THE PREVALENCE OF *BORRELIA BURGDORFERI* IN *IXODES RICINUS* TICKS DETECTED BY PCR IN LITHUANIA

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Abstract. *Borrelia burgdorferi* sensu lato (s.l.), the aethiological agent of the zoonosis Lyme borrelosis (LB) is transmitted by ticks *Ixodes ricinus*. Distributed virtually throughout Eurasia and N. America, it is considered the most important human tick borne disease. Our study was the first attempt to determine the prevalence of *B. burgdorferi* (s.l.) infection in ticks *I. ricinus* in Lithuania by using polymerase chain reaction (PCR). Questing 477 *I. ricinus* ticks from different locations throughout Lithuania were collected. Furthermore, DNA from individual ticks was extracted and PCR was performed. The primers FL6 and FL7 were used for amplification the flagellin gene fragment of the spirochete genome. The products were visualized by electrophoresis. The mean prevalence of *B. burgdorferi* s.l. was 6.9% (33 ticks were positive) with range from 0 to 33% at the different locations of Lithuania. Since the prevalence of infected ticks in different locations was very variable, further studies and more detail investigations in Lithuania are needed.

Keywords: *Borrelia burgdorferi,* PCR, *Ixodes ricinus*, prevalence.

ERKIŲ *IXODES RICINUS* UŽSIKRĖTIMO *BORRELIA BURGDORFERI* NUSTATYMAS LIETUVOJE, TAIKANT PGR METODĄ.

Santrauka. Laimo liga plačiai paplitusi infekcinė liga, kurią sukelia spirocheta *Borrelia burgdorferi s.l.* įsisiurbus infekuotai erkei į žmogaus kūną. Šios studijos tikslas buvo nustatyti *Ixodes ricinus* erkių užsikrėtimo lygį Laimo ligos sukėlėju spirocheta *Borrelia burgdorferi* sensu lato įvairiuose Lietuvos rajonuose, naudojant molekulinius genetinius metodus. Kiekviena erkė buvo analizuojama individualiai. Buvo ištirtos 477 *I. ricinus* erkės iš įvairių Lietuvos rajonų (išskirta DNR, tirta polimerazės grandininės reakcijos metodu, naudojant specifinius pradmenis FL6 ir FL7. PGR produktai vizualizuojami ir vertinami UV šviesoje, atlikus elektroforezę 2% agarozės gelyje). Erkių tyrimai parodė, kad Lietuvoje erkių užsikrėtimas *B. burgdorferi* s.l. sukėlėju yra 6,9% ir svyruoja įvairiuose rajonuose nuo 0% iki 33%.

Raktažodžiai: *Borrelia burgdorferi,* PGR*, Ixodes ricinus,* paplitimas

Introduction. The spirochete *Borrelia burgdorferi* sensu lato (s.l) is the causative agent of Lyme borreliosis and is transmitted to humans primarily by tick of the genus *Ixodes Ixodes ricinus* is the main vector, responsible for the transmission of Lyme borreliosis, the widely distributed disease in Europe caused by the spirochetes *Borrelia burgdorferi* sensu lato. The rate of *Borrelia* infection in ticks is high, both adult and nymphs are responsible of epidemiological importance for transmission of *Borrelia* to humans (Dubinina et al., 2000). In Europe, five different *B. burgdorferi* sensu lato species are found *B. burgdorferi* sensu stricto, *B. lusitaniae*, *B. valaisiana*, *B. afzelii* and *B. garinii*. *Borrelia afzelii* and *B. garinii* are present throughout the continent (Alekseev et al., 2001, Hubalek & Halouzka, 1997, Bunikis et al., 1996).

Ticks *I. ricinus* also is common and widespread in Lithuania and *B. burgdorferi* s.l. occurs throughout the country. The cases of Lyme borreliosis are identified each year (Žygutienė, 2000).

The aim of our study was to determinate the prevalence of *Borrelia burgdorferi* s. l. infection in ticks *Ixodes ricinus* from various parts of Lithuania by using molecular methods. Ticks were tested individually for the presence of the spirochetes using polymerase chain reaction (PCR) technique able to identify *Borrelia burgdorferi* s.l. No investigations have been carried out to assess the prevalence of *Borrelia burgdorferi* infection in *Ixodes ricinus* in Lithuania using PCR method before. Recently, PCR technique has proved to be sensitive and specific method to identify of *B. burgdorferi* in infected ticks (Mommert et al., 2001).

Materials and methods. The study was conducted from 2001 to 2002 year and sampling was carried out from early spring to the early frost, of each year. The ticks were collected by flagging undergrowth with 1 m^2 white towel (Методические указания, 1987). Attached nymphs and adults collected into vials. *Ixodes ricinus* abundance was counted separately for females, males and nymphs during they activity period.

The *Ixodes ricinus* ticks were collected from the Vilnius, Marijampole, Panevėžys, Varėna, Klaipėda, Ukmergė, Šiauliai, and Ignalina districts. Specimens were preserved in 70% ethanol or refrigerated until processed. All specimens were identified as *I. ricinus* - like by their morphological characteristics (Померанцев, 1950).

The detection of *Borrelia burgdorferi* sensu lato.

The preparation of the DNA samples for PCR

All ticks were analysed individually. DNR extraction was carried out from homogenized tissue of ticks. Two

DNR extraction methods were used. First, commercial set „*Genomic DNA Purification Kit"* (MBI Fermentas, Lithuania) was used to free the DNR. In the second method ammonium hydroxide was used: all specimens were removed from ethanol and dried. The each adult individually and nymphs individually were immersed in 100 µl of 0,7M NH_4OH and crushed with pipette tips. The suspensions were boiled for 15-20 min in a heating block in a sealed vial. Then caps were opened and heating was extended for another 10 min. to remove ammonia and reduce the volume to 50 µl. The lysates were then stored at - 20°C until use for PCR (Kirstein et al. 1997, Stańczak et al., 1999).

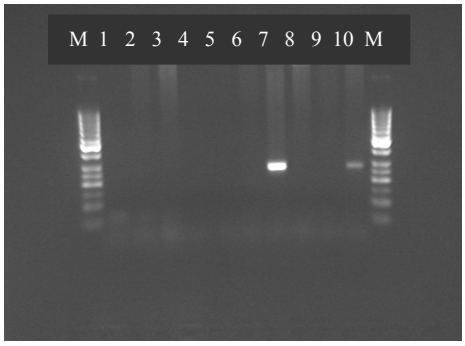
The DNA amplification and identification.

The ticks were tested individually for the presence of the spirochetes using polymerase chain reaction (PCR) techniques able to identify *Borrelia burgdorferi* s. l.. PCR was performed according to Stańczak et al., using the oligonucleotide primers: FL6: (5' TTC AGG GTC TCA AGC GTC TTG GAC T 3') and FL7 (5' GCA TTT TCA ATT TTA GCA AGT GAT G-3') in conserved regions of the *fla* gene of *B.burgdorferi.*

PCR was performed in reaction volume of 25 µl containing 0.2 µl *Taq* DNA polymerase (stock 5 U/µl), 2.5 µl 10x PCR Buffer, 2 µl $MgCl_2$ (stock 25mM), 2.5µl dNTPs mixture (stock 2.5 mM) (MBI Fermentas, Lithuania), 1.5 µl FL6 (stock 10 pmol/µl), 1.5 µl FL7 (stock 10 pmol/µl) (Roth, Germany), 10.8 µl double distilled water and 4 µl of the processed tick sample. In each PCR run we used positive and negative controls.

All reactions were carried out in Eppendorf PCR system "*Mastercycler personal"* thermal cycler. Samples were initially denatured for 1 min at 94°C. Subsequent cycles were at 94°C for 30 sec (denaturation), 55°C for 30 sec (annealing), and 72°C for 1 min (extension). The forty cycles were performed.

For the analysis of PCR amplification products, 10 µl aliquots of reaction mixtures were applied to 2 % agarose gels (MBI Fermentas, Lithuania) with Tris-Borate-EDTA (pH 8.2) as running buffer and electrophoresed for 1 h at 75 V. DNA bands were stained with ethidium bromide and visualized by UV transillumination (EASY Win32, Herolab, Germany). Achieved specific products of 276 base pares were considered as a positive result (Fig. 1).



M line – 50 bp. marker;
1 lane – negative control;
2-6 and 8, 9 lines – contains a negative *B. burgdorferi* s. l. PCR sample;
7 line – contains a positive *B. burgdorferi* s. l. PCR sample (276bp fragment);
10 line – positive control (276bp).

Figure 1. Results of PCR products analysis with specific oligonucleotide FL6 and FL7 primers.

Results and discusion. The four hundred seventy seven DNA samples were tested by PCR technique from ticks, which were collected in Vilnius, Marijampolė, Panevėžys, Klaipėda, Šiauliai, Ukmergė and Ignalina districts (Table 1). Achieved specific product of 276 base pairs was considered as a positive result (Fig. 1).

Table 1. Infection rate of *Ixodes ricinus* ticks with *Borrelia burgdorferi* s.l.

District	Examined (number)	Positive (number)	Positive (%)
Ignalina	43	0	0
Klaipėda	14	0	0
Marijampolė	36	12	33.3
Panevėžys	50	2	4.0
Šiauliai	45	0	0
Ukmergė	94	1	1.1
Varėna	32	2	6.3
Vilnius	163	16	9.8
Total	477	33	6.9

The 16 of 163 ticks from Vilnius district and 12 of 36 ticks from Marijampolė district were the majority of infected ticks by *Borrelia burgdorferi* sensu lato. In the Ukmergė, Panevėžys, Varėna districts the infection rates with *Borrelia burgdorferi* s. l. were 1.1%, 4%, 6.3% respectively, then in the Klaipėda, Ignalina and Šiauliai districts the investigated ticks were negative for *Borrelia burgdorferi* sensu lato by PCR (Table 1). Our findings show very different tick infection rates in surveyed areas, the percentages of infected ticks ranging from 0% to 33%. According the published data in the Lithuania in 1999, infection rates in *Ixodes ricinus* varied in different districts from 0 % to 27 % (Žygutienė, 1999). The high infection rates have been reported in a tick population in Marijampolė, Klaipėda and Tauragė districts (38.7%) (Motiejūnas, 1997). The infection rate of *Ixodes ricinus* was reported to be high in similar survey in Poland and Russia (Stańczak et al., 1999, Dubinina et al., 2000). Compared with data obtained in studies of the prevalence of *Borrelia burgdorferi* sensu lato in *Ixodes ricinus* ticks in other countries of Europe, which show the maximum values at about 43% (Cinco et al., 1998), our findings

showed the similar situation in Lithuania. The Lithuanian authors have been mentioned the high infection rate in Šiauliai – 18% (Žygutienė, 1999) and Klaipėda – 38.7% (Motiejūnas, 1997) districts. Nevertheless our findings show negative infection with *Borrelia burgdorferi* in these districts. The infection rates differences can be related to different years when ticks were collected. The variable percentages of *Borrelia burgdorferi* infected ticks can be observed during following years of investigations in the same locations (Stańczak et al., 1999). On the other hand, the reason can be in use of different methods of *Borrelia* detections in ticks. First studies of *Borrelia* detections in Lithuanian ticks were done by dark field microscopy. This might explain results of different rates of infection, because sometimes in dark-field-positive ticks the microorganisms seen may represent species other than *B.burgdorferi* sensu lato (Alekseev et al., 2001). Furthermore, the PCR have proved to be specific and sensitive method to detect the species-specific agent of Lyme borreliosis in infected ticks. Finally, it has been shown that in a large proportion of the dark-field-positive ticks no *Borrelia* DNA was detected by PCR (Liebisch et al., 2001). Infection of *Ixodes ricinus* with *Borrelia burgdorferi* sensu lato has been reported in Vilnius, Marijampolė, Ukmergė, Panevėžys, and Varėna districts in Lithuania (this study).

B. burgdorferi s.l. was detected in 6.9% of the ticks examined, and detection ranged from zero to 33% at the various sites. In conclusion, these results suggest that molecular tools can detect *Borrelia burgdorferi* in *Ixodes ricinus* and therefore provide a better understanding of the epidemiology of *Borrelia burgdorferi* sensu lato. This is the first extensive study of *Borrelia* in ticks from Lithuania using PCR. Further study and detail investigations are needed to clarify the epidemiology of Lyme disease agent in Lithuania.

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