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PREVALENCE OF *BORRELIA BURGDORFERI* GENOSPECIES IN *IXODES RICINUS* TICKS FROM LUBLIN REGION (EASTERN POLAND)

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Abstract: The objective of the study was to determine the prevalence rate of 3 *Borrelia burgdorferi* genospecies in *Ixodes ricinus* ticks collected from wooded areas of the Lublin region (eastern Poland). A group of 1,813 *I. ricinus* ticks from 6 districts were examined for the presence of *Borrelia burgdorferi* sensu lato (*B.b.*s.l.) by polymerase chain reaction (PCR). Another group of 438 *I. ricinus* ticks collected from 4 districts were examined for the presence of *B.b.*s.l. by culture on BSKH liquid medium confirmed by PCR, and for the presence of *Borrelia* spp. by dark field microscopy (DFM). *Borrelia burgdorferi* genospecies (*Borrelia burgdorferi* sensu stricto, *Borrelia afzelii* and *Borrelia garinii*) were determined by nested-PCR in 113 ticks lysates showing presence of *B.b.*s.l. (in PCR or in culture and PCR). 5.4% of *I. ricinus* ticks examined by PCR showed the presence of *B.b.*s.l. DNA. The infection rate was highest in females (12.1%), lower in males (6.0%) and the lowest in nymphs (1.7%) ($p<0.001$). The minimum infection rate of *I. ricinus* ticks with *B.b.*s.l. determined by culture was 3.4%, whereas the minimum infection rate of ticks with motile spirochetes morphologically resembling *Borrelia* spp., determined by DFM, amounted to 11.2%. The presence of all 3 *Borrelia burgdorferi* genospecies under investigation was found in ticks collected from 5 out of 6 examined districts. In 81.4% of infected ticks only single infection with 1 genospecies was observed, while coinfections with 2 or 3 genospecies were detected respectively in 16.8% and 1.8% of infected ticks. *Borrelia burgdorferi* sensu stricto was the dominant genospecies in all examined tick stages and districts, both in single infections and in coinfections, and found in a total of 62.8% of *I. ricinus* ticks infected with *B.b.*s.l. *Borrelia afzelii* and *Borrelia garinii* were less frequent and observed in respectively 39.8% and 17.8% of infected ticks.

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INTRODUCTION

Lyme borreliosis (LB) is a multisystemic disease caused by *Borrelia burgdorferi* sensu lato (*B.b.*s.l.) spirochete which is sustained mainly by wild animals and transmitted by ixodid ticks to humans. LB is considered the most common vector-borne infection of the northern hemisphere [3, 7, 13, 18, 20, 22, 26, 31, 32, 40, 48, 54, 57]. The spread of borreliosis depends on geographical, environmental and climatic factors and pathogenicity of *B.b.*s.l. strains [10, 12, 14, 17, 19, 21, 24, 26, 28, 32, 38,

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46, 48]. The annual number of LB cases in Poland shows a growing tendency and amounted in 2005 to 4,406 [35].

The main pathogenic genomic species responsible for human LB in Europe are: *Borrelia garinii*, *Borrelia afzelii*, and *Borrelia burgdorferi* sensu stricto [3, 6, 8, 13, 18, 21, 23, 26, 32, 42, 43, 46, 48, 52, 57]. The relationship between the 3 above-mentioned genospecies and clinical manifestations of LB has been demonstrated by many authors [16, 17, 21, 26, 32, 41, 46, 48, 50, 57].

The evaluation of prevalence of *B.b.*s.l. in *Ixodes ricinus* ticks, which are the main vector of the pathogen in Europe, as well as the determination of its genospecies diversity can be an indicator for risk of acquiring *Borrelia* infection in an ecosystem by humans. The prevalence of *B.b.*s.l. in *I. ricinus* ticks is relatively well known, whereas knowledge of the occurrence of genomic species of *B. burgdorferi* in various areas in Europe insufficient to date [6, 13, 17, 18, 23, 25, 26, 42, 44, 51, 59].

The objective of this study was to assess the risk of borreliosis in the Lublin region (eastern Poland) by the examination of *Ixodes ricinus* ticks, and to determine the rate of infection of the ticks with 3 pathogenic genospecies of *Borrelia burgdorferi* sensu lato using PCR and nested-PCR methods.

MATERIALS AND METHODS

Collection of ticks. Unfed *Ixodes ricinus* ticks (adults and nymphs) were collected in spring/summer seasons in 2005-2006 on the territory of 7 districts of the Lublin region (eastern Poland). Of these, 2 districts (Parczew, Włodawa) harboured wet lakeland forests, while the other 5 districts (Lublin, Zamość, Kraśnik, Puławy, Lubartów) harboured dry upland forests. Ticks were collected by flagging lower vegetation at peripheral and inner parts of deciduous and mixed forests, including suburban localities and picnic areas. Collected ticks were examined for the presence of *Borrelia burgdorferi* sensu lato (*B.b.*s.l.) with 2 different methods as described below:

• A group of 1,813 ticks collected from 6 districts of the Lublin region (Lublin, Zamość, Włodawa, Puławy, Parczew, Kraśnik) were examined by polymerase chain reaction (PCR).

• Another group of 438 ticks collected from 4 districts of the Lublin region (Lublin, Zamość, Włodawa, Lubartów) were examined by dark field microscopy (DFM) and culture. The identity of isolated *B.b.*s.l. strains was confirmed by PCR.

All tick lysates showing presence of *B.b.*s.l. were examined for the presence of 3 Borrelia pathogenic genomic species (*B. afzelii*, *B. garinii*, and *B. burgdorferi* sensu stricto) by nested-PCR method.

Examination of ticks for the presence of *Borrelia burgdorferi* sensu lato by polymerase chain reaction (PCR). 1,813 ticks collected from 6 districts of the Lublin region were placed in glass tubes with 70% ethanol for further investigation. Bacterial DNA was isolated according to Rijpkema *et al.* [45] by boiling in 0.7 M ammonium hydroxide and stored at -70ºC. The isolates were examined for the presence of *B.b.*s.l. DNA by polymerase chain reaction (PCR) with oligonucleotide primer set FLA1/FLA2 (Eurogentec, Seraing, Belgium) specific for DNA *fla* gene sequence [49, 59, 60]. All adult ticks were investigated separately and nymphs in pools of 5 specimens.

In each PCR reaction were applied: • matrix DNA, • FLA1/FLA2 primers, • Polish strain Bo-148c/2 (obtained by courtesy of Dr. B. Wodecka, University of Szczecin) as a positive control, • redistilled water as a negative control, • thermostable polymerase (DyNAzyme™ II DNA, Finnzymes Oy, Espoo, Finland), • mixture of dTP nucleotides (DNA, Gdańsk, Poland). The amplification was carried out in a PTC-150 thermal cycler (MJ Research Inc., Waltham, MA, USA) according to Wodecka & Skotarczak [59]. The size of the amplified DNA fragment was 482 base pairs (bp). Amplification products were identified in 1.5% agarose gel, after electrophoresis in standard conditions and staining with ethidium bromide solution (2 µg/ml). Minimum infection rate in nymphs was calculated according to Kahl [29].

Examination of ticks for the presence of *Borrelia burgdorferi* s.l. by DFM and culture. 438 ticks collected from 4 districts of the Lublin region were checked for the presence of *Borrelia* spp. by DFM according to Štěpánová-Tresová *et al.* [56]. Briefly, ticks, after removal from the cloth, were kept alive in glass tubes for several days until examination. After rinsing in 40% ethanol and next in phosphate buffered saline (PBS) ticks were examined for the presence of motile *Borrelia* spp. spirochetes by the observation of extracts from tick's mitgut in BSKH liquid medium in a dark microscopy field at 312 × magnification under a Jenamed 2 (Germany) microscope. Adult ticks were examined in pools of 2 specimens and nymphs in pools of 5 specimens. The isolates showing presence of motile *Borrelia* spp. spirochetes were inoculated on the liquid BSKH medium (Sigma) containing bovine albumin fraction V and HEPES buffer [1]. Cultures were incubated at 33ºC and examined microscopically for the presence of spirochetes every 7 days over a period of 1 month. Bacterial DNA from cultured spirochetes was isolated by the ammonium hydroxide lysis [45] and examined by PCR to confirm the identity of *Borrelia burgdorferi* s.l. [49, 60]. Minimum infection rate was calculated according to Kahl [29].

Species identification of *Borrelia burgdorferi* sensu lato by nested-PCR reaction. All tick lysates in which the presence of *B.b.*s.l. was detected by PCR or culture/PCR methods were examined for the presence of 3 pathogenic *Borrelia* genospecies by nested-PCR reaction.

Species-specific primers BB1/BB2, BA1/BA2 and BG1/BG3 (Eurogentec, Seraing, Belgium) designed for differentiation *B. burgdorferi* s.l. into 3 genospecies (*Borrelia burgdorferi* sensu stricto, *Borrelia afzelii*,

Borrelia garinii) were used in nested-PCR reaction of 482 bp fragment of the first PCR reaction product, obtained with FLA1/FLA2 primers [51, 59]. The reagents applied in nested-PCR reaction were: • each of the 3 above-mentioned pair primers, • thermostable polymerase (DyNAzyme™ II DNA, Finnzymes Oy, Espoo, Finland), • dNTPs (DNA, Gdańsk, Poland).

The nested-PCR reaction was carried out in a thermal cycler (MJ Research, USA), according to Stańczak *et al.* [52] and Wodecka & Skotarczak [59]. The sizes of amplified DNA fragments were: 76 bp for *B. burgdorferi* sensu stricto (*B. burgdorferi* s.s.), 103 bp for *B. afzelii* and 125 bp for *B. garinii*. Amplification products were identified in 4% agarose gel, after electrophoresis in standard conditions and staining with ethidium bromide solution (2 µg/ml).

Statistical analysis. The data were analysed by χ^2 test and t-Student test with the use of STATISTICA for Windows v. 5.0 package (StatSoft Inc., Tulsa, Oklahoma, USA).

RESULTS

Prevalence of *Borrelia burgdorferi* sensu lato in ticks determined by PCR. As seen in Table 1, 5.4% of a total number of 1,813 *Ixodes ricinus* ticks examined by PCR showed the presence of DNA of *Borrelia burgdorgferi* sensu lato (*B.b.*s.l.). Adult females were infected in the greatest proportion equal to 12.1%, males in 6.0%, and the minimum infection rate in nymphs amounted to 1.7%. The variability of infection rate in individual tick stages proved to be statistically significant ($p<0.001$). Among the examined 6 districts, the greatest infection rate of ticks with *B.b.*s.l. (10.9%) was found in Parczew district, characterised by the presence of wet lakeland forests. The infection rate of ticks in the remaining 5 districts was within a narrow range of 4.3-4.6%, and the mean value (4.5%) was significantly lower compared to that found in the Parczew district ($p<0.001$).

Prevalence of *Borrelia burgdorferi* sensu lato in ticks determined by DFM and culture. The minimum infection rate of motile spirochetes morphologically resembling *Borrelia* spp. amounted to 11.2% of the total examined ticks. Significantly higher infection rates were noted in females and males (15.8% and 13.8% respectively) than in nymphs (5.4%) ($p<0.001$) (Tab. 1). 15 *Borrelia* isolates from 2 out of 4 examined districts showed the ability to grow in BSKH medium (Tab. 1, 3) and the presence of *B.b.*s.l. in the culture was confirmed by PCR.

Table 1. Prevalence of *Borrelia burgdorferi* sensu lato and *Borrelia* spp. in *Ixodes ricinus* ticks determined by PCR, culture, and dark field microscopy (DFM).

Stage/sex	Proportion of positive/examined (percent)		
Positive for:	*Borrelia burgdorferi* s.l.		*Borrelia* spp.
	PCR	Culture	DFM
Females	55/455 (12.1%)	7/133* (5.3%)**	21/133* (15.8%)**
Males	28/463 (6.0%)	4/138* (2.9%)**	19/138* (13.8%)**
Nymphs	15/895* (1.7%)**	4/167* (2.4%)**	9/167* (5.4%)**
Total	98/1813 (5.4%)	15/438* (3.4%)**	49/438* (11.2%)**

*examined in pools; **minimum infection rate

Totally, strains of *Borrelia burgdorferi* sensu lato were cultured from 3.4% of examined ticks. Similarly as in other examinations, the infection rate determined by culture was highest in females (5.3%), lower in males (2.9%) and the lowest in nymphs (2.4%) but the differences between individual stages were smaller and not significant ($p>0.05$). In contrast, significant differences were found between the prevalence of positive cultures in individual districts ($p<0.05$). The greatest infection rate determined by culture (8.3%) was found in Włodawa district which, similar to the neighbouring Parczew district, is covered with wet lakeland forests.

Prevalence of 3 genospecies of *Borrelia burgdorferi* sensu lato in ticks determined by nested-PCR. The examination of 113 tick lysates, showing presence of *B.b.*s.l. in PCR or culture, by nested-PCR revealed the presence of all 3 pathogenic *Borrelia burgdorferi* genospecies under investigation (*B. burgdorferi* s.s., *B. afzelii*, *B. garinii*) in all examined stages of *Ixodes ricinus* ticks (Tab. 2) and in ticks from all but 1 district of the Lublin region with positive findings of *B.b.*s.l. (Tab. 3). In 81.4% of infected ticks only a single infection with 1

Table 2. Prevalence of *Borrelia burgdorferi* sensu lato (*B.b.*s.l.) genospecies in *Ixodes ricinus* ticks positive in PCR or in culture confirmed by PCR, determined by nested-PCR: results presented by stage/sex of ticks.

Stage/sex	Total positive for *B.b.*s.l. in PCR or culture	Genospecies (number of positive, percent of total ticks positive for *B.b.*s.l.)						
		ss	a	g	ss + a	ss + g	ss + a + g	Total
Females	62/588* (10.5%)	25 (40.3%)	18 (29.0%)	7 (11.3%)	10 (16.1%)	2 (3.2%)	0 (0)	62 (100%)
Males	32/601** (5.3%)	15 (46.9%)	6 (18.7%)	5 (15.6%)	4 (12.5%)	0 (0)	2 (6.3%)	32 (100%)
Nymphs	19/1062*** (1.8%)	10 (52.6%)	2 (10.5%)	4 (21.1%)	3 (15.8%)	0 (0)	0 (0)	19 (100%)
Total	113/2251**** (5.0%)	50 (44.2%)	26 (23.0%)	16 (14.2%)	17 (15.0%)	2 (1.8%)	2 (1.8%)	113 (100%)

ss = *Borrelia burgdorferi* sensu stricto; a = *Borrelia afzelii*; g = *Borrelia garinii*; *55 positive ticks were identified by PCR, 7 by culture confirmed by PCR; **28 positive ticks were identified by PCR, 4 by culture confirmed by PCR; ***15 positive ticks were identified by PCR, 4 by culture confirmed by PCR; ****98 positive ticks were identified by PCR, 15 by culture confirmed by PCR.

genospecies was observed, while coinfections with 2 or 3 genospecies were detected respectively in 16.8% and 1.8% of infected ticks (Tab. 2, 3). The dominant genospecies was *Borrelia burgdorferi* sensu stricto which was most common in all tick stages (Tab. 2) and in all examined districts with positive findings of *B.b.*s.l. (Tab. 3). *Borrelia burgdorferi* s.s. genospecies was dominant both in single infections and in coinfections, and was found in a total of 62.8% of *I. ricinus* ticks infected with *B.b.*s.l. *Borrelia afzelii* and *Borrelia garinii* were less frequent and observed in respectively 39.8% and 17.7% of infected ticks (Tab. 4).

Table 4. Total occurrence of individual *Borrelia burgdoferi* sensu lato (*B.b.*s.l.) genospecies in 113 infected *Ixodes ricinus* ticks with regard to summarized single and mixed infections.

Genospecies	Positive/total infected with *B.b.*s.l. (percent)
Borrelia burgdorferi sensu stricto	71/113 (62.8%)
Borrelia afzelii	45/113 (39.8%)
Borrelia garinii	20/113 (17.7%)

DISCUSSION

The total infection rates of *Ixodes ricinus* ticks (adults and nymphs) with *Borrelia burgdorgferi* sensu lato found in the present study varied from 3.4% in culture through 5.4% in PCR to 11.2% in DFM method. These data are very similar to rates reported in our previous research [11, 15] and to the results obtained by Wodecka [60] in northwestern Poland and Mäkinen *et al.* in southwestern Finland [34], but are lower compared to prevalence found by Stańczak *et al.* in 8 different regions of Poland [51, 52, 53]. The present data are also lower than the infection rates of ticks with *B.b.*s.l. reported from the Czech Republic [18], Slovakia [55], Norway [27], Spain [2], Germany [4, 30, 33, 44], France [42], and Italy [47].

There are only a few reports concerning occurrence of the particular borrelial genospecies in *Ixodes ricinus* ticks collected from different regions of Poland [6, 16, 40, 52, 59]. The results of the present study showing that *Borrelia burgdorferi* sensu stricto was the most prevalent genospecies on the examined territory are in agreement with those reported by Wodecka and Skotarczak [59] and Bukowska [6] from the Western Pomerania region in Poland, but not with the results of Stańczak *et al.* [52] who reported that *Borrelia afzelii* was the most dominant genomic species in *I. ricinus* ticks collected from various localities in Poland. Nevertheless, in the same study, the authors indicated *B. burgdorferi* s.s. as the most common pathogenic species in the Lublin region [52]. *Borrelia burgdorferi* s.s. was also the most prevalent genospecies in yellow-necked mice and nymphal *I. ricinus* ticks in the forest habitat of west-central Poland [36]. On the contrary, Pawełczyk *et al.* [40] revealed that *Borrelia garinii* moderately dominates *Borrelia afzelii* in ticks from the Mazury Lakes district of northeastern Poland.

Borrelia burgdorferi sensu stricto was found also to be the most frequent genospecies of *B.b.*s.l. in *I. ricinus* ticks in various European localities, i.e. in Southern Bohemia [56], central Hesse in Germany [58], in northeastern Italy [13], in the Eindhoven area in the Netherlands [25], in the Basque Country in Spain [2], and in eastern Slovakia [55]. Lenčáková *et al.* [31] found that the most prevalent *Borrelia* species in *I. ricinus* ticks from eastern Slovakia and southern Poland are *Borrelia garinii* and *Borrelia burgdorferi* s.s. According to a study by Danielová *et al.* [18] conducted in 2004 in South Bohemia, the high frequency of *Borrelia burgdorferi* s.s. exceeds the as yet reported occurrence in Central Europe. Ranka *et al.* [43] found *Borrelia afzelii* as a dominant *Borrelia* species in *I. ricinus* and *I. persulcatus* ticks from all regions of Latvia. This genospecies was also the most common in *I. ricinus* ticks collected in a number of other European countries, i.e. in urban and suburban localities of Bonn in western Germany and in the region of Konstanz (south Germany) [44, 48], in the area neighbouring the south and east coast

Table 3. Prevalence of *Borrelia burgdorferi* sensu lato (*B.b.*s.l.) genospecies in *Ixodes ricinus* ticks positive in PCR or in culture confirmed by PCR, determined by nested-PCR: results presented by examined districts of the Lublin region.

District	Total positive for *B.b.*s.l. in PCR or culture	Genospecies (number of positive, percent of total ticks positive for *B.b.*s.l.)						
		ss	a	g	ss + a	ss + g	ss + a + g	Total
Zamość	8/273* (2.9%)	4 (50.0%)	1 (12.5%)	3 (37.5%)				8 (100%)
Puławy	20/440* (4.5%)	9 (45.0%)	2 (10.0%)	2 (10.0%)	7 (35.0%)			20 (100%)
Parczew	29/265* (10.9%)	12 (41.4%)	3 (10.3%)	8 (27.6%)	3 (10.3%)	1 (3.5%)	2 (6.9%)	29 (100%)
Włodawa	12/213** (5.6%)	6 (50.0%)	1 (8.3%)	1 (8.3%)	3 (25.0%)	1 (8.4%)		12 (100%)
Lublin	35/796*** (4.4%)	14 (40.0%)	15 (42.9%)	2 (5.7%)	4 (11.4%)			35 (100%)
Kraśnik	9/207* (4.3%)	5 (55.6%)	4 (44.4%)					9 (100%)
Lubartów	0/57**** (0)							
Total	113/2251***** (5.0%)	50 (44.2%)	26 (23.0%)	16 (14.2%)	17 (15.0%)	2 (1.8%)	2 (1.8%)	113 (100%)

ss = *Borrelia burgdorferi* sensu stricto; a = *Borrelia afzelii*; g = *Borrelia garinii*; *positive ticks were identified only by PCR; **7 positive ticks were identified by PCR, 5 by culture confirmed by PCR; ***25 positive ticks were identified by PCR, 10 by culture confirmed by PCR; ****ticks were examined only by culture, all with negative result; *****98 positive ticks were identified by PCR, 15 by culture confirmed by PCR.

of Sweden [23], in the Lyon region of France [42], in southwestern Finland and Vormsi Island in Estonia [34], and in Switzerland [8]. By contrast, the majority of *B.b.*s.l. infections of *I. ricinus* collected from birds in Thuringia (Germany) was due to *Borrelia garinii* [30]. Maetzel *et al.* [33] indicated *Borrelia afzelii* and *Borrelia garinii* as the dominant borrelian genomic species in *I. ricinus* ticks from urban and suburban Bonn (Germany).

The genospecies *Borrelia burgdorferi* s.s was found also in other tick species from various continents. It was isolated from *Ixodes scapularis* ticks in Ontario (Canada) and from *Ixodes affinis* and *Ixodes minor* ticks in the southeastern United States [37, 39]. Brown *et al.* [5] suggest that the western gray squirrel (*Sciurus griseus*) may be an important reservoir of *B. burgdorferi* s.s. in northern Californian oak woodlands. Likewise, the Taiwan (China) isolates of *B.b.*s.l. were closely related to the genospecies of *Borrelia burgdorferi* s.s. [9].

The results obtained in this work are in accordance with our earlier serologic study, which demonstrated that forestry workers from the Lublin region who showed the positive response to *B.b.*s.l. antigen reacted most frequently to *Borrelia burgdorferi* s.s. [16].

The above-mentioned study also confirmed a relationship between infection with a particular genomic species of *Borrelia* and clinical symptoms. In forestry workers showing the presence of antibodies against *B. burgdorferi* s.s., the most common were arthritis symptoms [16].

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