JOURNAL OF CLINICAL MICROBIOLOGY, June 2001, p. 2237–2242 Vol. 39, No. 6
0095-1137/01/$04.00+0 DOI: 10.1128/JCM.39.6.2237–2242.2001

Identification of *Ehrlichia* spp. and *Borrelia burgdorferi* in *Ixodes* Ticks in the Baltic Regions of Russia

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Received 13 November 2000/Returned for modification 4 March 2001/Accepted 8 April 2001

The presence and distribution of *Ehrlichia* spp. and *Borrelia burgdorferi* sensu lato was demonstrated among ixodid ticks collected in the Baltic regions of Russia, where Lyme borreliosis is endemic. A total of 3,426 *Ixodes ricinus* and 1,267 *Ixodes persulcatus* specimens were collected, and dark-field microscopy showed that 265 (11.5%) *I. ricinus* and 333 (26.3%) *I. persulcatus* ticks were positive. From these samples, 472 dark-field-positive and 159 dark-field-negative ticks were subjected to PCR and subsequent reverse line blot hybridization. Fifty-four ticks (8.6%) carried *Ehrlichia* species, and 4 (0.6%) carried ehrlichiae belonging to the *Ehrlichia phagocytophila* complex, which includes the human granulocytic ehrlichiosis agent. The *E. phagocytophila* complex and an *Ehrlichia*-like species were detected only in *I. ricinus* whereas *Ehrlichia muris* was found exclusively in *I. persulcatus*, indicating a possible vector-specific infection. *Borrelia garinii* was found predominantly in *I. persulcatus*, but *Borrelia afzelii* was evenly distributed among the two tick species. Only two *I. ricinus* ticks carried *B. burgdorferi* sensu stricto, while *Borrelia valaisiana* and a newly identified *B. afzelii*-like species were found in 1.7 and 2.5% of all ticks, respectively. Of the dark-field-positive ticks, only 64.8% yielded a *Borrelia* PCR product, indicating that dark-field microscopy may detect organisms other than *B. burgdorferi* sensu lato. These observations show that the agent of human granulocytic ehrlichiosis may be present in ticks in the Baltic regions of Russia and that clinicians should be aware of this agent as a cause of febrile disease.

Borrelia burgdorferi sensu lato, the causative agent of Lyme disease, is frequently found in a variety of tick species throughout the world. The widespread distribution has made Lyme borreliosis the most prevalent tick-transmitted zoonotic disease in humans (29). In Europe, five different *B. burgdorferi* sensu lato species are found, of which *Borrelia afzelii* and *Borrelia garinii* are present throughout the continent. In countries of central Europe, such as The Netherlands, Germany, Italy, and France, *B. burgdorferi* sensu stricto and *Borrelia valaisiana* are found (28). *Borrelia lusitaniae* is mainly found in *Ixodes ricinus* ticks in Portugal but has also been detected in ticks from the Czech Republic, Moldavia, Ukraine, and Belorussia (10). Previous surveys have shown 10 to 30% of the *Ixodes* ticks from the St. Petersburg and Kaliningrad regions of Russia carried *B. afzelii* and *B. garinii,* but not *B. burgdorferi* sensu stricto (1). In a similar study it was shown that the prevalence of *B. burgdorferi* sensu lato infection of *I. ricinus* ticks from nearby Helsinki, Finland, varied from 19 to 55% (7). In addition, various other studies have shown that ticks in different regions of Russia are infected with *B. burgdorferi* sensu lato (8, 11, 26).

Ehrlichioses are known as important tick-borne diseases in animals, particularly in wildlife and domestic ruminants (25). During the last decade, two previously unknown *Ehrlichia* species have emerged as agents causing considerable public health problems in the United States (2, 5, 32). The *Ehrlichia* species that was identified in 1986 as causing human monocytic ehrlichiosis is designated *Ehrlichia chaffeensis*. Initially it was assumed that this species was transmitted by *Amblyomma americanum* only, but in a recent study researchers have also identified this *Ehrlichia* species in *Ixodes* ticks (9). Until now there have been no reports of confirmed cases of human monocytic ehrlichiosis outside the United States. More recently, the agent causing human granulocytic ehrlichiosis (HGE) was identified. This species belongs to the *Ehrlichia* genogroup 2, which includes *Ehrlichia phagocytophila*, an organism isolated from ruminants like sheep, cattle, and deer, and *Ehrlichia equi*, which is found in horses. These organisms, designated the *E. phagocytophila* complex, are closely related and may even represent the same species. There have been several reports that described the presence of the HGE agent in ticks in Europe (3, 12, 20, 27). However, there seems to be a paucity of reports of confirmed cases of HGE in Europe, and most of these cases are found in Slovenia where Lyme borreliosis is highly prevalent (14, 17, 19, 20, 30).

The aim of this study was to determine the prevalence of *Ehrlichia* infection in ticks in a region where diseases like tick-borne encephalitis and Lyme borreliosis are highly prevalent. For this reason, *I. ricinus* and *Ixodes persulcatus* ticks were collected from the Baltic regions of Russia and screened for the presence of *B. burgdorferi* sensu lato by dark-field microscopy, followed by PCR and subsequent reverse line blot hybridization to identify the *Borrelia* and *Ehrlichia* species present in these ticks.

MATERIALS AND METHODS

Ticks. From 1997 to 1998, 4,693 ticks were collected by flagging in forested areas near Morskaja and Lisy Nos in the vicinity of St. Petersburg and along the Curonian Spit in the Kaliningrad enclave of Russia. Ticks were stored alive and analyzed by dark-field microscopy within hours after collection.

Dark-field examination of ticks. For dark-field inspection the contents of the gut from a dissected tick were ejected into a drop of saline. After being immediately covered with a thin cover glass, the slide was inspected under a micro-

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scope for the presence of live spirochetes, with 250 fields viewed. The remainder of the tick was stored in 70% ethanol at 4°C for PCR analysis.

Preparation of DNA extracts from ticks. Ticks were processed as described before (23). Briefly, ticks were taken from the 70% ethanol solution, briefly dried, and boiled for 20 min in 100 μl of 0.7 M ammonium hydroxide to free the DNA. After being cooled, the vial with the lysate was left open and incubated for 20 min at 90°C to evaporate the ammonia. The tick lysate was either used directly for PCR or stored at −20°C until use.

Construction of spike DNA. To assess the efficiency of the PCRs we constructed plasmids that carried the sequences complementary to the primer sequences and used these as internal spikes in the *Ehrlichia* and *Borrelia* PCRs. For construction of the *Ehrlichia* spike, primers were designed that carried a hybrid of the sequences of a restriction site, the *Ehrlichia* 16S rRNA priming sites, and primer sequences encompassing a 508-bp region of the *tmpB* gene of *Treponema pallidum*. The restriction sites were incorporated into the primers to facilitate cloning and subcloning of the PCR products but were not used in this study. For the construction of a spike control for the *Borrelia* PCR, a similar approach was used. In the latter case, hybrids between the sequences of a restriction site, the *Borrelia* primer sequences, and primer sequences encompassing a 589-bp region of the *tmpA* gene of *T. pallidum* were used. PCRs were run with *T. pallidum* DNA, and the PCR products were cloned directly into a TA-TOPO vector (Invitrogen, Groningen, The Netherlands). The plasmids were isolated and purified with the Qiagen Plasmid Minikit (Hilden, Germany) and used as spike controls with the *Ehrlichia* (*tmpB* spike) and *Borrelia* (*tmpA* spike) PCR. The appropriate spike concentration was determined by titrating the amount of spike in a PCR with serial dilutions of *Ehrlichia* and *Borrelia* DNA. The amount of spike DNA used allowed the detection of a single copy of the specific target. The composition of the spike construction primers and the spike probes is presented in Table 1.

PCR amplifications. PCR amplifications were performed with an Omnigene thermal cycler (Hybaid, Ltd., Teddington, United Kingdom). DNA amplification was done with 50-μl reaction volumes. For the amplification of *Ehrlichia* DNA, each reaction mixture contained 10 fg of *tmpB* spike DNA, 80 pmol of primers 16S8FE and B-GA1B, 1.2 U of SuperTaq DNA polymerase (HT Biotechnology, Ltd., Cambridge, United Kingdom), 0.26 μg of the TaqStart antibody (Clontech Laboratories, Palo Alto, Calif.), 0.1 U of uracil-DNA glycosylase (UDG) (GibcoBRL, Life Technologies B.V., Breda, The Netherlands), deoxynucleoside triphosphates (100 μM dUTP, 100 μM dTTP, 200 μM dATP, 200 μM dGTP, and 200 μM dCTP), and SuperTaq buffer (10 mM Tris-HCl [pH 9.0], 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% stabilizer, 0.1% Triton X-100). A 25-μl overlay of paraffin oil was added to the tubes, followed by 5 μl of the tick DNA extract. The mixture was incubated for 3 min at 37°C to promote UDG activity, followed by a 10-min incubation at 94°C to inactivate the UDG. To minimize nonspecific amplification a touchdown-up PCR program was used: two cycles of 20 s at 94°C, 30 s at 65°C, and 30 s at 72°C; and then two cycles with conditions identical to the previous cycles, but with an annealing temperature of 63°C. During the subsequent two cycle sets, the annealing temperature was lowered by 2°C until it reached 55°C. Then, an additional 20 cycles, each 20 s at 94°C, 30 s at 55°C, and 30 s at 72°C, and 20 cycles, each 20 s at 94°C, 30 s at 63°C, and 30 s at 72°C, followed the touchdown program. The PCR was ended by an extra incubation for 7 min at 72°C and held at 65°C to keep the UDG inactive. For the amplification of *B. burgdorferi* sensu lato DNA, similar conditions were used. However, in this PCR, 1 fg of *tmpA* spike DNA, 20 pmol of the primers 23SBor and B-5SBor, 2.5 U of SuperTaq, and 0.55 μg of TaqStart were used. In addition, the DNA was amplified in a regular touchdown PCR ranging from 60 to 50°C, with 40 cycles at the touchdown temperature.

To monitor for the occurrence of false-positive PCR results, negative controls were included during extraction of the tick samples: one control sample for each five tick samples with a minimum of two controls. In addition, each time that PCR was performed, negative- and positive-control samples were included. To minimize contamination, the reagent setup, the extraction and sample additions, and the PCR and sample analyses were performed in three separate rooms, the first two rooms of which were kept at positive pressure and had airlocks.

Reverse line blot hybridization. The reverse line blotting technique was performed as described before (23, 24) with some modifications. Briefly, solutions with 5′-amino-linked oligonucleotide probes ranging from 6 to 800 pmol were coupled covalently to an activated Biodyne C membrane in a line pattern with a miniblotter (Immunetics, Cambridge, Mass.). After the oligonucleotide probes were bound, the membrane was taken from the miniblotter, washed in 2× SSPE (360 mM NaCl, 20 mM $Na_2HPO_4{\cdot}H_2O$, 2 mM EDTA) with 0.1% sodium dodecyl sulfate (SDS) at 60°C and placed in the miniblotter again with the oligonucleotide lines perpendicular to the slots. Ten microliters of the biotin-labeled PCR product was diluted in 150 μl of 2× SSPE–0.1% SDS, denatured for 10 min at 99°C, and cooled rapidly on ice. The slots of the miniblotter were filled with the denatured PCR product, and hybridization was performed for 1 h at 42°C. The membrane was removed from the miniblotter and washed twice for 10 min in 2× SSPE–0.1% SDS at 51°C. Subsequently, the membrane was incubated for 30 min at 42°C with streptavidin-peroxidase (Boehringer GmbH, Mannheim, Germany), diluted 1:4,000 in 2× SSPE–0.5% SDS, and washed twice for 10 min in 2× SSPE–0.5% SDS. Hybridization was visualized by incubating the membrane with ECL detection liquid (Amersham International plc, Den Bosch, The Netherlands) and exposing an X-ray film (Hyperfilm; Amersham) to the membrane. For species identification the biotinylated *Ehrlichia* PCR product was hybridized with seven different oligonucleotide probes in the reverse line blot assay. Similarly, the biotinylated spacer fragment of the *Borrelia* PCR was hybridized with five *B. burgdorferi* genospecies-specific oligonucleotide probes. All primers and probes are displayed in Table 1.

DNA sequencing and data analysis. PCR products, used for DNA sequencing, were purified with Qiaquick PCR purification kits (Qiagen). For DNA sequencing reactions, the fluorescence-labeled dideoxynucleotide technology was used. The sequenced fragments were separated, and the data was collected with ABI 377 and ABI 3700 automated DNA sequencers (PE Biosystems, Nieuwerkerk aan de Ijssel, The Netherlands). The collected sequences were assembled, edited, and analyzed with the DNAStar package (Madison, Wis.). The phylogenetic tree and multiple alignments were constructed with the alignment and clustering modules in the Bionumerics package (Applied Maths, Kortrijk, Belgium).

Nucleotide sequence accession numbers. The partial 16S rRNA gene sequence of *Ehrlichia muris* and the 5S-23S intergenic spacer region of the *B. afzelii*-like organism found in this study are available in the GenBank database under accession numbers AF312907 and AF312906, respectively.

RESULTS

PCR detection of *Borrelia* and *Ehrlichia* DNAs in ticks. In this study 2,305 *I. ricinus* and 1,267 *I. persulcatus* ticks were collected by flagging, and screening by dark-field microscopy for the presence of spirochetes showed that 265 (11.5%) of the *I. ricinus* ticks and 333 (26.3%) of the *I. persulcatus* ticks were dark-field positive. By random choice, 215 dark-field-positive and 80 dark-field-negative *I. ricinus* ticks and 257 dark-field-positive and 79 dark-field-negative *I. persulcatus* ticks were selected and analyzed by PCR and subsequent reverse line blot hybridization to detect and identify *Borrelia* and *Ehrlichia* species. In 338 (53.6%) of 631 ticks analyzed, *Borrelia* DNA was detected whereas *Ehrlichia* DNA was found in 54 (8.6%) ticks (Table 2). In 296 samples (46.9%), only *Borrelia* DNA was found, and in 12 samples (1.9%) only *Ehrlichia* DNA was detected. Forty-two (6.7%) extracts of all 631 ticks carried both *Borrelia* and *Ehrlichia* DNA.

Comparison of dark-field results and detection of *Borrelia* and *Ehrlichia* by PCR. In 209 of the 257 (81.3%) dark-field-positive *I. persulcatus* ticks, *B. burgdorferi* sensu lato DNA was detected (Table 2). In contrast, only 97 of the 215 (45.1%) dark-field-positive *I. ricinus* ticks carried *B. burgdorferi* sensu lato DNA. Fifteen of the 80 (18.8%) dark-field-negative *I. ricinus* and 17 of the 79 (21.5%) dark-field-negative *I. persulcatus* ticks carried *B. burgdorferi* sensu lato DNA. *Ehrlichia* DNA was detected in 29 (11.2%) dark-field-positive *I. persulcatus* ticks and in 24 (11.1%) dark-field-positive *I. ricinus* ticks. Only one (1.3%) dark-field-negative *I. ricinus* tick carried *Ehrlichia*, and none of the dark-field-negative *I. persulcatus* ticks carried detectable *Ehrlichia* DNA.

Distribution of *Borrelia* and *Ehrlichia* species in ticks. The majority of the *Borrelia*-positive ticks carried *B. afzelii* and/or *B. garinii* DNA. There was a remarkable difference in distribution of the various *Borrelia* species between *I. persulcatus* and *I. ricinus* (Table 3). Of the 226 *Borrelia*-positive *I. persulcatus* ticks 107 (47.3%) contained *B. garinii*, 50 (22.1%) carried *B.*

TABLE 1. Oligonucleotide primers and probes used in PCR and hybridization assays

Oligonucleotide primer or probe	Sequence	Target organism	Target gene	Nucleotide position	Reference or source
Borrelia-specific primers					
B-5SBor	5′biotin-GAGTTCGCGGGAGAGTAGGTTATT	*B. burgdorferi* sensu lato	5S rRNA (*rrfA*)	82–105	This study
23SBor	TCAGGGTACTTAGATGGTTCACTT	*B. burgdorferi* sensu lato	23S rRNA (*rrlB*)	208–185	This study
Probes					
SL	5′amino-CTTTGACCATATTTTTATCTTCCA	*B. burgdorferi* sensu lato	23S-5S spacer[a]	182–167	24
SS	5′amino-AACACCAATATTTAAAAAACATAA	*B. burgdorferi* sensu stricto	23S-5S spacer	59–36	24
GA	5′amino-AACATGAACATCTAAAAACATAAA	*B. garinii*	23S-5S spacer	58–35	24
AF	5′amino-AACATTTAAAAAATAAATTCAAGG	*B. afzelii*	23S-5S spacer	44–21	24
VS	5′amino-CATTAAAAAAATATAAAAAATAAATTTAAGG	*B. valaisiana*	23S-5S spacer	51–21	24
Ruski	5′amino-GAATAAAACATTCAAATAATATAAAC	*B. afzelii* like	23S-5S spacer	67–42	This study
Ehrlichia-specific primers					
16S8FE	GGAATTCAGAGTTGGATCMTGGYTCAG	Eubacteria	16S rRNA gene	8–27	27
B-GA1B	5′biotin-CGGGATCCCGAGTTTGCCGGGACTTCTTCT	*Ehrlichia* genus	16S rRNA gene	476–456	27
Probes					
A-EhrAll	5′amino-TTATCGCTATTAGATGAGCC	*Ehrlichia* genus	16S rRNA gene	203–222	27
A-HGE	5′amino-GCTATAAAGAATAGTTAGTGG	HGE agent	16S rRNA gene	87–107	27
A-Phago	5′amino-TTGCTATAAAGAATAATTAGTGG	*E. phagocytophila*	16S rRNA gene	85–107	27
A-D-HGE	5′amino-GCTATGAAGAATAGTTAGTG	HGE agent variant	16S rRNA gene	87–106	27
A-D-Phago	5′amino-TTGCTATGAAGAATAATTAGTG	*E. phagocytophila* variant	16S rRNA gene	87–106	27
A-ESchot	5′amino-GCTGTAGTTTACTATGGGTA	*Ehrlichia* like	16S rRNA gene	76–95	27
A-ECan	5′amino-TCTGGCTATAGGAAATTGTTA	*Ehrlichia canis*	16S rRNA gene	85–105	27
A-EChaf	5′amino-ACCTTTTGGTTATAAATAATTGTTA	*E. chaffeensis*	16S rRNA gene	83–107	27
A-EmurisC	5′amino-GCTATAGGTTCGCTATTAG	*E. muris* C variant	16S rRNA gene	85–103	This study
A-EmurisT	5′amino-AGCTATAGGTTTGCTATTAGT	*E. muris* T variant	16S rRNA gene	86–104	This study
Spike primers					
23SBortmpA	CGGGATCCC-TCAGGGTACTTAGATGGTTCACTT-ACCCCGAACTTTTCTCC[b]	*T. pallidum*	*tmpA*	199–215	This study
5SBortmpA	GGAATT-CGAGTTCGCGGGAGAGTAGGTTATT-CTTCCCCAGTTCTGTGTAGA	*T. pallidum*	*tmpA*	725–706	This study
EhrFtmpB	GCTCTAGAG-AGAGTTTGATCCTGGCTCAG-CGCCTATCCGACCGAGTAT	*T. pallidum*	*tmpB*	308–326	This study
EhrRtmpB	CGGGATCCC-GAGTTTGCCGGGACTTCTTCTA-CCACGTAGTCACCTTGTATT	*T. pallidum*	*tmpB*	756–736	This study
Probes					
A-TmpABor	5′amino-TACAATCTAAGATCCAGACT	*T. pallidum*	*tmpA*	346–365	This study
A-TmpBEhr	5′amino-GAAAACTCGAAGAACAAAGAA	*T. pallidum*	*tmpB*	502–522	This study

[a] The SL probe targets the spacer and the first eight bases of the 23S gene sequence (*rrlB*).
[b] The dashes in the spike primers indicate the separation between the restriction site sequences, the *Borrelia* or *Ehrlichia* sequence, and the *tmpA*- or *tmpB*-specific sequence.

TABLE 2. Comparison of dark-field microscopy and reverse line blot detection of *Borrelia* and *Ehrlichia* DNA in ticks

Tick species and result by dark-field microscopy	No. of ticks	Reverse line blot[a]			
		Borrelia		*Ehrlichia*	
		Positive	Negative	Positive	Negative
I. ricinus					
Positive	215	97	118	24	191
Negative	80	15	65	1	79
Total	295	112	183	25	270
I. persulcatus					
Positive	257	209	48	29	228
Negative	79	17	62	0	79
Total	336	226	110	29	307
Total	631	338	293	54	577

[a] Values are numbers of ticks.

afzelii, and 57 (25.2%) carried both *B. afzelii* and *B. garinii*. In contrast, 80 (71.4%) out of 112 *Borrelia*-positive *I. ricinus* ticks carried *B. afzelii*, 13 (11.6%) carried *B. garinii* DNA, and only 1 tick (0.9%) was infected with both *B. afzelii* and *B. garinii*. Only one *I. persulcatus* tick was triple infected by *B. afzelii*, *B. garinii,* and *B. valaisiana,* and two *I. ricinus* ticks carried *B. burgdorferi* sensu stricto DNA.

Sixteen of all *Borrelia*-positive ticks reacted with the *B. burgdorferi* sensu lato probe only. Therefore, we sequenced the 5S-23S intergenic spacer of six of these ticks and found that they carried *Borrelia* species with identical spacer sequences which were similar to but distinct from the spacer of *B. afzelii* (Fig. 1). We therefore designated this species as *B. afzelii* like, designed a new probe (Ruski) for use with the reverse line blot, and hybridized the PCR products of all 16 samples with the new probe. As expected, all 16 samples reacted with this probe, and no cross-hybridization with other species-specific probes was seen.

TABLE 3. Distribution of *Ehrlichia* and *Borrelia* spp. in *I. ricinus* and *I. persulcatus* ticks

Reverse line blot result	Tick species		Total
	I. ricinus	*I. persulcatus*	
Borrelia positive	112	226	338
B. afzelii	80	50	130
B. afzelii + *B. valaisiana*	6	2	8
B. afzelii like	9	7	16
B. garinii	13	107	120
B. garinii + *B. afzelii*	1	57	58
B. garinii + *B. afzelii* + *B. valaisiana*		1	1
B. garinii + *B. valaisiana*	1	2	3
B. burgdorferi sensu stricto	2		2
Borrelia negative	183	110	293
Ehrlichia positive	25	29	54
E. muris		29	29
E. phagocytophila	1		1
HGE	3		3
Ehrlichia like	21		21
Ehrlichia negative	270	307	577
Total no. of ticks	295	336	631

Of the 25 *Ehrlichia*-positive *I. ricinus* ticks, 3 carried HGE DNA, 1 contained *E. phagocytophila* DNA, and 21 carried DNA from an organism that was identified before in Dutch ticks and designated as an *Ehrlichia*-like organism (27). In 29 *I. persulcatus* ticks, DNA from *Ehrlichia* species was found that initially reacted with the *Ehrlichia* genus-specific probe only. From these samples the PCR products were sequenced, and comparison showed that their sequences were identical. Alignment with DNA sequences in the GenBank data bank revealed a close similarity (>99%) with *E. muris* 16S rRNA gene sequences. We thereafter designed two *E. muris*-specific probes that differed only in one residue targeting position 95 of the 16S rRNA gene and hybridized all 29 above-mentioned PCR products with these probes. All samples reacted with the *E. muris* C probe but not with the *E. muris* T probe.

There was no significant difference between *Borrelia* or *Ehrlichia* infection rates in the different sexes or stages of the *I. ricinus* ticks investigated (Table 4). However, in *I. persulcatus* ticks approximately 75% of the adults and only 35.5% of the nymphs were infected with *Borrelia*. Similarly, about 10% of adult *I. persulcatus* ticks carried *Ehrlichia* DNA, whereas only 1.6% of nymphs did. None of the 24 larvae carried any detectable *Borrelia* or *Ehrlichia* DNA.

DISCUSSION

In the study presented here we have shown that *Ehrlichia* species were found in 8.6% of ixodid ticks collected from vegetation in the Baltic region of Russia. Members of the granulocytic *E. phagocytophila* group, including some reacting with our HGE agent probe, were found in *I. ricinus* but not in *I. persulcatus*. However, the rate of HGE infection in these ticks was low (1%). This prevalence of infection is comparable to that found by several other research groups in Europe (13, 16, 18, 21). However, other European studies have reported infection rates ranging from 3.2% in Slovenia to 28.9% in The Netherlands (6, 20, 27, 31), and some studies from the United States even reported a prevalence of infection in *Ixodes scapularis* up to 50% (4, 15). The explanation for these large differences in prevalence of infection needs to be established with comparative studies. About 7.1% of the *I. ricinus* ticks were

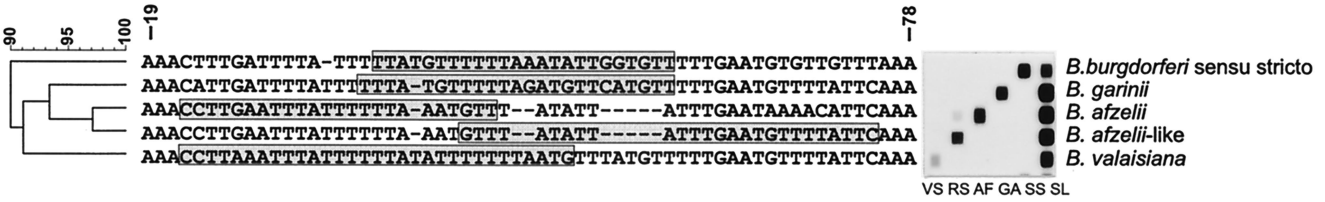

FIG. 1. Multiple aligment and clustering of the 23S-5S intergenic spacer region of *B. burgdorferi* sensu lato and reverse line blot result using the amplified spacer sequences. Multiple alignment and clustering were performed with the complete 174- to 182-bp spacer region and are only partially displayed. The coordinates of the *B. burgdorferi* sensu stricto spacer sequence are indicated above the sequences, and the probe regions are denoted by grey boxes. Reverse line blot probes are indicated as follows: VS, *B. valaisiana*; RS, *B. afzelii* like; AF, *B. afzelii*; GA, *B. garinii*; SS, *B. burgdorferi* sensu stricto; SL, *B. burgdorferi* sensu lato.

infected with an *Ehrlichia*-like organism previously identified in *I. ricinus* ticks found in The Netherlands and Italy (27). Remarkably, this species was not found in *I. persulcatus* ticks from the same regions, suggesting a vector specificity of this *Ehrlichia* species. Although the true nature of this species remains unknown, phylogenetic analysis based on the 16S rRNA gene sequence indicates a close relationship with the monocytic group of ehrlichiae. Nearly 9% of the *I. persulcatus* ticks were infected with an *Ehrlichia* species with a 16S rRNA gene sequence nearly identical to one of the published *E. muris* sequences. This monocytic *Ehrlichia* species was identified only in *I. persulcatus* ticks, again suggesting a vector-specific *Ehrlichia* infection. In a recent study, *I. persulcatus* ticks from Perm, Russia (region of Ural Mountains), were tested for the presence of *Ehrlichia* by PCR using *Ehrlichia*-specific primers (22). None of the ticks carried detectable HGE DNA. However, 5 out of 35 ticks tested yielded a 16S rRNA PCR product of which the DNA sequence was shown to be identical to that of *E. muris*. This result confirms our finding that *E. muris* was the only *Ehrlichia* species found in *I. persulcatus* ticks.

Analysis of the ticks for the presence of *Borrelia* species corroborated earlier findings on infection rates of ticks in the Baltic region. Speciation by reverse line blot hybridization showed that many ticks (10.6%) carried more than one *Borrelia* species. The majority of the ticks were infected with *B. afzelii* and/or *B. garinii*; *B. burgdorferi* sensu stricto was found in only two tick extracts. In 2.5% of the ticks a *B. burgdorferi* species was found which carried a 5S-23S rRNA spacer sequence similar to, but distinct from, that of *B. afzelii*. This *B. afzelii*-like species was found both in *I. persulcatus* and *I. ricinus*. We have not investigated whether coinfection of this species with other *B. burgdorferi* sensu lato species occurs. Furthermore, we have no data that indicates that this species is pathogenic for humans. There was no significant difference between infection rates of *I. persulcatus* and *I. ricinus* with *B. afzelii*. In contrast, of the 182 ticks infected with *B. garinii* only 15 (8.2%) were *I. ricinus* ticks. As with the distribution of the *Ehrlichia* species this suggests a vector-specific infection. It is uncertain whether this represents true vector specificity or a relationship between the vector and its host range. To determine whether an association between pathogen and tick species exists, additional studies, including studies of the vector hosts such as large and small mammals and birds, are required.

In our study, none of the larvae contained any detectable *Borrelia* or *Ehrlichia* DNA, but the number of larvae included in this study is too low to draw any conclusions from this result. However, a more significant observation was that *I. persulcatus* adults are infected with either *Borrelia* or *Ehrlichia* species more than twice as often as nymphs. This was true if either the dark-field examination or the PCR result was used as an indicator of *Borrelia* infection. Such a stage-dependent prevalence of infection was not seen in the *I. ricinus* ticks. In fact, 60 to 80% of the *I. ricinus* adults and nymphs included in the study were dark-field positive, and 38 to 43% of the same ticks were positive with the *Borrelia* PCR.

The latter finding showed that in a large proportion of the dark-field-positive ticks no *Borrelia* DNA was detected. A possible explanation for this observation is that dark-field microscopy is more sensitive than *Borrelia* PCR in detecting *Borrelia*. However, the detection of *Borrelia* DNA in 18% of the dark-field-negative samples argues against that. Furthermore, we have shown in previous studies that PCR, followed by the

TABLE 4. Distribution of infection type, stratified by tick species, sex, and stage

Infection status	Tick species	Sex		Stage		Total
		Female	Male	Nymph	Larva	
Infected + noninfected	*I. ricinus*	107	89	77	22	295
	I. persulcatus	154	118	62	2	336
Borrelia infected	*I. ricinus*	47	35	30	0	112
	I. persulcatus	112	92	22	0	226
Ehrlichia infected	*I. ricinus*	13	4	8	0	25
	I. persulcatus	15	13	1	0	29
Double infected	*I. ricinus*	8	4	6	0	18
	I. persulcatus	13	11	0	0	24

reverse line blot method, is both sensitive and specific. False-negative results due to inhibition of the PCR were excluded by the use of internal spike controls. There is a possibility that the DNA of the *Borrelia* species was degraded during storage, but earlier experiments in our lab have shown that *Borrelia* DNA in ticks stored in ethanol is extremely stable. In addition, the fact that there is a significant difference in *Borrelia* DNA detection between *I. persulcatus* and *I. ricinus* dark-field-positive ticks also suggests that the microorganisms seen in the dark-field analysis may represent species other than *B. burgdorferi* sensu lato and that this species is found more frequently in *I. ricinus* than in *I. persulcatus*. If this hypothesis is correct, studies that use dark-field microscopy as the sole method of determining the rate of *Borrelia* infection in ticks may yield an overestimated prevalence of infection. Further PCR studies using primers that will amplify the 16S rRNA gene of a broad range of bacteria and subsequent sequence analyses will have to corroborate this theory and identify the species.

To our knowledge, there have been no reports until now in the scientific literature in English of cases of ehrlichiosis in any part of Russia. However, in the city of St. Petersburg, many cases of Lyme disease are reported (250 cases per year; 5 cases per 100,000 inhabitants), and the majority of these cases are patients who sustained tick bites in the suburban region of the city. It is this region where we collected the *Borrelia*- and *Ehrlichia*-infected ticks used in this study. It is therefore conceivable that some people in the St. Petersburg region may have been infected with the HGE agent. Due to the lack of sufficient diagnostic tools and the lack of awareness, these cases of human ehrlichiosis may have gone unnoted. However, the rate of *Ehrlichia* infection in the ticks is about 10- to 20-fold lower than the rate of *Borrelia* infection. This would suggest that the chance of infection with *Ehrlichia* after a tick bite is significantly lower than the chance of being infected with *Borrelia* but that such exposure may still account for 10 to 25 cases of ehrlichiosis in the St. Petersburg area. Therefore, awareness of possible cases of ehrlichiosis remains important in tick-infested areas like the suburban region of St. Petersburg.

ACKNOWLEDGMENTS

The research described in this paper was made possible in part by grant N98-04-49899 from the Russian Basic Research Foundation and in part by grant 9600864 from the Danish Research Councils.

REFERENCES

1. **Alekseev, A. N., H. V. Dubinina, L. P. Antykova, T. I. Dzhivanyan, S. G. Rijpkema, N. V. Kruif, and M. Cinco.** 1998. Tick-borne borrelioses pathogen identification in *Ixodes* ticks (Acarina, Ixodidae) collected in St. Petersburg and Kaliningrad Baltic regions of Russia. J. Med. Entomol. **35:**136–142.
2. **Anderson, B. E., J. E. Dawson, D. C. Jones, and K. H. Wilson.** 1991. *Ehrlichia chaffeensis*, a new species associated with human ehrlichiosis. J. Clin. Microbiol. **29:**2838–2842.
3. **Baumgarten, B. U., M. Rollinghoff, and C. Bogdan.** 1999. Prevalence of *Borrelia burgdorferi* and granulocytic and monocytic ehrlichiae in *Ixodes ricinus* ticks from southern Germany. J. Clin. Microbiol. **37:**3448–3451.
4. **Chang, Y. F., V. Novosel, C. F. Chang, J. B. Kim, S. J. Shin, and D. H. Lein.** 1998. Detection of human granulocytic ehrlichiosis agent and *Borrelia burgdorferi* in ticks by polymerase chain reaction. J. Vet. Diagn. Investig. **10:**56–59.
5. **Chen, S. M., J. S. Dumler, J. S. Bakken, and D. H. Walker.** 1994. Identification of a granulocytotropic *Ehrlichia* species as the etiologic agent of human disease. J. Clin. Microbiol. **32:**589–595.
6. **Cinco, M., D. Padovan, R. Murgia, M. Maroli, L. Frusteri, M. Heldtander, K. E. Johansson, and E. O. Engvall.** 1997. Coexistence of *Ehrlichia phagocytophila* and *Borrelia burgdorferi* sensu lato in *Ixodes ricinus* ticks from Italy as determined by 16S rRNA gene sequencing. J. Clin. Microbiol. **35:**3365–3366.
7. **Junttila, T., M. Peltomaa, H. Soini, M. Marjamaki, and M. K. Viljanen.** 1999. Prevalence of *Borrelia burgdorferi* in *Ixodes ricinus* ticks in urban recreational areas of Helsinki. J. Clin. Microbiol. **37:**1361–1365.
8. **Kovalevskii, Y. V., and E. I. Korenberg.** 1995. Differences in *Borrelia* infections in adult *Ixodes persulcatus* and *Ixodes ricinus* ticks (Acari: Ixodidae) in populations of north-western Russia. Exp. Appl. Acarol. **19:**19–29.
9. **Kramer, V. L., M. P. Randolph, L. T. Hui, W. E. Irwin, A. G. Gutierrez, and D. J. Vugia.** 1999. Detection of the agents of human ehrlichioses in ixodid ticks from California. Am. J. Trop. Med. Hyg. **60:**62–65.
10. **Lefleche, A., D. Postic, K. Girardet, O. Peter, and G. Baranton.** 1997. Characterization of *Borrelia lusitaniae* sp. nov. by 16S ribosomal DNA sequence analysis. Int. J. Syst. Bacteriol. **47:**921–925.
11. **Lesnyak, O., E. Laikovskaya, I. Kufko, H. Bruinink, N. Baranova, and S. Rijpkema.** 1998. Clinical features of Lyme borreliosis in the middle Urals and distribution of *Borrelia burgdorferi* sensu lato species in local *Ixodes persulcatus* ticks. Zentralbl. Bakteriol. **288:**111–119.
12. **Leutenegger, C. M., N. Pusterla, C. N. Mislin, R. Weber, and H. Lutz.** 1999. Molecular evidence of coinfection of ticks with *Borrelia burgdorferi* sensu lato and the human granulocytic ehrlichiosis agent in Switzerland. J. Clin. Microbiol. **37:**3390–3391.
13. **Liz, J. S., L. Anderes, J. W. Sumner, R. F. Massung, L. Gern, B. Rutti, and M. Brossard.** 2000. PCR detection of granulocytic ehrlichiae in *Ixodes ricinus* ticks and wild small mammals in western Switzerland. J. Clin. Microbiol. **38:**1002–1007.
14. **Lotric-Furlan, S., M. Petrovec, T. Avsic-Zupanc, W. L. Nicholson, J. W. Sumner, J. E. Childs, and F. Strle.** 1998. Human ehrlichiosis in central Europe. Wien. Klin. Wochenschr. **110:**894–897.
15. **Magnarelli, L. A., K. C. Stafford III, T. N. Mather, M.-T. Yeh, K. D. Horn, and J. S. Dumler.** 1995. Hemocytic rickettsia-like organisms in ticks: serologic reactivity with antisera to ehrlichiae and detection of DNA of agent of human granulocytic ehrlichiosis by PCR. J. Clin. Microbiol. **33:**2710–2714.
16. **Ogden, N. H., K. Bown, B. K. Horrocks, Z. Woldehiwet, and M. Bennett.** 1998. Granulocytic *Ehrlichia* infection in ixodid ticks and mammals in woodlands and uplands of the U. K. Med. Vet. Entomol. **12:**423–429.
17. **Oteo, J. A., J. R. Blanco, V. Martinez de Artola, and V. Ibarra.** 2000. First report of human granulocytic ehrlichiosis from southern Europe (Spain). Emerg. Infect. Dis. **6:**430–432.
18. **Parola, P., L. Beati, M. Cambon, P. Brouqui, and D. Raoult.** 1998. Ehrlichial DNA amplified from *Ixodes ricinus* (Acari: Ixodidae) in France. J. Med. Entomol. **35:**180–183.
19. **Petrovec, M., S. Lotric Furlan, T. A. Zupanc, F. Strle, P. Brouqui, V. Roux, and J. S. Dumler.** 1997. Human disease in Europe caused by a granulocytic *Ehrlichia* species. J. Clin. Microbiol. **35:**1556–1559.
20. **Petrovec, M., J. W. Sumner, W. L. Nicholson, J. E. Childs, F. Strle, J. Barlic, S. Lotric-Furlan, and T. Avsic Zupanc.** 1999. Identity of ehrlichial DNA sequences derived from *Ixodes ricinus* ticks with those obtained from patients with human granulocytic ehrlichiosis in Slovenia. J. Clin. Microbiol. **37:**209–210.
21. **Pusterla, N., J. B. Huder, H. Lutz, and U. Braun.** 1998. Detection of *Ehrlichia phagocytophila* DNA in *Ixodes ricinus* ticks from areas in Switzerland where tick-borne fever is endemic. J. Clin. Microbiol. **36:**2735–2736.
22. **Ravyn, M. D., E. I. Korenberg, J. A. Oeding, Y. V. Kovalevskii, and R. C. Johnson.** 1999. Monocytic Ehrlichia in *Ixodes persulcatus* ticks from Perm, Russia. Lancet **353:**722–723.
23. **Rijpkema, S., D. Golubic, M. Molkenboer, N. Verbeek De Kruif, and J. Schellekens.** 1996. Identification of four genomic groups of *Borrelia burgdorferi* sensu lato in *Ixodes ricinus* ticks collected in a Lyme borreliosis endemic region of northern Croatia. Exp. Appl. Acarol. **20:**23–30.
24. **Rijpkema, S. G., M. J. Molkenboer, L. M. Schouls, F. Jongejan, and J. F. Schellekens.** 1995. Simultaneous detection and genotyping of three genomic groups of *Borrelia burgdorferi* sensu lato in Dutch *Ixodes ricinus* ticks by characterization of the amplified intergenic spacer region between 5S and 23S rRNA genes. J. Clin. Microbiol. **33:**3091–3095.
25. **Rikihisa, Y.** 1991. The tribe *Ehrlichieae* and ehrlichial diseases. Clin. Microbiol. Rev. **4:**286–308.
26. **Sato, Y., K. Miyamoto, A. Iwaki, T. Masuzawa, Y. Yanagihara, E. I. Korenberg, N. B. Gorelova, V. I. Volkov, L. I. Ivanov, and R. N. Liberova.** 1996. Prevalence of Lyme disease spirochetes in *Ixodes persulcatus* and wild rodents in far eastern Russia. Appl. Environ. Microbiol. **62:**3887–3889.
27. **Schouls, L. M., I. Van De Pol, S. G. T. Rijpkema, and C. S. Schot.** 1999. Detection and identification of *Ehrlichia*, *Borrelia burgdorferi* sensu lato, and *Bartonella* species in Dutch *Ixodes ricinus* ticks. J. Clin. Microbiol. **37:**2215–2222.
28. **Smith, M., G. Gettinby, M. Granstrom, J. S. Gray, E. C. Guy, C. Revie, J. N. Robertson, and G. Stanek.** 1998. The European union concerted action world wide web site for Lyme borreliosis. Zentralbl. Bakteriol. **287:**266–269.
29. **Steere, A. C.** 1989. Lyme disease. N. Engl. J. Med. **321:**586–596.
30. **van Dobbenburgh, A., A. P. van Dam, and E. Fikrig.** 1999. Human granulocytic ehrlichiosis in western Europe. N. Engl. J. Med. **340:**1214–1216.
31. **von Stedingk, L. V., M. Gurtelschmid, H. S. Hanson, R. Gustafson, L. Dotevall, E. O. Engval, and M. Granstrom.** 1997. The human granulocytic ehrlichiosis (HGE) agent in Swedish ticks. Clin. Microbiol. Infect. **3:**573–574.
32. **Walker, D. H., and J. S. Dumler.** 1996. Emergence of the ehrlichioses as human health problems. Emerg. Infect. Dis. **2:**18–29.