

Prevalence of various *Borrelia burgdorferi* sensu lato species in *Ixodes* ticks in three Baltic countries

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Abstract

Borrelia burgdorferi sensu lato, a tick-borne spirochete, is the causative agent of Lyme disease, the most prevalent vector-borne disease in Europe and United States. However, the incidence of this disease is variable and the clinical picture depends on the pathogen species. The infectivity of *Ixodes* ticks with *Borrelia*, was 46 % and 35 % in 2000 and 2001 in Latvia, respectively, and 14 % in 2002 in Lithuania, assessed by nested polymerase chain reaction (PCR) amplification of the plasmid OspA gene fragment of *Borrelia*. PCR-restriction fragment length polymorphism (RFLP) analysis of the 16S-23S (*rrs-rrlA*) rRNA intergenic spacer was used for typing of *Borrelia* directly in ticks. Species-specific primers and subsequent sequences analysis were used as another approach for *Borrelia* species typing. All three clinically relevant *B. burgdorferi* sensu lato genospecies (*B. afzelii*, *B. garinii*, *B. burgdorferi* sensu stricto) were detected in the ticks collected in Latvia. The same result was obtained earlier in Estonia. *B. valaisiana*, a possible infectious agent of Lyme borreliosis, was detected only in Latvia. Only *B. afzelii* and *B. garinii* species were detected in ticks from Lithuania. Different subspecies were also identified. This study demonstrates the predominance of the genospecies *B. afzelii* in all three Baltic countries, and the circulation of different *B. burgdorferi* sensu lato subspecies in the environment. This knowledge might have a significant importance for monitoring of Lyme disease in Europe.

Key words: Baltic region, *Borrelia burgdorferi*, Lyme disease, tick.

Introduction

Lyme disease (LD, Lyme borreliosis) is a multisystem and multistage infection caused by tick-borne spirochetes of the *Borrelia burgdorferi* sensu lato genogroup.

Three species of this group have been confirmed as pathogenic for humans. These include *Borrelia burgdorferi* sensu stricto (distributed mostly in North America), *Borrelia afzelii* (distributed in Western Europe, Central Europe and Russia), and *Borrelia garinii* (distributed in Europe, Russia and northern Asia). Symptoms of LD include arthritis, carditis, dermal symptoms and neurological symptoms, usually preceded by erythema migrans, a characteristic rash that begins days to weeks and spreading the bite site (Steere 2001). Some new cutaneous (alopecia) and ocular manifestations recently have been described (Schwarzenbach et al. 1998; Kostler et al. 1999; Pleyer et al. 2001;

Krist, Wenkel 2002), and a solitary borrelial lymphocytoma was reported from Slovenia (Maraspin et al. 2002). LD has become the most common vector-borne disease in North America and Eurasia (Wang 1999).

Lyme borreliosis in all locations is transmitted by ticks of the *Ixodes ricinus* complex (Lane et al. 1991; Spielman 1994). These ticks have larval, nymphal, and adult stages, they require a blood meal at each stage. In Europe, the principal vector is *I. ricinus*, and in Asia it is *I. persulcatus*. Notably, both of these species are common in Latvia and Estonia, and in the Baltic region of Russia (St. Petersburg region). *I. ricinus* is the main vector of Lyme disease in Lithuania.

PCR-based methods have become the most popular methods in detection and typing of *Borrelia burgdorferi* sensu lato in different biological samples and clinical materials in the world. Sensitive nested PCR method targeted *B. burgdorferi* specific OspA gene was used for the detection of the pathogen in field-collected ticks in our study. Molecular typing of *B. burgdorferi* from infected ticks was performed by restriction fragment length polymorphism (RFLP) analysis of PCR-amplified fragments of 16S-23S (*rrs-rrlA*) rRNA intergenic spacer. For PCR-RFLP analysis the restriction enzyme *Hinf* I was used. This typing method is fast and sensitive, and allows the differentiation of *B. burgdorferi* species directly in tick material without the need for isolation and culture of the microorganism.

The prevalence rates of *B. burgdorferi* sensu lato species in ticks was suspected as a major factor in assessing the transmission risk of Lyme borreliosis in endemic areas (Matuschka et al. 1992; Hubalek et al. 1996). The aim of this study was to perform molecular typing of *B. burgdorferi* sensu lato, to investigate the prevalence of clinically relevant *B. burgdorferi* species in host-seeking *Ixodes* tick populations in Latvia and Lithuania, and to compare these data to those obtained in Europe. This type of data can show the relative risk of infection with Lyme disease in the Baltic countries and offer a basis for comparative clinico-epidemiological studies of Lyme borreliosis in Europe.

Materials and methods

Collection of ticks

Questing ticks were collected by flagging of low vegetation in Latvia in 2000 and 2001 and in Lithuania in 2002. A total of 210 and 450 ticks were collected in different regions of Lithuania and Latvia, respectively. All ticks were identified for species, sex and stage; then each tick was placed in a separate plastic tube and frozen at -20 °C until further use.

DNA preparation

Each tick was mechanically crushed with a sterile plastic rod in a tube with 100 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.6), and DNA was extracted by phenol-chloroform extraction method. Briefly, buffer with tick was extracted with a 100 µl phenol/chloroform/isoamyl alcohol mixture (25:24:1, pH 8.0) by vortexing and subsequent centrifugation to separate DNA from proteins. The upper layer after centrifugation was transferred to a new tube and the DNA was reextracted with 100 µl chloroform by vortexing and centrifugation. The upper layer was again transferred to a new tube, and heated for 15 minutes (90 °C). Aliquots were frozen and stored at -20 °C until further use.

Reference DNA

DNA samples isolated from five reference strains (*B. burgdorferi sensu stricto* B31, *B. afzelii* ACA I, *B. afzelii* VS-461, *B. garinii* Ip90, *B. valaisiana* VS-116), kindly donated by S. Bergstrom, Umeå, Sweden, and D. Postic, Pasteur Institute, France, were used as positive controls in all PCR-based methods.

B. burgdorferi detection by PCR amplification

DNA amplification targeting the *OspA* gene located in linear plasmid lp54 was performed as described by Priem et al. (1997); reaction conditions were modified. Briefly, a 50- μ l PCR reaction volume contained a 3 μ l aliquot of isolated DNA, 100 mM (each) deoxynucleoside triphosphates (NBI Fermentas, Lithuania), 1.5 U of Taq DNA polymerase (NBI Fermentas, Lithuania), and 30 pmol of each primer. First-round amplification employed primers PrZS7 (5'-GGGAATAGGTCTAATATTAGCC-3'; positions 18-39 of the *OspA* gene) as the forward primer and Osp5 (5'-CACTAATTGTTAAAGTGGAAGT-3', positions 660-682 of the *OspA* gene) as the reverse primer. The amplification profile for the first-round PCR consisted of 35 cycles of denaturation at 95 °C for 15 s, annealing at 50 °C for 20 s, and extension at 72 °C for 60 s. Three microlitres of the first-round PCR product was employed as a template in a second-round PCR with primers Osp6 (5'-GCAAAATGTTAGCAGCCTTGAT-3'; positions 54-75 of the *OspA* gene) as the forward primer and Osp8 (5'-CGTTGTATTCAAGTCTGGTTCC-3', positions 423-444 of the *OspA* gene) as the reverse primer. The amplification profile for the second-round PCR consisted of 25 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 20 s, and extension at 72 °C for 30 s. PCR amplification resulted in a 391-bp product. Amplicons were visualized on a 1.5% agarose gel stained with ethidium bromide.

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 twenty tick samples. Negative and positive control samples were included each time that the PCR was performed. To avoid cross-contamination and sample carryover, pre- and post-PCR sample processing and PCR amplification were performed in separate rooms, and plugged pipette tips were used for all fluid transfers.

B. burgdorferi typing by 16S-23S rDNA spacer PCR-RFLP

OspA gene-positive DNA samples were used in further analysis. Nested PCR targeting the 16S-23S (*rrs-rrlA*) rDNA spacer region was performed as described by Liveris et al. (1999). Ten-microlitre aliquots of the nested-PCR amplification products were subjected to RFLP analysis by digestion with 2 U of *Hinf* I (NBI Fermentas, Lithuania) according to the manufacturer's instructions. *Hinf* I-digested fragments were analyzed by electrophoresis in a 1.5 % agarose gel stained with ethidium bromide.

Species-specific PCR

Species-specific PCR targeted 16S rRNA gene with *B. garinii*, *B. burgdorferi sensu stricto* and *B. valaisiana* specific primers were performed as described elsewhere. (Liebisch et al. 1998)

PCR-RFLP of 5S-23S rDNA spacer amplicons

In order to compare *B. garinii* strains detected in ticks from Latvia to those typed in

Europe, the 5S-23S rDNA PCR-RFLP typing method was used as described elsewhere (Postic et al. 1994).

DNA sequencing

PCR amplicons were purified with a DNA extraction kit (NBI Fermentas, Lithuania) according to the manufacturer's instructions. For DNA sequencing reactions, the fluorescence-labeled dideoxynucleotide technology was used (Perkin-Elmer, Applied Biosystems Division). The sequenced fragments were separated, and data were collected on an ABI automated DNA sequencer (Perkin-Elmer, Applied Biosystems Division).

Nucleotide sequence accession number

The 5S-23S rDNA intergenic spacer sequence of the *B. garinii* that was found in this study is available in the GenBank database under the accession number AY163784.

Statistical methods

Statistical insignificant differences were calculated using the χ^2 test.

Results

Prevalence of *Borrelia* in ticks

Altogether, 450 ticks collected in different regions of Latvia (years 2000 and 2001) and 204 ticks collected in different regions of Lithuania (year 2002) were analyzed. The overall prevalence of *Borrelia* in ticks in Latvia in the year 2000 was 46 % which was significantly higher ($P < 0.05$) than in 2001 (35 %). The overall prevalence of *Borrelia* in ticks in Lithuania was 14 %, significantly lower ($P < 0.05$) than in Latvia. In Table 1 the obtained results are compared with those from Estonia in 1999.

Typing of *B. burgdorferi* directly in ticks by RFLP analysis

The *rrn* cluster of most *B. burgdorferi* sensu lato strains contains a single copy of 16S rRNA (*rrs*) and tandem repeated 23S rRNA (*rrlA* and *rrlB*) and 5S rRNA (Fukunaga et al. 1992; Schwartz et al. 1992; Gazumyan et al. 1994; Ojami et al. 1994). The rDNA gene cluster is located at the center of the linear chromosome and is arranged in the following order: *rrs-rrlA-rfa-rrlB-rrfB*. The *rrs-rrlA* intergenic spacer is about 3.2 kb in *B. burgdorferi* sensu stricto and 5 kb in *B. garinii* and *B. afzelii* (Schwartz et al. 1992;

Table 1. The prevalence of different *B. burgdorferi* genospecies in Latvia, Lithuania and Estonia. *, Data from this study. **, Data from T. Prück et al. 1999

Country	No. of ticks tested	<i>Borrelia</i> positive ticks (%)	% (No.) of ticks infected by different genospecies			
			<i>B. afzelii</i>	<i>B. garinii</i>	<i>B. burgdorferi</i> sensu stricto	<i>B. valaisiana</i>
Lithuania*	204	13.7	68 (19)	18 (5)	0	0
Latvia*	450	46.0 (year 2000) 34.9 (year 2001)	76 (80)	18 (19)	2 (2)	2 (2)
Estonia**	422	5.5	65 (15)	17 (4)	13 (3)	0

Table 2. The number of cases of Lyme disease in North-European countries. Data are from *EpiNorth - Bulletin of the Network for Communicable Disease Control in Northern Europe*

Country or region	1999		2000		2001	
	Total No	Per 100000 inhabitants	Total No	Per 100000 inhabitants	Total No	Per 100000 inhabitants
Norway	146	3.3	138	3.1	124	2.8
Finland	404	7.8	895	17.2	691	13.3
Estonia	321	22.2	601	43.8	342	25.0
Latvia	281	11.5	472	19.4	379	16.0
Lithuania	766	20.7	1713	46.3	1153	33.0
St. Petersburg region	265	5.6	541	11.3	323	6.7

Gazumyan et al. 1994; Ojami et al. 1994). We used the typing method based on the PCR-RFLP analysis of a highly variable 16S-23S (*rrs-rrlA*) rDNA spacer previously described by Liveris et al. (1999). This method can be performed rapidly with small DNA amounts, thus obviating the need for culture isolation. Amplification of the partial *rrs-rrlA* spacer, followed by digestion with *Hinf*I produces species-specific RFLP patterns.

A total of 135 and 28 ticks from Latvia and Lithuania, that were positive after *B. burgdorferi* detection, respectively, were employed for further analysis. The amplicon was obtained from 106 and 24 samples, respectively. PCR-RFLP species-specific pattern analysis showed that in Latvia 76 % of samples belonged to *B. afzelii* (80 samples), 18 % - to *B. garinii* (19 samples), 2% - to *B. burgdorferi sensu stricto* (2 samples), and 2 % were *B. valaisiana* (2 samples, Table 1). Results were confirmed by species-specific PCR analysis (data not shown). Two different *B. garinii* subtypes were identified. Those samples differing from the reference strain Ip90 were employed for the 5S-23S rDNA PCR-RFLP analysis and sequencing of the 5S-23S rDNA PCR amplicon. The obtained sequence was compared with data in the GenBank. The results confirmed these samples as *B. garinii* and closely related to the NT29 isolate from Russia. In Lithuania, 68 % (19 samples) belonged to *B. afzelii*, 18 % (5 samples) to *B. garinii*.

Discussion

In Europe, Lyme borreliosis is widely established in forested areas (Steere 2001). The highest reported frequencies of the disease are in middle Europe and Scandinavia, particularly in German, Austria, Slovenia, and Sweden. The infection is also found in Russia, China, and Japan (Steere 2001). Epidemiological data presented in *EpiNorth - Bulletin of the Network for Communicable Disease Control in Northern Europe* shows that the status of Lyme disease in the three Baltic countries is endemic (Table 2). The number of cases of Lyme disease in this region is higher than in neighbouring Northern European countries excepting the St. Petersburg region of Russia.

We investigated the prevalence of *B. burgdorferi sensu lato* genotypes in questing ticks from Latvia and Lithuania, and compared the results with those obtained in Estonia and the Baltic region of Russia (St. Petersburg region). The study shows that there is a

considerable risk of contracting a borrelia infection in all three Baltic countries, and that a large human population is at risk. The extremely high percentage of infected ticks in 2000 in Latvia correlates with the high number of registered cases of Lyme disease that year (Table 2).

Not all strains of the described *Borrelia* species are pathogenic for humans. Currently, only *B. burgdorferi* sensu stricto, *B. garinii* and *B. afzelii* have been cultured from Lyme disease patients in Northern America and Europe (Wang 1999). However, recently two other species, *B. bissettii* and *B. valaisiana*, were reported to have been cultured from a LD patient in Europe (Schaarschmidt et al. 2001; Maraspin et al. 2002). DNA specific for *B. valaisiana* has been detected by PCR from patients with LB (Probert et al. 1995), and *B. valaisiana* specific antibodies have been determined in patients sera (Ryffel et al. 1999). Therefore, we can expect that all *B. burgdorferi* sensu lato species mentioned above can cause Lyme disease in Europe.

It has been proposed that the different species of *B. burgdorferi* sensu lato are associated with distinct clinical manifestations of LB: Lyme arthritis with *B. burgdorferi* sensu stricto infection, neuroborreliosis with *B. garinii* infection, and acrodermatitis chronica et atroficans (ACA) with *B. afzelii* infection (Wang 1999). Literature indicates that the clinical features of Lyme borreliosis may depend on the species of the causative agent. Nevertheless, in everyday clinical practice, the determination of *Borrelia burgdorferi* species cannot be made. Therefore, the analysis of *Borrelia* species in ticks and interpolation of their relative incidence to the clinical cases is the only single possibility to foresee the pathogenesis of definite clinical cases today. The latter is significant for elaboration of prophylactic measures (including vaccination with the single commercially available vaccine against the *B. burgdorferi* sensu stricto) and for therapeutic strategy in Lyme borreliosis cases in the Baltic region.

This study shows that *B. afzelii* is the most prevalent genospecies of *B. burgdorferi* complex in Latvia and Lithuania. *B. garinii*, *B. burgdorferi* sensu stricto and *B. valaisiana* have also been detected in questing ticks from Latvia. This finding is in agreement with a previous study by Kurtenbach et al. (2001), but our study covered a wider region. However, in the former study *B. valaisiana* was detected in 18 % of ticks compared to 2 % in our study. This may be explained with the differences of the collection strategy of ticks, since only ticks from the Riga region were included in study by Kurtenbach et al. (2001).

In summary, *B. afzelii*, *B. garinii*, *B. burgdorferi* sensu stricto and *B. valaisiana* species were detected in *Ixodes* ticks in Latvia. All of these four genospecies were detected also in the St. Petersburg region of Russia (Alekseev et al. 2001). In Estonia, *B. afzelii*, *B. garinii*, *B. burgdorferi* sensu stricto were detected in questing ticks (Postic et al. 1997; Prükk et al. 1999). Only *B. afzelii* and *B. garinii* were detected in ticks from Lithuania. The differences might be explained by an uneven distribution of *B. burgdorferi* sensu lato in Europe, or simply by an insufficient tick sample size investigated.

The most prevalent *B. afzelii* and *B. garinii* genospecies are the most probable aetiological agents responsible for the more than an 2000 cases per year of Lyme disease in the three Baltic countries.

5S-23S rDNA spacer amplicon sequence analysis showed that the second *B. garinii* subtype is closely related (99 % similarity, BLAST) to the *B. garinii* NT29 variant. The NT29 subtype is most frequently isolated by culture from *I. persulcatus* ticks in Russia

and Estonia, and 7 out of 8 isolates from human skin biopsies in Russia were identified as this type (Postic et al. 1997). Interestingly, this variant, frequently isolated in Japan (Fukunaga et al. 1993; Fukunaga et al. 1996), is absent in Western and Central Europe (Postic et al. 1997). Sequence analysis of variable regions could be a useful tool for understanding the evolution of different species and subspecies, and probably could help to explain the different pathogenesis of different *B. burgdorferi* species. Our results also clearly show that the distribution of different *B. burgdorferi* genospecies in Europe depends on the distribution of the *Ixodes* vector. With the exception of the most northern regions, the distribution area of ixodid ticks covers all of Europe. The study in Finland demonstrates that even very urban parks can serve as habitats for *I. ricinus* ticks (Junttila et al. 1999). Interestingly, Postic et al. (1997) suggested that the absence of *B. burgdorferi sensu stricto* from all regions where *I. persulcatus* is the single vector could be explained by the inability of *I. persulcatus* to harbour and to transmit this genospecies. Further investigations of this observation are required, and knowledge of the epidemiology of the LB vectors and their infestation rate is essential for understanding the risk of LB in a local setting.

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Dažādu *Borrelia burgdorferi sensu lato* sugu prevalence *Ixodes* ērcēs trijās Baltijas valstīs

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Kopsavilkums

Laima slimību (Laima boreliozī) izraisa *Borrelia burgdorferi sensu lato* spirohetas, ko dabā pārnēsā ērces. Pētījumā ar 16S-23S rDNA starpgēnu rajona polimerāzes ķēdes reakcijas-restrīkcijas fragmentu polimorfisma analīzes (PCR-RFLP) metodi tika noteiktas tās *Borrelia* sugas, kas cirkulē Latvijā un Lietuvā. Dati tika salīdzināti ar Igaunijas un Krievijas (Sanktpēterburgas apgabals) datiem. Visas trīs *B. burgdorferi sensu lato* sugas (*B. afzelii*, *B. garini* un *B. burgdorferi sensu stricto*), kas šobrīd tiek uzskatītas par galvenām Laima slimības izraisītājām, ir atrastas Latvijas ērcēs, līdzīgs rezultāts tika ziņots no Igaunijas. Lietuvā ērcēs tika atrastas tikai *B. afzelii* un *B. garini* sugas. Šis pētījums skaidri parāda *B. afzelii* sugas dominēšanu visās trīs Baltijas valstīs. Pētījumi par dažādu *Borrelia* sugu cirkulāciju apkārtējā vidē var būt ļoti būtiski Laima slimības monitoringam Eiropā.