Prevalence of *Anaplasma phagocytophilum* and its coinfection with *Borrelia afzelii* in *Ixodes ricinus* and *Ixodes persulcatus* ticks inhabiting Tver Province (Russia) – a sympatric region for both tick species

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Human granulocytic anaplasmosis (HGA) and Lyme borreliosis (LB) are tick-borne infectious diseases caused by *Anaplasma phagocytophilum* and *Borrelia burgdorferi sensu lato* species, respectively. In this study, *p44/msp2* paralogues specific to *A. phagocytophilum* and 5S–23S rRNA gene-intergenic spacers specific to *B. burgdorferi sensu lato* species were detected by PCR in ticks collected in two regions, Tver (Kalinin) and Konakovo, of the Tver (Kalinin) Province located 150 km north-west of Moscow. The PCR amplicons obtained were further characterized by sequencing and RFLP analysis. In the total of 199 ticks collected, 8.8 % (7/80) and 33.8 % (27/80) of *Ixodes ricinus*, and 2.5 % (3/119) and 45.4 % (54/119) of *Ixodes persulcatus*, were found to be infected with *A. phagocytophilum* and *B. burgdorferi sensu lato* spp., respectively. Of those 199 ticks, 5 (2.5 %) were coinfecting with *A. phagocytophilum* and *Borrelia afzelii*. Phylogenetic analysis revealed unique *p44/msp2* paralogous genes in *A. phagocytophilum*-infected Russian ticks. The sequence similarities with those of *A. phagocytophilum* in the United States, UK and Japan ranged from 42 % to 80.4 %, and there were no sequences showing more than 90 % similarity with those sequences from the other countries. The results showed that the *p44/msp2* sequence similarity groups may provide an index of adaptation of *A. phagocytophilum* strains to specific vector ticks or reservoir hosts in different countries and areas. These findings suggest that there is a public health threat from HGA and LB in Tver Province surrounding Moscow.

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INTRODUCTION

Human granulocytic anaplasmosis (HGA) and Lyme borreliosis (LB) are tick-borne and emerging infectious diseases caused by *Anaplasma phagocytophilum* (formerly *Ehrlichia phagocytophila*, revised by Dumler *et al.*, 2001) and *Borrelia burgdorferi sensu lato* species. HGA is a febrile

systemic illness and the severity of this disease ranges from asymptomatic seroconversion to death. The first case of human infection by *A. phagocytophilum* was found in the United States in 1994 (Chen *et al.*, 1994). Since then, the number of patients has increased in the United States (Dumler *et al.*, 2005). In Europe, the first human cases of this disease were described in 1997 (Petrovec *et al.*, 1997), and serological and PCR analyses suggest that *A. phagocytophilum* is distributed throughout Europe and in some parts of the Middle East and Asia (Blanco & Oteo, 2002; Cao *et al.*, 2000; Heo *et al.*, 2002; Keysary *et al.*, 1999). LB is the most common tick-borne infectious disease in the Northern Hemisphere (Masuzawa, 2004; Steere, 2001). The aetiological agents, *Borrelia burgdorferi*

Abbreviations: HGA, human granulocytic anaplasmosis; LB, Lyme borreliosis.

The GenBank/EMBL/DDBJ accession numbers for *p44/msp2* gene sequences of *Anaplasma phagocytophilum* detected from ticks collected in Moscow are AB262195, AB262196, AB262198, AB262199, AB262201–AB262209, AB262211–AB262224, AB262226–AB262232.

sensu lato, are classified into at least 13 species. Among them, *Borrelia burgdorferi sensu stricto*, *Borrelia garinii* (Eurasian type), *Borrelia afzelii*, *Borrelia lusitaniae*, *Borrelia valaisiana* and *Borrelia spielmanii* are found to infect *Ixodes ricinus* ticks in Europe. Since both pathogens are transmitted by *Ixodes* tick species, human cases coinfecting with both LB and HGA pathogens have been reported (Ahkee & Ramirez, 1996; Bakken *et al.*, 1996; Lebech *et al.*, 1998).

Prevalence rates of *A. phagocytophilum* in *I. ricinus* were found to be different among European countries (Blanco & Oteo, 2002). In Russia, *A. phagocytophilum* were detected from *I. ricinus* ticks collected in the Baltic region (Alekseev *et al.* 2001) and from *Ixodes persulcatus* ticks collected in Vologda Province (Eremeeva *et al.*, 2006), in Western Siberia (Rar *et al.*, 2005), and in a survey area from the Urals to the Far East of Russia (Shpynov *et al.*, 2006).

We previously showed the prevalence rate of *B. burgdorferi sensu lato* species by their isolation from *I. ricinus* and *I. persulcatus* collected in Moscow Province (Masuzawa *et al.*, 2005). Moscow and its suburbs are unique ecosystems with respect to tick habitat. *I. ricinus* ticks, which primarily inhabit the Western part of Eurasia, and *I. persulcatus* ticks, which primarily inhabit Eastern part of Eurasia, overlap around the Moscow region. However, little information is available regarding the epidemiological features and the prevalence rate of *A. phagocytophilum* in the area surrounding Moscow. The aim of this study was to determine the prevalence of *A. phagocytophilum* and the possibility of coinfection with LB *Borrelia* species and *A. phagocytophilum* in *I. persulcatus* and *I. ricinus* in Tver Province near Moscow.

METHODS

Survey area and tick sampling. During the summer of 2002, unfed adult ticks (*I. persulcatus* and *I. ricinus*) were collected from vegetation by a dragging method using 1 m² flannel flags, in the Tver (Kalinin) and Konakovo regions of the Tver Province, which are located 110–150 km north-west from Moscow. Vegetation in the survey area consists of Russian plain and forest (aspen, firs and birches) with bushes. The areas are mostly forest with limited human attendance and no livestock. Konakovo is located on the bank of a large reservoir (Ivan'kovskoe reservoir on the Volga river); other environmental features are similar for the two regions.

DNA preparation and qualification of tick DNA. After collection, the ticks were placed in a refrigerator, where they were naturally killed and dried in test tubes under low moisture conditions. Dry ticks were kept at 4 °C for 6 months prior to analysis.

The dried tick samples were disrupted for 30 s with 3 mm tungsten carbide beads in 1.5 ml tubes by TissueLyser (Qiagen). Total DNA was isolated from whole tick tissues by using the QuickGene-800 Nucleic-acid Isolation System with the QuickGene DNA tissue kit (both Fuji film). Quality of the extracted DNA was evaluated by PCR targeting the tick rDNA internal transcribed spacer 2 (ITS2) with primers 5.8S F3/1 (5'-GGG TCG ATG AAG AAC GCA GCC AGC-3') and 28S R1/1 (5'-TTC AGG GGG TTG TCT CGC CTG ATG-3'), according to the method described by Fukunaga *et al.* (2000). Briefly,

PCRs were performed with denaturation at 94 °C for 5 min, followed by 35 cycles consisting of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min and extension at 72 °C for 2 min with a T-gradient thermocycler (BioMetra). The PCR products were electrophoresed on a 1% agarose gel, stained with ethidium bromide and visualized under UV light. After confirmation of the tick DNA quality by the PCR, the tick DNA was used as a template for the following experiments.

To prevent carryover of amplified DNA, the PCR reaction mix before and after PCR was handled in a safety cabinet which was UV illuminated before and after use to degrade contaminated DNA. Electrophoresis was done in a separate room. To detect carryover caused by amplified or contaminated DNA in the PCR, one negative control tube was prepared for each seven samples. All PCRs were repeated at least three times to confirm results.

Detection of *A. phagocytophilum*-specific *p44/msp2* DNA by PCR. To detect *A. phagocytophilum*, a nested-PCR was conducted using primers p3726F (5'-GCT AAG GAG TTA GCT TAT GA-3'), p3761F (5'-CTG CTC T[T/G]G CCA A[A/G]A CCT C-3'), p4183R (5'-CAA TAG T[C/T]T TAG CTA GTA ACC-3') and p4257R (5'-AGA AGA TCA TAA CAA GCA TTG-3') (Ohashi *et al.*, 2005; Lin *et al.*, 2002; Zhi *et al.*, 1999), which were previously designed based on the highly conserved region of *p44/msp2* paralogues. Briefly, the PCR conditions were 1 cycle of denaturation at 94 °C for 5 min, followed by 35 cycles consisting of denaturation at 94 °C for 30 s, 1 min of annealing at 58 °C and 1 min of extension at 72 °C. The second-step PCR was conducted with 1 µl of the first-step PCR product as template DNA under the same conditions. The ~300–400 bp nested-PCR products of *p44/msp2* obtained from 10 PCR-positive samples were cloned into the pCR2.1 vector using TA Cloning kit (Invitrogen) and introduced into *Escherichia coli* DH5α; *E. coli* containing recombinant plasmids were identified by PCR targeted to *p44/msp2* genes.

DNA sequencing and phylogenetic analysis. For sequencing, recombinant *E. coli* clones were randomly selected and the plasmid DNA was amplified by using the Illustra Templiphi DNA amplification kit (GE Healthcare) at 30 °C for 18 h according to the manufacturer's protocol. Sequencing was carried out by using BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) with an ABI 3130-Avant Genetic Analyzer (Applied Biosystems). A phylogenetic tree was constructed based on the alignment of Russian *p44/msp2* sequences and the previously published sequences by CLUSTAL W algorithm using sequence analysis software, MEGALIGN (DNASTAR Inc., Madison, WI, USA) followed by the neighbour-joining method with 1000 bootstrap resamplings. All DNA sequences determined in this study were deposited in DDBJ/EMBL/GenBank under the following accession numbers. Sequences (accession numbers given in parentheses) derived from seven *I. ricinus* ticks were R10-13 clones a, b and d (AB262195, AB262196 and AB262198), R10-14 clones a, c and d (AB262199, AB262201 and AB262202), R13-10 clones a, b and c (AB262207 to AB262209), R18-8 clones a to d (AB262211 to AB262214), R17-3 clones a to d (AB262215 to AB262218), R18-9 clones a to d (AB262219 to AB262222) and R7-10 clones a to d (AB262229 to AB262232). The sequences derived from three *I. persulcatus* ticks were R10-16 clones a to d (AB262203 to AB262206), R29-6 clones a, b and d (AB262223, AB262224 and AB262226) and R29-9 clones a and b (AB262227 and AB262228).

Detection and genotyping of *Borrelia* spp. For detection of *B. burgdorferi sensu lato* species, the 5S–23S rDNA intergenic spacer was amplified with primers RIS1 corresponding to the 3' end of 5S rDNA (*rfl*) (5'-CTG CGA GTT CGC GGG AGA-3') and RIS2 corresponding to the 5' end of 23S rDNA (*rml*) (5'-TCC TAG GCA TTC ACC ATA-3') (Postic *et al.*, 1994) in the first-step PCR. For the nested

PCR, inner primer sets, RIS3 (5'-GGA GAG TAG GTT ATT GCC AGG-3') and RIS4 (5'-GAC TCT TAT TAC TTT GAC C-3') were used, which amplified about 200 bp DNA. The initial amplification consisted of 45 cycles, each consisting of 30 s at 95 °C, 30 s at 50 °C and 30 s at 72 °C. To perform the second-step PCR, 1 µl of the product from the first-step PCR was used for amplification in a 25 µl reaction mixture. The amplification was carried out under the same condition as the first-step PCR. The products amplified were electrophoresed in 1 % agarose gels stained with ethidium bromide. To identify *B. burgdorferi sensu lato* species that were infecting the ticks, the PCR products were digested with *Mse*I and *Dra*I, and the digested DNA was electrophoresed through 10–20 % gradient polyacrylamide gels (Atto) as described previously (Masuzawa *et al.*, 1996). The DNA bands were visualized by ethidium bromide staining and the RFLP pattern of these amplicons was compared with those of the reference strains: *B. burgdorferi* B31, *B. garinii* 20047, *B. afzelii* P/Gau, *B. lusitanae* PotiB2, *B. valaisiana* VS116 and *B. spielmanii* PsigII.

RESULTS AND DISCUSSION

A total of 199 unfed adult *Ixodes* ticks (119 *I. persulcatus* and 80 *I. ricinus*) were collected in the Tver region and the Konakovo region of Tver Province, Russia, during the summer of 2002. The ITS2-PCR specific to tick DNA was positive in all of the extracted DNA samples (data not shown) and the DNAs extracted from ticks were analysed for the presence of *A. phagocytophilum* and *B. burgdorferi sensu lato* species genes. By PCR of *p44/msp2*, we found that 8.8 % of *I. ricinus* ticks (7/80) and 2.5 % of *I. persulcatus* ticks (3/119) were infected with *A. phagocytophilum*, while by PCR of the 5S–23S rDNA spacer, 33.8 % of *I. ricinus* ticks (27/80) and 45.4 % of *I. persulcatus* ticks (54/119) were infected with *B. burgdorferi sensu lato* species. Among 100 male and 99 female ticks, five ticks of each sex were infected with *A. phagocytophilum*. No differences between prevalence rate for male and female ticks were found. In Konakovo region, 10 % of *I. ricinus* (7/70) and 4.3 % of *I. persulcatus* (3/70) were infected with *A. phagocytophilum*. We believe that this is the first record showing ticks infected with *A. phagocytophilum* in the region. However, *A. phagocytophilum*-infected ticks were not found in the Tver region located to the north-west of the Konakovo region. The prevalence rates were similar to those previously reported in other European countries (Blanco & Oteo, 2002) and other regions in Russia (Aleksiev *et al.*, 2001; Ereemeeva *et al.*, 2006; Rar *et al.*, 2005; Shpynov *et al.*, 2006). This finding indicates that both *I. ricinus* and *I. persulcatus* maintain this pathogen, and leads us to speculate that the infected ticks are distributed in a limited area in the region. However, further study is necessary to reach this conclusion.

The *p44/msp2* amplicons obtained from ten *A. phagocytophilum*-infected ticks (three *I. persulcatus* and seven *I. ricinus*) were cloned, and a total of 38 randomly selected clones (two to four per tick) were sequenced, yielding 34 different sequences (accession numbers AB262195 to AB262232). A phylogenetic tree was constructed based on the *p44/msp2* sequences derived from Russian ticks in this

study and the previously published sequences from the United States, UK and Japan (Fig. 1). *p44/msp2* sequences derived from *I. ricinus* (Ir) and *I. persulcatus* (Ip) clustered together on the phylogenetic tree (Fig. 1), indicating that there are no differences between *p44* genes detected from *I. ricinus* and *I. persulcatus*. All *p44/msp2* gene sequences detected from the Russian ticks showed some similarity to the sequences from the United States, UK and Japan, which ranged from 46.2 % to 73.2 %, 46.6 % to 80.4 %, and 42 % to 78.1 %, respectively. Among the Russian sequences, 25 out of 34 sequences which were grouped into seven clusters had more than 90 % similarity as shown in Fig. 1, whereas

Fig. 1. Phylogenetic tree of *A. phagocytophilum p44/msp2* gene sequences in Russia, the United States, UK and Japan. The phylogenetic tree was constructed based on the alignment of *p44/msp2* sequences by CLUSTAL W algorithm and followed by the neighbour-joining method with 1000 bootstrap resamplings. The sequences obtained from Russian ticks are indicated in bold type. J, US and UK indicate Japan, the United States and UK, respectively. Ip and Ir indicate tick species and the origin of the sequence, *I. persulcatus* and *I. ricinus*, respectively. Bar, sequence divergence of 5 %.

there was no sequence showing more than 90 % similarity with sequences from the United States, UK and Japan.

The 44 kDa major surface protein, P44/Msp2, of *A. phagocytophilum*, encoded by the *p44/msp2* multigene family, is well-characterized; *p44/msp2* contains a central hypervariable region (Zhi *et al.*, 1999) and is expressed from a genomic expression site subject to frequent gene conversion. The complete genome sequence of strain HZ isolated from a patient in the United States has been published and 113 *p44/msp2* loci were found in its genome, some of which are truncated (Hotopp *et al.*, 2006). Casey *et al.* (2004) compared partial 16S rRNA genes and *p44/msp2* paralogous sequences from 10 ruminant or tick isolates in the UK with those in the United States. Significant clustering among these sequences could not be established. Furthermore, the *p44/msp2* sequences were not clustered by countries, hosts and vector ticks, but most *p44/msp2* paralogue sequences showed 30–70 % similarity. Groups showing more than 95 % similarity comprised the sequences from either the United States or the UK. Similar results were obtained in this study, as groups composed of Russian sequences showing more than 90 % similarity did not include the sequences from the United States and UK. Thus, our findings appear to support the hypothesis advocated by Casey *et al.* (2004), that the *p44/msp2* sequence similarity groups may provide an index of adaptation of *A. phagocytophilum* strains to specific vector ticks or reservoir hosts in different countries and areas. Among 34 different sequences, five (R10-13-b, R10-14-a, R10-16-a, R10-16-c and R29-6-a) included termination codons, suggesting that these are probably pseudogenes or truncated genes. A one-base deletion was found in four sequences (R10-13-b, R10-16-a, R10-16-c, and R29-6-a) and resulted in truncated P44/Msp2 proteins due to frame shift. In the present study, we did not find any evidence

that this feature is specific to the agents detected in Russia. Since few *p44*-positive samples were obtained in this study, further study will be necessary to characterize Russian *A. phagocytophilum* in detail.

In the Tver and Konakovo regions, 33.8 % of *I. ricinus* (27/80) and 45.4 % of *I. persulcatus* (54/119) were infected with *B. burgdorferi sensu lato* species (Table 1). Difference in the prevalence rates was not observed between the two regions. We have previously demonstrated the relationship between *Borrelia* species and the vector ticks, *I. persulcatus* and *I. ricinus*, that were collected in Moscow Province, a sympatric region for both species of ticks, by isolation in BSK II medium (Masuzawa *et al.* 2005). The isolation rates of *Borrelia* species in *I. persulcatus* and *I. ricinus* collected in Moscow Province were 42.3 % and 20 %, respectively, the equivalent to the current prevalence rates estimated by PCR analysis in both tick species collected in the Tver Province. The prevalence of *B. burgdorferi sensu lato* species was higher in the Tver Province than in other European countries (Hubálek & Halouzka, 1998). To clarify the reason for this, we need to conduct further surveys in this area to determine reservoir host animals and their prevalence rates.

To identify the *Borrelia* species infecting ticks collected in the current study, RFLP analysis of the intergenic spacer amplicons was performed. The predominant *Borrelia* species detected were *B. afzelii*, followed by the Eurasian type of *B. garinii* (20047 type) (Table 1). The Asian type of *B. garinii* (NT29 type) was detected from *I. persulcatus* but not from *I. ricinus*. On the contrary, *B. afzelii* and *B. garinii* (Eurasian type) infected both tick species. We previously obtained findings as follows: (i) most of the isolates from *I. ricinus* were identified as *B. afzelii* and the Eurasian type of *B. garinii*, which were found in both *I. ricinus* and *I.*

Table 1. Prevalence of *A. phagocytophilum* and *B. burgdorferi sensu lato* in ticks collected in two regions of Tver Province in Russia in 2002

Ap, *A. phagocytophilum*; Bbsl, *B. burgdorferi sensu lato*; Bbss, *B. burgdorferi sensu stricto*; Baf, *B. afzelii*; Bg(A), *B. garinii* Asia (NT29 type); Bg(E), *B. garinii* Eurasia (20047 type).

Place	Tick species	No. of ticks examined	No. of PCR-positive ticks (%)		No. of ticks infected with one <i>Borrelia</i> species				No. of ticks dually infected with different <i>Borrelia</i> species and/or <i>A. phagocytophilum</i>			
			Ap	Bbsl	Bbss	Baf	Bg(A)	Bg(E)	Baf + Bg(A)	Baf + Bg(E)	Bg(A) + Bg(E)	Ap + Baf
Tver (Kalinin) region	<i>I. ricinus</i>	10	0	2 (20)	0	1	0	1	0	0	0	0
	<i>I. persulcatus</i>	49	0	22 (44.9)	1	9	4	4	2	0	2	0
Konakovo region	<i>I. ricinus</i>	70	7 (10)	25 (35.7)	0	14	0	10	0	1	0	3
	<i>I. persulcatus</i>	70	3 (4.3)	32 (45.7)	0	15	9	6	1	0	1	2
Total	<i>I. ricinus</i>	80	7 (8.8)	27 (33.8)	0	15	0	11	0	1	0	3
	<i>I. persulcatus</i>	119	3 (2.5)	54 (45.4)	1	24	13	10	3	0	3	2
	Both ticks	199	10 (5)	81 (40.7)	1	39	13	21	3	1	3	5

persulcatus; no Asian type of *B. garinii*, which is only found in *I. persulcatus*, was isolated; (ii) the isolates from *I. persulcatus* were identified as both Eurasian type and Asian type of *B. garinii* and *B. afzelii*; and (iii) no *B. burgdorferi sensu stricto* was isolated from *I. persulcatus* (Masuzawa *et al.* 2005). However, in the current study, we detected DNA of *B. burgdorferi sensu stricto* from one *I. persulcatus* tick collected in Tver region. *I. ricinus* was also collected in the Tver region, indicating that the enzootic transmission cycle between vector ticks, *I. ricinus* and *I. persulcatus*, and reservoir animals is established at this site. We speculated that the *I. persulcatus* was accidentally infected with the *B. burgdorferi sensu stricto* via blood feeding from an animal reservoir infected with *B. burgdorferi sensu stricto* transmitted from *I. ricinus* ticks. Since the vector potential and competence of *I. persulcatus* for *B. burgdorferi sensu stricto* have not been fully understood, further study of experimental transmission is necessary to prove this theory. No *B. valaisiana*-infected tick was detected in this study whereas one (of 72) *I. ricinus* infected with *B. valaisiana* was isolated in our previous study (Masuzawa *et al.*, 2005). These facts suggest that *B. valaisiana* is sparsely distributed in the survey area.

Among ticks infected with *B. burgdorferi sensu lato* or *A. phagocytophilum*, five were coinfecting with *A. phagocytophilum* and *B. afzelii*, but not with the other species of the genus *Borrelia* (Table 1). It has been reported that rodents are reservoir hosts for *B. afzelii* and some types of *B. garinii*, while the majority of *B. garinii* types and *B. valaisiana* appear to be associated with birds (Kurtenbach *et al.*, 2002). We previously detected *B. afzelii* and *B. garinii* (type 20047, Eurasia) from the wild mammals *Clethrionomys glareolus* and *Apodemus flavicollis* in Russia (Masuzawa *et al.*, 2005). Reservoirs for *A. phagocytophilum* are likely to comprise some ruminants, rodents, and perhaps other small and intermediate-size mammals (Dumler *et al.*, 2005). However, to determine the reservoir host for *A. phagocytophilum* in the survey area, further study is needed.

Coinfection with both pathogens has been reported in humans (Ahkee & Ramirez, 1996; Bakken *et al.*, 1996; Lebech *et al.*, 1998), *Ixodes* ticks (Daniels *et al.*, 1997; Pancholi *et al.*, 1995; Telford *et al.*, 1996) and wild rodents (Levin *et al.*, 1999). In experimentally infected mice, the coinfection of *A. phagocytophilum* and *B. burgdorferi sensu stricto* resulted in increased levels of both pathogens and more severe Lyme arthritis compared with those in mice experimentally infected with *B. burgdorferi sensu stricto* alone (Thomas *et al.*, 2001; Grab *et al.*, 2007). It was suggested that the severity of disease was associated with diminished interleukin-12, gamma interferon and tumour necrosis factor alpha levels, and elevation of interleukin-6 levels; it was also suggested that coinfection induced the modulation of host immune responses, resulting in increased severity of the diseases.

In conclusion, we demonstrated that LB and HGA agents infected *I. persulcatus* and *I. ricinus* ticks in Tver Province.

This study indicates the public health significance of both LB and HGA, and that further risk assessment and awareness are important for clarifying the current status of these diseases in the Moscow region.

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