgdorferi* was more prevalent in ticks in the Small Xingan Mountains (39.3%) than that in the Great Xingan Mountains ($\chi^2 = 19.80$, df = 1, $P < 0.001$). There was a significant difference in *B. burgdorferi* infection between adult and nymphal ticks from the Small Xingan Mountains. ($\chi^2 = 8.95$, df = 1, $P = 0.003$). Among 62 *A. phagocytophila*-positive *I. persulcatus* ticks, seven were coinfected with *B. burgdorferi*. The overall coinfection rate was 0.5%. No difference in proportions of coinfected ticks was demonstrated between the two studied areas.

Ten ticks positive for *A. phagocytophila* by the nested PCR were randomly selected for sequence analysis. Of the 10 positive specimens, six (3 males, 2 females, and 1 nymph) were from the Great Xingan Mountains and four (2 males and 2 females) were from the Small Xingan Mountains. A 919-basepair nucleotide sequence amplified with primer pair GE9f and GE10r[4] was obtained from each tick specimen. Sequence analysis of the 10 PCR amplicons revealed three variants of *A. phagocytophila* (Table 2). The nucleotide sequences from two male and one female ticks from the Great Xingan Mountains and one male tick from the Small Xingan Mountains were identical to the published sequences of the HGE agent. In addition, two sequence variants were detected. Variant 1, isolated from a male, a female, and a nymphal tick from the Great Xingan Mountains, had a T instead of an A at position 81 according to the corresponding sequence of the HGE agent (GenBank accession no. U02521). Variant 2, isolated from a male and two female ticks from the Small Xingan Mountains, differed from the HGE agent sequence by four bases at position 76, 77, 80, and 84 (Table 2).

DISCUSSION

The prevalence of *A. phagocytophila* infection in *I. persulcatus* ticks was investigated in the forest areas of northeastern China where Lyme disease the tick-borne encephalitis are endemic. As a result, 4.6% ticks were found to be infected, which further confirmed the existence of *A. phagocytophila* in the region. However, the overall infection rate of ticks determined in the present study is remarkably higher than that in our previous investigation.[12] A significant difference in the positive rate was also demonstrated between ticks from the Great Xingan Mountains and ticks from the Small Xingan Mountains. Recently, discrepant infection rates of *A. phagocytophila* in ticks from different areas around the world have been reported. The prevalence of *A. phagocytophila* infection was 0.8% in adult *I. pacificus* from California,[7] and in free-living adult *I. ricinus* from areas endemic for tick-borne fever in Switzerland,[11] which are comparable to our previous findings.[12] Higher prevalences were reported in *I. scapularis* and *I. pacificus* in the United States,[5,6,8,9,22] and in *I. ricinus* in Europe.[10,11,15,16,23] This discrepancy in positive rates could be attributable to differences in sampling approaches, tick species, geographic and seasonal variations of infected ticks, or to

Table 1

Results of the nested polymerase chain reaction for the identification of *Anaplasma phagocytophila* and *Borrelia burgdorferi* in *Ixodes persulcatus* ticks from northeastern China

Origin	Sex or stage	No. of ticks	No. (%) of ticks infected with		
			A. phagocytophila	*B. burgdorferi*	*A. phagocytophila* and *B. burgdorferi*
Great Xingan Mountains	Male	323	18 (5.6)	106 (32.8)	3 (0.9)
	Female	199	16 (8.0)	63 (31.7)	0
	Nymph	121	6 (5.0)	9 (7.4)	1 (0.8)
Small Xingan Mountains	Male	283	12 (4.2)	108 (38.2)	1 (0.4)
	Female	341	10 (2.9)	150 (44.0)	2 (0.6)
	Nymph	78	0	18 (23.1)	0
Total		1,345	62 (4.6)	454 (33.8)	7 (0.5)

TABLE 2

Comparison of partial 16S rRNA gene sequences of *Anaplasma phagocytophila* in *Ixodes persulcatus* from northeastern China with published sequences of *A. phagocytophila* of different origins

Geographic origin	Biological origin	Nucleotide difference at position*						GenBank accession no.	Reference
		76	77	80	81	84	886		
USA	Human	A	A	A	A	G	G	U02521	4
USA	Horse	A	A	A	A	A	—†	M73223	31
USA	Goat	A	A	A	A	A	—†	M73220	31
USA	Roe deer	G	A	A	A	A	G	AF384213	20
China	*I. persulcatus*	A	A	A	T	G	G	AY079425	This study
China	*I. persulcatus*	G	G	G	A	A	G	AF205140	This study
Sweden	*I. ricinus*	A	A	A	A	G	G	AJ242785	23
Sweden	*I. ricinus*	G	A	A	A	G	G	AJ242783	23
Sweden	*I. ricinus*	G	A	A	A	A	G	AJ242784	23
Canada	*I. scapularis*	G	A	A	A	A	G	AF311343	30

* The position of the nucleotide relative to the sequence of the agent for human granulocytic ehrlichiosis (HGE) reported by Chen and others.[4]
† — indicates no nucleotide corresponds to the HGE agent; a gap was required at this position to align the adjacent sequence.

limits of PCR sensitivity. A study carried out in southern Norway showed that tick samples taken from different locations and at different time points might have different rates of infection with *A. phagocytophila*.[24] All these findings imply that estimates based on spot investigations may have only local and temporary applicability, and have limited value in forming public health policy.

Ixodes persulcatus ticks are distributed over an extensive area from Russia to eastern Asia, where approximately one-fifth of the human population of the world resides. The results obtained in the present survey demonstrate that *A. phagocytophila* infection poses a potential health threat to both humans and animals where *I. persulcatus* is abundant, and should be useful in alerting public health officials and clinicians about the presence of ehrlichiosis in northeastern China. In contrast to our study, an investigation conducted in the Baltic region of Russia failed to demonstrate *A. phagocytophila* infection in *I. persulcatus*.[25] In southern Germany, ehrlichia-positive ticks were only found in one of five surveyed regions, arguing that the distribution of *A. phagocytophila* seems to be focal.[26] Further epidemiologic studies are required to clarify the diversity of *A. phagocytophila*, to identify its natural foci, and especially to define potential human and animal infection risks following tick bites in areas infested with *I. persulcatus*.

In forest areas of northeastern China, *I. persulcatus* is the most abundant tick species and is responsible for the majority of tick bites in humans. This tick species infests multiple animal hosts, and therefore, may acquire more than one pathogen from different reservoirs. Coinfection of ixodid ticks with *A. phagocytophila* and *B. burgdorferi* has been reported in the United States and many European countries with various prevalences.[9,10,15,16,24] In our study, seven of 62 ehrlichia-positive ticks were found to harbor both *A. phagocytophila* and *B. burgdorferi*. Coexistence of the two pathogens in *I. persulcatus* ticks from Asia has not been previously reported. This finding suggests that humans may become coinfected with the two pathogens as a consequence of a single tick bite. In fact, simultaneous human infection with the two agents has already been reported,[27] and it may lead to variations in clinical symptoms and signs.[28,29]

If one considers that *A. phagocytophila* can cause immunosuppression in its mammalian hosts, coinfection with two or more tick-borne agents may aggravate the clinical pictures of Lyme disease and tick-borne encephalitis. The identification of *A. phagocytophila* in *I. persulcatus*, and the finding of coinfection with *B. burgdorferi* in the current study imply that the possible occurrence of ehrlichiosis should be considered in the differential diagnosis of febrile patients with a history of tick bite in the forest areas of northeastern China, particularly when clinical manifestations are atypical for Lyme disease. Unfortunately, the *B. burgdorferi* genospecies was not determined in the present investigation. A previous study reported 64.4% *B. garinii* and 35.6% *B. afzelii* based on a PCR-restriction fragment length polymorphism analysis of 45 *B. burgdorferi* isolates from this area.[18] Whether the coinfection was associated with *B. burgdorferi* genospecies remains to be determined.

The nucleotide sequences of PCR products from tick samples were all identified as part of *A. phagocytophila* 16S rRNA gene, and showed high level of identity (99.6–100%) with published sequences of the HGE agent (Table 2). Three types of *A. phagocytophila* sequences were found: one with the identical sequence of the HGE agent, and two variants with nucleotide differences from the HGE agent by one and four basepairs, respectively. A variable region was found near the 5′ end of 16S rRNA gene at the position from 76 to 84 (according to HGE agent [GenBank accession no. U02521]). This result is consistent with findings of previous studies in other places, in which nucleotide differences were also identified in this variable region but at different positions.[13,20,23,30,31] It remains to be determined whether each molecular variant of *A. phagocytophila* can cause a disease in humans or animals.

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