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Molecular biology of *Borrelia burgdorferi* sensu lato in Latvia

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1. LIST OF ABBREVIATIONS AND TERMS.

ACA	Acrodermatitis chronica atrophicans
BSK	Barbour-Stroenner-Kelly
Da	Dalton
DbpAB	Decorin-binding prortein A and B
ELISA	Enzyme-linked immunosorbent assay
EM	Erythema migrans
Fla	flagellin
His	Histidine
kb	Kilobase
LA	Lyme arthritis
LB	Lyme borreliosis
LD	Lyme disease
ORF	Open reading frame
Osp	Outer surface protein
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism analysis
SDS	Sodium dodecyl sulphate
s.l.	Sensu lato
s.s.	Sensu stricto
VlsE	Variable major protein-like sequence expression site
WB	Western blotting
WCL	Whole-cell lysate

2. INTRODUCTION.

Lyme disease (Lyme borreliosis, LB) is the most prevalent vector-borne disease in Europe and the United States. Symptoms of LB have been known for more than 100 years. In 1975, the rheumatologist Allen C. Steere began to investigate patients with arthritis in Connecticut close to the town of Old Lyme, and a tick-transmitted infectious agent was suspected. However, only in 1981 the aetiological spirochaete of LB was isolated by Dr. Willy Burgdorfer from *Ixodes dammini* ticks (now called *Ixodes scapularis*) collected on Long Island, New York (Burgdorfer et al., 1982). The spirochaete was identified as a new species of *Borrelia* and was named *Borrelia burgdorferi* in 1984 (Johnson et al., 1984). Since that 11 species of *Borrelia burgdorferi* sensu lato complex were identified.

Lyme borreliosis in all locations is transmitted by ticks of the *Ixodes ricinus* complex. These ticks have larval, nymphal, and adult stages, and require a blood meal at each stage. In Europe, the principal vector is *I. ricinus,* and in Asia it is *I. persulcatus.* Three Baltic States are located in Northeastern Europe, bordered to the east by the Russian Federation to the southeast by Belarus and to the southwest by Poland. Situated on the Baltic Sea coast, Lithuania is the most southerly followed by Latvia and Estonia in the north. Forests, the natural habitat of ticks, cover a large territory of the Baltic countries. Notably, both of *Ixodes* 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. It is also significant that through territoria of three Baltic countries the active bird migration proceedes and the role of birds in transmission of *B.burgdorferi* was confirmed (Humair, 2002). The first cases of LB in Latvia were registered in 1986. There were 379 cases in 2001 (an incidence of 16.02 cases per 100,000 inhabitants), 328 (14.0) in 2002 and 714 (30.62) cases in 2003. Cases of LB were registered in all four regions of Latvia (Kurzeme, Vidzeme, Zemgale, and Latgale).

Three species of *B. burgdorferi* sensu lato 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. Since the spirochete *Borrelia burgdorferi*, responsible for this zoonotic infection, is

phenotypically and genotypically heterogeneous, it causes variability in the clinical aspects of the disease. Arthritis and carditis are preferentially associated with *B. burgdorferi* sensu stricto, the degenerative skin disorder acrodermatitis chronica et atrophicans with *B. afzelii* and neuroborreliosis with *B. garinii*. However, *B. burgdorferi* sensu stricto is also responsible for human neuroborreliosis in the United States.

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. Various methods have been used to type *B. burgdorferi* sensu lato in culture isolates but only a few of them are useful for typing of *Borrelia* directly in ticks. The most popular are the PCR – based methods, such as species-specific PCR in which the conserved 16S rRNA gene or species-specific plasmid gene loci are targeted, the 5S-23S rRNA spacer region PCR hybridization method, and the rDNA PCR-RFLP analysis of the *rrs-rrlA* intergenic spacer.

In this study the presence and distribution of different *Borrelia burgdorferi* sensu lato genospecies was studied among field-collected *Ixodid* ticks in Latvia and Lithuania; nested polymerase chain reaction (PCR) targeted chromosomal *Fla* and plasmid *OspA* gene fragments of *Borrelia* was used. The presence of *B. burgdorferi* in host-seeking ticks was detected in Latvia and Lithuania within 6 years (1997-2002) and in year 2002, respectively. In order to develop a simple and fast method for typing European *B. burgdorferi* isolates directly in tick material without the need for isolation and culture of the microorganism, restriction fragment lenght polymorphism (RFLP) analysis of PCR-amplified fragments of 16S-23S *(rrs-rrlA)* rRNA intergenic spacer was modified, main restriction patterns of *Borrelia* isolates presented in Latvia were characterized. The prevalence of clinically relevant *B. burgdorferi* sensu lato species in *Ixodes ricinus* and *Ixodes persulcatus* ticks collected from all four regions of Latvia were compared, vector-associated distribution of different *B. burgdorferi* subspecies was studied. Epidemiological situation of Lyme borreliosis in Latvia was analysed in the light of obtained results. Data show the relative risk of infection with Lyme disease in Latvia and Lithuania and offer a basis for comparative clinico-epidemiological studies of Lyme borreliosis in Europe.

In recent years, several recombinant borrelial proteins have been tested to improve serodiagnosis of Lyme disease and as targets for new vaccine development. Recombinant His-tagged BBK32 protein of *Borrelia afzelii* was cloned, expressed in *E.coli* and partially purificated in order to characterize main immunogenic properties and investigate it's possible use in serodiagnosis of Lyme disease in Latvia.

3. PAPERS IN THIS THESIS.

I. **Bormane, A., R. Ranka, I. Lucenko, M. Lavrinoviča, J. Kloviņš, V. Kalniņa, A. Puzuka, K. Vetce-Vecmane, and V. Baumanis.** 2001. *Borrelia burgdorferi* – infected ticks and incidence of Lyme borreliosis in Latvia. *Proceedings of the Latvian Academy of Sciences*. 55(4):164 – 170.

II. **Ranka R., K. Salmina, M. Žygutiene, B. Morkunas, A. Bormane, and V. Baumanis.** 2003. Prevalence of various *Borrelia burgdorferi* sensu lato species in *Ixodes* ticks in tree Baltic countries. *Acta Biologica Universitatis Latviensis*. 662: 7-15.

III. **Ranka, R., A. Bormane, K. Salmina and V. Baumanis.** 2004. Identification of three clinically relevant *Borrelia burgdorferi* sensu lato genospecies by PCR-Restriction fragment length polymorphism analysis of 16S-23S ribosomal DNA spacer amplicon. *J Clin Microbiol*. 42(4)

IV. **Bormane A., I. Lucenko, A. Duks, V. Mavtchoutko, R. Ranka, K. Salmina, and V. Baumanis**. 2003. Vectors of tick-borne diseases and epidemiological situation in Latvia in 1993-2002. *Int J Med Microbiol*. 293, Suppl. 37, 36-47.

V. **Žygutiene M., R. Ranka, K. Salmiņa**. 2003. Genospecies of *Borrelia burgdorferi* s.l. in *Ixodes ricinus* ticks in Northern Lithuania. *Acta zoologica Lithuanica*. Vol. 13, Num.4, 385-389.

4. REVIEW OF THE LITERATURE.

4.1. Main characteristics of *Borrelia burgdorferi* spirochetes.

Borrelia burgorferi belongs to the family *Spirochaetaceae* in the bacterial order *Spirochaetales* (Paster and Dewhirst, 2000). *Serpulinaceae* and *Leptospiraceae* are two other families of his order. *Spirochaetaceae* include species of the genera *Borrelia*, *Brevinema*, *Cristispira*, *Spirochaeta*, *Spironema*, and *Treponema*. Genera *Borreliae* include several *Borrelia* species that are associated with diseases of humans and animals. One of the characteristics of *Borreliae* is that all species are transmitted to vertebrates by haematophagous arthropods. All known species of *Borrelia* are transmitted by ticks, with only one exception, *B. recurrentis*, which is transmitted by the human body louse. *Borreliae* species causing disease in humans are *B. hermsii*, *B. recurrentis* and some other less frequent species which cause relapsing fever, and *B. burgdorferi* sensu lato causing Lyme disease (Schwan et al., 1986, Paster and Dewhirst, 2000). Molecular analysis has indicated that different *B. burgdorferi* sensu lato isolates are phenotypically and genotypically diverse and at present, 11 different species or genomic groups are identified (Table 1).

Borrelia isolates showing less than 70% homology with each other in genotyping methods are suggested to belong to different genospecies (Baranton et al., 1992; Le Fleche et al., 1997). The loss of some plasmids occurring during in vitro cultivation may result in DNA-DNA relatedness below 100% between an isolate and its subcultured variants and also among closely related strains.

Table 1. Species of *Borrelia burgdorferi* sensu lato and their association with causing Lyme borreliosis in man (Modified from Lebech, 2002).

Species	Causing Lyme disease in man	Geographic distribution	References
B.burgdorferi sensu stricto	Yes	US, Europe (little)	Baranton et al., 1992
B.afzelii	Yes	Europe, Asia	Baranton et al., 1992; Canica et al., 1993.
B.garinii	Yes	Europe, Asia	Baranton et al., 1992
B. valaisiana	Uncertain	Europe, Asia	Wang et al., 1997
B.lusitaniae	No	Europe	Le Fleche et al., 1997.
B.bissettii	Uncertain	US, Slovenia	Postic et al., 1998; Picken et al., 1996.
B.andersonii	No	US	Marconi et al., 1995.
B.japonica	No	Japan	Kawabata et al., 1993.
B. turdi	No	Japan	Fukunaga et al., 1996.
B.tanukii	No	Japan	Fukunaga et al., 1996.
B.sinica	No	China	Masuzawa et al., 2001.

B.burgdorferi is a microaerofilic bacterium that can be grown in the Barbour-Stoenner-Kelly medium (BSK medium) at 33°C-35°C (Barbour, 1984), supplement with rabbit serum is needed. The in vitro generation time is 12-24 hours. Borreliae require long-chain fatty acids for growth, and they produce lactic acid through glycolysis. Under unfavorable conditions (for example, in distilled water, in BSK media without rabbit serum, during long-time cultivation) *B.burgdorferi* forms spheroids or bleb-like structures (Brorson and Brorson, 1997, 1998; Murgia and Cinco, 2004). It was demonstrated that such cystic forms have an ability to reconvert into normal motile infective spirochetes not only in vitro, but also in vivo, in cyst-inoculated mice (Gruntar et al., 2001). Cystic forms of *Borrelia burgdorferi* might represent a low metabolic activity state or phase of *B. burgdorferi* cells that allows the spirochete to survive in a hostile environment until conditions are favourable to multiply again. The cyst formation may help explain how cells of *B. burgdorferi* can survive in different hosts and host tissues.

Morphologically, *B.burgdorferi* sensu lato is motile helically shaped bacterium with approximately 7-11 flagellae in its periplasmic space (Figure 1). The flagella are found beneath the outer membrane, attached subterminally to opposite ends of the protoplasmic

cylinder, and they mediate both the motility and the shape of borreliae. The cells are long, thin with dimensions of 0.2-0.3 μm (diameter) by 10-30 μm (length) and composed of 3-10 loose coils (Goldstein et al., 1996). The cytoplasmic membrane is trilaminar and closely approximated to the cell wall (Barbour and Hayes, 1986). There are outer membrane with numerous lipoproteins on the surface, a peptidoglycan layer in the periplasmic space, and an inner membrane. Although *B.burgdorferi* bacteria are generally considered to be Gram-negative bacteria, the architecture of the membrane shares characteristics of both Gram-positive and Gram-negative bacteria (Haake, 2000). As in Gram-positive bacteria, the cytoplasmic membrane of spirochaetes is closely associated with the peptidoglycan cell wall. Spirochaetes also have an outer membrane; however it appears to be fluid and labile which contrasts it with the outer membrane of Gram-negative bacteria (Haake, 2000). Also the absence of lipopolysaccharide and the presence of membrane glycolipids containing a single hexose saccharide molecule are similar to Gram-positive bacteria (Takayama et al., 1987; Hossain et al., 2001). The major outer surface proteins are lipoproteins.

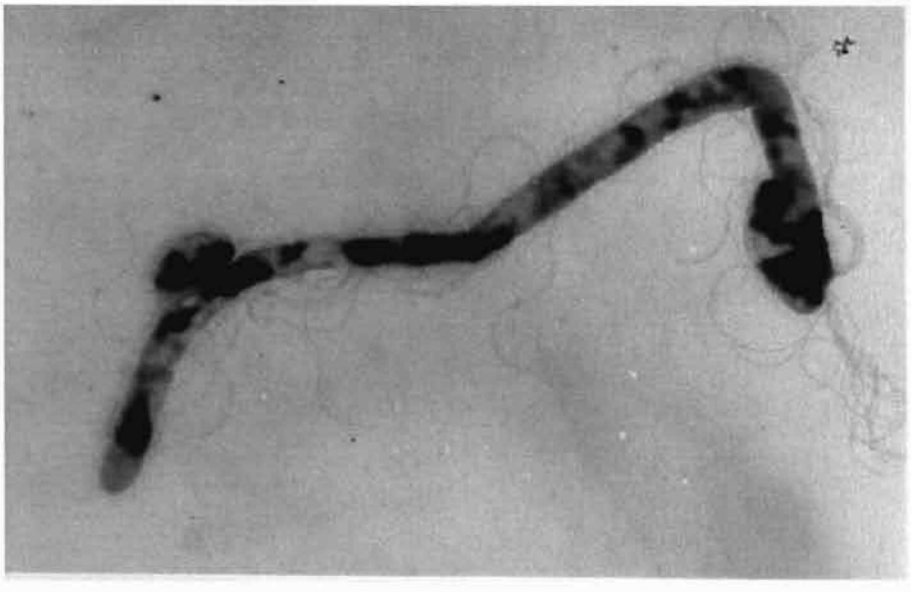

Figure 1. Flagellae of *B. burgdorferi* (lysed cell), electron microscopy. (Photo: V. Ose, R. Ranka, Biomedical Research and Study Centre).

4.2. Molecular biology of *Borrelia burgdorferi* sensu lato.

The whole genome of *B. burgdorferi* sensu stricto species strain B31 has been sequenced (Fraser et al., 1997). The genome is composed of a linear chromosome of 910,725 bp with an unusually low average guanine + cytosine (G+C) content of 28.6% and 21

plasmids (9 circular and 12 linear) with a combined size of more than 613,00 bp. The linear chromosome contains approximately 853 genes encoding a basic set of proteins, however, no genes for the synthesis of fatty acids, amino acids or nucleotides have been identified. Roughly 8% of *B. burgdorferi* open reading frames are predicted to encode lipoproteins (approximately 105 potential lipoproteins), which is a significantly higher frequency than that of any other bacterial genome sequenced to date (Haake, 2000). Many plasmids contain homologous DNA regions, the number and size of the plasmids can vary among strains (Barbour et al., 1996; Casjens et al., 2000; Palmer et al., 2000; Zückert and Meyer, 1996). The linear plasmids are present at a copy number of approximately one per chromosome (Fraser et al., 1997; Hinnebusch et al., 1992). Linear plasmid content analysis of tick and wildlife isolates showed overall uniformity (Casjens, 2000; Palmer et al., 2000). Circular plasmid contents are more difficult to display, but in the isolates that have been analyzed, multiple, related plasmid types are always present. A curious feature of some plasmids is they often appear to share regions of homologous DNA (Casjens et al., 1997; Porcella et al., 1996; Simpson et al., 1990; Stalhammar-Carlemalm et al., 1990; Stevenson et al., 1996; Tilly et al., 1997; Xu et al., 1995; Zückert et al., 1994; Zückert and Meyer, 1996). Several of the plasmids appear to be present in all natural isolates of *Borrelia burgdorferi*; therefore, the term minichromosome may be more correct for the description of their nature. For example, a 49- to 54-kb linear plasmid and 26-kb circular plasmid, which carry the outer surface protein genes *OspAB* and *OspC*, respectively, are ubiquitous (Barbour, 1988; Livey et al., 1995; Marconi et al., 1993; Sadziene et al., 1993; Samuels et al., 1993; Stevenson and Barthold, 1994; Xu and Johnson, 1995).

Long-term culture of *B. burgdorferi* results in the loss of some plasmids and has been associated with the inability of the spirochete to infect laboratory animals (McDowell et al., 2001; Schwan et al., 1988; Xu et al., 1996), suggesting that the plasmids encode important proteins involved in virulence. *B. burgdorferi* plasmids are also of interest because they contain multiple paralogous genes which appear to have undergone extensive recombination during their evolution (Akins et al., 1999; Barbour et al., 1996; Caimano et al., 2000; Carlyon et al., 1998; Fraser et al., 1997; Stevenson et al., 1998; Zückert and Meyer, 1996). Most of the differentially expressed borrelial genes identified to date are plasmid encoded (Porcella et al., 1996; Stevenson et al., 1995; Suk et al., 1995)

A total of 93% of the chromosome represents predicted coding sequences (open reading frames, ORFs), in contrast to 71% of the plasmid DNA. Another distinction between plasmid

and chromosomal sequences is the relative proportion of coding sequences that are similar to identified sequences in the database: 59% of the chromosomal ORFs are homologous to known sequences, whereas only 16% of the plasmid ORFs are homologous. Plasmid coding sequences exhibit a high degree of redundancy, with more than one-third of the ORFs belonging to paralogous gene families. These differences suggest that the chromosome and plasmids are not necessarily equivalent components of a segmented genome and may reflect different evolutionary histories and constraints (Murray et al., 1999). The complete genomic sequence indicates that *B. burgdorferi* contains genes encoding a basic set of proteins for DNA replication, transcription, translation, and energy metabolism but is lacking most cellular biosynthetic pathways. This limited metabolic capacity presumably necessitates the acquisition of many essential components from the media. The level of DNA relatedness among different *B. burgdorferi* sensu lato species is about 48% to 70% (Baranton et al., 1992; Johnson et al., 1984b; Postic et al., 1993). DNA homology between *B. burgdorferi* and other human pathogenic relapsing fever *Borrelia* species is 30%-60%, whereas *B. burgdorferi* exhibits less than 2% DNA homology to *Treponema pallidum* and *Leptospirae* species (Johnson et al., 1984a).

4.3. Natural cycle and vectors of *Borrelia burgdorferi.*

B. burgdorferi sensu lato bacteria are maintained in enzootic spirochete – tick vector – vertebrate cycles and incidental human exposure occurs when ticks bite humans. The primary bridging vectors of LB spirochetes to humans include four tick species of the *Ixodes ricinus* complex (Acari: Ixodidae: *Ixodes*: subgenus *Ixodes*) (Filippova, 1999; Keirans et al., 1999). These are the castor bean tick, *Ixodes ricinus*, and the taiga tick, *Ixodes persulcatus*, in Eurasia and the western black-legged tick, *Ixodes pacificus*, and the black-legged tick, *Ixodes scapularis*, in North America (Table 2). However, vector competence for *B. burgdorferi* s.l. has been experimentally confirmed for 12 tick species (Eisen and Lane, 2002).

Table 2. Main vectors of different *B. burgdorferi* species (Modified from Eisen and Lane, 2002; Ryffel et al., 2003; Alekseev et al., 2001)

Ixodes vector	Geographical distribution	*B.burgdorferi* sensu stricto	*B. afzelii*	*B.garinii*	*B.valaisiana*
Ixodes ricinus	Europe, far-western Asia, far-northern Africa	X	X	X	X
Ixodes persulcatus	Far-eastern Europe, Asia		X	X	X
Ixodes pacificus	Western North America	X			
Ixodes scapularis	Eastern North America	X			

Ixodes ticks develop through a two to three year life cycle. All ixodid ticks undergo four developmental stages: egg, larva, nymph and adult. A single large blood meal is ingested in the larval, nymphal and adult female stages. Adult male *I. ricinus* and *I. persulcatus* are considered facultative blood feeders. However, males may repeatedly take small blood meals (Oliver, 1989). Each stage parasite a large number of different animal species. Deer and small rodents, especially mice, constitute main reservoir hosts. In Europe 9 small mammals, 7 medium-sized mammals and 16 bird species are known to be capable of acting as sources of spirochetes to ticks (Gern et al., 1998).

The European *B. burgdorferi* genospecies are considered to be preferentially associated with different reservoir hosts (Gylfe et al., 1999; Hanincova et al., 2003a, 2003b; Humair et al., 1998, 1999; Humair and Gern, 1998, 2000; Kurtenbach et al., 1998a, 1998b; Olsén et al., 1993). *B.garinii* and *B. valaisiana* occur preferentially in birds, *B. afzelii* in small mammals and *B. burgdorferi* sensu stricto and *B.garinii* in squirrels. Interestingly, unlike the other OspA serotypes of *B. garinii*, OspA serotype 4 strains, that are hyperinvasive and mainly been obtained from human patients, are associated with rodents (Etti et al., 2003; Hu et al., 2001; Huegli et al., 2002). Hovewer, this serotype seems to be rare for Europe. Analysis of the genospecies in individual ticks showed that *B. garinii* and *B. valaisiana* segregate from *B. afzelii* (Kurtenbach et al., 2002). The hypothesized origin of such specific associations is attributed to the fact that different groups of vertebrates (rodents and birds) exhibit distinct types of innate immunity, which may either destroy or tolerate certain *Borrelia* genospecies.

Differences in serum complement sensitivity among *Borrelia* species was shown as a possible key factor in LB ecology (Kraiczy et al., 2004; Kurtenbach et al., 1998b, 2001, 2002; Stevenson et al., 2002). Other observations seem to disagree with these hypotheses (Christova et al., 2001; Jenkins et al., 2001; Misonne et al., 1998; Richter et al., 1999), implying that within *Borrelia* genospecies, the interactions with different vertebrate hosts may be more diverse than expected. Interestingly, according to differences in complement sensitivity, *B. afzelii* strains as well as *B. garinii* serotype 4 are resistant to normal human serum. *B. garinii* serotypes 3, 5, 6, 7 and 8 are sensitive to normal human serum, but *B. burgdorferi* sensu stricto strains have intermediate complement-sensitivity (van Dam et al., 1997; Kurtenbach et al., 1998b).

B. burgdorferi infection is passed transtadially from larvae to nymphs, and from nymphs to adult ticks, there is no transovarial transmission (Hayes and Piesman, 2003; Patrican, 1997). Some findings indicate the increasing of *Borrelia* prevalence through the life stages of ticks (Hubalek and Halouzka, 1998; Derdakova et al., 2003) indicating the possibility of *Borrelia* infection in each stage of tick because of feeding on infected host. Infected nymphs and adult ticks transmit spirochetes to the host during meal. Efficient transmission of *Borrelia burgdorferi* between cofeeding *Ixodes ricinus* ticks was also observed (Gern and Rais, 1996).

In moulted unfed *Ixodes* species ticks the spirochetes are generally restricted to the midgut; during a blood meal the spirochetes multiply in the gut before they escape through the gut wall into the haemocoel and migrate to and invade the salivary glands (Gern et al., 1990; de Silva and Fikrig, 1995; Ribeiro et al., 1987). The transmission of *B. burgdorferi* into laboratory animals usually occurs 36-48 hours after tick attachment (Piesman, 1993; Shih et al, 1995). However, the time elapsed from tick attachment to spirochete transmission may differ between vector species, between *B. burgdorferi* sensu lato genospecies, and even between strains of the same genospecies. It was noted, in Europe, *B. afzelii* may be transmitted from *I.ricinus* ticks more rapidly (Crippa et al., 2002). The time differs even between different host species, and the factor(s) ultimately determining vector competence are still unknown. One of these factors may be antigenic changes *B. burgdorferi* undergoes during the blood meal; immunosuppressive factors present in tick saliva also may be related to the ability of spirochetes to infect the tick host (Ribeiro and Mather, 1998; Valenzuela et al., 2000).

Some studies were performed in order to determinate the role of different life stages of *Ixodes* ticks as bridging vectors to humans. The nymphal stage is considered the primary

bridging vector for *I.ricinus* in Europe (Eisen and Lane, 2002). Three studies showed that nymphs accounted for as much as 54, 70 and 82%, respectively, of all detected human bites by *I. ricinus* ticks, as compared with 43, 22 and 3% for female ticks (Maiwald et al., 1998; Robertson et al., 2000). In contrast, *I. persulcatus* females are the primary bridging vector of LB spirochetes to humans in far-eastern Europe and Asia because nymphs only rarely infest humans (Ai et al., 1990; Korenberg, 1994). The questing behavior of the adult stage is more aggressive in *I. persulcatus* than in *I. ricinus* (Uspensky, 1993). Generalized infections were more common for *B. afzelii* or *B. garinii* in *I. persulcatus* adults than for *B. burgdorferi* sensu stricto in *I. scapularis* (12.9% versus 2.4%, Korenberg and Moskvitina, 1996). Theese findings suggest that *I. persulcarus* females may be more likely to transmit spirochetes shortly after attachment.

Identification of the pathogenic *B. burgdorferi* species in ticks has epidemiological importance since they are prime factors in determining the risk of acquiring Lyme disease and its clinical presentation. However, in Latvia only *Ixodes ricinus* ticks collected in the vicinity of Riga area have been examined for the presence of *Borrelia* species in previous studies (Kurtenbach et al., 2001).

4.4. Lyme disease.

Lyme disease (Lyme borreliosis, LB) is the most prevalent vector-borne disease in Europe and the United States causing by spirochaetes of *Borrelia burgdorferi* sensu lato complex. *B. burgdorferi* enters the skin at the site of the tick bite. If left untreated the early infection may spread from an initially localized erythema migrans (EM) typically occurring at the site of tick bite in lymph, producing regional adenopathy, or disseminate in blood to organs or other skin sites and involve arthritic, neurological, dermatological, or cardiac manifestations (Steere, 2001). Disseminated infection occurs within days or weeks (stage 2), and late or chronic infection (stage 3) may occur within weeks, months, or even years. It was suggested that most manifestations of persistant infection are due to host immune response rather than to the destructive properties of the organism, because of the relative paucity of organisms in the involved tissue and failure of antibiotic treatment in some patients with Lyme arthritis (The Merck manual, 1999; Gross et al., 1998). Recent findings of Heikkila et al. (2003) showed that the rates of decline in antibody levels in patients with acute, episodic, or chronic LA did not differ, supports this theory.

Another theory is based on the fact that *B. burgdorferi* forms cystic structures in exposure of antibiotics. Morphologically intact borrelia parts seen after incubation with antibiotics, however, may also persist in humans during antibiotic treatment. (Kersten et al., 1995; Mursic et al., 1996). These atypical forms without cell walls can be a possible reason why *Borrelia* survives in the organism for a long time and the cell-wall-dependent antibody titers disappear and emerge after reversion. Some researchers suggest that formation of *B. burgdorferi* cystic forms in the human organism may explain the long periods of latency, the frequent failures of antibiotic therapy, negative serological results, the commonly reported relapses of Lyme disease and low PCR sensitivity in clinical materials (Brorson and Brorson, 1998; Gruntar et al., 2001).

Early symptoms of Lyme disease are nonspecific and can include malaise, fever, headache, stiff neck, myalgias and chills. Because these symptoms are not specific to Lyme disease alone, combined with the fact that patients often do not recall being bitten (Sigal, 1997), individuals may experience debilitating late manifestations of untreated Lyme disease weeks to months later, including musculoskeletal, cardiologic, and neurologic dysfunctions before diagnosis (Steere et al., 1984). So that prevention is the best method for avoiding infection and subsequent disease – related complications. Use of repellents, screening for ticks after potential exposure and removing them can help prevent infection. However, personal protective strategies have not always proven to be successful (Hayes et al., 1999; Smith et al., 1988) indicating a need for an efficacious vaccine.

Not all *Borrelia burgdorferi* species are pathogenic to man. Currently, only *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* have been isolated frequently from patients with Lyme borreliosis (Table 1). *B. valaisiana* and *B. bissettii* status is still uncertain (Godfroid, E. et al. 2003; Picken et al., 1996; Rijpkema, S.G. et al. 1997; Ryffel, K. et al.1999; Strle et al., 1997), however, it appeared some strains of *B. valaisiana* may be pathogenic for humans.

The heterogeneity among *B. burgdorferi* isolates had important implications for understanding the epidemiology and the clinical diversity of Lyme borreliosis. Approximately 90% of patients with untreated erythema migrans recovered spontaneously whereas 10% continue to disseminate disease. Some LB patients developed neurological disease but other patients developed arthritic symptoms. Understanding of strain differences was thought important to explain these questions. There are differences between the clinical picture of Lyme borreliosis seen in Europe and the US. Lyme arthritis is more frequently reported among US patients, whereas neuroborreliosis and acrodermatitis chronica atrophicans (ACA)

are primarily observed in Europe. Studies of the prevalence of different *B. burgdorferi* species in US and Europe and isolation of numerous *B. burgdorferi* s.l. strains from LB patients supported a concept of species-dependent Lyme disease. *B. burgdorferi* sensu stricto seems to be predominating etiological agent of Lyme arthritis, *B. afzelii* – of ACA, but *B. garinii* infections seems to be associated with neuroborreliosis (Wang et al, 1999).

4.5. Immunologic testing in diagnosis of Lyme disease.

The diagnosis of LB is based on clinical evaluation of the patients, but serologic assays are often used to provide supporting evidence of infection with *B. burgdorferi*. The immune response to *B. burgdorferi* sensu lato infection begins with appearance of specific immunoglobulin M (IgM) antibodies, usually within the first several weeks after initial exposure. Most patients will have detectable IgG antibodies after 1 month of active infection. Both the IgM and the IgG responses may persist for many months or years after LD symptoms have resolved. Thus, the presence of specific IgM or IgG antibodies cannot be used as the sole criteria to diagnose a resent infection. Both IgG and IgM responses can be greatly diminished or absent in patients receiving antimicrobial therapy early in the course of disease (Reed, 2002).

The earliest immunoserologic tests for LB were indirect FA (IFA) assays and ELISAs using whole-cell preparations of *B. burgdorferi* sensu stricto. However, when results from different laboratories for well-characterized proficiency samples are compared, significant differences in the sensitivities and specificities of ELISA and IFA have been observed (Bakken et al., 1997). Also the numerous antigens present in whole-cell assays can result in cross-reaction with antibodies to other microorganisms or tissue components. The antigens predominantly used in the present routine LB serodiagnostic tests are borrelial flagellin protein or whole-cell lysate (WCL) of the microbes cultured in vitro. Some immunoserologic tests that utilize whole-cell preparations of *B.burgdorferi* are modified in order to improve sensitivity and specificity. However despite the improvements they have the drawback of lacking sensitivity for early disease (Reed, 2002).

Resently, recombinant antigens have been applied to improve the laboratory diagnosis of LB. Use of recombinant proteins increased the specificity of ELISA assays, but sensitivity with single antigens has so far remained insufficient.

4.6. *Borrelia burgdorferi* proteins and their immunogenetic properties.

Approximately 150 lipoprotein genes have been described in *B. burgdorferi* (Casjens et al., 2000). Of these lipoproteins, only 19 have recognized counterparts in other bacteria (Liang et al., 2002). It was suggested that plasmid-encoded proteins are involved in the physiological and antigenic changes which underlie the Lyme disease spirochete's adaptation to different host environments as well as virulence expression within the mammalian host. During blood meal *B. burgdorferi* sensu stricto and *B. afzelii* downregulate expression of outer surface protein A (OspA) and upregulate OspC (Schwan et al., 1995; de Silva et al., 1996; Leuba-Garcia et al., 1998; Schwan and Piesman, 2000). Significant increasing of the mRNA level of outer surface protein vlsE was determined in tick salivary glands during tick feeding (Piesman et al., 2003). Interestingly, the *B. burgdorferi* genome contains no homologues for systems that specialize in the secretion of toxins or other virulence factors. The only known virulence factors of *B. burgdorferi* are surface proteins that allow the spirochete to attach to mammalian cells (Steere et al., 2001). Numerous lipoproteins are exposed on its surface and outer membrane so that offers a wide array of potential targets for immunoserologic testing. Elevated temperature, reduced pH, and an increase in *B. burgdorferi* cell density, conditions similar to those during tick engorgement or mammalian environment, are used in differential gene expression studies in order to determine these potential targets. Several genes, e.g., *bbk32*, *bbk50*, *vlsE*, and *OspE* and *OspF* homologs have been shown to be selectively expressed in vivo (Akins et al., 1995; Das et al., 1997; Fikrig et al., 1997; Probert and Johnson, 1998; Suk et al., 1995). Although the three genospecies of *B. burgdorferi* sensu lato express generally similar antigens, significant differences do occur; these differences complicate development of a single immunoserologic assay that is optimal for laboratory testing for Lyme disease that has resulted from infection with any one of the genospecies (Barbour, 1998; Nadelman and Wormser, 1998; Steere, 2001). Search of conserved immunogenic regions of target proteins seems to be important in development of new serodiagnosis tests.

Prevention is the best method for avoiding infection and subsequent disease-related complications. However, personal protective strategies have not always proven to be successful, up to 75% of patients with erythema migrans do not notice the tick bite before the rash develops (Shapiro, 2001; Berger, 1989; Nadelman et al., 1996) thus indicating a need for an efficacious vaccine. Lyme disease vaccine development has primarily targeted the outer surface protein A (OspA) of *Borrelia burgdorferi* (Sadziene and Barbour, 1996). OspA

vaccine (LYMErix, GlaxoSmithKline, Research Triangle Park, NC) used in humans have demonstrated a 76% efficacy after three doses and function by inducing anti-OspA antibodies that block the transmission of spirochetes from the tick to the mammalian host (Steere et al., 1998). However, if *B.burgdorferi* escape killing in the tick and are successfully transferred to the human host, they may escape elimination in the host because *Borrelia* down-regulate OspA expression (Liang et al., 2002). Recently LYMErix vaccine has been removed from the market (February 2002).

To avoid immune evasion in the host, designing a multivalent vaccine as effective approach was proposed, as decorin-binding protein A (DbpA) in combination with OspA (Hanson et al., 2000). Other immunogenic proteins examined as vaccine candidates include OspC, p53/BBK32, Vra/BB116 (Zhong et al., 1997; Fikrig et al., 1997; Labandeira-Rey et al., 2001). The serious problem in vaccine design is protection against heterologous *B.burgdorferi* strains due to heterogeneity of most *Borrelia* proteins. If vaccines against *Borrelia* species are to be designed, it is crucial to know the prevalent infecting species.

OspE/F related proteins (Erps) are surface exposed lipoproteins that are expressed during the initial stages of mammalian infection (Akins et al., 1995; Stevenson et al., 1998b; El-Hage et al., 2001). The erp genes are known by various names, including *ospE*, *ospF*, *p21*, *elp*, *pG*, *bbk2.10*, *bbk2.11* and are located on different homologous 32-kb circular plasmids that are suggested to be prophages (Casjens et al., 1997b, 2000; Stevenson et al., 1998, Eggers et al., 2001; Hefty et al., 2002). It was suggested that erp proteins play a role in host-pathogen interactions during mammalian infection by binding complement inhibitory factor H (Hellwage et al., 2001; Stevenson et al., 2002).

The *vls* (*vmp* (variable major proteins of *B. hermsii*)-like sequence) expression site (*vlsE*) is a lipoprotein gene with a cassette region bounded by 17bp direct repeats located on 28 kb linear plasmid (lp28) (Zhang and Norris, 1998a). The *vls* system has been reported to be involved in antigenic variation in *B.burgdorferi* when spirochetes are in the vertebrate host (Zhang et al., 1997). Recently studies of Liang et al. (2002) and Heikkilä et al. (2003) showed that an ELISA based on a peptide antigen corresponding to the invariable region 6 (IR_6) of the borrelial VlsE protein has high sensitivity and specificity.

DbpBA operon is located on linear plasmid lp54. The two-gene operon encoding the lipoprotein DbpA and the related lipoprotein DbpB. Recombinant DbpA binds decorin, collagen-associated proteoglycan found in various tissues, including skin and joints, sites typically associated with Lyme disease, and inhibits the adherence of *B. burgdorferi* to decorin (Guo et al., 1998). The increased expression of *DbpA* at 37°C consists with the

importance of decorin binding after *B. burgdorferi* is transmitted from the tick to the mammalian host. *DbpA* gene demonstrates considerable heterogeneity among *B.burgdorferi* strains, however, DbpA recombinant protein was proposed to have diagnostic potential as antigen in the serology of LB (Heikkilä et al., 2003, Schulte-Spechtel et al., 2003).

BBK32 (p47) is a 47 kDa fibronectin-binding protein expressed by the spirochaetes preferentially in vivo. *BBK32* gene is located on linear plasmid lp36. It is detectable in spirochetes during tick feeding even before transmission to the host but not in unfed ticks. Bbk32 is up-regulated during the transmission to mammalian tissue but decreases thereafter to low levels (Probert and Johnson, 1998; Fikrig et al., 2000; Revel et al., 2002). Antibodies to BBK32 have been observed in mice (Akins et al., 1999) and in humans (Fikrig et al., 1997; Heikkilä et al., 2002). These findings indicate that the BBK32 proteins are promising serodiagnostic antigens for the detection of early and disseminated LB, but that variant BBK32 proteins may be needed either in parallel or in combination with an immunoassay for LB to cover all the relevant borrelial species that cause the disease.

In this study recombinant antigen of BBK32 from the most prevalent *B. burgdorferi* species in Latvia and Europe, *B. afzelii*, was cloned and expressed in *E. coli* cells.

5. AIMS OF THIS THESIS.

1. Investigate the prevalence of Lyme disease aethiologic agent, *Borrelia burgdorferi*, in field-collected *Ixodes* ticks in Latvia in different years in order to characterise possible risk of acquire Lyme disease for population.
2. Develop a new approach for the typing of *Borrelia burgdorferi* in field-collected ticks without cultivation.
3. Perform molecular typing of *B. burgdorferi* in *Ixodes* ticks in Latvia and Lithuania in order to investigate the spread of clinically relevant *B. burgdorferi* genospecies in Baltic countries.
4. Compare obtained data with those from Europe and carry out epidemiological studies.
5. Perform cloning and expression of *B. burgdorferi* BBK32 protein in order to consider its possible use in serodiagnosis of Lyme disease.

6. METHODS.

6.1. Collection of ticks.

Questing ticks were collected by flagging in the four regions of Latvia (Kurzeme, Vidzeme, Zemgale, Latgale) in years 1997, 1998, 1999, 2000, 2001 and 2002. An average of 400 ticks was collected in each region of Latvia each year.

In total 210 ticks were collected in Lithuania in 2002.

6.2. Microscopic detection of *Borrelia* in questing ticks.

For microscopic detection of *Borrelia* in questing ticks after the identification of the tick gender, stage and species they were killed and two smear lines was made on the preparation glass. The fixed tick smears were stained in the solutions of Gymsa-Romanovski and Chrystalviolet and after were looked through with microscope in the oil-immerse system at the total magnification 750 times. Initially for the comparative studies ticks were dissected and midguts were divided into two parts. One part was studied by microscopic examination whereas another was kept in refrigerator -20°C or in 70% ethyl alcohol and used for DNA extraction and nested-PCR analysis. Later collected ticks were analysed separately.

6.3. Cultivation of *Borrelia burgdorferi.*

Alive ticks were crushed with a sterile plastic rod in sterile cultivation tube containing 5 ml of Barbour-Stoenner-Kelly (BSK) media supplied with rabbit serum (Sigma, St.Louis, Mo). Borrelia were cultivated at 34°C. As motile, living organisms *B. burgdorferi* were detected by dark-field microscopy.

6.4. Reference DNA.

DNA samples isolated from seven reference strains (*B. burgdorferi* sensu stricto B31, *B. burgdorferi* sensu stricto N40, *B. afzelii* F1, *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.

6.5. DNA extraction.

By random choice, DNA was extracted by phenol-chloroform method from approximately 260 ticks collected in Latvia each year. In total 204 ticks from Lithuania (year 2002) were analyzed. Each tick was crushed with a sterile plastic rod in sample tube containing100 µl of TE buffer. DNA was extracted by adding 100 µl of phenol/chloroform/isoamyl alcohol mixture (25:24:1, pH 8,0) by vortexing and subsequent centrifugation. Extracted DNA samples were stored at -20°C until further use.

6.6. *B. burgdorferi* detection by PCR amplification.

B. burgdorferi DNA was detected in samples by nested PCR amplification carried out by the method described by Priem et al., 1997. *OspA* gene fragment PCR amplification resulted in a 391-bp product, flagellin gene fragment PCR amplification resulted in a 410–bp product. Sequences of primers used in this study are shown in Table 3.

Table 3. The sequences of the oligonucleotide primers used in *B.burgdorferi* detection in ticks.

Target Gene	Primer	Use in PCR	Location in gene (*B. burgdorferi* B31 strain)	Sequence (5'-3')
OspA (linear plasmid lp54)	PrZS7	1.PCR Forward primer	18-39	GGGAATAGGTCTT AATATTAGCC
	OspA5	1.PCR Reverse primer	660-682	CACTAATTGTTAAA GTGGAAGT
	OspA6	2.PCR Forward primer	54-75	GCAAAATGTTAGC AGCCTTGACG
	OspA8	2.PCR Reverse primer	423-444	CTGTGTATTCAAGT CTGGTTCC
Flagellin (chromosome)	Fla1	1.PCR Forward primer	128-147	CTGCTGGCATGGG AGTTTCT
	Fla2	1.PCR Reverse primer	838-857	TCAATTGCATACTC AGTACT
	Fla3	2.PCR Forward primer	280-300	GCAGTTCAATCAG GTAACGGC
	Fla4	2.PCR Reverse primer	671-690	AGAAGGTGCTGTA GCAGGTG

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. Each time the PCR was performed, negative and positive control samples were included. False-negative results due to inhibition of the PCR were excluded by the use of internal spike controls. To avoid cross-contamination and sample carryover, pre- and post-PCR sample processing and PCR amplification were performed in separate rooms.

6.7. Molecular typing of *B. burgdorferi* by 16S-23S ribosomal DNA spacer PCR-RFLP.

A region of the *B. burgdorferi* 16S-23S (*rrs-rrlA*) rDNA spacer region was amplified by nested PCR with primers originally described by Liveris at al. (1999). The use of the nested-PCR procedure resulted in an increased yield of product, allowing the use of this method directly on DNA extracted from ticks, thus obviating the necessity of culture. Six microlitres of isolated DNA were employed for the PCR. Ten-microlitre aliquots of the nested-PCR amplification products were subjected to RFLP analysis by digestion with 2 U of either *Hinf*I or *Tru*1I (MBI Fermentas, Lithuania). *Hinf*I-digested fragments were analyzed by electrophoresis in a 1.5% agarose gel stained with ethidium bromide. *Tru*1I-digested fragments were analyzed by electrophoresis in 18% PAAG gel stained with ethidium bromide or silver. Sequences of primers used in this study are shown in Table 4.

6.8. Molecular typing of *B. burgdorferi* by species-specific PCR targeted 16S rRNA gene.

Species-specific PCR targeted the conserved 16S rRNA gene with *B. garinii*, *B. burgdorferi* sensu stricto and *B. valaisiana* specific primers were performed (Liebisch et al., 1998). 15 microlitres of isolated DNA were employed for the PCR, and 40 PCR cycles were performed in order to obtain sufficient amount of the amplicon. Sequences of primers used in this study are shown in Table 4.

6.9. Molecular typing of *B. burgdorferi* by 5S-23S ribosomal DNA spacer amplicon sequencing.

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). 15 microlitres of isolated DNA were employed for the PCR, and 40 PCR cycles

were performed in order to obtain sufficient amount of the amplicon. Sequences of primers used in this study are shown in Table 4. Sequencing of the amplicons were performed, and sequences were compared to those in GenBank.

Table 4. The sequences of the oligonucleotide primers used in *B.burgdorferi* typing

Target Gene	Primer	Use in PCR	Location in gene (*B. burgdorferi* B31 strain)	Sequence (5'-3')
16S-23S (*rrs-rrlA*) rDNA intergenic spacer	Pa	1.PCR Forward primer	positions 1465 to 1481 in the mature 16S rRNA sequence	GGTATGTTTAGTGAGGG
	P95	1.PCR Reverse primer	positions 941 to 924 of the spacer	GGTTAGAGCGCAGGTCTG
	Pb	2.PCR Forward primer	positions1505 to 1524 in the mature 16S rRNA sequence	CGTACTGGAAAGTGCGGCTG
	P97	2.PCR Reverse primer	positions 908 to 886 of the spacer	GATGTTCAACTCATCCTGGTCCC
16S (*rrs*) rRNA gene, *B.garinii*	Bgar1	Forward primer	positions 74 to 92 in the 16S rRNA sequence	GGGATGTAGCAATACATCT
	Bgar 2	Reverse primer	Positions 648 to 630 in the 16S rRNA sequence	ATATAGTTTCCAACATAGT
16S (*rrs*) rRNA gene, *B.burgdorferi* s.s.	Bbss 1	Forward primer	positions 74 to 92 in the 16S rRNA sequence	GGGATGTAGCAATACATTC
	Bbss 2	Reverse primer	positions 648 to 630 in the 16S rRNA sequence	ATATAGTTTCCAACATAGG
16S (*rrs*) rRNA gene, *B.valaisiana*	Bval 1 (P1)	Forward primer	Positions 63 to 83 in the 16S rRNA sequence	GCAAGTCAAACGGGATGTAGT
	B val 2 (P2)	Reverse primer	positions 612 to 589 in the 16S rRNA sequence	GTATTTTATGCATAGACTTATATG
5S-23S (*rrfA-rrlB*) rDNA intergenic spacer	Primer 1	Forward primer	Positions 229 to 246 of the spacer	CTGCGAGTTCGCGGGAGA
	Primer 2	Reverse primer	Positions 481 to 464 of the spacer	TCCTAGGCATTCACCATA

6.10. Sequencing of PCR amplicons.

For the questionable samples DNA sequencing was used. DNA extraction kit (MBI Fermentas, Lithuania) was used to purify PCR products from agarose gels according to manufacturer's instructions. If only one PCR product was obtained (as confirmed gel analysis), amplicon was purified directly from the reaction mixture by Exonuclease I (USB, USA) and Shrimp Alkaline Phosphatase (USB, USA). Four or five microlitres of the purified PCR product, depending on the amount of DNA, were used for DNA-sequencing reactions.

For sequencing reactions, the fluorescence-labeled dideoxynucleotide technology was used (Applied Biosystems, Inc., Foster City, Calif.). The sequenced fragments were separated, and the data were registered on an ABI PRISM 3100 automated DNA sequencer (Applied Biosystems, Inc., Foster City, Calif.).

6.11. Cloning, expression and purification of His-tagged recombinant BBK32 protein.

A PCR- based approach was used to amplify and sequence *Bbk32* genes, as genomic sources for recombinant proteins reference strains of *B. afzelii* ACAI and F1 were used. A primer set (forward primer [5'CCCCATGGGAAAAATTAAAAGTAAATG] and reverse primer with encoded 6 Histidine (His) aminoacids in 3'end [5'GGCTGCAGTCAAT GGTGATGGTGATGGTGGTACCAAACACCATTCTT]) for gene amplification and constructs were designed on the basis of published data (Heikkilä et al., 2002), *BBK32* gene sequence in GenBank (accession numbers AF472528, AF472525) and pBAD/Thio-TOPO (Invitrogen, The Netherlands) vector cloning site sequence. Approximately 1 ng of template DNA was used under standart PCR conditions: 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1.5 min. The PCR products (1092 nt) were digested with restriction endonucleases *Nco*I and *Pst*I and purified from agarose gel by DNA purification kit (Fermentas, Lithuania). His tagged *Bbk32* gene (1086 nt) was ligated in similarly prepared modified pBAD/Thio-TOPO vector (Invitrogen, The Netherlands, kindly donated by Dr. J.Klovins with T4 ligase. The ligation mixture was used to transform *E.coli* host cells. The transformation mixture was plated onto Luria-Bertani agar containing 100 μg ampicillin per ml. A primary culture was started by inoculating a single colony from a fresh transformant plate to 5 ml of Luria-Bertani media containing 100 μg ampicillin per ml. Cultures were incubated at 37°C overnight with shaking. Positive clones with correct

constructs were identified by both restriction analysis of plasmid DNA and PCR with subsequent sequence analysis.

A primary culture for expression of recombinant BBK32 protein was started by inoculating the positive culture to 150 ml of Luria-Bertani media containing antibiotic as above. Recombinant protein expression was induced by arabinose in accordance with manufacturer's instructions. His-tagged protein expression was detected by Western Blot after SDS-PAGE with Anti-His antibodies (Invitrogen, The Netherlands) in accordance with manufacturer's instructions.

The HisBBK32 protein was initially purified in the ProBond^{TM} Purification System (Invitrogen, The Netherlands), the elution fractions were tested for the content of recombinant protein by Western Blot as described above. The final purity of the recombinant protein was tested by SDS-PAGE.

7. RESULTS AND DISCUSSION.

7.1. Comparison of microscopic examination, *OspA* gene and *Fla* gene nested PCR as tools for detecting of *B. burgdorferi* in field-collected ticks.

Percentage of *Borrelia* infected ticks determined by microscopic examination and PCR analysis (*OspA* gene fragments) are shown in the Table 5. These data indicates more than twofold higher sensitivity of molecular detection method and likely reflects the real percentage of infected ticks. The *Borrelia* prevalence in ticks detected with the means of PCR reveal approximately 2 times higher infectivity level. Comparative studies showed that in every case, when borrelias were detected by microscopic examinations, PCR analysis was positive with any of amplified gene fragment. Dark-field microscopy is another method used for the detection of *B. burgdorferi* directly in ticks. However, the study of Alekseev et al. (2001) suggests that the microorganisms seen in the dark-field analysis may represent species other than *B. burgdorferi* sensu lato. These comparisons indicate that molecular detection methods when used with appropriate positive and negative controls and adequate DNA isolation and storage are more precise and sensitive than microscopic examination for the detection of Lyme disease spirochete in field-collected ticks.

Table 5. Percentage of *Borrelia* infected *Ixodes* ticks evaluated by microscopic examination and PCR (different samples).

	1997		1998		1999	
	Microscopy*	PCR	Microscopy*	PCR	Microscopy*	PCR
Borrelia prevalence in *Ixodes* ticks	**12.5 %**	**30 %**	**9 %**	**18,9%**	**11,4 %**	**22.4 %**
♀	12.9 %		7.9 %	23,6%	13,1 %	15,6 %
♂	11.9 %		10 %	13,4%	9,6 %	32,6 %

* Data from A.Bormane, State Agency "Public Health Agency".

When two nested PCR methods were compared, the most sensitive was the determination by *OspA* gene fragment. This finding is in agreement with conclusion that PCR targets carried on plasmids, such as *OspA*, *OspC*, and *vlsE*, are present in multiple copies within each bacterium, and assays with these targets have greater sensitivity that those

employing single-copy chromosomal targets (Reed, 2002). So that in further analysis nested PCR targeted plasmid *OspA* gene was used (Figure 2), tests of the sensitivity of the method showed that the smallest amount of DNA that gave a positive result represented 3 spirochetes in each PCR mixture.

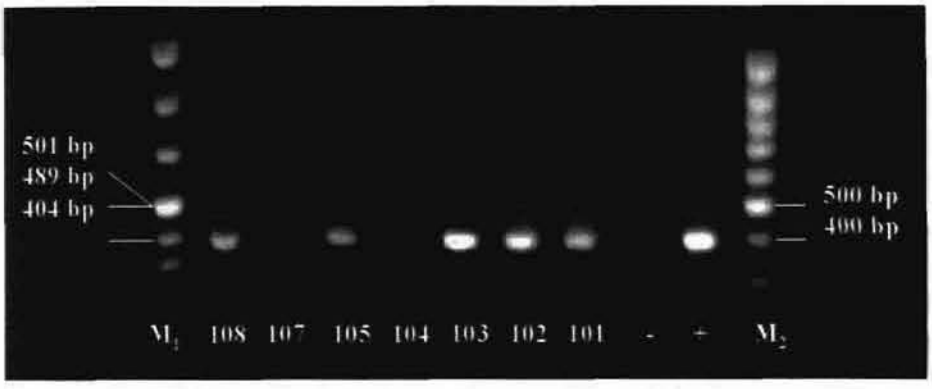

Figure 2. *B. burgdorferi* detection in ticks. *OspA* gene PCR amplification product analyzed in 1,5% agarose gel. Lanes M1, M2 - DNA size markers; lane +, positive control sample; lane - , negative control sample; lanes 107, 104, DNA samples prepared from ticks, negative result; lanes 108, 105, 103, 102, 101, DNA samples prepared from ticks, positive result (infected ticks)

7.2. Correlation observed between the Lyme disease cases and the prevalence of *B. burgdorferi* in ticks in Latvia.

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 6). The number of cases of Lyme disease in this region is higher than in neighbouring Northern Europe countries excepting the St. Petersburg region of Russia.

Table 6. 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		2002	
	Total No	Per 100000 inhabitants	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	109	2.4
Finland	404	7.8	895	17.2	691	13.3	884	17.0
Estonia	321	22.2	601	43.8	342	25.0	319	23.3
Latvia	281	11.5	472	19.4	379	16.0	328	14.0
Lithuania	766	20.7	1713	46.3	1153	33.0	894	25.7
St. Petersburg region	265	5.6	541	11.3	323	6.7	398	8.5

In order to investigate the possible correlation of the Lyme disease cases and the prevalence of *B. burgdorferi* in field-collected ticks, data from 5-year period was compared (1998-2002) (Figure 3).

As it is seen in Figure 3 there is the tight correlation between registered Lyme disease cases and the prevalence of *B. burgdorferi* in field-collected ticks. The only except is the year 1998. The extremely high percentage of infected ticks in 2000 in Latvia correlates with the high number of registered cases of Lyme disease that year. However, the tick activity (average tick numbers in forests) and weather conditions that can influence people number visiting forests must be taken in account before final conclusions. Figure 4 illustrate the mean distribution of *Ixodes* tick number in monitoring sites where the average tick number is estimated each year. As it can be seen the highest activity of *Ixodes* ticks was in year 1998 and the number of active ticks was high during the whole season. In that year there was the highest incidence of established LB in Latvia. For primary prevention people can be counseled to avoid areas that are infested with ticks. However, most patients get infected in the vicinity of their homes (I. Lucenko, A. Bormane, Abstracts of 5th Baltic-Nordic Conference on Tick-Borne Zoonosis; abstract Nr 4, 1998), and study of Junttila et al. (1999) demonstrated high prevalence of *Borrelia burgdorferi* in *Ixodes* ticks in urban areas of Helsinki. So that tick checks every time when tick-infested habitat is visited, removement any attached ticks and repellent use seems to be helpful for prevention of Lyme disease. A new vaccine will be the best prevention method, but it has to be safe, inexpensive, confer long-lasting immunity with only a few doses and be highly efficacious.

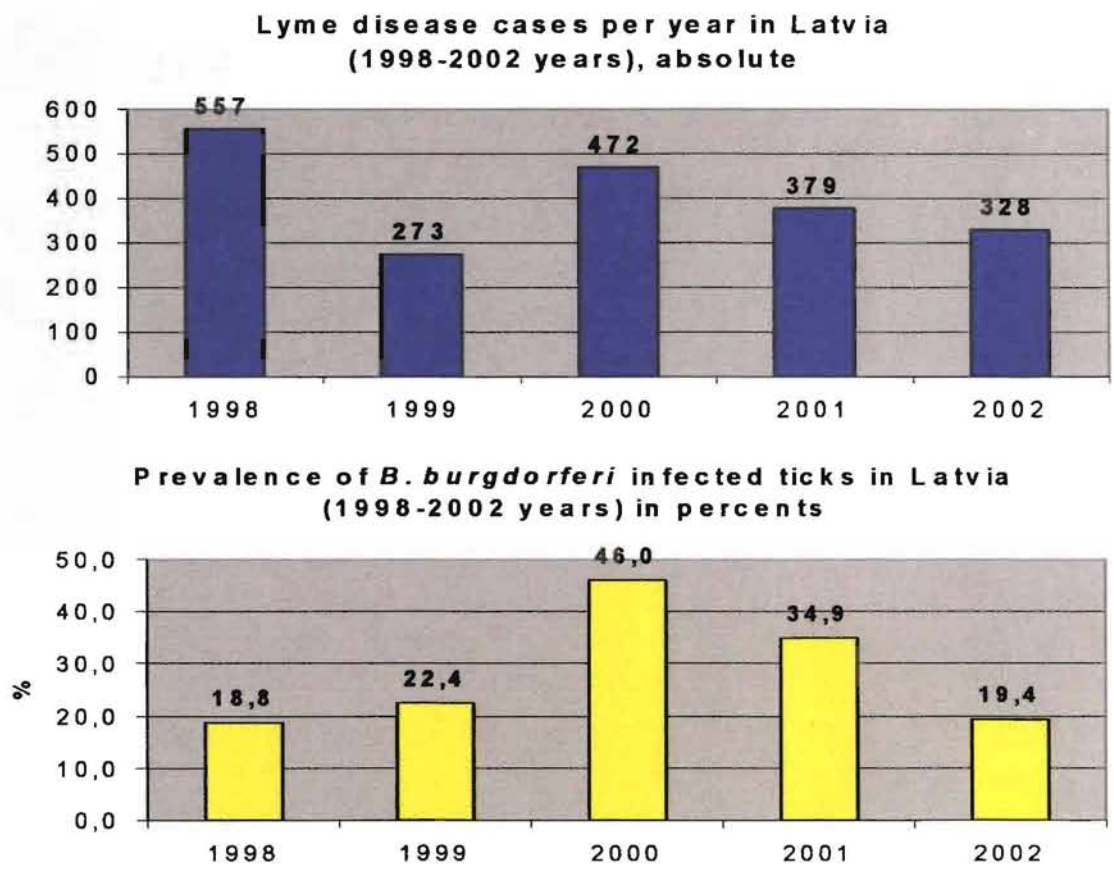

Figure 3. Lyme disease cases and the prevalence of *B.burgdorferi* in questing ticks in Latvia (1998-2002).

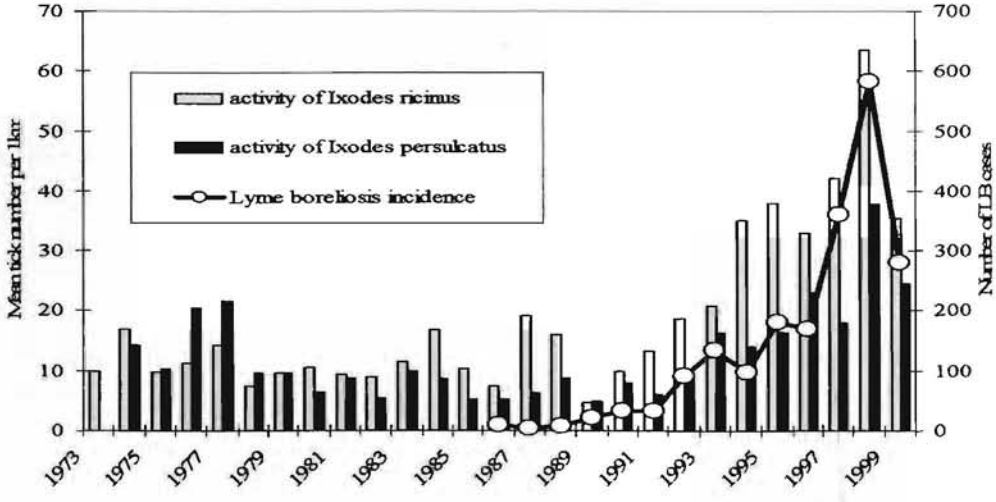

Figure 4. Mean distribution of *Ixodes* tick number and Lyme borreliosis incidence by years. (Paper I).

7.3. Prevalence of *B. burgdorferi* in different *Ixodes* species in Latvia.

On the territory of Latvia both *I.ricinus* and *I.persulcatus* ticks are distributed (Figure 5). The prevalence of *B. burgdorferi* in different *Ixodes* species in Latvia was studied. In order to minimize the influence of collection time and place on the result, ticks collected in all four different Latvia regions during four years were analysed. In average, 22.6% of the *I. ricinus* and 27.9% of the *I. persulcatus* ticks were PCR- positive. In the Baltic St. Petersburg and Kaliningrad regions of Russia an infection rate of 26.3% for *I. persulcatus* and 11.5% for *I. ricinus* has been reported whereas in the Western Siberian region of Russia 38% of the *I. persulcatus* ticks where infected (Alekseev et al., 2001; Morozova et al., 2002). The infection rate of *I. persulcatus* ticks in our study (27.9%) is similar to that obtained in the neighbouring Baltic regions of Russia, but the prevalence of *Borrelia* in *I. ricinus* in Latvia is higher (22.6%). There was no significant difference between infection rates of *I. ricinus* and *I. persulcatus* with *B. burgdorferi* in Latvia. However, an infection rate of 31.3% was reported in *Ixodes ricinus* ticks from the Riga area of Latvia in a study of Kurtenbach et al., 2001. These differences can be explained by differences in the strategy used in collecting the ticks.

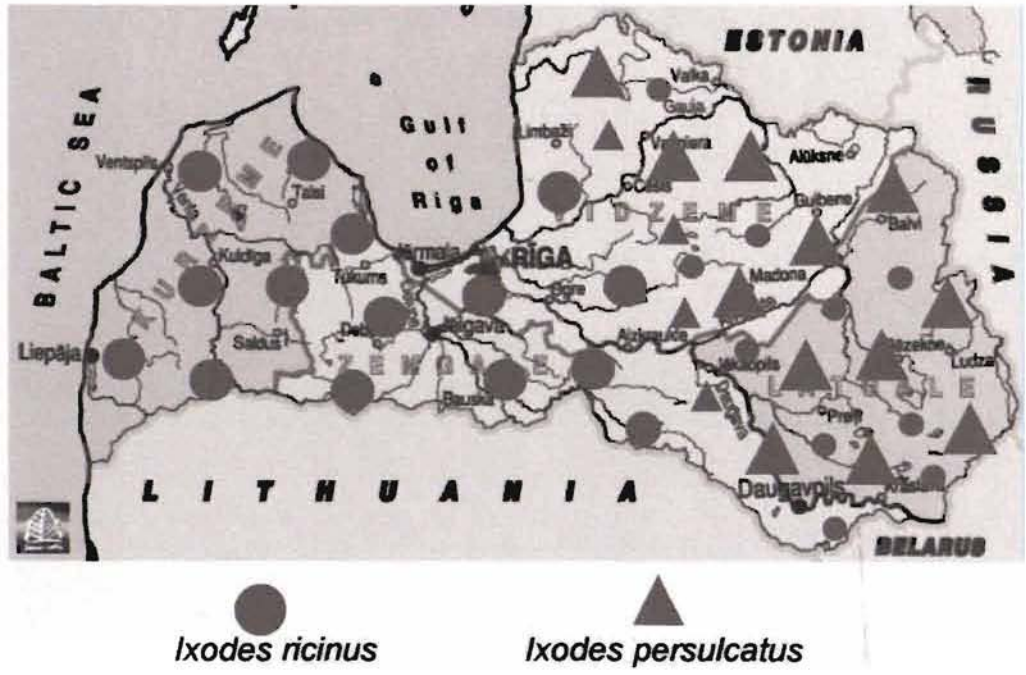

Figure 5. Distribution of *Ixodes* ticks in Latvia. (Data from A.Bormane, State Agency "Public Health Agency").

7.4. Prevalence of *B.burgdorferi* in ticks in three Baltic countries.

Lyme disease is endemic in all three Baltic countries, Latvia, Lithuania and Estonia. In order to compare the prevalence of *B.burgdorferi* in *Ixodes* ticks in all three Baltic countries in total 210 ticks were collected in Lithuania in 2002 and 204 ticks were analyzed. The average prevalence of *Borrelia* in field-collected ticks in Latvia was 25.3%. The overall prevalence of *Borrelia* in ticks in Lithuania was 14 %, significantly lower ($P<0.05$) than in Latvia. In Table 7 the obtained results are compared with those from Estonia in 1999.

Resently the study of Etti et al. shows that the migration rates of *I.ricinus* are not sufficient to homogenize the frequency distribution of *B.burgdorferi* sensu lato genotypes between ecologically distinct sites (Etti et al., 2003). In this study the distribution of *B.burgdorferi* in different regions of Latvia was also compared. The higher prevalence level was found in the Eastern part of country, neighbouring to Russia and Belarus (36%), also in North-Eastern part, neighbouring to Estonia (33%). The borrelia prevalence level in central (neighbouring to Lithuania) and Western (along the Baltic sea coast) parts of country was 11 and 22%, respectively. These findings could be explained with different vegetation types presented in different areas, and also uneven distribution of hosts (small mammals and sea birds) may have an influence on the *B.burgdorferi* prevalence rates in ticks.

Table 7. The prevalence of different *B. burgdorferi* genospecies in Latvia, Lithuania and Estonia. *, Data from this study. **, Data from T. Prükk et al. 1999

Country	*Borrelia* positive ticks (%)	% (No.) of ticks infected by different genospecies			
		B. afzelii	*B. garinii*	*B. burgdorferi* sensu stricto	*B. valaisiana*
Lithuania*	13.7	68 (19)	18(5)	0	0
Latvia*	25,3 (average)	76 (80)	18 (19)	2(2)	2(2)
	18,9 (year 1998) 22.4 (year 1999) 46.0 (year 2000) 34.9 (year 2001) 19.4 (year 2002)				
Estonia**	5.5	65 (15)	17(4)	13(3)	0

These findings are in general agreement with the results obtained in other European countries where the reported mean rates for adult unfed *I. ricinus* ticks infected with

B.burgdorferi vary from 3 to 58% (Hubalek and Halouzka, 1997). An extremely high prevalence of *B. burgdorferi* infected ticks was found in Portugal (75%), where the majority belonged to the *B. lusitanie* type (De Mishelis et al., 2000).

Data 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.

7.5. Genetic diversity of *B. burgdorferi* species in field-collected *Ixodes* ticks.

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, however, further studies and confirmations are needed.

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). Litherature 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 and for therapeutic strategy in Lyme borreliosis cases in the Baltic region.

Various methods have been used to type *B. burgdorferi* sensu lato in culture isolates but only a few of them are useful for typing of *Borrelia* directly in ticks. The most popular are the PCR – based methods, such as species-specific PCR in which the conserved 16S rRNA gene (Liebisch et al., 1998) or species-specific plasmid gene loci (Misonne et al., 1998, Misonne and Hoet, 1998) are targeted, the 5S-23S rRNA spacer region PCR hybridization method (Schouls et al., 1999), and the rDNA PCR-RFLP analysis of the *rrs-rrlA* intergenic spacer

(Liveris et al., 1999). In our study the 16S-23S rDNA nested PCR-RFLP method was used for molecular typing of *B. burgorferi* directly in ticks in Europe for the first time.

In this study *OspA* gene-positive DNA tick samples were used for further analysis. A region of the *B. burgdorferi* 16S-23S (*rrs-rrlA*) rDNA spacer region was amplified by nested PCR with primers originally described by Liveris at al., 1999. Aliquots of the nested-PCR amplification products were subjected to RFLP analysis by digestion with either *Hin*fI or *Tru*1I restriction enzymes. In order to develop a simple epidemiological and diagnostic tool to assess rapidly the genetic diversity of *B. burgdorferi* the 16S-23S rDNA nested PCR-RFLP typing method, originally used for the typing of *B. burgdorferi* in clinical specimens from early Lyme disease patients (Iyer et al., 2001; Liveris et al., 1999), was extended to include the typing of *B. garinii, B. afzelii* and *B.valaisiana* species directly in field-collected ticks. PCR-RFLP patterns obtained from tick samples were compared with those obtained from reference strains. The smallest amount of DNA that gave a positive result represented 6 spirochetes in each PCR mixture The observed RFLP patterns from the DNA samples were completely consistent with the classification of the *B. burgdorferi* into four distinct species (*B. afzelii, B. garinii, B. burgdorferi* sensu stricto and *B. valaisiana*) (Figure 6 and 7).

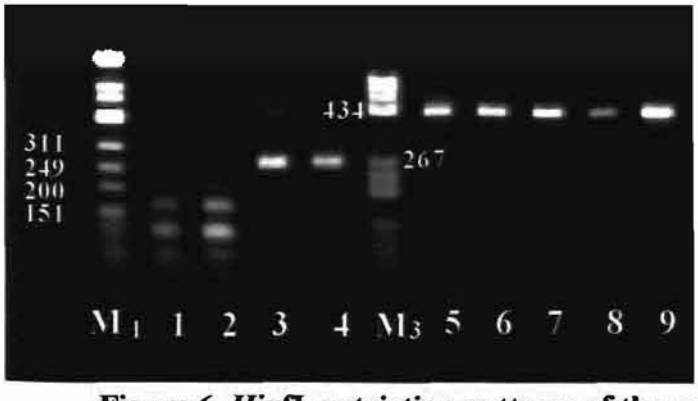

Figure 6. *Hin*fI restriction patterns of the amplified 16S-23S rDNA spacer from *B. burgdorferi* tick isolates. Lane 1, tick Nr77; lane 2, tick Nr22; lane 3, tick Nr65; lane 4, *B. garinii* strain IP_{90}; lane 5, *B. afzellii* strain ACAI; lane 6, tick Nr27; lane 7, tick nr26; lane 8; tick Nr25; lane 9, tick Nr21; lanes M_1, M_3 - DNA size markers.

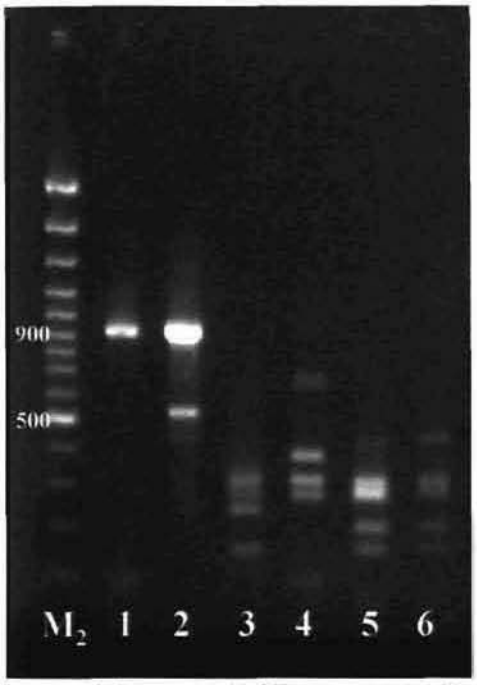

Figure 7. PCR products (lines 1 and 2) and *Hin*fI restriction patterns (lines 3 - 6) of the 16S-23S rDNA spacer from *B. burgdorferi* sensu stricto tick isolates. Lane 1, *B. burgdorferi* sensu stricto strain B31; lane 2, tick Nr201; lane 3, *B. burgdorferi* sensu stricto strain N40; lane 4, *B. burgdorferi* sensu stricto strain B31; lane 5, tick Nr201; lane 6, tick Nr211; lane M_2, - DNA size marker.

Typing patterns were confirmed by species-specific PCR that targeted the 16S rRNA gene for the typing of *B. garinii*, *B. burgdorferi* sensu stricto and *B. valaisiana* (Liebisch et al., 1998) (Figure 8).

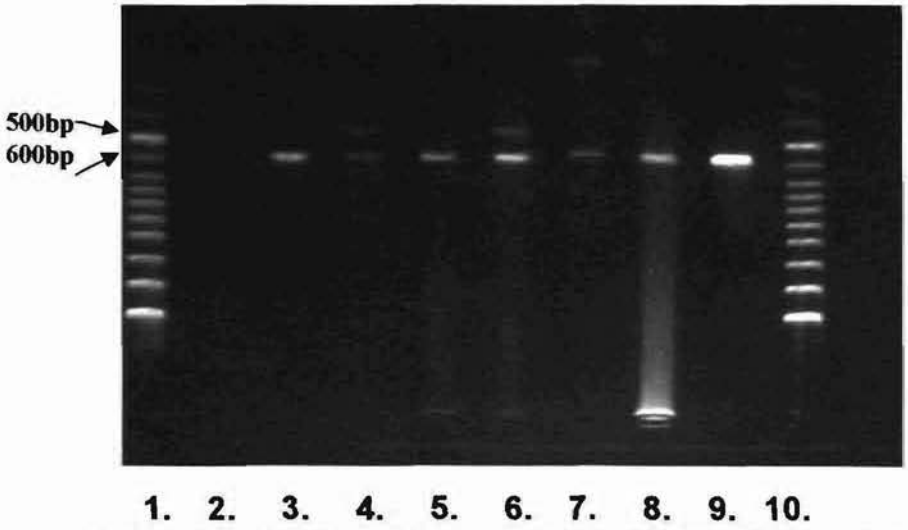

Figure 8. Analysis of *B. garinii* species-specific PCR products in 1.5% agarose gel. Line 1 - DNA length marker, line 2 - negative control sample, line 3 - *B.garinii* IP 90, line 4 - tick Nr.192, line 5 - tick Nr.195, line 6 - tick Nr.216, line 7 - tick Nr.217, line 8 - tick Nr.87, line 9 - tick Nr.77, line 10 - DNA length marker.

In Latvia 76 % of samples belonged to *B. afzelii*, 18 % - to *B. garinii*, 2% - to *B. burgdorferi* sensu stricto, and 2 % were *B. valaisiana* (Table 7). In Lithuania, 68 % belonged to *B. afzelii*, 18 % to *B. garinii*. The data demonstrate that all clinically relevant *B. burgdorferi* sensu lato genotypes are found in Latvia.

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). 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 unsufficient tick sample size investigated.

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

Notably, used 16S-23S rDNA PCR-RFLP method was able to identify also subspecies-specific paterns. Two different *B. garinii* subtypes were identified in Latvia. Those samples differing from the reference strain Ip90 were employed for the 5S-23S rDNA PCR-RFLP analysis (Postic et al. 1994) 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 (99 % similarity, BLAST) to the NT29 isolate from Russia.

Two different *B. afzelii* subtypes were identified in Latvia, and PCR-RFLP pattern of *B.burgdorferi* sensu stricto and *B. valaisiana* isolates differed from those obtained from reference strains. The presence of different subtypes in Latvia is not surprising, since many previous studies have identified strain heterogeneity within genomic groups (Mathiesen et al., 1997; Wang et al., 1998). To determine if genetic heterogeneity may be responsible for the wide variation in symptomatology and severity of Lyme disease, there is need for further studies on the pathogenity of different subtypes of *Borrelia*.

The present study established that 16S-23S rDNA spacer analysis is both species- and subspecies specific and can serve to identify *B. burgdorferi* sensu lato genotypes.

7.6. Vector-associated distribution of different *B.burgdorferi* species.

Two different *B. afzelii* subspecies were identified in Latvia: 54.9% of all infected *Ixodes* ticks carried the first subtype, and 9.9% had the second one (Table 8). Interestingly, the *B. afzelii* second subtype was detected only in *I. persulcatus* ticks, common in Far-Easter Europe and Asia (P = 0.0142). There are also significant differences in numbers of tick species carrying different *B. garinii* RFLP types: the subtype, similar to *B. garinii* IP90 was found predominantly in *I. ricimus* (8 versus 1 tick, P = 0.003), whereas the subtype, similar to *B. garinii* NT29 was more common in *I. persulcatus* (8 versus 1 tick, P = 0.035). 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).

The *B. burgdorferi* sensu stricto was detected only in two *I. ricimus* ticks from Latvia, but *B. valaisiana* in two *I. persulcatus* ticks. Our results also clearly show that the distribution of different *B. burgdorferi* genospecies in Europe depends on the distribution of the *Ixodes* vector. 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 genospecie. This finding suggests that the infection is vector-specific. To determine to what extent an association between a pathogen and a tick species exists, additional studies are required.

Table 8. PCR-RFLP typing of *B. burgdorferi* isolates from *Ixodes* ticks.

Digestion patterns		No(%) of DNA samples total	No of DNA samples of *I. ricimus*	No of DNA samples of *I. persulcatus*	Genospecies of *B. burgdorferi* sensu lato
*Hin*fI	*Tru*1I				
H1	A	39 (54.9)	18	21	*B. afzelii* (variant 1)
H1	B	7 (9.9)	0	7	*B. afzelii* (variant 2)
H2	C	9 (12.7)	8	1	*B. garinii* (similar to IP90)
H3	D	9(12.7)	1	8	*B. garinii* (similar to NT29)
H4	E	2 (2,8)	2	0	*B. burgdorferi* sensu stricto
H5	F	2 (2,8)	0	2	*B. valaisiana*
H1 and H2	A and C	3 (4,2)	2	1	*B. afzelii* + *B. garinii*

7.7. Mixed infections of *B. burgdorferi* sensu lato species in ticks.

Some mixed infections were also detected in our study. Three ticks were infected with *B. garinii* and *B. afzelii*, one with *B. burgdorferi* sensu stricto and *B. garinii*, and one with *B. burgdorferi* sensu stricto and *B. afzelii* spirochetes. In the world, the prevalence of mixed infections in ticks varies from 5 to 40% (Wang, 1999). Such mixed infections may result directly from feeding on a host infected with multiple *Borrelia* species or through cofeeding transmission. It could be possible also that ticks may acquire different *Borrelia* species from different individual hosts during their life cycle stages. Notably, mixed infections have been found in patients with Lyme disease also (Demaerschalck et al., 1995; Liveris et al., 1999; Rijpkema et al., 1997). So that we can propose that there is a possibility to be infected with different *B. burgdorferi* species for humans. This finding may be important in clinical studies, however the number of ticks with mixed *Borrelia* infections in Latvia is low.

7.8. The genetic heterogeneity of 16S-23S rDNA spacer in different *B.burgdorferi* species.

The rDNA cluster of most *B. burgdorferi* sensu lato strains is located in the center of the linear chromosome and is arranged in the following order: *rrs-rrlA-rrfA-rrlB-rrfB* (Fukumaga et al., 1992; Gazumyan et al., 1994; Ojami et al., 1994; Schwartz et al., 1992). One of the unusual properties of the rRNA gene cluster is the presence of a large (> 3 kb) and highly variable intergenic spacer between the 16S (*rrs*) and 23S (*rrl*A) rRNA genes (Liveris et al., 1996). The amplification of the partial *rrs-rrlA* spacer, followed by digestion with *Hin*fI and *Tru*1I gave both species- and subspecies-specific RFLP patterns. In agreement with the original reference describing this method (Liveris et al., 1999) the amplicon size of *B. burgdorferi* sensu stricto B31, *B. burgdorferi* sensu stricto N40, and two of our tick samples, classified as *B. burgdorferi* sensu stricto, was 940 bp. However, the amplicon sizes of the three *B. afzelii* reference strains, the *B. garinii* reference strain and most of our tick samples belonging to these strains were much smaller, 532-430 bp. This finding does not agree with the results of Liveris et al. (1996) reported that both *B. afzelii* and *B. garinii* have larger 16S-23S rDNA spacers than *B. burgdorferi* sensu stricto. However, more detailed information about the 16S-23S rDNA intergenic spacer sequences in strains other than those of *B. burgdorferi* sensu stricto is lacking. To answer this requires the sequencing of the whole 16S-23S rRNA spacer in *B. burgdorferi* genotypes other than *B. burgdorferi* sensu stricto. One of the objectives of our study was to partially sequence this spacer in the *B. garinii* and *B. afzelii* genotypes and subtypes by using PCR amplicons. The sequences obtained were submitted to

the GenBank. Sequence analysis of variable regions could be also a useful tool for understanding the evolution of different species and subspecies of *B. burgdorferi* sensu lato because it is now well established that recombinatorial processes have conferred a remarkable degree of plasticity upon the *B.burgdorferi* genome, presumably in the context of enhancing its adoption to diverse ecological niches.

7.9. Cloning of BBK32 protein of *Borrelia afzelii.*

Two reference strains of *B. afzelii* ACAI and F1 were used as DNA template for PCR amplification of 6X His tagged BBK32 protein. *B. afzelii* strains similar to ACAI strain as confirmed 16S-23S rDNA PCR-RFLP typing method were the most prevalent in field-collected *Ixodes* ticks in Latvia and Lithuania. Hovever, as confirmed sequence analysis cloned *bbk32* genes of both strains are identical. This finding is in accordance with those of Heikkilä et al (2002) showed that sequences of *bbk32* gene of four different *B.afzelii* isolates were from 99 to 100% identical.

Western Blot analysis performed after SDS-PAGE with Anti-His antibodies (Invitrogen, The Netherlands) confirmed the expression of suspected His-tagged recombinant protein with weight of approximately 47 kDa (Figure 9).

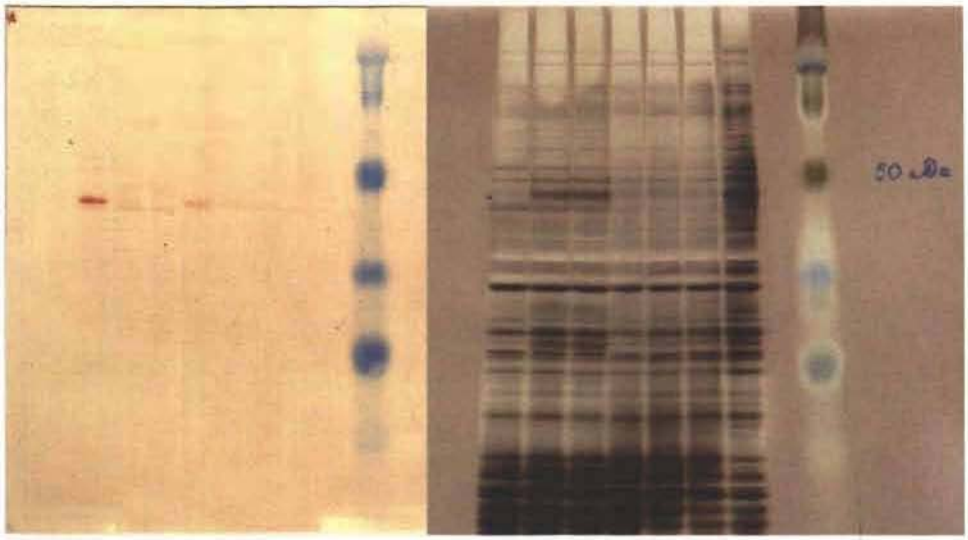

Figure 9. Western Blot and SDS-PAGE analysis of different *E.coli* samples containing recombinant HisBBK32 protein.

However, the expression level was low, so that further modifying of expression conditions are required.

In order to obtain pure recombinant His-tagged BBK32 protein for further studies, bacteria lysate was initially purified in the ProBond™ Purification System (Invitrogen, The Netherlands). The elution fractions were tested for the content of recombinant protein by Western Blot as described above. SDS-PAGE analysis of elution fractions showed good recombinant protein recovery but still contaminated with non-recombinant proteins (Figure 10).

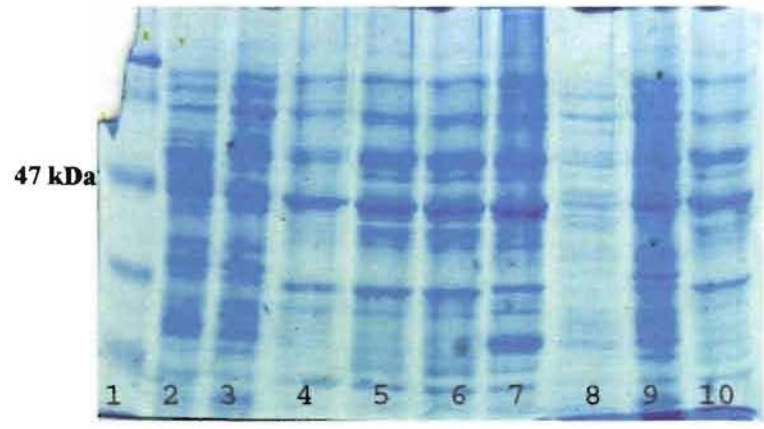

Figure 10. SDS-PAGE analysis of elution fractions of HisBBK32 protein from the ProBond™ Purification column. Line 1 – Prestained protein molecular weight marker, line 2 - negative control sample (*E.coli* total proteins without BBK32 insert), line 3 – total *E.coli* proteins with BBK32 before initial purification, line 4 – first elution fraction after initial purification, line 5 – elution fraction 2 after initial purification, line 6 – elution fraction 3 after initial purification, line 7 – elution fractions 5 and 6 together after initial purification, line 8 – total *E.coli* soluble proteins with BBK32 before initial purification, line 9 – total *E.coli* insoluble proteins with BBK32 before initial purification, line 10 – elution fraction 2 after initial purification.

BBK32 (p47) is a 47 kDa fibronectin-binding protein expressed by the spirochaetes preferentially in vivo. Resent findings indicate that the BBK32 proteins are promising serodiagnostic antigens for the detection of early and disseminated LB (Fikrig et al., 1997; Heikkilä et al., 2002). In this study Western Blot analysis confirmed successful cloning and expression of recombinant HisBBK32 protein of *B.afzelii* in *E.coli*. Further analysis of BBK32 protein immunogenetic and other properties may have important implications in serodiagnosis of different clinical forms of Lyme borreliosis.

7.10. Nucleotide sequence accession number.

The *rrs-rrlA* intergenic spacer sequences of *B. burgdorferi* that were found in this study are available in the GenBank database under the accession numbers AY121817, AY121816, AY163781, AY163782 and AY163783.

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.

8. CONCLUSIONS.

- Distribution of *B.burgdorferi* infected ticks in Latvia and neighbouring country Lithuania have been studied by molecular methods – PCR amplification of the fragment of *OspA* and *Fla* genes. The average prevalence of *B.burgdorferi* in field-collected ticks in Latvia is 25.3 %, the average prevalence of *B.burgdorferi* in ticks in Lithuania is 13.7 %.
- Various *B. burgdorferi* detection methods – microscopy, PCR amplification of fragment *OspA* and *Fla* genes were compared. The most sensitive was nested PCR method targeted plasmid-encoded *OspA* gene. This method also confirmed the growth of *B. burgdorferi* in BSK media, spirochetes were visualised later by electron microscopy.
- The prevalence of *B. burgdorferi* in field-collected ticks was compared in different years. The corelation between obtained data and Lyme disease cases in different years in Latvia was confirmed. Significant difference in the prevalence of borrelia in *Ixodes ricinus* versus *Ixodes persulcatus* ticks, and in females versus males ticks were not observed.
- Molecular typing of *Borrelia burgdorferi* in field-collected ticks was performed by 16S-23S rDNA nested PCR-RFLP typing method without preliminary culture of microorganisms. The applied method was found to be fast and useful tool for typing of borrelia and it is able to distinguish differences within all *B.burgdorferi* genomic groups investigated. All clinically relevant *B. burgdorferi* species are distributed in Latvia, the dominant genospecies being *B. afzelii*. Only *B. afzelii* and *B. garinii* species were detected in ticks from Lithuania.
- Different subtypes of borrelia are distributed on the teritory of Latvia as confirmed used typing method. Significant genetic heterogeneity exists among the *B. burgdorferi* subtypes in *I. ricinus* and *I. persulcatus* ticks, this finding will be usefull in understanding uneven distribution of different *B. burgdorferi* genotypes in Europa and Asia.
- Cloning of His-tagged BBK32 protein of *B. afzelii* in *E. coli* were performed and the expression product with molecular weight approximately 47 kDa was obtained and partially purified.

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PAPER 1.

PROCEEDINGS OF THE LATVIAN ACADEMY OF SCIENCES. Section B. Vol. 55 (2001), No. 4 (615), pp. 164–170

Borrelia burgdorferi-INFECTED TICKS AND INCIDENCE OF LYME BORRELIOSIS IN LATVIA

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The density and spread of pathogen Ixodes *ticks in Latvia has been increasing since 1994, and a concomitant increase of Lyme borreliosis during the last five years has been observed. The tick activity, its distribution, and characteristics of pathogen and clinical consequences, were reviewed and compared with the results of microscopic examinations and that of nested polymerase chain reaction (PCR amplification) of chromosomal* FlaB *and plasmid* OspA *and* OspC *gene fragments of* Borrelia burgdorferi *sensu lato. The proportion of infected ticks in* Ixodes ricinus *and* Ixodes persulcatus *collected in Latvia was found to be about 20–30 % in each of the last three years studied. The epidemiological situation of Lyme borreliosis was analysed in the light of the obtained results.*

Key words: *Lyme disease,* Borrelia, Ixodes ricinus, Ixodes persulcatus, *polymerase chain reaction, Latvia.*

INTRODUCTION

Lyme borreliosis (LB) is a tick-borne zoonotic disease caused by the spirochete *Borrelia burgdorferi* complex, transmitted by *Ixodes* tick species (Burgdorfer *et al.*, 1982). Ticks acquire *Borrelia* from their hosts (the *Borrelia* reservoir), which are mainly small mammals and some birds. The circulation of *B. burgdorferi* in nature is based on the long-term co-existence of specific vertebrates which serve as tick hosts and pathogen reservoirs, and *Ixodes* ticks as vectors become infected in the larval or nymphal stage by sucking blood from an infected host. After transstadial transmission, they transmit *Borrelia* to another host. LB has been reported from almost all European countries within the *I. ricinus* distribution area (Oschmann *et al.*, 1999).

The first cases of LB in Latvia were registered in 1986, and since then, a total of 2008 cases were registered. The morbidity of LB started to increase in 1995. In 1999, it was officially registered as 281 Lyme borreliosis patients (incidence 11.5 per 100.000 population) in Latvia (Anonymous, 2000).

LB is a multisystem disorder with a potentially chronic neurological, cardiac, cutaneous, and arthritic manifestation (Steere, 1989). LB is characterised by systemic manifestations (as malaise, fatigue, fever, headache, stiff neck, myalgia, migratory arthralgias and/or lymphadenopathy) and neurologic, rheumatologic, and cardiatic involvement that occurs in varying combinations over a period of months to years (Chin, 2000). The distinctive skin lesion in the first stage of disease is common and is called "*erythema migrans*". Patients may have a mild disease only with *erythema migrans* without other manifestations, or they may show also skin, nerve, heart, and joint involvement.

Borrelia burgdorferi is a complex of spirochetes containing different (at least nine) genospecies that may cause distinct clinical manifestations (Kurtenbach *et al.*, 1997). The investigation of *B. burgdorferi* strains of North America and Europe suggested that there is more than only one agent causing LB. The frequency of certain typical LB symptoms differs in specific geographic regions (Kahl, 1991). *B. burgdorferi* sensu stricto predominates in North America where arthritis is common, whereas *Borrelia garinii* and *Borrelia afzelii* in Eurasia could be the cause of Lyme borreliosis clinical manifestations such as the common neuroborreliosis and acrodermatitis. There are four genospecies (*Borrelia lusitaniae, Borrelia valaisiana, Borrelia japonica* and *Borrelia andersonii*) which have not been reported as pathogenic for humans. Recent scientific investigations showed that geographic variation in the distribution of different *Borrelia burgdorferi* sensu lato genospecies is associated with host-specific differential spirochete transmission between hosts and ticks, e.g. *B. garinii* and *B. valaisiana* are transmitted to ticks mostly by avian hosts, but *B. afzelii*, by rodent species (Kurtenbach *et al.*, 1997). However, no microbiological typing of existing *Borrelia* species has been performed in Latvia yet.

Spirochete has a small genome with an unusual organisation. The main genetic information is located in the one linear chromosome with size approximately of 960 kbp; the rest is located in the set of linear and circular plasmids, at least 17, with size from 5 to 60 kbp and 2 to 30 kbp, respectively. The full nucleotide sequence of *Borrelia burgdorferi* B31 strain has been determined (Fraser *et al.*, 1997). The most typical *Borrelia burgdorferi* surface-expressed membrane proteins are *OspA* (822 bp) and *OspB*; these genes are located in the 50 kbp linear plasmid lp54. The *OspC* gene is located in the 27 kbp circular plasmid (Marconi *et al.*, 1994; Jonsson and Bergstrom, 1995). Flagelline gene *FlaB* (1011 bp long) is located in the chromosomal DNA at positions 147.649-148.659.

The aim of this work was to show the topicality of LB, study the tick activity and percentage of infected ticks in Latvia by various methods, and, on the basis of obtained results of three years of studies, to design outlines for monitoring programmes for this important tick-borne disease in Latvia.

MATERIALS AND METHODS

Tick collection. Tick collection was conducted in all the administrative districts of Latvia in peridomestic and sylvatic tick habitats. Ticks were collected from vegetation by flagging. There are two monitor sites of *Ixodes ricinus* in Rīga and Rīga District: in Mežaparks (Rīga center) and in the Babīte Forestry District, where ticks were collected by flagging in the transects periodically three times a month over a season. Some activity observations were conducted in the *I. persulcatus* distribution area, in Saikava (Madona District). Approximately 20 ticks from each geographical district of Latvia were prepared for the PCR investigations in each of the three last years.

Tick processing for analysis. Ticks were analysed for the presence of *Borrelia* by two methods: microscopic examination and polymerase chain reaction (PCR). Initially, for the comparative studies (1997), ticks were dissected and midguts were divided into two parts. One part was studied by microscopic examination, and the other was stored in a refrigerator (–20 °C) or in 70 % ethyl alcohol and used for DNA extraction and nested-PCR analysis. Later, collected ticks (years 1998–1999) were analysed separately by microscopy and PCR method.

Microscopic examinations. After the identification of the tick gender, stage and species, they were killed and two smear lines were made on a preparation glass. The fixed tick smears were stained in Giemsa-Romanovski and chrystalviolet solutions and then were examined under a microscope in an oil-immersion system (total magnification 750 times)

DNA preparation. Each tick or its part was transferred to 100 µl of TE buffer (10 mM Tris-HCl (pH 7,6), 1 mM ethylenediaminetetraacetate (EDTA) (pH 8,0)), and carefully crushed. Buffer with tick was extracted with 100 µl phenol/chloroform/isoamyl alcohol mixture (25:24:1), re-extracted with chloroform, and after centrifugation, the upper layer was transferred to a new tube and heated for 15 min (90 °C). DNA material was stored at –20 °C until use.

PCR. The method used was modified from literature (Priem *et al.*, 1997) for direct use in ticks without cultivation of *Borrelia*. We used four primers sets, two targeting the *B. burgdorferi* specific segment of flagellar filament 41 kDa core protein *FlaB* gene and two of linear plasmid lp54 located in the *OspA* gene (Figure 1, Table 1).

Oligonucleotide primers were synthesised in the BMC on an automated DNA synthesiser (Pharmacia, Sweden). PCR amplifications were performed in a Thermal Cycler (Biometra). Both first- and second-round nested PCR amplification were conducted in 50 µl reaction volumes. Each reaction mixture contained 26 µl deionised water, 5 µl 10x PCR am-

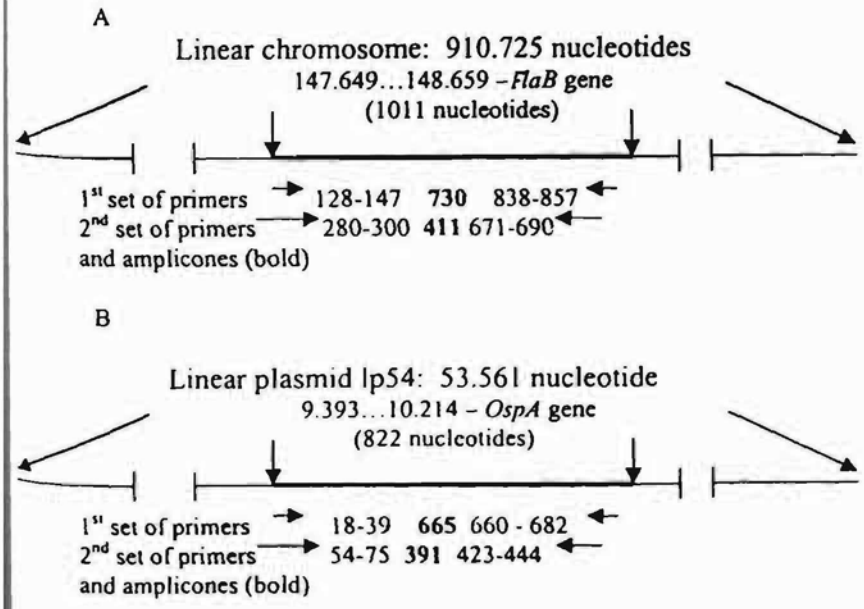

Fig 1. Borrelia burgdorferi B31 chromosome (A) and linear plasmid lp54 (B) coding *FlaB* and *OspA* genes, respectively, and used primers and products of amplification.

Table 1

SEQUENCES OF THE OLIGONUCLEOTIDE PRIMERS USED (Priem *et al.* 1997)

Gene	Primer	Use in PCR	Length Nt	Location in the gene	Sequence (5'-3')
OspA	PtZS7	1.PCR Forward primer	22	18–39	GGGAATAGGTCTAATATTAGCC
	OspA5	1.PCR Reverse primer	22	660–682	CACTAATTGTTAAAGTGGAAGT
	OspA6	2.PCR Forward primer	23	54–75	GCAAAATGTTAGCAGCCTTGACG
	OspA8	2.PCR Reverse primer	22	423–444	CTGTGTATTCAAGTCTGGTTCC
FlaB	Fla1	1.PCR Forward primer	20	128–147	CTGCTGGCATGGGAGTTTCT
	Fla2	1.PCR Reverse primer	20	838–857	TCAATTGCATACTCAGTACT
	Fla3	2.PCR Forward primer	21	280–300	GCAGTTCAATCAGGTAACGGC
	Fla4	2.PCR Reverse primer	20	671–690	AGAAGGTGCTGTAGCAGGTG

plification buffer (NBI Fermentas, Lithuania), 7.5 µl (25 mM) $MgCl_2$, 2.5 µl (2 mg·ml^{-1}) bovine serum albumin (BSA) (NBI Fermentas, Lithuania), 2.5 µl (4mM) dNTP (NBI Fermentas, Lithuania), 0.2 µl Taq DNA polymerase (4 u·µl^{-1}, NBI Fermentas, Lithuania), and 2 µl of each primer (20 µM). A 50 µl overlay of mineral oil (SIGMA) was added to the PCR tubes. 3 µl DNA prepared from ticks was added for PCR first-round amplification. 3 µl of the first-round PCR amplification product was employed as a template in a second PCR.

The amplification profile for the *OspA* gene first-round PCR consisted of 35 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 20 s, and extension at 72 °C for 60 s.

The amplification profile for the *Fla* gene first-round PCR consisted of 35 cycles of denaturation at 95 °C for 15 s, annealing at 46 °C for 20 s, and extension at 72 °C for 60 s.

The amplification profile for the *OspA* gene second-round PCR consisted of 25 cycles of denaturation at 95 °C for 15 s, annealing at 58 °C for 20 s, and extension at 72 °C for 30 s.

The amplification profile for the *Fla* gene second-round PCR consisted of 25 cycles of denaturation at 95 °C for 15 s, annealing at 54 °C for 20 s, and extension at 72 °C for 30 s. PCR was started with denaturation at 94 °C for 2 minutes, and ended with extra extension at 72 °C for 5 minutes.

An aliquot of each sample were analysed by electrophoresis in 1.5 % agarose and stained in ethidium bromide, visualised and documented by a Kodak imaging and analysis system (Figure 2). *OspA* gene fragment amplification by nested PCR resulted in a 391-bp product; flagellin gene fragment amplification by nested PCR resulted in a 411-bp product.

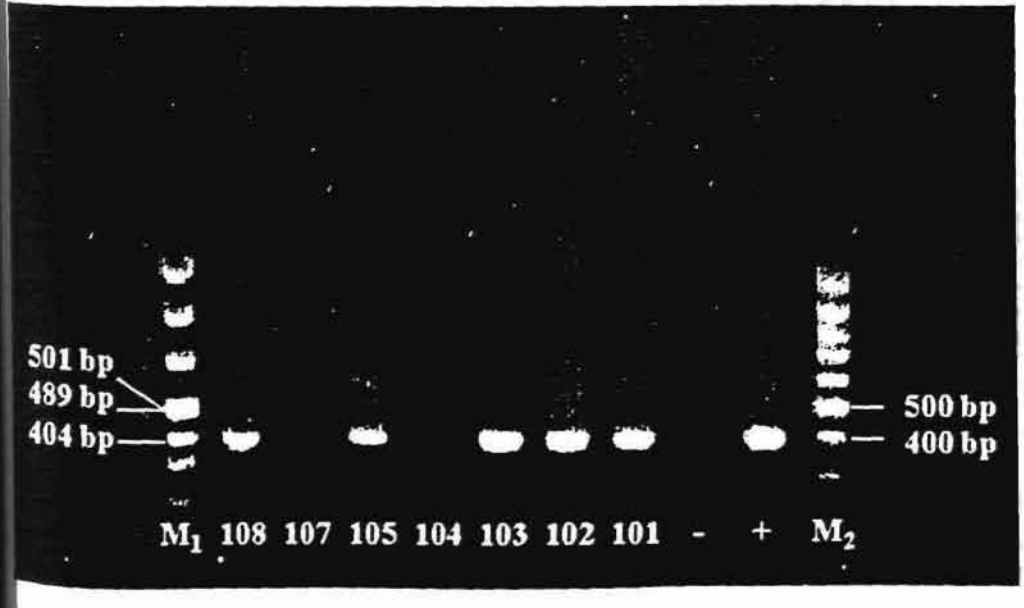

Fig. 2. OspA gene PCR amplification product analysed in 1.5% agarose gel.

Lane M_1, DNA size marker pUC Mix Marker, 8 (NBI Fermentas, Lithuania); lane M_2, DNA size marker GeneRuler^{tm} 100bp DNA Ladder Plus (NBI Fermentas, Lithuania). lane +, positive control sample (3 µl TE buffer with DNA of the *B burgdorferi* sensu stricto strain B31 was used for PCR amplification). lane –, negative control sample (3 µl TE buffer without DNA was used for PCR amplification). lanes 107, 104 DNA samples prepared from ticks, negative result. lanes 108, 105, 103, 102, 101, DNA samples prepared from ticks, positive result (infected ticks)

Statistical evaluation. Significant differences among percentages of infected ticks were determined using mathematical paired Student's test method (Bailey, 1959).

RESULTS

There are two *Ixodes* species in Latvia: *Ixodes ricinus* and *Ixodes persulcatus*. Tick activity starts when the soil temperature rises to 5–7 ^{0}C. For mature *I. ricinus*, a long period of seasonal activity is typical in Latvia: from the end of March to the middle of November with two activity peaks in May (or June) and August (September), depending on meteorological conditions. The period of seasonal activity of mature *I. persulcatus* lasts till July, with one activity peak usually in May (June).

Long-term *Ixodes* tick observations showed a gradual increase of tick activity during 1993–2000 (Figure 3). Since 1990, the average seasonal tick number per 1 km (data from monitoring sites) of *I. ricinus* (imagos) has increased more than sixfold and that of *I. persulcatus* almost fivefold, with the exception in 1999, when the average tick density was considerably decreased due to weather conditions (frosts in May, dry and hot weather during summer). For *I. ricinus*, the first peak of the activity was always higher then the second one.

The highest activity of *I. ricinus* was in 1998, and the number of active ticks was high during the whole season, even in July when the tick activity usually decreases (Figure 3). That year, there was the highest incidence—583 cases—of LB recorded in Latvia. The activity of *Ixodes persulcatus* during 1998–2000 remained stable, but in 1999, the period of their activity began in the middle of April, earlier than usual. In both cases, the highest activity was observed from May till June.

The spirochete *Borrelia burgdorferi* in ticks *Ixodes ricinus* is usually located in the midgut; and in *Ixodes persulcatus*, mainly in salivary glands. Changes of the annual average infectivity rate of *Ixodes* with *Borrelia* are rather similar for both species. The average tick infectivity rate with *Borrelia* s.l. detected by means of microscopy of stained fixed tick preparations in 1997 was 12.5 %; in 1998, 9.0 %; and in 1999, 11.4 %. The infectivity level of *I. ricinus* was slightly higher (or similar) than that of *I. persulcatus* during 1997–2000.

To check the sensitivity of the nested PCR method, we used standard DNA of the reference strain *B. burgdorferi sensu stricto* (kindly provided by S. Bergstrom, Umeå, Sweden). The initial concentration of the DNA was 20 ng·μl^{-1}, and we ran nested PCR with 10-fold serial dilutions of this DNA. Equal sensitivities were achieved with the plasmid *OspA* gene primers and chromosomal *FlaB* gene primers. The smallest amount of the standard DNA that gave a positive result was 3 μl for first-round PCR, with a concentration of 1 fg·μl^{-1}, which was calculated to represent the a single microbial cell. This DNA was also used as a positive control for each PCR reaction.

The percentages of *Borrelia*-infected ticks determined by microscopic examination and PCR analysis (*OspA, FlaB* gene fragments) are shown in the Table 3. These data indicated more than twofold higher sensitivity of the molecular detection method, and likely reflects the real percentage of infected ticks. Our comparative studies showed that, in every case when borrelias were detected by microscopic examinations, PCR analysis was positive with any of the amplified gene fragments. However, not all genetic markers studied were with identical sensitivity. The most sensitive was the determination by *OspA* gene fragment (Figure 4), which was more than twofold of that of *FlaB* and *OspC* (not shown).

Statistical evaluation. In all years, the percentages obtained by microscopy and PCR method were significantly different.

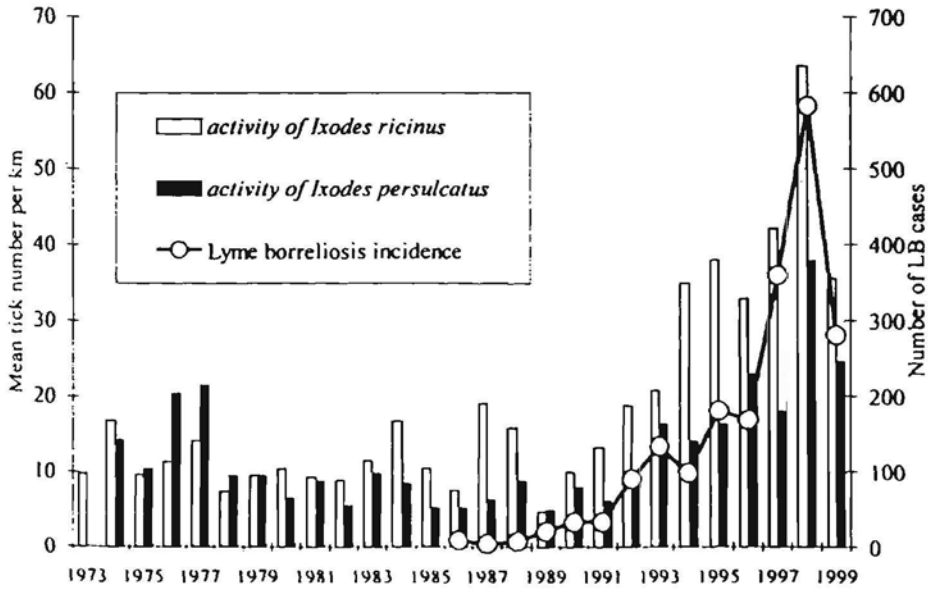

Fig. 3. Mean distribution of *Ixodes* tick number and Lyme borreliosis (LB) incidence by years.

Table 2

PERCENTAGE OF *Borrelia*-INFECTED *Ixodes ricinus* AND *Ixodes persulcatus* TICKS, EVALUATED BY MICROSCOPIC EXAMINATION AND POLYMEROSE CHAIN REACTION (PCR) (different samples)

		1997		1998		1999	
		Microscopy	PCR	Microscopy	PCR	Microscopy	PCR
Borrelia prevalence in both species together	total	M±SEM	M±SEM	M±SEM	M±SEM	M±SEM	M±SEM
		12.5%*# ±1.13	30.0 %♂ ±3.8	9.0 %* ±0.95	18.9% ±1.76	11.4 %* ±1.31	28.0 % ±4.51
	♀	12.9 %	nd	7.9 %	23.6%	13.1 %	25.0 %
	♂	11.9 %	nd	10.0 %	13.4%	9.6 %	31.25 %
Borrelia prevalence in *I.ricinus*	total	13.3 %	32.3 %	9.0 %	19.8%	11.6 %	25.9 %
	♀	13.8 %	nd	5.7 %	23.8%	13.1 %	20.0 %
	♂	12.8 %	nd	11.9 %	15.2%	10.0 %	27.3 %
Borrelia prevalence in *I.persulcatus*	total	10.8 %	18.9 %	9.0 %	17.9%	17.1 %	28.8 %
	♀	11.2 %	nd	10.4 %	23.3%	13.1 %	25.5 %
	♂	10.5 %	nd	7.6 %	10.9%	22.8 %	34.6 %
Number of investigated ticks	*imagos*	864	223	911	495	587	100

nd – not detected separately by sex.
* – $P<0.05$ microscopy vs PCR
Difference between percentage of infected ticks:
– $P<0.05$, year 1997 vs 1998, detected by microscopy
♂ – $P<0.05$, year 1997 vs 1998, detected by PCR
not significant, year 1997 vs 1999, detected by microscopy
not significant, year 1997 vs 1999, detected by PCR
not significant, year 1998 vs 1999, detected by microscopy
not significant, year 1998 vs 1999, detected by PCR

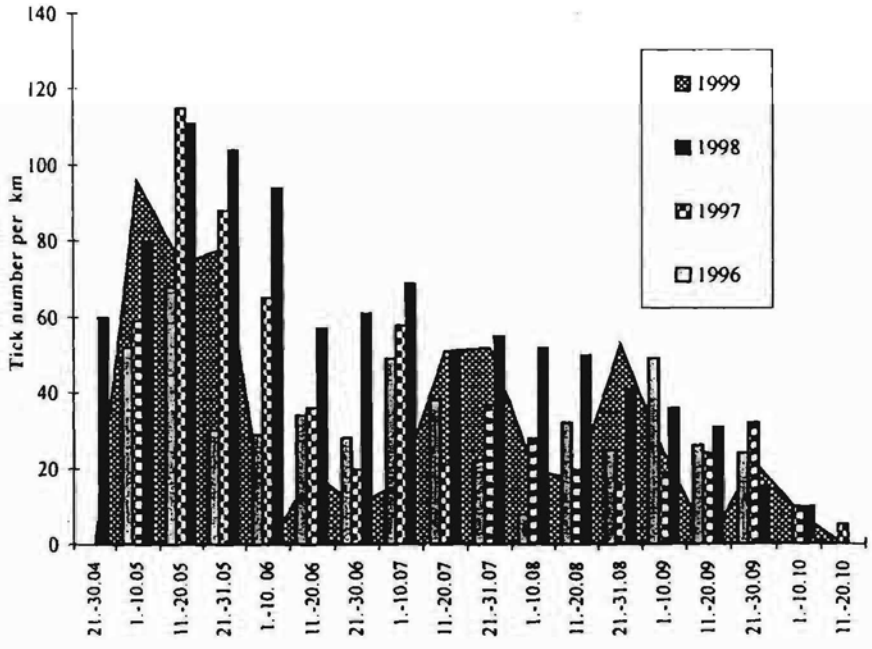

Fig. 4. Seasonal activity of *Ixodes ricinus* imagos in 1996 / 1999 (sylvatic tick habitat)

DISCUSSION

Tick-borne infections are an important medical problem in Latvia. Lyme borreliosis and tick-borne encephalitis cases have been registered in all of the administrative units of Latvia, but geographical distribution of cases has not been uniform. Also, the tick distribution throughout the territory is not even: their density varies strongly in dependence of the landscape type and vegetation (Bormane, 1999).

Observations during 1973–2000 show that a significant increase in density and spread of *I. ricinus*, as well as morbidity due to TBE and LB, occurred beginning from 1994, and since that time, the tick activity has not declined to the rate of the previous years. These data are consistent with observations in neighbouring countries (Postic *et al.*, 1997). The trends completely differ for the imago activities of *I. ricinus* and *I. persulcatus*, but they become somewhat similar in 1998–2000. The study of *I. persulcatus* habitats suggests an

increased *I. ricinus* proportion in eastern Latvia. Until 1995, *I ricinus* ticks were rare even in sites of persistent observations of *I. persulcatus*, but in 1996, during the first maximum activity, *I. ricinus* contributed 3 % of collected ticks; in 1997, 11 %; in 1998, 2 %; and in 1999, 2.4 %. These changes in the proportion of *I. ricinus* should be considered as different localisation of *Borrelia* in the tick body of a suitable species, which may modulate the infection process.

The microscopic detection of *Borrelia* shows differences in the annual infectivity level for tick males and females, which changes between years. This method has low sensitivity in comparison with the results of PCR, but is simple and serves as a rapid tool for screening of changes of the infectivity level.

The *Borrelia* prevalence in ticks detected by PCR showed an approximately two times higher infectivity level, which can be explained by the high sensitivity of this method. Theoretically, the presence of only one Borrelia in the tick is sufficient for detection. Therefore, rapid screening of ticks for the presence of borrelia genes by PCR after tick bites may be used in medical practice and prophylactic chemotherapy as one of the monitoring measures for Lyme borreliosis in Latvia.

The higher sensitivity of plasmid *OspA* gene, in comparison with the chromosomal *FlaB* gene in PCR reaction, may be explained by differences in their denaturation rate due to their length. Also higher mobility of the *OspA* gene has been shown by analysis of its expression rate depending on environmental temperature. The gene product *OspA* lipoprotein is immunogenic, and it has been used to construct a recombinant vaccine for American type borrelia. However, due to the heterogenity of *OspA* gene products in European borrelia types (Barbour, 1993), they have no vaccine, and hence further molecular analysis of the appropriate genes and immunogenicity of their products is the only means for development of their specific vaccines.

The clinical manifestation of Lyme borreliosis is likely to be tightly connected with a specific type of pathogen (Oksi, 1996). The pathogenic mechanisms and factors are unexplored, but a wide variety of clinical symptoms and outcomes of the disease strongly support such a speculation (Rowe, 2000). Molecular methods are the main tool for typing of *Borrelia* (Wang, *et al.*, 1999). Our studies have confirmed a high incidence of *Borrelia*-infected ticks in Latvia, and the existence of unnoticed clinical cases of Lyme borreliosis seems to be much higher than officially recorded. The clinical picture of observed cases is very variable, and hence the pathogenic role of different borrelia types must be suspected. Therefore, molecular typing of *Borrelia burgdorferi* s. l. in Latvia is a goal in establishing of the relationships between the molecular (antigenic) structure of the pathogen and the clinical picture of Lyme borreliosis. Further knowledge in this field would promote elaboration of rational therapeutic and prophylactic measures, taking into account the absence of specific prophylaxis against European variants of this tick-borne disease.

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ĒRČU INFICĒTĪBA AR *Borrelia burgdorferi* UN LAIMA BORELIOŽU IZPLATĪBA LATVIJĀ

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Rakstā aplūkota Laima boreliožu problēma Latvijā. *Ixodes* ērču izplatība un aktivitāte Latvijā kopš 1994. gada ir pieaugusi un līdztekus tai pēdējos piecos gados novērota pieaugoša saslimstība ar Laima boreliozēm. Analizēta ērču aktivitāte, izplatība, patogēna raksturojums un slimības klīniskie aspekti. Latvijā savāktām *Ixodes ricinus* un *Ixodes persulcatus* ērcēm eksperimentāli noteikta to inficētība gan mikroskopējot, gan ar divpakāpju polimerāzes ķēdes reakciju. Amplificējot chromosomālo *FlaB* un plazmīdu *OspA* un *OspC* gēnu fragmentus, atrasts apmēram 20–30 % ar *Borrelia burgdorferi sensu lato* inficētu ērču katrā no trim pēdējiem novērojumu gadiem. Balstoties uz iegūtajiem rezultātiem, tika veikta Laima boreliožu epidemioloģiskā analīze.

PAPER 2.

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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 became 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 lenght 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-μl PCR reaction volume contained a 3 μl 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 purifying with a DNA extraction kit (NBI Fermentas, Lithuania) according to the manufacturers 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 x^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. Prükk et al. 1999

Country	No. of ticks tested	*Borrelia* positive ticks (%)	% (No.) of ticks infected by different genospecies			
			B. afzelii	*B. garinii*	*B. burgdorferi* sensu stricto	*B. valaisiana*
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 Europe 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). Litherature 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 unsufficient 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 genospecie. 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 boreliozi) izraisa *Borrelia burgdorferi* sensu lato spirohetas, ko dabā pārnēsā ērces. Pētījumā ar 16S-23S rDNA starpgēnu rajona polimerāzes ķēdes reakcijas-restrikcijas 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.

PAPER 3.

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Identification of Three Clinically Relevant *Borrelia burgdorferi* Sensu Lato Genospecies by PCR-Restriction Fragment Length Polymorphism Analysis of 16S-23S Ribosomal DNA Spacer Amplicons

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We report the results of a study of the prevalences of three clinically relevant *Borrelia burgdorferi* sensu lato genospecies (*Borrelia burgdorferi* sensu stricto, *Borrelia afzelii*, and *Borrelia garinii*) in 1,040 questing *Ixodes* ticks from all regions of Latvia, where Lyme borreliosis is endemic. The prevalences of *Borrelia* in *Ixodes ricinus* and *Ixodes persulcatus* were 22.6 and 27.9%, respectively. Molecular typing of *B. burgdorferi* from infected ticks was performed by restriction fragment length polymorphism (RFLP) analysis of PCR-amplified fragments of the 16S-23S (*rrs-rrlA*) rRNA intergenic spacer by using species-specific primers and subsequent sequencing. The dominant *Borrelia* species in both *Ixodes* species was *B. afzelii*. In addition, different restriction patterns of *B. garinii* and *B. afzelii* were also identified. This study demonstrates that the 16S-23S rRNA PCR-RFLP typing method is simple, sensitive, and fast and that it allows one to differentiate among *B. burgdorferi* species and subspecies with various degrees of pathogenic potential directly in ticks. These features are important in monitoring Lyme disease.

Lyme disease (Lyme borreliosis [LB]), a disorder that can affect multiple organ systems, results from an infection with the spirochete *Borrelia burgdorferi* sensu lato and is the most common tick-borne human disease in Europe and the United States. Since *B. burgdorferi*, the spirochete responsible for this zoonotic infection, is phenotypically and genotypically heterogeneous, it causes variability in the clinical aspects of the disease. Arthritis and carditis are preferentially associated with *B. burgdorferi* sensu stricto, the degenerative skin disorder acrodermatitis chronica et atrophicans is primarily associated with *Borrelia afzelii*, and neuroborreliosis is primarily associated with *Borrelia garinii* (13, 18). However, *B. burgdorferi* sensu stricto is also responsible for human neuroborreliosis in the United States (31).

B. burgdorferi sensu lato spirochetes are maintained in enzootic spirochete-tick vector-vertebrate cycles. Incidental human exposure occurs when enzootic ticks from such maintenance cycles bite humans. The principal vectors of *B. burgdorferi* sensu lato are ticks of the *Ixodes ricinus* complex (2). Two tick species, *I. ricinus* and *Ixodes persulcatus*, are present in Latvia, and vector competence for these two species has been experimentally confirmed (3, 5, 8, 10, 24, 28). Identification of the pathogenic *B. burgdorferi* species in ticks has epidemiological importance, since they are prime factors in determining the risk of acquiring LB and its clinical presentation. However, in Latvia, only *I. ricinus* ticks collected in the vicinity of Riga have been examined for the presence of *Borrelia* species in previous studies (14).

The first cases of LB in Latvia were registered in 1986. There were 583 cases of LB in Latvia in 1998, 281 cases in 1999 (an incidence of 11.52 cases per 100,000 inhabitants), 472 cases in 2000 (19.35 cases per 100,000 inhabitants), and 379 cases in 2001 (16.02 cases per 100,000 inhabitants). Cases of LB were registered in all four regions of Latvia (Kurzeme, Vidzeme, Zemgale, and Latgale). In spite of the relatively small territories occupied by each of these regions, some of their differences could influence the prevalence of LB. First, only *I. ricinus* is found in the western part of Latvia (Kurzeme and Zemgale), whereas both tick species, *I. ricinus* and *I. persulcatus*, are found in the central and eastern parts of Latvia (Vidzeme and Latgale). Secondly, many colonial seabirds that may be reservoirs for LB, especially for *B. garinii* (9, 26), migrate primarily along the coast of western and central Latvia (Kurzeme and Vidzeme). Most patients get infected in the vicinity of their homes, and erythema migrans appears in the early stages of *B. burgdorferi* sensu lato infections in most Latvian LB patients (I. Lucenko and A. Bormane, Abstr. 5th Baltic-Nordic Conf. Tick-Borne Zoonosis, abstr. 4, 1998). Neuroborreliosis is a frequent manifestation of disseminated infection in Latvia, as confirmed by serologic testing in all of the cases (I. Logina and I. Supe, Abstr. 5th Baltic-Nordic Conf. Tick-Borne Zoonosis, abstr. 6, 1998). However, there are no data about the *Borrelia* genospecies causing neuroborreliosis in Latvia.

In order to establish an epidemiological context for the prevalence of LB in Latvia, we surveyed the prevalence of clinically relevant *B. burgdorferi* sensu lato species in *I. ricinus* and *I. persulcatus* ticks collected during 4 years from all four regions of Latvia.

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MATERIALS AND METHODS

Collection of ticks. Questing ticks were collected by flagging in the four regions of Latvia (Kurzeme, Vidzeme, Zemgale, and Latgale) in 1998, 1999, 2000, and

2001. Ticks attached to the flag were removed with tweezers, placed singly in a 1.5-ml Eppendorf tube, and kept frozen at −20°C until they were used. An average of 400 ticks was collected in each region of Latvia each year, altogether >6,000. By random choice, 1,040 ticks were selected (260 ticks from each year) for the studies of *Borrelia* prevalence.

Extraction of DNA from ticks. One hundred microliters of Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.6) was added to each sample tube, and each tick was crushed with a sterile plastic rod. Buffer containing the tick triturate was extracted with 100 μl of phenol-chloroform–isoamyl alcohol mixture (25:24:1; pH 8.0) by vortexing and subsequent centrifugation (11,000 rpm for 2 min; Eppendorf centrifuge 5417C; rotor FA 45-24-11) to separate the DNA from proteins. After centrifugation, the upper layer was transferred to a new tube and the DNA was reextracted with 100 μl of chloroform by vortexing and centrifugation (11,000 rpm for 2 min). The upper layer was again transferred to a new tube and heated for 15 min (90°C). Aliquots were frozen at −20°C until they were used.

Reference DNA. DNA samples isolated from six reference strains (*B. burgdorferi* sensu stricto B31, *B. burgdorferi* sensu stricto N40, *B. afzelii* F1, *B. afzelii* ACA 1, *B. afzelii* VS461, and *B. garinii* IP90) kindly donated by S. Bergstrom, Umeå, Sweden, were used as positive controls in all PCR-based methods.

***B. burgdorferi* detection by PCR amplification.** Nested PCR was carried out by the method described by Priem et al. (27). We used a primer set targeting the *B. burgdorferi*-specific segment of the *ospA* gene, located in linear plasmid lp54. A 3-μl aliquot of isolated DNA was employed for the PCR. The 50-μl reaction mixture contained 5 μl of 10-fold PCR buffer with $(NH_4)_2SO_4$ (MBI Fermentas, Vilnius, Lithuania) [final concentrations, 75 mM Tris-HCl, 20 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.01% Tween 20], 2.5 μl of bovine serum albumin (0.1 mg · ml^{-1}; MBI Fermentas), 100 mM each deoxynucleoside triphosphate, 1.5 U of *Taq* DNA polymerase (MBI Fermentas), and 30 pmol of each primer. First-round amplification employed PrZS7 (5′-GGGAATAGGTCTAATATTAGCC-3′; positions 18 to 39 of the *ospA* gene) as the forward primer and Osp5 (5′-CACTA ATTGTTAAAGTGGAAGT-3′; positions 660 to 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 microliters of the first-round PCR product was employed as a template in a second-round PCR with Osp6 (5′-GCAAAATGTTAGCAG CCTTGAT-3′; positions 54 to 75 of the *ospA* gene) as the forward primer and Osp8 (5′-CGTTGTATTCAAGTCTGGTTCC-3′; positions 423 to 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 the occurrence of false-positive PCR results, negative controls were included during extraction of the tick samples (1 control sample for every 20 tick samples). Each time the PCR was performed, negative and positive control samples were included. False-negative results due to inhibition of the PCR were excluded by the use of internal spike controls.

To avoid cross-contamination and sample carryover, pre- and post-PCR sample processing and PCR amplification were performed in separate rooms.

***B. burgdorferi* typing by PCR-restriction fragment length polymorphism (RFLP).** *ospA* gene-positive DNA samples were used for further analysis.

(i) PCR. A region of the *B. burgdorferi* 16S-23S (*rrs-rrlA*) ribosomal DNA (rDNA) spacer region was amplified by nested PCR with primers originally described by Liveris at al. (17). The use of the nested-PCR procedure resulted in an increased yield of product, allowing the use of this method directly on DNA extracted from ticks, thus obviating the necessity of culture. Six microliters of isolated DNA were employed for the PCR. The 50-μl reaction mixture contained 5 μl of 10-fold PCR buffer with $(NH_4)_2SO_4$ [final concentrations, 75 mM Tris-HCl, 20 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.01% Tween 20; MBI Fermentas], 2.5 μl of bovine serum albumin (final concentration, 0.1 mg · ml^{-1}; MBI Fermentas), 100 mM each deoxynucleoside triphosphate, 1.5 U of *Taq* DNA polymerase (MBI Fermentas), and 30 pmol of each primer. First-round amplification employed Pa (5′-GGTATGTTTAGTGAGGG-3′) as the forward primer and P95 (5′-GGTTAGAGCGCAGGTCTG-3′) 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 microliters of the first-round PCR product was used as a template in a second-round PCR with Pb (5′-CGTACTGGAAAGTGCGGCTG-3′) as the forward primer and P97 (5′-GATGTTCAACTCATCCTGGTCCC-3′) as the reverse primer. The amplification profile for the second-round PCR consisted of 25 cycles of denaturation at 95°C for 15s, annealing at 55°C for 20 s, and extension at 72°C for 30 s. A final extension step at 72°C for 5 min was carried out after both PCRs. Amplicons were visualized on a 1.5% agarose gel stained with ethidium bromide.

(ii) RFLP analysis. Ten-microliter aliquots of the nested-PCR amplification products were subjected to RFLP analysis by digestion with 2 U of either HinfI or TruI (MBI Fermentas). HinfI-digested fragments were analyzed by electrophoresis in a 1.5% agarose gel stained with ethidium bromide. TruI-digested fragments were analyzed by electrophoresis in 18% polyacrylamide gel stained with ethidium bromide or silver.

DNA sequencing. For questionable samples, DNA sequencing was used. A DNA extraction kit (MBI Fermentas) was used to purify PCR products from agarose gels according to the manufacturer's instructions.

If only one PCR product was obtained (as a confirmed gel analysis), the DNA product was purified directly from the reaction mixture. Purification of the PCR products was carried out in 26-μl reaction volumes. Each reaction volume contained 25 μl of nested PCR product, 1 U of exonuclease I (U.S. Biochemicals, Cleveland, Ohio), and 0.9 U of shrimp alkaline phosphatase (U.S. Biochemicals). The reaction profile was 20 min at 37°C, 15 min at 80°C, and 5 min at 4°C. Four or 5 ml of the PCR product, depending on the amount of DNA, was used for DNA-sequencing reactions. For these reactions, fluorescence-labeled dideoxynucleotide technology was used (Applied Biosystems, Inc., Foster City, Calif.). The sequenced fragments were separated, and the data were registered on a PRISM 3100 automated DNA sequencer (Applied Biosystems, Inc.).

Species-specific PCR. Species-specific PCR that targeted the 16S rRNA gene for the typing of *B. garinii* and *B. burgdorferi* sensu stricto was performed as described elsewhere (15).

Statistical methods. *Statistical significance was calculated using the* χ^2 *test.*

Nucleotide sequence accession numbers. The *rrs-rrlA* intergenic spacer sequences of *B. burgdorferi* found in this study are available in the GenBank database under accession numbers AY121817, AY121816, AY163781, AY163782, and AY163783.

RESULTS

Prevalence of *Borrelia* in ticks. In this study, 517 *I. ricinus* and 523 *I. persulcatus* ticks were analyzed by nested PCR for the presence of *B. burgdorferi* sensu lato. The results showed that 117 (22.6%) of the *I. ricinus* and 146 (27.9%) of the *I. persulcatus* ticks were PCR positive. In summary, 263 (25.3%) of the 1,040 ticks collected in four regions of Latvia in different years were infected with *B. burgdorferi* sensu lato species.

Tests of the sensitivity of the method showed that the smallest amount of DNA that gave a positive result represented three spirochetes in each PCR mixture (data not shown).

Typing of *B. burgdorferi* directly in ticks by RFLP analysis. By random choice, a total of 100 DNA samples prepared from field-collected ticks that were positive for the presence of *Borrelia* and six reference strains (*B. burgdorferi* sensu stricto strain B31, *B. burgdorferi* sensu stricto strain N40, *B. afzelii* strains F1, ACAI, and VS461, and *B. garinii* strain IP90) were analyzed by PCR-RFLP using the two restriction enzymes HinfI and TruI. The smallest amount of DNA that gave a positive result represented six spirochetes in each PCR mixture (data not shown). A PCR amplicon was obtained from each of 71 DNA samples (71%). The PCR amplicon sizes differed and were species specific (Fig. 1). Five different HinfI (H1 to H5) and six different TruI (A, B, C, D, E, and F) restriction patterns were obtained from isolated tick DNAs. A typical result of HinfI RFLP analysis is shown in Fig. 2, and the results of all the analyses are summarized in Table 1.

PCR-RFLP patterns obtained from tick samples were compared with those obtained from reference strains. Interestingly, only the two *B. burgdorferi* sensu stricto reference strains used and two of the tick samples gave the expected 940-bp amplicon with the Pa, Pb, P95, and P97 primers. After digestion with HinfI, three different PCR-RFLP patterns were observed; both

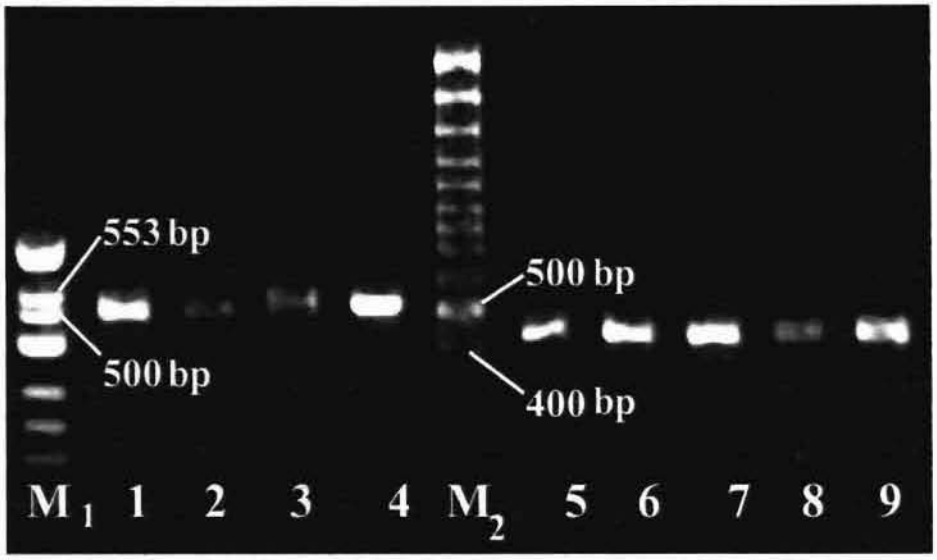

FIG. 1. PCR products of the 16S-23S rDNA spacer from *B. burgdorferi* sensu lato tick isolates. The species assignments of the strains are given in Table 1. Amplification was carried out using the Pa, Pb, P95, and P97 primer set. DNAs were electrophoresed on a 1.5% agarose gel in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA), stained with ethidium bromide, and visualized by UV illumination. A ϕX174 DNA/HinfI marker (M_1; Fermentas) and a 100-bp Gene Ruler (M_2; Fermentas) were used as DNA molecular size markers. Lane 1, tick Nr77; lane 2, tick Nr22; lane 3, tick Nr65; lane 4, *B. garinii* strain IP90; lane 5, *B. afzelii* strain ACAI; lane 6, tick Nr27; lane 7, tick Nr26; lane 8; tick Nr25; lane 9, tick Nr21.

tick samples gave identical patterns (pattern H4E [Fig. 3, lanes 5 and 6]). The *B. burgdorferi* sensu stricto B31 PCR-RFLP pattern with digestion enzyme HinfI corresponded to its sequence in GenBank (372, 306, and 262 bp).

All three *B. afzelii* reference strains (F1, ACAI, and VS461), *B. garinii* reference strain IP90, and most samples from ticks (except those with the H4E and H5F patterns [four samples]) gave much shorter amplicons (532 to 430 bp). The H1A and the H1B RFLP types, obtained from 39 (54.9%) and 6 (8.5%) of the *Borrelia*-infected ticks, respectively, were sequenced. These patterns showed 100 and 98% similarity to the *B. afzelii* reference strains ACAI and VS461 (the two reference strains showed identical PCR-RFLP patterns). The H2C RFLP pattern, obtained from nine (12.7%) samples, was identical to the

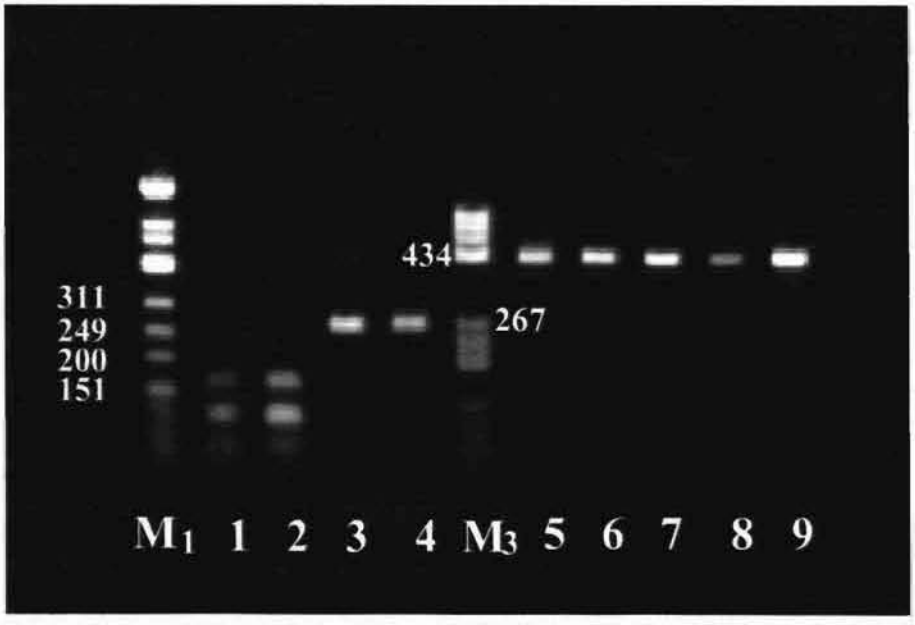

FIG. 2. HinfI restriction patterns of the amplified 16S-23S rDNA spacer from *B. burgdorferi* tick isolates. The species assignments of isolates are indicated in Table 1. DNAs were electrophoresed on a 1.5% agarose gel in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA), stained with ethidium bromide, and visualized by UV illumination. A ϕX174 DNA/HinfI marker (M_1; Fermentas) and a pBR322 DNA/BsuRI marker, (M3; Fermentas) were used as DNA molecular size markers. Lane 1, tick Nr77; lane 2, tick Nr22; lane 3, tick Nr65; lane 4, *B. garinii* strain IP90; lane 5, *B. afzellii* strain ACAI; lane 6, tick Nr27; lane 7, tick Nr26; lane 8, tick Nr25; lane 9, tick Nr21.

TABLE 1. PCR-RFLP typing of *B. burgdorferi* isolates from *Ixodes* ticks

Digestion pattern[a]		No. (%) of DNA samples			Genospecies of *B. burgdorferi* sensu lato
HinfI	TruI	Total	*I. ricinus*	*I. persulcatus*	
H1	A	39 (54.9)	18	21	*B. afzelii*
H1	B	7 (9.9)	0	7	*B. afzelii*
H2	C	9 (12.7)	8	1	*B. garinii*
H3	D	9 (12.7)	1	8	*B. garinii*
H4	E	2 (2.8)	2	0	*B. burgdorferi* sensu stricto
H5	F	2 (2.8)	0	2	Untyped
H1 and H2	A and C	3 (4.2)	2	1	*B. afzelii* + *B. garinii*

[a] Based on digestion pattern with either HinfI or TruI as defined in the text.

restriction pattern obtained from the *B. garinii* reference strain IP90. The PCR amplicon obtained from the *B. afzelii* reference strain F1 was ~500 bp long. However, similar PCR-RFLP patterns were not obtained from any of the tick samples (data not shown).

The H3D and the H4E RFLP patterns were obtained from 10 (14.1%) and 2 (2.8%) of the tick DNA samples, respectively. These patterns differed from the patterns given by the reference strains used in this study, and the strains were classified as *B. garinii* (H3D samples) and *B. burgdorferi* sensu stricto (H4E samples) based on the results of the species-specific PCR analysis.

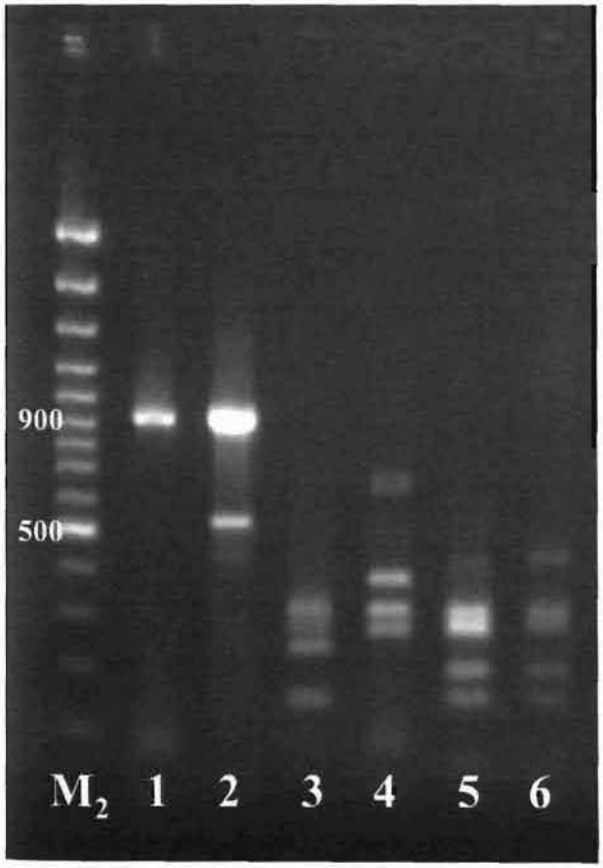

FIG. 3. PCR products (lines 1 and 2) and HinfI restriction patterns (lines 3 to 6) of the 16S-23S rDNA spacer from *B. burgdorferi* sensu stricto tick isolates. The species assignments of strains are given in Table 1. DNAs were electrophoresed on a 1.5% agarose gel in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA), stained with ethidium bromide, and visualized by UV illumination. A 100-bp Gene Ruler (M_2; Fermentas) was used as a DNA molecular size marker. Lane 1, *B. burgdorferi* sensu stricto strain B31; lane 2, tick Nr201; lane 3, *B. burgdorferi* sensu stricto strain N40; lane 4, *B. burgdorferi* sensu stricto strain B31; lane 5, tick Nr201; lane 6, tick Nr211.

Two of the DNA samples (2.8%) had an identical and definitely atypical H5F RFLP pattern that was not classified in this study.

Three tick samples (4.2%) had mixed RFLP patterns (both H1A and H2C), suggesting a double infection with both *B. afzelii* and *B. garinii* in these ticks. Both of the *B. burgdorferi* sensu stricto-infected ticks also showed evidence of a mixed infection, one with the *B. afzelii* H1A RFLP type and the second with the *B. garinii* H2C RFLP type.

The H1A RFLP pattern of *B. afzelii* was dominant in both *Ixodes* species. Interestingly, the H1B RFLP pattern was observed only in *I. persulcatus* ticks. The H2C RFLP pattern was observed mainly in *I. ricinus* ticks (eight ticks versus one tick; $P = 0.003$), whereas the H3D pattern predominated in *I. persulcatus* ticks (eight ticks versus one tick; $P = 0.035$).

The observed RFLP patterns from the DNA samples were completely consistent with the classification of *B. burgdorferi* into three distinct species (*B. afzelii*, *B. garinii*, and *B. burgdorferi* sensu stricto). The data presented in Table 1 demonstrate that all three clinically relevant *B. burgdorferi* sensu lato genotypes are found in Latvia.

The present study established that 16S-23S rDNA spacer analysis is both species and subspecies specific and can serve to identify *B. burgdorferi* sensu lato genotypes.

DISCUSSION

In the present study, for the first time in Latvia, *Ixodes* ticks collected from ecologically different regions of the country were examined and the prevalences of *B. burgdorferi* species in *Ixodes persulcatus* ticks were determined. Also in this study, the 16S-23S rDNA nested-PCR–RFLP typing method, originally used for the typing of *B. burgdorferi* in clinical specimens from early LB patients (12, 17), was extended to include the typing of *B. garinii* and *B. afzelii* species directly in field-collected ticks. Only a brief mention of such an approach was found in the literature (17).

In our study, the prevalence of *Borrelia* in field-collected ticks was 25.3%. This is in general agreement with the results obtained in other European countries, where the reported

mean rates for adult unfed *I. ricinus* ticks infected with *B. burgdorferi* vary from 3 to 58% (11). An extremely high prevalence of *B. burgdorferi*-infected ticks was found in Portugal (75%), where the majority belonged to the *Borrelia lusitanie* type (4). In the Baltic St. Petersburg and Kaliningrad regions of Russia, infection rates of 26.3% for *I. persulcatus* and 11.5% for *I. ricinus* have been reported, whereas in the Western Siberian region of Russia, 38% of the *I. persulcatus* ticks were infected (1, 22). The infection rate of *I. persulcatus* ticks in our study (27.9%) is similar to that obtained in the neighboring Baltic regions of Russia, but the prevalence of *Borrelia* in *I. ricinus* in Latvia is higher (22.6%). There was no significant difference between rates of infection of *I. ricinus* and *I. persulcatus* with *B. burgdorferi* in Latvia. However, an infection rate of 31.3% was reported in *I. ricinus* ticks from the Riga area of Latvia in a previous study (14). These differences can be explained by differences in the strategy used in collecting the ticks. A recent study by Jenkins et al. (13) shows that samples taken at different times and from different locations may differ greatly in the prevalence of *Borrelia*. Therefore, in order to minimize these differences, we examined ticks randomly chosen from those collected in all four regions of Latvia throughout the 4-year period studied.

Various methods have been used to type *B. burgdorferi* sensu lato in culture isolates, but only a few of them are useful for typing *Borrelia* directly in ticks. The most popular are the PCR-based methods, such as species-specific PCR, in which the conserved 16S rRNA gene (15) or species-specific plasmid gene locus (20, 21) is targeted, the 23S-5S rRNA spacer region PCR hybridization method (29), and the rDNA PCR-RFLP analysis of the *rrs-rrlA* intergenic spacer (17). The 16S-23S rDNA nested-PCR–RFLP method used in our study was chosen because it is relatively inexpensive and could be applied without isolating *Borrelia* from culture. The rDNA cluster of most *B. burgdorferi* sensu lato strains is located in the center of the linear chromosome and is arranged in the following order: *rrs-rrlA-rrfA-rrlB-rrfB* (6, 7, 25, 30). One of the unusual properties of the rRNA gene cluster is the presence of a large (>3-kb) and highly variable intergenic spacer between the 16S (*rrs*) and 23S (*rrlA*) rRNA genes (16). To our knowledge, this method had not been used previously for typing *B. garinii* and *B. afzelii* directly in ticks from Europe. The amplification of the partial *rrs-rrlA* spacer, followed by digestion with HinfI and TruI, gave both species- and subspecies-specific RFLP patterns. In agreement with the original reference describing this method (17), the amplicon size of *B. burgdorferi* sensu stricto B31, *B. burgdorferi* sensu stricto N40, and two of our tick samples, classified as *B. burgdorferi* sensu stricto, was 940 bp. However, the amplicon sizes of the three *B. afzelii* reference strains, the *B. garinii* reference strain, and most of our tick samples belonging to these strains were much smaller, 532 to 430 bp. This finding does not agree with the results of Liveris et al. (16), who reported that both *B. afzelii* and *B. garinii* have larger 16S-23S rDNA spacers than *B. burgdorferi* sensu stricto. However, more detailed information about the 16S-23S rDNA intergenic spacer sequences in strains other than those of *B. burgdorferi* sensu stricto is lacking. To address this requires the sequencing of the whole 16S-23S rRNA spacer in *B. burgdorferi* genotypes other than *B. burgdorferi* sensu stricto. One of the objectives of our study was to partially sequence this spacer in the *B. garinii* and *B. afzelii* genotypes and subtypes by using PCR amplicons. The sequences obtained were submitted to GenBank.

Five different HinfI (H1 to H5) and six different TruI (A, B, C, D, E, and F) restriction patterns were obtained from the isolated tick DNA. Two different *B. afzelii* subspecies were identified in Latvia: 54.9% of all infected *Ixodes* ticks carried the H1A RFLP type, and 9.9% had the H1B RFLP type. Interestingly, the *B. afzelii* H1B RFLP type was detected only in *I. persulcatus* ticks, common in far eastern Europe and Asia ($P = 0.0142$). There are also significant differences in the numbers of tick species carrying different *B. garinii* RFLP types: the H2C RFLP type was found predominantly in *I. ricinus* (eight ticks versus one tick; $P = 0.003$), whereas the H3D RFLP type was more common in *I. persulcatus* (eight ticks versus one tick; $P = 0.035$). This finding suggests that the infection is vector specific. To determine to what extent an association between a pathogen and a tick species exists, additional studies are required.

The *B. burgdorferi* sensu stricto strain detected only in two *I. ricinus* ticks from Latvia differed from the reference strains B31 and N40. The presence of different subtypes in Latvia is not surprising, since many previous studies have identified strain heterogeneity within these genomic groups (19, 32). To determine if genetic heterogeneity may be responsible for the wide variation in the symptomatology and severity of LB (23), there is a need for further studies of the pathogenicities of different subtypes of *Borrelia*.

Some mixed infections were also detected in our study. Three ticks were infected with *B. garinii* and *B. afzelii*, one with *B. burgdorferi* sensu stricto and *B. garinii*, and one with *B. burgdorferi* sensu stricto and *B. afzelii* spirochetes. Two *I. persulcatus* tick isolates with identical but atypical RFLP patterns that could not be identified require further investigation.

The seroepidemiological studies of the population living in LB areas of endemicity in Latvia showed that 2.3% had positive (immunoglobulin G) serum samples, indicating the existence of abortive, subclinical, and/or unnoticed clinical forms of LB. In contrast, 21.2% of neurological patients whose exposure to an infected tick in the area of endemicity was suspected were seropositive (Logina and Supe, Abstr. 5th Baltic-Nordic Conf. Tick-Borne Zoonosis). The high incidence of *Borrelia*-infected ticks and the prevalence of clinically relevant *B. burgdorferi* sensu lato genospecies in these ticks, together with the existence of different clinical forms of LB in Latvia, indicates that there may be a correlation between the clinical form and the genospecies of the causative agent.

This study showed that a relatively large human population is at considerable risk of contracting a *Borrelia* infection in Latvia. All three clinically relevant *B. burgdorferi* species are distributed in Latvia, with the dominant genospecies being *B. afzelii*. The 16S-23S rDNA nested-PCR–RFLP typing method was able to distinguish differences within all three genomic groups investigated and demonstrated that significant genetic heterogeneity exists among the *B. burgdorferi* subtypes in *I. ricinus* and *I. persulcatus* ticks. This study confirms that simple RFLP analysis can be a fast and useful tool for typing of *B. burgdorferi* isolates without culturing.

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PAPER 4.

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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 became 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 lenght 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-μl PCR reaction volume contained a 3 μl 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 purifying with a DNA extraction kit (NBI Fermentas, Lithuania) according to the manufacturers 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 x^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. Prükk et al. 1999

Country	No. of ticks tested	*Borrelia* positive ticks (%)	% (No.) of ticks infected by different genospecies			
			B. afzelii	*B. garinii*	*B. burgdorferi* sensu stricto	*B. valaisiana*
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 Europe 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). Litherature 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 unsufficient 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 genospecie. 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 boreliozi) izraisa *Borrelia burgdorferi* sensu lato spirohetas, ko dabā pārnēsā ērces. Pētījumā ar 16S-23S rDNA starpgēnu rajona polimerāzes ķēdes reakcijas-restrikcijas 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.

PAPER 5.

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GENOSPECIES OF *BORRELIA BURGDORFERI* S. L. IN *IXODES RICINUS* TICKS IN LITHUANIA

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Abstract. The tick *Ixodes ricinus*, the most important vector of Lyme disease caused by *Borrelia burgdorferi*, has spread throughout the territory of Lithuania. The Lyme disease is the most frequently reported vector-borne zoonosis in this country. The aim of the study was to estimate the density of *I. ricinus* and the prevalence of *B. burgdorferi* sensu lato in *I. ricinus* in Lithuania as well as to determine the bacterial genospecies. Two-hundred and four ticks were collected in eight districts of Lithuania and tested individually for *Borrelia* genospecies using polymerase chain reaction (PCR) techniques to identify both *B. burgdorferi* s. l. and its genospecies (*Borrelia garinii*, *B. afzelii*, *B. burgdorferi* sensu stricto). Prevalence of *Borrelia* infection in adult ticks fluctuated between 5% and 35% in different districts of Lithuania. *Borrelia afzelii* (9.3%) dominated over *B. garinii* (2.5%). Infection of *I. ricinus* with *B. afzelii* was recorded for the first time in Lithuania.
Key words: *Ixodes ricinus*, Lyme disease, *Borrelia burgdorferi* genospecies

Introduction

In Europe, in endemic foci the three-host tick *Ixodes ricinus* (Acari: Ixodidae) is the main vector of *Borrelia burgdorferi* sensu lato, the agent of Lyme diseases (LD). In Asia, *I. persulcatus* and *I. ovatus*, and in the USA, *I. scapularis* and *I. pacificus* are principal vectors of LD (Oksi 1996). Infected ticks transmit the *Borrelia* genospecies to animals, which serve as a source of infection for other ticks (Cinco *et al.* 1998).

Borrelia burgdorferi is a spirochete originally isolated by William Burgdorfer from the *I. dammini* tick from Shelter Island in New York in 1982. The spirochete was first characterised in 1984 and found to be a new species of the genus *Borrelia*. Since then, many strains of *B. burgdorferi* have been isolated from ticks, humans, and reservoir hosts (Tresova *et al.* 1998). It is now recognised that *B. burgdorferi* consist of a complex related genospecies: *B. afzelii*, *B. garinii*, *B. burgdorferi* sensu stricto, *B. lusitaniae*, and *B. valaisiana* (Hubalek & Holuzka 1997; Alekseev *et al.* 2001). It was shown that infection of *B. afzelii* could cause a chronic atrophic acrodermatitis in man, while that of *B. garinii* may lead to neuroborreliosis (Dubinina *et al.* 2000).

The *Ixodes ricinus* parasitises over 200 species of animals in Europe. The major reservoir hosts of *B. burgdorferi* are medium sized and small mammals, including rodents of the genera *Peromyscus*, *Apodemus*, and *Clethrionomys* (Oksi 1996). In Europe, particularly, yellow-necked field mouse (*Apodemus flavicollis*) and bank vole (*Clethrionomys glareolus*) are important reservoir hosts (Peltomaa 1999; Korenberg *et al.* 2002; Pawelczyk & Sinski 2002). These mammals are common in Lithuania (Motiejūnas 1974).

Ticks activity starts when the temperature of soil rises to 5–7°C. In Lithuania, the highest period of activity of ticks is from April to October (Žygutienė 2001).

Material and methods

Collection of ticks

Ixodes ricinus ticks were collected by flagging of low vegetation. Collection of ticks was carried out once between 2 and 21 May 2002. A total of 204 adult ticks (104 females and 100 males) were collected from eight districts of Lithuania (Fig. 1, Table 1). Tick density was estimated as a total number of collected ticks per 1 km of a route. Species, sex and stage of the development of all collected ticks were identified. Each tick was placed in a separate plastic tube and frozen at -20°C until further analysis. This investigation was a part of the investigation programme of *Borrelia burgdorferi* s. l. carried out in Latvia. Only, therefore ticks were collected in districts bordering Latvia.

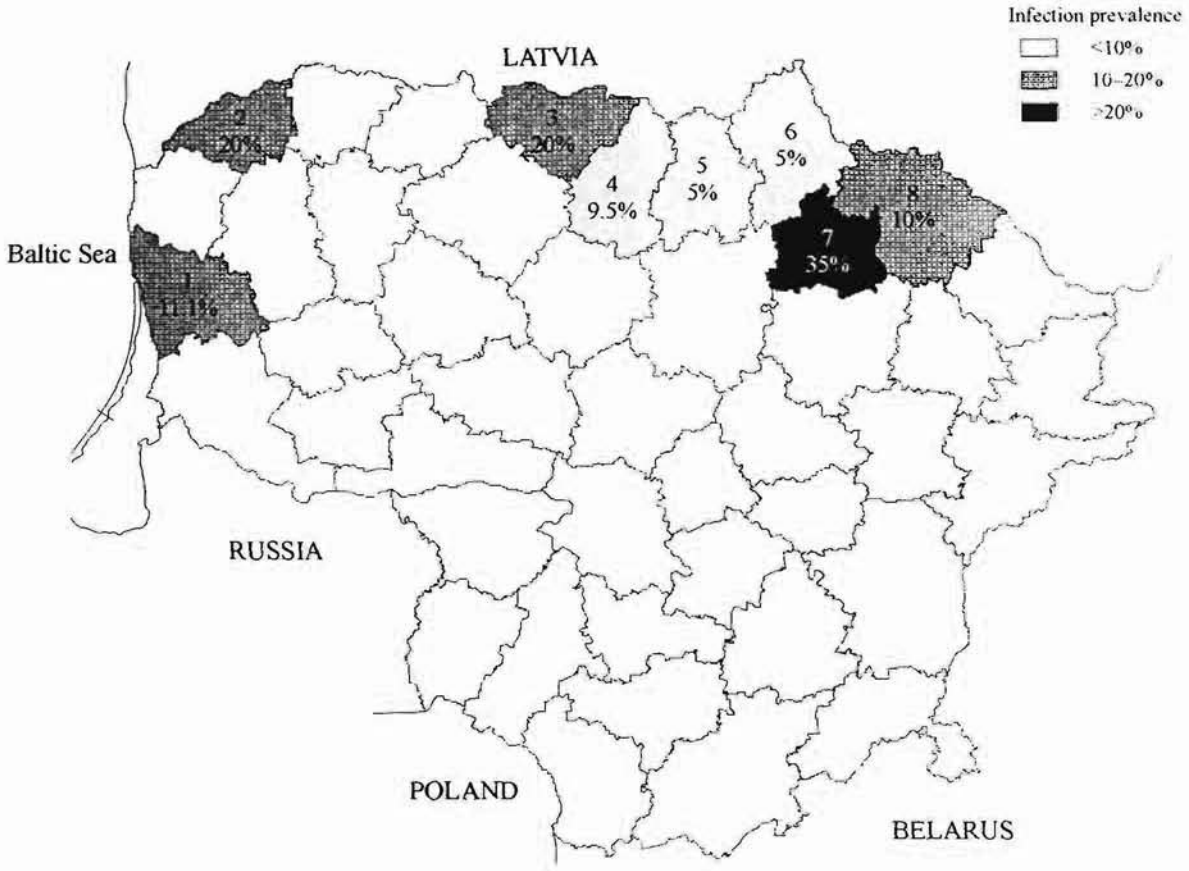

Figure 1. Distribution of tick collecting localities and the prevalence (percentage) of *Borrelia burgdorferi* s. l. infection in Lithuania. Investigated districts: 1 – Klaipėda, 2 – Skuodas, 3 – Joniškis, 4 – Pakruojis, 5 – Pasvalys, 6 – Biržai, 7 – Kupiškis, 8 – Rokiškis.

DNA preparation

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

Reference DNA

DNA samples isolated from four reference strains (*B. burgdorferi* s. s. B31, *B. afzelii* ACA 1, *B. afzelii* VS-461, and *B. garinii* Ip90), kindly donated by S. Bergstrom, Umeå, Sweden, were used as positive controls in all PCR-based methods.

***Borrelia burgdorferi* detection by PCR**

Two-hundred and four DNA samples isolated from *Ixodes* ticks were screened for the presence of *Borrelia burgdorferi* s. l. DNA.

DNA amplification targeted at the *OspA* gene located in linear plasmid lp54 was performed as described by Priem *et al.* (1997). The reaction conditions were modified. 50 µl PCR reaction volume contained 3 µl aliquot of isolated DNA, 100 mM deoxynucleoside triphosphates (NBI Fermentas, Lithuania), 1.5 U of *Taq* DNA polymerase (NBI Fermentas, Lithuania), and 30 pmol of each primer. The 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 were 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. Amplicons were visualised on a 1.5% agarose gel stained with ethidium bromide. The amplicon size was 391 bp for all *Borrelia* strains.

To monitor the occurrence of false-positive PCR results, negative controls were included during extraction of the tick samples: one control sample for each sample containing twenty ticks. Each time that the PCR was performed, negative and positive control samples were included. To avoid cross-contamination and sample carryover, pre- and post-PCR sample processing and PCR were performed in separate rooms, and plugged pipette tips were used for all fluid transfers.

Borrelia burgdorferi typing by polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP)

OspA gene-positive DNA samples were involved in further analysis in order to determine genospecies of *Borrelia*. Nested PCR targeting 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*1 (NBI Fermentas, Lithuania) according to the manufacturer's instructions. *Hinf*1-digested fragments were analysed by electrophoresis in a 1.5% agarose gel stained with ethidium bromide (Fig. 2).

Prevalence data were compared by Yates corrected chi-square test. A p value of 0.05 or less was considered significant.

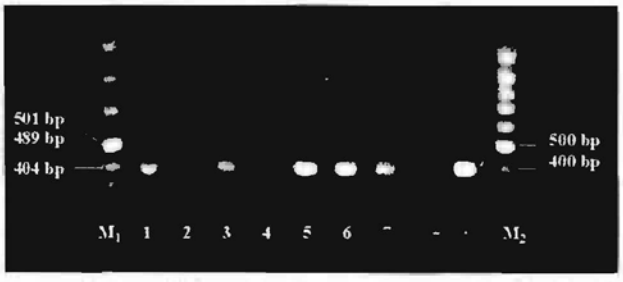

Figure 2. Borrelia burgdorferi s. l. detection in ticks. *OspA* gene PCR amplification product analysed in 1.5% agarose gel. Lanes M_1, M_2 – DNA size markers; lanes 1, 3, 5, 6, 7 – DNA samples prepared from infected ticks (positive result); lanes 2, 4 – DNA samples from uninfected ticks (negative result); lane - – negative control sample; lane + – positive control sample.

Results

All four *B. burgdorferi* reference strains, which represent three clinically relevant *Borrelia* genospecies (*B. burgdorferi* s. s., *B. afzelii* and *B. garinii*) gave a positive result in the PCR targeted *OspA* gene. Of the 204 DNA samples tested, 28 were positive.

16S–23S rDNA PCR-RFLP method used for the genotyping of positive *B. burgdorferi* tick samples gave species-specific PCR-RFLP patterns. It was approved by the use of three different *Borrelia* genospecies DNA. Only two PCR-RFLP patterns were obtained from *Borrelia* genospecies positive *I. ricinus* ticks. One of them represented *B. afzelii* (19 samples) and the other – *B. garinii* (five samples). Four tick samples remained untyped because of an insufficient amount of DNA. No mixed infections with different *Borrelia* genospecies were detected in the study.

Table 1 shows the density of ticks and the prevalence of their infection with different genospecies of *Borrelia* in eight districts of Lithuania. The density of ticks varied from one to 13 individuals per 1 km of a route (Table 1). The infection prevalence of *Borrelia burgdorferi* s. l. varied between 5% and 35% with the overall prevalence of 13.7%. Prevalences of two different *Borrelia* genospecies were detected. *Borrelia afzelii* was detected in all investigated localities, except Rokiškis district. *Borrelia garinii* was less frequently found. *Borrelia burgdorferi* s. s. was not detected. We were unable to detect *Borrelia burgdorferi* s. l. in 2% of infected ticks. The highest prevalence of infection was recorded in Kupiškis district (Table 1). The prevalence of *B. burgdorferi* s. l. in that area was significantly higher than in Biržai ($p < 0.005$), Klaipėda ($p < 0.05$), and Pasvalys ($p < 0.05$) districts, but the difference in the infection prevalence was insignificant compared to the data from Kupiškis, Joniškis, Pakruojis, Rokiškis, and Skuodas districts. There were no significant differences in the prevalence of *B. afzelii* and *B. garinii* in different districts of Lithuania (Table 1). However, in total, *B. afzelii* was significantly more prevalent than *B. garinii* ($p < 0.001$).

Discussion

The study shows that ticks infected with *B. burgdorferi* s. l. are common in Lithuania.

The increase of LD cases has been observed in Lithuania since 1993. The highest morbidity rate was registered in 2000 when over 1,713 cases of LD were recorded (on average, 46.3 cases per 100,000 inhabitants). Natural infection of *I. ricinus* with *B. burgdorferi* s. l. has

Table 1. Distribution of *Borrelia burgdorferi* genospecies isolated from the *Ixodes ricinus* ticks collected in different districts in Lithuania.

District	Tick density ind/km	n	Number of infected ticks (infection prevalence, %)	Number (%) of infections		
				Borrelia afzelii	*Borrelia garinii*	Not identified
Biržai	6	20	1 (5)	1 (5)	0	0
Joniškis	6	20	4 (20)	3 (15)	1 (5)	0
Klaipėda	10	63	7 (11.1)	5 (7.9)	0	2 (3.2)
Kupiškis	1	20	7 (35)	4 (20)	3 (15)	0
Pakruojis	3	21	2 (9.5)	2 (10)	0	0
Pasvalys	9	20	1 (5)	1 (5)	0	0
Rokiškis	13	20	2 (10)	0	0	2 (10)
Skuodas	3	20	4 (20)	3 (15)	1 (5)	0
Total		204	28 (13.7)	19 (9.3)	5 (2.5)	4 (2)

been reported in foci of LD in all districts of Lithuania, especially in Kaunas, Vilnius and Panevėžys districts (Žygutienė 2001). The overall prevalence of *B. burgdorferi* s. l. infection in *I. ricinus* was 8%, ranging between 0% and 24% in different districts (Žygutienė 2001). In Europe, the reported overall prevalence of *B. burgdorferi* s. l. infection in unfed adult *I. ricinus* ticks varied between 3% and 58% (on average 17.4%; Peltomaa 1999).

Two *Borrelia* genospecies, *B. afzelii* and *B. garinii*, are common all over Europe (Hubalek & Holuzka 1997). In Lithuania, like in other European countries such as Finland (Peltomaa 1999), Poland (Tresova *et al.* 1998), Italy (Cinco *et al.* 1998), and Russia (Tokarevich *et al.* 2002), *B. afzelii* and *B. garinii* appear to be the most common genospecies. Only two records of *B. garinii* had been known in Lithuania from ixodid ticks until the study (Hubalek & Holuzka 1997).

It is noteworthy that differences between the presence and prevalence of different *Borrelia* genospecies in *I. ricinus* and *I. persulcatus* ticks were recorded by Alekseev *et al.* (2001). They found that *I. ricinus* carried a mixed *B. garinii* and *B. afzelii* infection only in 0.9% cases (1 sample of 112) contrary to *I. persulcatus* (25.2% of infections in that tick were mixed). Mixed infections were not detected in *I. ricinus* in our study, which is in accordance to the study of Alekseev *et al.* (2001).

Ticks infected with *B. burgdorferi* s. s. were not found in northern Lithuania during the study. This confirms uneven distribution of different *Borrelia* species in European countries. In different studies, it was noticed that *B. burgdorferi* s. s is distributed mainly in western European countries and rarely found in eastern Europe (Wang *et al.* 1999). Infection of *I. ricinus* with *B. afzelii* was recorded for the first time in Lithuania. The occurrence of *B. afzelii* or *B. garinii* and the absence of *B. burgdorferi* s. s. may affect a clinical understanding of Lyme disease in Lithuania.

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BORRELIA BURGDORFERI S. L. GENOTIPAI *IXODES RICINUS* ERKĖSE LIETUVOJE

M. Žygutienė, R. Ranka, K. Salmina

SANTRAUKA

Tyrimų tikslas – nustatyti *Ixodes ricinus* erkių paplitimą, jų užkrėstumą Laimo ligos sukėlėju *Borrelia burgdorferi* s. l. bei jo genotipus. Ištirtos 204 erkės, surinktos aštuoniuose Lietuvos rajonuose (Biržų, Joniškio, Klaipėdos, Kupiškio, Pakruojo, Pasvalio, Rokiškio ir Skuodo), naudojant polimerazės grandininę reakciją. Tirtose vietovėse rasti 5–35% erkių, užsikrėtusių Laimo ligos sukėlėju. Daugiausiai rasta erkių, infekuotų *Borrelia afzelii* (9,3%) ir *Borrelia garinii* (2,5%). *Borrelia afzelii* aptiktas pirmą kartą Lietuvoje.

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