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Coexistence of *Borrelia burgdorferi* s.l. genospecies within *Ixodes ricinus* ticks from central and eastern Poland

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Abstract

The purpose of the study was to assess the prevalence and coinfection rates of *Borrelia burgdorferi* sensu lato genotypes in *Ixodes ricinus* (L.) ticks sampled from diverse localities in central and eastern regions of Poland. In years 2009-2011, questing nymphs and adults of *I. ricinus* were collected using a flagging method at 18 localities representing distinct ecosystem types: urban green areas, suburban forests and rural woodlands. Molecular detection of *B. burgdorferi* s.l. genospecies was based on amplification of a *fla* gene using nested PCR technique, subsequent PCR-RFLP analysis and bidirectional sequencing. It was revealed that 45 samples (2.1%) harboured two different *B. burgdorferi* s.l. genospecies, whereas triple infections with various spirochetes was found in 11 (0.5%) individuals. Generally, the highest average coinfection rates were evidenced in arachnids gathered at rural woodlands, intermediate at suburban forests, while the lowest were recorded at urban green areas. Overall, single spirochete infections were noted in 16.3% (n = 352/2,153) ticks. Importantly, it is the first report evidencing the occurrence of *Borrelia miyamotoi* (0.3%, n = 7/2153) in *I. ricinus* populations within central Poland. Circumstantial variability of *B. burgdorferi* s.l. genospecies in the common tick individuals sampled at various habitat types in central and eastern Poland was displayed. The coexistence of two or three different spirochete genospecies in single adult ticks, as well as the presence of *B. miyamotoi* were demonstrated. Therefore, further studies uncovering the co-circulation of the tested bacteria and other human pathogens in *I. ricinus* ticks are required.

Keywords

Borrelia burgdorferi sensu lato genospecies, *Borrelia miyamotoi*, *Ixodes ricinus*, coinfection, prevalence, molecular diagnostics

Introduction

The common tick (*Ixodes ricinus* L.) serves as a primary European vector of *Borrelia burgdorferi* sensu lato (s.l.), a complex group of the causative agents of Lyme disease (borreliosis). It is the most frequent tick-borne infection in humans affecting a wide range of organs, e.g. skin, joints, heart or nervous system (Fingerle *et al*. 2008). In recent years, there has been reported a steady increment in population densities of these hematophagous ectoparasites in parallel with the elevated prevalence and coinfection rates of many *I. ricinus*-borne pathogens (Schwarz *et al*. 2012; Pangrácová *et al*. 2013). Tick abundance depends on multitude of environmental factors, such as air temperature, relative humidity, precipitation, host cenosis, degree of anthropogenic interference, heterogeneity and buffer capacity of habitat (Derdáková and Lencáková 2005; Schwarz *et al*. 2012; Pangrácová *et al*. 2013; Schulz *et al*. 2014). Larval, nymphal and adult tick individuals may acquire spirochetes by feeding on the infected hosts. Although a transstadial transmission of *B. burgdorferi* s.l. is predominant in ticks, a transovarial route of transmission has also been postulated by some authors (Derdáková and Lencáková 2005).

Until now, the application of advanced molecular tools allowed the detection of a variety of *B. burgdorferi* s.l. genospecies *I. ricinus* ticks collected at diverse habitats in

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many European countries: *B. afzelii*, *B. bavariensis*, *B. bissetti*, *B. burgdorferi* sensu stricto, *B. garinii*, *B. lusitaniae*, *B. spielmanii* and *B. valaisiana* (Skotarczak *et al*. 2002; Wodecka *et al*. 2014). Additionally, combined infections with *Borrelia* genospecies in ixodid ticks as well as biological samples obtained from Lyme disease patients have been increasingly identified during the last decade. It has been reported that genetic variability of spirochete genotypes implies specific symptomatology of the borreliosis (Schwarz *et al*. 2012). Furthermore, it was demonstrated that *B. afzelii* was predominantly associated with *erythrema migrans*, while *B. garinii* was mainly isolated from cerebrospinal fluid (CSF) that indicates its involvement with Lyme neuroborreliosis (LNB) (Rosef *et al*. 2014).

Despite conducting numerous surveys determining the prevalence levels of *B. burgdorferi* s.l. single infections in hard ticks, there is scarce available information regarding the coexistence patterns of many spirochetes in these arachnids. The main purpose of the performed molecular investigation was to recognize genospecies variability of the causative agents of Lyme disease in the host-seeking nymphal and adult *I. ricinus* ticks collected from a variety of sampling areas throughout central and eastern parts of Poland. Furthermore, the present study was also aimed at comparing the coinfection and prevalence rates of the identified *Borrelia burgdorferi* s.l. genospecies in tick samples gathered from diverse spectrum of habitat types with dissimilar anthropogenic impact (urban green zones, suburban forests and rural woodlands). It may be assumed that hard tick populations inhabiting the examined ecosystems are differentially infected with the pathogenic spirochete genospecies. Hence, the conducted survey was divided into four consecutive phases: i) assessment of ixodid ticks abundance within the assorted habitat types, ii) identification of *B. burgdorferi* s.l. genospecies in the tested tick specimens, iii) evaluation of the spirochete distribution in developmental stages of *I. ricinus*, iv) risk assessment of Lyme disease acquisition from the coinfected and infected ticks dwelling the studied localities.

Materials and Methods

Study sites and collection of questing ticks

Individuals of *I. ricinus* (nymphs, females and males) were gathered two times a month from April to June in 2009–2011. Ticks were sampled every time in the morning hours at the same locations by the same number of persons. Host-seeking hard ticks were collected using a white woollen flag (1.0 × 1.0 m) over the vegetation at 18 localities in central and eastern regions of Poland. Sampling sites encompassed urban green areas (allotment gardens, municipal parks, and squares) situated in Łódź, Tomaszów Mazowiecki, Pułtusk, Warszawa, Węgrów and Biała Podlaska, suburban forests off these towns and rural woodlands in the respective district zones. Tick specimens were preserved in 70% ethanol solution in sterile Falcon tubes, and species verification of the samples was achieved on the basis of morphological characteristics of the individual ticks.

DNA isolation from tick lysates

Extraction of genomic DNA (gDNA) from tick lysates and its subsequent purification were carried out using Genomic Mini kit (A&A Biotechnology, Gdynia, Poland), following the protocol's guidelines. Adult ticks were processed individually, whereas nymphs were homogenized in pools of 5 individuals each. Qualitative-quantitative evaluation of DNA eluates was accomplished with the use of a NanoVue spectrophotometer (GE Healthcare). Only intact DNA samples of high purity were subjected to molecular screening for the tested tick-borne pathogens. DNA isolates were stored at –20°C until the further molecular analyses.

Nested PCR assay

Molecular detection of *B. burgdorferi.* s.l. was based on the two-step PCR amplification of a *fla* gene fragment, according to the method described by Wodecka *et al*. (2010). The following pairs of primers were applied during the relevant PCR reactions: the first round – 132f (5'-TGGTATGGGAGTTTCTGG-3') and 905r (5'-TCTGTCATTGTAGCATCTTT-3'), the second round - 220f (5'-CAGACAACAGAGGGAAAT-3') and 823r (5'-TCAAGTCTATTTTGGAAAGCACC-3'). The length of the specific amplicons after the completion of PCR reactions were 774 and 604 bp, accordingly. Additionally, each set of PCR reactions included both positive and negative (no template) controls.

PCR-RFLP analysis

Molecular identification of *B. burgdorferi* s.l. genospecies was based on the restriction fragment length polymorphism (RFLP) of nested PCR products in accordance with a method designed by Wodecka *et al*. (2010). Amplicons of a *fla* gene obtained with the inner primers (220f and 823r) were subjected to the enzymatic cleavage with *Hpy*F3I endonuclease (Fermentas, Lithuania) that recognizes the specific DNA sequence: 5'...C↓T N A G...3'. The characteristic patterns of restriction enzyme digestion were used for identification of several spirochete DNA in mixed and single infections in the investigated tick specimens.

Electrophoretic separation of DNA fragments

Horizontal gel electrophoresis (2 and 3% of agarose, TBE buffer) was accomplished to separate nested PCR and PCR-RFLP products. The amplicons of *fla* gene and the restriction fragments were visualized with ethidium bromide (Sigma-

Aldrich, Germany) staining. DNA Molecular Weight Markers 26–501 bp and 100–1000 bp (BLIRT, Poland) were used to determine the relative molecular size of the analysed nucleic acids.

Sequencing of PCR products

Borrelia-positive nested PCR products were purified and sequenced in both directions as described previously (Sytykiewicz *et al*. 2012). The Basic Local Alignment Search Tool (BLASTn) was used to assess similarity between the obtained sequences and previously deposited records in the GenBank® database (NCBI, Bethesda, USA).

Statistical analysis

The differences between values of the tested parameters were compared using chi-square test with Yate's correction. All calculations were accomplished using STATISTICA 10.0 software (StatSoft, Poland). *P*-values less than 0.01 were considered statistically significant.

Results

In total, 2,153 questing ticks (1,160 nymphs, 534 females, 459 males) were sampled at 18 distinct localities representing various ecosystem types (urban green areas, suburban forests and rural woodlands) in central and eastern parts of Poland (Table I). It should be underlined that the presence of *I. ricinus* individuals was confirmed in all tested habitats, however, the abundance of tick populations varied greatly between the sampling areas. The highest number of ticks occurred within rural woodlands in Warszawa district (n = 366) and suburban forests surrounding Warszawa (n = 276). Moderate size of *I. ricinus* populations was noted at rural woodlands localized in Łódź district (n = 224), suburban forests of Łódź (n = 197) and green zones in Warszawa (n = 150), whereas the lowest density of populations revealed at urban green areas of Biała Podlaska (n = 29), Tomaszów Mazowiecki (n = 44) and Węgrów (n = 46). Generally, rural woodlands characterized with the greatest levels of the common ticks abundance (n = 565), intermediate number of individuals was found at suburban forests (n = 355), whereas the least density occurred at urban green zones (n = 240).

Table I. Prevalence of single and mixed spirochete infections in the common tick individuals sampled at various natural and urban habitats (central and eastern regions of Poland, 2009-2011)

Sampling site	Ecosystem type	No. of collected ticks	No. of infected (%) ticks		
			S	D	T
Łódź	G	117	16 (13.7)	1 (0.9)	0 (0.0)
Tomaszów Mazowiecki	G	44	5 (11.4)	1 (2.3)	0 (0.0)
Pułtusk	G	63	8 (12.7)	0 (0.0)	0 (0.0)
Warszawa	G	150	24 (16.0)	4 (2.7)	1 (0.7)
Węgrów	G	46	4 (8.7)	0 (0.0)	0 (0.0)
Biała Podlaska	G	29	3 (10.3)	0 (0.0)	0 (0.0)
Total	G	449	60 (13.4)	6 (1.4)	1 (0.2)
Łódź	F	197	28 (14.2)	4 (2.0)	1 (0.5)
Tomaszów Mazowiecki	F	55	7 (12.7)	1 (1.8)	0 (0.0)
Pułtusk	F	79	8 (10.1)	0 (0.0)	0 (0.0)
Warszawa	F	276	55 (19.9)	7 (2.5)	2 (0.7)
Węgrów	F	70	10 (14.3)	1 (1.4)	0 (0.0)
Biała Podlaska	F	49	6 (12.2)	2 (4.1)	0 (0.0)
Total	F	726	114 (15.7)	15 (2.0)	3 (0.4)
Łódź d	W	224	40 (17.9)	7 (3.1)	1 (0.5)
Tomaszów Mazowiecki d	W	83	13 (15.7)	2 (2.4)	1 (1.2)
Pułtusk d	W	120	15 (12.5)	1 (0.8)	0 (0.0)
Warszawa d	W	366	83 (22.7)	13 (3.6)	5 (1.4)
Węgrów d	W	109	19 (17.4)	0 (0.0)	0 (0.0)
Biała Podlaska d	W	76	8 (10.5)	1 (1.3)	0 (0.0)
Total	W	978	178 (18.2)	24 (2.5)	7 (0.7)
Σ	G+F+W	2,153	352 (16.3)	45 (2.1)	11 (0.5)

d – district; G – urban green areas (municipal parks, squares and allotment gardens); F – suburban forest areas; W – rural woodlands; S –occurrence of a single genospecies of *B. burgdorferi* s.l. in the hard tick specimens; D – co-circulation of two different spirochete genospecies; T – coexistence of three various spirochete genospecies

Table II. Distribution of *B. burgdorferi* s.l. genospecies in developmental stages of *I. ricinus* ticks gathered in central and eastern regions of Poland (2009-2011)

Tick stage	No. of sampled ticks	No. of infected (%) ticks									
		BA	BB	BG	BV	S	BB+BG	BB+BV	BG+BV	D	T
Nymphs*	1,160	80 (6.9)	10 (0.9)	30 (2.6)	5 (0.4)	125 (10.8)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Females	534	58 (10.9)	15 (2.8)	45 (8.4)	16 (3.0)	134 (25.1)	15 (2.8)	9 (1.7)	2 (0.4)	26 (4.9)	9 (1.7)
Males	459	43 (9.4)	11 (2.4)	27 (5.9)	12 (2.6)	93 (20.3)	11 (2.4)	5 (1.1)	3 (0.6)	19 (4.1)	2 (0.4)
Total	2,153	181 (8.4)	36 (1.7)	102 (4.7)	33 (1.5)	352 (16.3)	26 (1.2)	14 (0.6)	5 (0.2)	45 (2.1)	11 (0.5)

*– nymphs were analysed in pools of five tick individuals each; (%) – prevalence of spirochete infection; BA – *Borrelia afzelii*; BB – *Borrelia burgdorferi* sensu stricto; BG – *Borrelia garinii*; BV – *Borrelia valaisiana*; S – occurrence of a single genospecies of *B. burgdorferi* sensu lato; D – co-circulation of two different spirochete genospecies; T – triple infection with *B. burgdorferi* s.s., *B. garinii* and *B. valaisiana*

Table III. Distribution of *B. burgdorferi* s.l. genospecies in hard ticks sampled within the investigated habitats (central and eastern regions of Poland, 2009-2011)

Ecosystem type	No. of sampled ticks	No. of infected (%) ticks									
		BA	BB	BG	BV	S	BB+BG	BB+BV	BG+BV	D	T
Urban green areas	449	80 (6.9)	10 (0.9)	17 (3.8)	8 (1.8)	60 (13.4)	3 (0.7)	2 (0.5)	1 (0.2)	6 (1.3)	1 (0.2)
Suburban forests	726	58 (10.9)	15 (2.8)	40 (5.5)	14 (1.9)	114 (15.7)	10 (1.4)	4 (0.5)	1 (0.1)	15 (2.1)	3 (0.4)
Rural woodlands	978	43 (9.4)	11 (2.4)	45 (4.6)	11 (1.1)	178 (18.2)	13 (1.3)	8 (0.8)	3 (0.3)	24 (2.5)	7 (0.7)
Total	2,153	181 (8.4)	36 (1.7)	102 (4.7)	33 (1.5)	352 (16.3)	26 (1.2)	14 (0.6)	5 (0.2)	45 (2.1)	11 (0.5)

(%) – prevalence of spirochete infection; BA – *Borrelia afzelii*; BB – *Borrelia burgdorferi* sensu stricto; BG – *Borrelia garinii*; BV – *Borrelia valaisiana*; S – occurrence of a single genospecies of *B. burgdorferi* sensu lato; D – co-circulation of two different spirochete genospecies; T – triple infection with *B. burgdorferi* s.s., *B. garinii* and *B. valaisiana*

It was uncovered that 2.1% ticks (n = 45) harboured two different *B. burgdorferi* s.l. genospecies, whereas triple infections were found in 0.5% (n = 11) individuals (Table II). Importantly, polymicrobial infections were present only within adult individuals of *I. ricinus*. Prevalence of dual (4.9%; n = 26/534) and triple (1.7%; n = 9/534) infections in females were statistically different ($p<0.001$) from levels of the estimated parameters in males (4.1%, n = 19/459 – double infection; 0.4%, n = 2/459 – triple infection). Simultaneous occurrence of the following pairs of spirochete genospecies was identified: *B. burgdorferi* s.s./*B. garinii* (BB+BG), *B. burgdorferi* s.s./*B. valaisiana* (BB+BV), and *B. garinii*/*B. valaisiana* (BG+BV). Among ticks infected with two spirochete genospecies, coexistence of BB+BG was the most frequent (57.8%; n = 26/45), following BB+BV coinfection pattern (31.1%; n = 16/45), while BG+BV mixed infection was rarely identified (11.1%; n = 14/45) (Table III). Females characterized with greater prevalence of BB+BG (non-significant) and BB+BV ($p<0.01$) coinfections when compared to male individuals. The opposite tendency was ascertained in case of males that possessed a slightly higher rate of BG+BV coinfection than females, however the differences were not statistically significant. Dual infection with *B. burgdorferi* s.l. was not confirmed in tick specimens collected in 5 of 18 examined localities (green areas of Węgrów, Biała Podlaska and Pułtusk towns, forests surrounding Pułtusk and rural woodlands of Pułtusk district), whereas the arachnids gathered from other sampling sites were coinfected with the analysed microorganisms. The performed analyses evidenced that *I. ricinus* populations at rural woodlands possessed the highest average prevalence of spirochetes (2.5%; n = 24/978), intermediate levels were noted at suburban forests (2.1%; n = 15/726), while the lowest values were recorded at urban green areas (1.3%; n = 6/449). The comparative data revealed that the highest frequency of dual infections occurred in ticks collected at suburban forests of Biała Podlaska (4.1%; n = 2/49) and rural woodlands of Warszawa Voivodeship (3.6%, n = 13/366). Moderate prevalence of the coinfections with various spirochete genospecies was noted in ticks inhabiting woodlands of Łódź district (3.1%; n = 7/224), urban green areas in Warszawa (2.7%; n = 4/150) and suburban forests of the city (2.5%; n = 7/276). The lowest number of ticks infected with two different *B. burgdorferi* s.l. genospecies was ascertained at green zones in Łódź (0.9%; n = 1/117) and rural woodlands of Pułtusk district (0.8%; n = 1/120). Triple infections were detected in 11 tick individuals (9 females and 2 males) gathered at 6/18 sampling sites. It was identified a homogenic pattern of this infection type in hard ticks involved with the concurrent presence of *B. burgdorferi* sensu stricto, *B. garinii* and *B. valaisiana*. The highest frequency of triple infections was noted in rural woodlands of Warszawa Voivodeship (1.4%; n = 5/366), whereas other localities (suburban forests of Warszawa and Łódź, green zones in Warszawa, rural woodlands in Tomaszów Mazowiecki and Łódź districts) characterized with minor prevalence levels (0.5–0.7%, 1–2 positive tick samples per site).

Molecular investigations revealed that 352 of 2153 ticks (16.3%) were infected with single spirochete genospecies (Tables II and III). It is of high importance that presence of *B. burgdorferi* s.l. was confirmed in *I. ricinus* populations inhabiting all the investigated locations. Among the positive samples, four *B. burgdorferi* s.l. genospecies were identified: *B. afzelii* (51.4%; n = 181/352), *B. garinii* (29.0%, 102/352), *B. burgdorferi* sensu stricto (10.2%, 36/352) and *B. valaisiana* (9.4%, n = 33/352) (Tables II and III). The highest mean infection rate was noted in females (21.9%, 134/534), slightly lower in males (20.3%, 93/459), and the lowest in nymphal individuals (10.8%, 125/1160). Significance of differences in prevalence between tested developmental stages of *I. ricinus* ticks was statistically confirmed ($p<0.01$). It was established that the highest average frequency of single spirochete infections appeared at rural woodlands (18.2%, 178/978), whereas the lowest one at urban green areas (13.4%, 60/449) (Table III). Furthermore, it was demonstrated that tick specimens collected at rural woodlands in Warszawa district and forests surrounding Warszawa characterized with the highest prevalence (22.7 and 19.9%, respectively). The lowest infection rates were obtained for ticks collected at green zones in Węgrów and Biała Podlaska, suburban forests of Pułtusk and woodlands of Biała Podlaska district (8.7–10.5%, depending on habitat types) (Table II). Additionally, the applied molecular methods revealed the occurrence of *Borrelia miyamotoi* DNA in 5 nymphs (0.4%) and 2 females (0.3%) collected at suburban forests surrounding Warszawa agglomeration. The overall prevalence of this spirochete species was ascertained at 0.3% level (n = 7/2153).

Eight randomly selected *Borrelia*-positive DNA amplicons were subjected to bidirectional sequencing. BLASTn search revealed 99.2-100% similarity of the analysed nucleotide sequences in comparison with formerly published GenBank® entries of *B. burgdorferi* s.l. genospecies and 100% homology with *B. miyamotoi* strain ZL27-07.

Discussion

In the last decades, the profound extension of the geographical distribution and noticeable rise in densities of *I. ricinus* populations at various European locations have been increasingly reported (Ferquel *et al*. 2006; Capelli *et al*. 2012; Lommano *et al*. 2013). The common tick has become an extremely important vector involved in transmission of a wide spectrum of human pathogens, such as *Anaplasma phagocytophilum*, *Babesia microti*, *Babesia venatorum* (*Babesia* sp. EU1), *Bartonella henselae*, *B. burgdorferi* s.l. complex, *Coxiella burnetii*, *Francisella tularensis*, *Rickettsiae* spp. or tick-borne encephalitis virus (TBE). Development of the sophisticated molecular techniques enabled identification and genetic characterization of newly discovered tick-transmitted microorganisms in human-derived samples and arachnids individuals gathered at various habitat types (e.g. *Candidatus*

Neoehrlichia mikurensis responsible for a systemic inflammatory syndrome in immunocompromised patients, *B. miyamotoi* causing a tick-borne relapsing fever) (Pejchalová *et al*. 2007; Cisak *et al*. 2008; Jenkins *et al*. 2012; Geller *et al*. 2013; Cosson *et al*. 2014; Dzięgiel *et al*. 2014). The coexistence of two or more different contagious agents may significantly affect the clinical manifestations leading to misdiagnosis, improper selection of antibiotic therapy that implies possible treatment failure. Although several authors emphasized an urgent need to evaluate the frequency of co-circulation of diverse human pathogens in tick populations, very limited molecular investigations were focused on identification of the specific coinfection patterns in tick specimens (Stańczak *et al*. 2000; Cisak *et al*. 2008; Lommano *et al*. 2012; Sytykiewicz *et al*. 2012).

Wodecka *et al*. (2010) developed a reliable and sensitive molecular method (nested PCR with subsequent RFLP analysis of amplicons) that was applied in this study in order to identify different *B. burgdorferi* s.l. genospecies in hard ticks. It is the first report evaluating the prevalence of multiple and single spirochete infections in *I. ricinus* specimens gathered at three ecosystem types (urban green zones, suburban forests and rural woodlands) within central and eastern regions of Poland. The performed analyses revealed that 2.1% ticks were coinfected with two spirochetes, while co-circulation of three genospecies was confirmed in 0.5% tested arachnids. Three distinct patterns of dual infections in tick samples were identified: *B. burgdorferi* s.s./*B. garinii* (57.8%), *B. burgdorferi* s.s./*B. valaisiana* (31.1%), and *B. garinii*/*B. valaisiana* (11.1%), whereas triple infection was associated with concurrent presence of the following spirochetes: *B. burgdorferi* s.s., *B. garinii* and *B. valaisiana*. Importantly, there were no confirmed spirochete coinfections in nymphal individuals, whereas polymicrobial infections were found more frequently in females when compared to males. Intriguingly, *B. burgdorferi* s.l. DNA was evidenced in ticks inhabiting all investigating sampling areas in central and eastern parts of Poland. Within the urban green areas, 6 ticks harboured two different spirochete genospecies, and 1 individual was infected with three genospecies. It should be underlined that the greatest coinfection rates with different *B. burgdorferi* s.l. genospecies in *I. ricinus* ticks were noted at various localities in Warszawa Voivodeship. This phenomenon may be associated with larger urban green zones (city parks, squares, gardens) and woodland areas, population density of ticks and their hosts, considerable anthropogenic impact, favorable microclimate conditions, heterogeneity and buffer capacity of habitats. Cisak *et al*. (2006) revealed that 16.8% of the *Borrelia*-positive samples were coinfected with two spirochete genospecies, whereas triple infections occurred in 1.8% of tick specimens gathered at woody habitats in Lublin region (eastern part of Poland). Stańczak *et al*. (2000) confirmed the triple infection with *B. burgdorferi* s.s., *B. afzelii* and *B. garinii* in 1.6% of adults ticks sampled in years 1996-1998 at different Polish woodlands. The same pattern of spirochetes coexistence was recognized by Cisak *et al*. (2008) in 0.9% ticks collected within the Lublin district. Researcher groups from other European countries reported a broad range of coinfection rates of different spirochete genospecies. Rosef *et al*. identified double infections (*B. afzelii*/*B. burgdorferi* s.s. and *B. afzelii*/*B. garinii*) in 7% of ticks collected in Norway (Rosef *et al*. 2014). Moreover, Lommano *et al*. (2012) revealed that polymicrobial infections with two or three spirochete genospecies were identified in 2.1% ticks gathered in western Switzerland, and further observation revealed that coexistence of *B. garinii* and *B. valaisiana* was most frequent. According to Derdáková and Lencáková (2005), identification of spirochete genospecies within the vectors is important issue in unravelling the intricate ecological and epidemiological aspects of Lyme disease. Importantly, the molecular survey conducted by Vennestrøm *et al*. (2008) documented very high coinfection rates of nymphal *I. ricinus* ticks collected at different Danish localities (51% of PCR-positive ticks were infected with two spirochetes, triple infections confirmed in 7.1%, and co-circulation of four spirochete genospecies occurred in 5.3%). These authors postulate that the described phenomenon may be due to high density of the reservoir hosts, as well as the specific non-systemic mode of spirochetes transmission from infected to uninfected recipient ticks by simultaneous co-feeding upon the same host.

In Europe, *B. garinii* and *B. afzelii* occurred predominantly in single infections in hard ticks, while *B. burgdorferi* s.s. and *B. valaisiana* were seldom recorded (Derdáková and Lencáková 2005; Ferquel *et al*. 2006; Pejchalová *et al*. 2007; Capelli *et al*. 2012; Pangrácová *et al*. 2013; Dzięgiel *et al*. 2014). However, Cisak *et al*. (2006) evidenced the highest percentage share of *B. burgdorferi* s.s. (44.2%) in *Borrelia*-positive ticks collected from woodland areas in Lublin region. Environmental studies revealed that rodents are important hosts of *B. afzelii*, birds are mainly associated with *B. garinii* and *B. valaisiana*, whereas rodents and birds are considered as competent reservoirs of *B. burgdorferi* s.s. (Wodecka *et al*. 2014). Prevalence of single infections with *B. burgdorferi* s.l. in *I. ricinus* ticks varied significantly between the various European countries, and surprisingly, even between closely situated localities (Poland – 5.4–13.1%, Estonia – 8.2%, Denmark – 11%, Norway – 13%, Lithuania – 13.3; Germany 15.8%; north-eastern Italy – 17.6%, western Switzerland – 22.5%, Belgium – 23%; Latvia – 28%; Slovakia – 31.9% (Skotarczak *et al*. 2002; Cisak *et al*. 2006; Smetanová *et al*. 2007; Cisak *et al*. 2008; Capelli *et al*. 2012; Lommano *et al*. 2012; Schwarz *et al*. 2012; Sytykiewicz *et al*. 2012; Geller *et al*. 2013; Pangrácová *et al*. 2013).

Borrelia miyamotoi is a newly recognized etiological agent of tick-borne relapsing fever and characterizes with a distant genetic similarity to *B. burgdorferi* s.l. complex. This spirochete species was identified in 1995 from *Ixodes persulcatus* ticks gathered in Japan. Recently, humans infections caused by this microorganism were reported in the United States, the Netherlands and Russia (Platonov *et al*. 2011; Geller *et al*. 2012; Cosson *et al*. 2014). In this work, occurrence of *B.*

miyamotoi was identified in 7 tick individuals (0.3%) sampled in suburban forests of Warszawa. Similar results were obtained by Wodecka *et al*. (2014) who identified this pathogen in 6/880 *I. ricinus* nymphs (0.7%) collected in northwestern Poland.

Summarizing, the present study demonstrated a circumstantial variability in occurrence of *B. burgdorferi* s.l. genospecies in *I. ricinus* ticks sampled in the examined habitat types in central and eastern Poland. Comprehensive molecular survey identified the coexistence of two or three different spirochete genospecies in adult ticks. In the context of public health, emergence and spread of the newly discovered *B. miyamotoi* and multiple infections with *B. burgdorferi* s.l. genospecies needs to be scrupulously monitored in tick populations, with special emphasis on urban and suburban habitats.

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