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The prevalence and distribution of *Borrelia burgdorferi* sensu lato in host seeking *Ixodes ricinus* ticks in Lithuania

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A total of 1559 unfed *Ixodes ricinus* (L., 1758) ticks were collected from the vegetation by flagging in 18 locations of Lithuania. The ticks were tested individually for the presence of spirochetes using polymerase chain reaction (PCR) techniques able to identify *Borrelia burgdorferi* sensu lato (s. l.). The overall prevalence of *B. burgdorferi* s. l. infection by PCR was shown to be 13.4%. Both nymph and adult stages were infected. There was no significant difference in the prevalence of *B. burgdorferi* s. l. in female compared to male ($p = 0.355$). Arrangement of data according to landscape type into 3 groups (group I – agricultural land; group II – pine forest; group III – deciduous and mixed forest) showed that differences in the prevalence of *B. burgdorferi* s. l. among these groups are significant. There was no correlation between the prevalence of *B. burgdorferi* s. l. in ticks and geographical areas in Lithuania.

Key words: *Ixodes ricinus* ticks, *Borrelia burgdorferi* sensu lato, PCR, prevalence, distribution

INTRODUCTION

Human Lyme borreliosis (LB) is the most prevalent arthropod-borne infection in temperate climate zones around the world and is caused by *Borrelia* spirochetes. In 1982, the bacterium that causes LB was first isolated by Willy Burgdorfer and colleagues from the hard tick *Ixodes dammini* Say, 1821 (now *Ixodes scapularis* Say, 1821 [1]) collected on Long Island, N. Y. [2]. The isolate was subsequently identified as a new species of the genus *Borrelia* and was named *Borrelia burgdorferi* in 1984 [3]. Since then, hundreds of *B. burgdorferi* isolates have been cultured worldwide from various geographic regions and biological sources, including *Ixodes* ticks, their reservoir hosts, and specimens from patients with different clinical syndromes. Molecular analysis has indicated that these *B. burgdorferi* isolates are genetically divergent. A closely related cluster containing several tick-borne *Borrelia* species and genomic groups associated with LB have been defined [3–7]. The term "*B. burgdorferi* sensu lato" is now collectively used to refer to all *Borrelia* isolates within this cluster [4].

The principal vectors of *B. burgdorferi* s. l. are ticks of the *Ixodes ricinus* complex: *I. ricinus* (L., 1758) in Europe, *I. persulcatus* Schulze, 1930 in Asian Russia, China and Japan, and *I. scapularis, I. pacificus* Cooley and Kohls, 1943 in the United States [2]. In Europe, the reported mean rates of unfed *I. ricinus* ticks infected with *B. burgdorferi* vary from 0 to 11% (mean, 1.9%) for larvae, from 2 to 43% (mean, 10.8%) for nymphs, and from 3 to 58% (mean, 17.4%) for adults [8].

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The life cycles of *I. ricinus* comprises three stages (three host tick). Each stage (larva, nymph and adults) feeds for a few days on the host and then detaches and develops in the vegetation to the next stage; this process takes approximately one year. Both the questing and developing stages are sensitive to desiccation and require a relative humidity of at least 80% throughout the year, so that they are confined to areas where a good cover of vegetation is present. The differences in vegetation could influence tick abundance and the circulation of pathogens [9]. Peculiarities of vegetation in Lithuania are determined by its special position as a crossroad of boreal coniferous and temperate deciduous forest zones**.** Agrarian ecosystems occupy the largest land area in Lithuania (53.7%) [10], and forests occupy 30.1%. Pine woods comprise 37.6%, spruce groves 24.0% and birch groves 19.5% of all forests. The dominant trees in deciduous and mixed forests are spruce (*Picea abies*) and birch (*Betula pendula*). According to some authors, reservoir host abundance is an important but not a limiting factor for the maintenance of tick populations [11]. Ticks acquire spirochaetes from a wide variety of mammals and birds, but large mammals, for example, in some agricultural habitats, are rarely infected. The greatest tick infection prevalences occur in deciduous woodlands harbouring a diverse mix of host species [12].

Since information on the *Borrelia* present in the tick population is essential to our understanding of the epidemiology and prevention of LB, we conducted this study to determine the prevalence and distribution of *B. burgdorferi* among ground host-seeking *I. ricinus* ticks in different regions of Lithuania.

MATERIALS AND METHODS

Collection of ticks

In summer of 2003 and 2004 ticks were collected by flagging undergrowth with 1 m^2 white towel in 18 locations of Lithuania with different landscapes (agricultural land – type I of landscape; pine forest – type II of landscape; deciduous and mixed forest – type III of landscape) (Fig. 1, Table).

Immediately after collection, the ticks (males, females and nymphs) were immersed in 70% ethanol

Table. **Tick sampling locations and the prevalence of ticks**

Location	Total prevalence (% and number of infected ticks)	Number of tested ticks	Prevalence (no. of infected / no. of tested)		
			Females	Males	Nymphs
Group I					
Agricultural land					
Klaipėda	2.0(1)	51	1/19	0/21	0/11
Kretinga	2.4(1)	41	1/20	0/17	0/4
Panevėžys	4.0(2)	50	0/20	2/30	0/0
Ukmergė	1.1(1)	94	1/41	0/44	0/9
Average	2.1		3/100	2/112	0/24
Group II					
Pine forest					
Ignalina	8.2(10)	125	8/87	2/38	0/0
Vilnius	8.5(29)	343	20/177	9/146	0/20
Varėna	5.4(2)	37	1/16	0/18	1/3
Average	8.1		29/280	11/202	1/23
Group III					
Deciduous and mixed forest					
Šilutė	28.6(6)	21	6/18	0/3	0/0
Mažeikiai	12.9(4)	31	1/16	3/15	0/0
Kaunas	12.6(15)	119	9/38	6/39	0/42
Joniškis	11.1(5)	45	4/24	1/18	0/3
Šiauliai	15.5(18)	116	10/53	6/50	2/13
Kelmė	17.4(12)	69	1/37	11/27	0/5
Radviliškis	31.9(30)	94	13/54	13/35	4/5
Biržai	22.5(27)	120	17/73	10/44	0/3
Prienai	14.5 (9)	62	6/27	1/21	2/14
Marijampolė	25.0(15)	60	4/17	5/24	6/19
Utena	27.2(22)	81	10/40	12/41	0/0
Average	19.9		81/397	68/317	14/104
Total	**13.4(209)**	**1559**	**113/777**	**81/631**	**15/151**

Fig. 1. Map of Lithuania showing the location of tick collection and the prevalence of *B. burgdorferi* sensu lato infection (%)

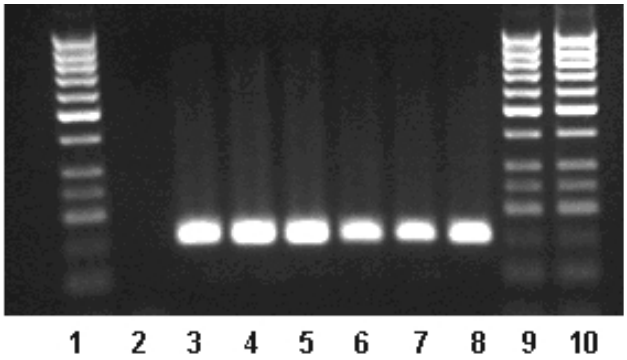

Fig. 2. Specificity of the *Ixodes ricinus* by PCR assay: 150 bp product of the 5.8s rRNA gene are specific for *I. ricinus.* Line 1, 9, 10: 50 bp marker; Line 2: negative control; Line 2–7: 150 bp specific fragment for *Ixodes ricinus*; Line 8: positive control (150 bp)

and stored at 4 °C until proceeded. All specimens were identified as *I. ricinus*-like by their morphological characteristics. A total of 1559 ticks (151 nymphs, 777 females and 631 males) were analyzed by molecular methods.

DNA extraction

All ticks were analysed individually. The ammonium hydroxide solution (2.5%) method with modifications was used for DNA extraction as described by Stańczak et al. [13]. The ticks were taken from ethanol solution, briefly dried on the paper towel and transferred to a test tube (Eppendorf; 1.5 ml) containing 100 µl of 2.5% NH4OH solution. The tubes were closed and placed in a heating block. The tubes with the mixture were incubated at 100 °C for 20 min or 99 °C for 25 min. After a brief centrifugation (in order to collect condensate from the cap and sides of the tube) all tubes with the lysate were placed back in the heating block with the cap open and incubated at 95 °C for 15 min to evaporate the ammonia. After incubation the tubes were closed and placed on ice for 1 min. After a brief centrifugation the tick lysate was stored at 4 °C until use as templates for PCR, or at –20 °C for longer periods.

Molecular identification of ticks

In the northern part of Lithuania in 1972 *I. persulcatus* another species of genus *Ixodes* was found [14]. Differences in the morphology of these two species are very small [9]. Besides, tick mouthparts and adjacent structures that are usually essential for identification may become damaged during the removal of ticks from its host. Consequently, these difficulties could be resolved by using keys based on molecular genetic markers.

Ticks were analysed using PCR techniques for taxonomic identification and for inhibition detection [15]. There were used Ixri-F (5' GGA AAT CCC GTC GCA CG 3') and Ixri-R (5' CAA ACg CgC CAA CgA AC 3') oligonucleotide primers. The 150 bp segment of the 5.8s rRNA gene, which is specific of *I. ricinus*, was amplified (Fig. 2).

Detection of *B. burgdorferi* s. l.

The ticks were tested individually for the presence of spirochetes using polymerase chain reaction (PCR) techniques able to identify *B. burgdorferi* s. l. PCR amplifications were performed with Mastercycler personal thermal cycler (Eppendorf, Germany). 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') of the conserved regions of the *fla* gene of *B. burgdorferi*, PCR was performed [13]. 25 µl of the reaction mixture containing 12.5 µl 2X PCR Master Mix (MBI Fermentas, Lithuania), 1.5 µl FL6 and 1.5 µl FL7 (stock 10 pmol/µl) (Roth, Germany), 5.5 µl double-distilled water and 4 µl of the processed tick sample. In each PCR run we used positive and negative controls. The cycle consisted of initial denaturation at 94 °C for 1 min, denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72°C for 1 min. The procedure was repeated 37 times, and the final extension was done at 72 °C for 3 min. For the analysis of PCR amplification products, 10 µl aliquots of reaction mixtures and 2 µl 6× loading day (MBI Fermentas, Lithuania), were applied to 1.5% agarose gels with Tris-Borate-EDTA (pH 8.2) as a running buffer and electrophoresed for 45 min at 75 V. The DNA bands were stained with ethidium bromide and visualized by UV transillumination (EASY Win32, Herolab, Germany). The obtained specific products of 276 base pairs were considered as a positive result (Fig. 3).

Statistical analysis

All calculations were done using the STATSOFT statistical package STATISTICA for WINDOWS 5.1, 1995.

RESULTS AND DISCUSSION

All specimens were identified as *I. ricinus*-like by their morphological characters [9] and by PCR with species-specific primers (Fig. 2).

The overall prevalence of *B. burgdorferi* s. l. infection by PCR was shown to be 13.4%. Both nymph and adult stages were infected. There was no significant difference in the prevalence of *B. burgdorferi* s. l. in female compared to male ($p = 0.355$). The prevalence of infection in the nymphs and adults is highly variable in Europe [16], adults generally showing a higher infection rate in a particular habitat, presumably because they have had the chance to become infected at both the larval and nymphal stages. Our data (Table) show that the infection rate increases with progressive tick instars: 10% of the nymphs and 13.8% of the adults were infected, but the difference was not significant ($\chi^2 = 1.74$, $p = 0.19$).

The distribution of Lyme disease is determined by the distribution of its vector *I. ricinus*, and environmental factors may limit tick population densities and the number of infected ticks [17].

It was important to take into account the distribution of vegetation, especially landscapes with southern-taiga dark coniferous forests or secondary forests replacing them and open nemoral broadleaved-mixed forests, which could influence not only the life cycle of *I. ricinus*, but also the density and distribution of the reservoir hosts. Both ecological factors could influence tick density and pathogen density also.

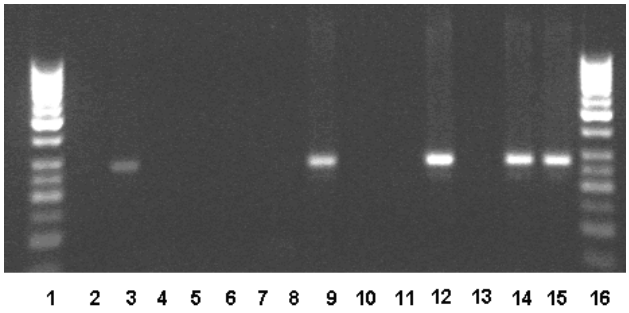

Fig. 3. Amplification (*fla* gene, FL6/FL7) of *B. burgdorferi* s.l. of *Ixodes ricinus* lysates demonstrated by agarose gel electrophoresis after etidium bromide staining. Line 1, 16: 50 bp marker; Line 2: negative control; Line 3, 9, 12, 14: contains a positive *B. burgdorferi* s. l. PCR sample (276bp fragment); Line 4–8, 10, 11, 13 contains a negative *B. burgdorferi* s. l. PCR sample; Line 15: positive control (276 bp)

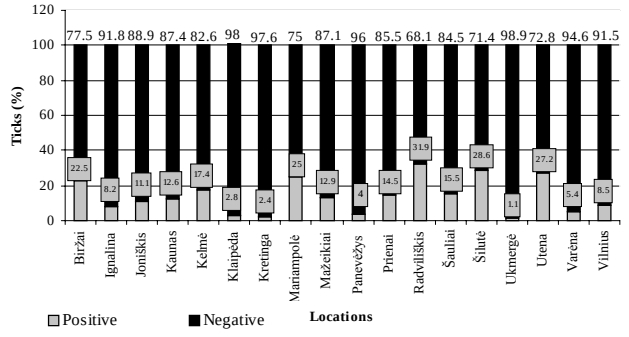

Fig. 4. The prevalence (%) of *B. burgdorferi* s.l. in *I. ricinus* ticks in different location of Lithuania

We arranged our data according to landscape type into three groups: group I – agricultural land; group II – pine forest; group III – deciduous and mixed forest. Differences in the prevalence of *B. burgdorferi* s. l. among these groups were significant ($p \leq 0.002$). The prevalence of *B. burgdorferi* s. l. in deciduous and mixed forests was highest (20%) (Table). Data on agricultural land suggests some evidence [17] for ixodid ticks that their mortality is higher in open, less humid habitat types and the density of ticks could correlate with *Borrelia* prevalence and distribution. The prevalence of *B. burgdorferi* s. l. was positively associated with deciduous and mixed forests, with spruce (*Picea abies*) and birch (*Betula pendula*) prevailing, although the largest wildlife biomass is found in deciduous and mixed forests [18]. In these types of landscape are dominant the primary and secondary hosts of ticks: yellow-necked mouse (*Apodemus flavicollis*), bank vole (*Clethrionomys grareolus*), roe deer (*Capreolus capreolus*), wild boar (*Sus scrofa*), moose (*Alces alces*) and white hare (*Lepus timidus*). In these forests migratory birds such as redstart (*Phoenicurus phoenicurus*), robin (*Erithacus rubecula*), thrush Nightingale (*Luscinia luscinia*), blackbird (*Turdus merula*), song Thrush (*T. philomelus*) nest. Several investigations in Europe and the Middle East have examined the role of birds as carriers of ticks infected with medically important pathogens [19, 20] and indicated that these species of migratory birds may play an important role in the dispersal of *I. ricinus* infected with the Lyme borreliosis agent, *B. burgdorferi* s. l.

A less prevalence (7.4%) was determined in pine forests with less humidity, minimal amounts of leaf litter, and very low numbers (2.4%) of infected ticks were found in agricultural land. This confirms findings [11] that the areas of suitable habitats for *I. ricinus* correspond to areas of a higher prevalence of *B. burgdorferi* s. l.

The geographical differences in the prevalence of *B. burgdorferi* s. l. were also detected (Fig. 1, 4, Table 1). The lowest prevalence was recorded in Ukmergė (1.1%), Klaipėda (2%), Kretinga (2.4%), Panevėžys (4%) and Varėna (5.4%) locations. The highest prevalence was recorded in Radviliškis (31.9%), Šilutė (28.6%), Utena (27%), Biržai (22.5%) and Marijampolė – 25% locations. In accordance with the previous studies in Europe [21], we have shown that the infection rate of *B. burgdorferi* s. l. spirochetes in *I. ricinus* ticks varies considerably among locations. The prevalence

of *B. burgdorferi* s. l. was shown to range from 1.1% (Ukmergė) to 31.9% (Radviliškis) (Table).

In conclusion, our results show that the prevalence of *B. burgdorferi* s. l. in ticks is variable and related with vegetation type, but there is no correlation between the prevalence of infection and the geographical area.

It is important to note that vegetation may be an important determinant of the tick, the main vector of *Borrelia burgdorferi* s. l. The prevalence and distribution of infection in the ticks may be dynamic in different locations, and investigation of landscape, ecology of Lyme disease vectors and reservoir hosts will continue to provide information potentially useful in reducing the prevalence of Lyme disease.

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Ixodes ricinus ERKIŲ UŽKRĖSTUMAS *Borrelia burgdorferi* SENSU LATO IR PASISKIRSTYMAS LIETUVOJE

Santrauka

Tirtas erkių *Ixodes ricinus* (L., 1758) užsikrėtimas Laimo ligos sukėlėju *Borrelia burgdorferi* sensu lato 18 Lietuvos vietovių. 1559 erkės (151 nimfa, 777 patelės ir 631patinėlis) ištirtos polimerazės grandininės reakcijos (PGR) metodu su specifiniais pradmenimis. Nustatytas vidutinis užkrėstumo *B. burgdorferi* s. l. lygis Lietuvoje – 13,4%. Tiek erkių nimfos, tiek suaugėliai buvo užsikrėtę, tačiau patikimų skirtumų tarp patelių ir patinų, taip pat skirtingose erkių vystymose stadijose nenustatyta. Užkrėstų erkių paplitimas siejamas su skirtingais augalijos tipais ir rezervuarinių šeimininkų gausa. Lapuočių ir mišriuose miškuose infekuotų erkių yra patikimai daugiau nei spygliuočių miškuose, o agrarinės aplinkos ekosistemose erkės yra mažiausiai užsikrėtusios. Ryšio tarp geografinės vietovės ir erkių užkrėstumo *B. burgdorferi* s. l. nenustatyta.