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Prevalence of *Borrelia burgdorferi* Sensu Lato Genospecies in *Ixodes ricinus* Ticks from Recreational Areas of Silesia

Częstość występowania genogatunków *Borrelia burgdorferi* sensu lato w populacji kleszczy *Ixodes ricinus* na terenach rekreacyjnych województwa śląskiego

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Abstract

Background. Lyme borreliosis is a multisystem disorder caused by the genetically diverse spirochetes *Borrelia burgdorferi* sensu lato. Different species of pathogenic *Borrelia* are able to cause various symptoms. *Borrelia* is transmitted to humans by ticks.
Objectives. The aim of the study was to analyze the prevalence of *B. burgdorferi* s.l. genospecies in *Ixodes ricinus* ticks.
Material and Methods. PCR-RFLP analysis of the flagellin gene was performed. The study was conducted in recreational areas of Silesia (Tarnowskie Góry administrative district).
Results. Eleven monoinfections and two dual infections were detected: six (40.0%) were infected with *B. burgdorferi* sensu stricto, five (33.3%) with *B. garinii*, and four (26.7%) with *B. afzelii.* Two of the analyzed ticks were infected simultaneously with *B. burgdorferi* s.s. and *B. garinii*.
Conclusions. Investigations of the prevalence of *B. burgdorferi* s.l. genospecies in tick populations of recreational areas are a basis for evaluating the risk of infection. Moreover, the PCR-RFLP technique is an easy and rapid method for genotyping *Borrelia* species (**Adv Clin Exp Med 2006, 15, 6, 1003–1008**).

Key words: *Borrelia burgdorferi,* genospecies, flagellin gene, PCR-RFLP analysis, ticks.

Streszczenie

Wprowadzenie. Borrelioza z Lyme jest wieloukładową chorobą wywołaną przez zróżnicowane genetycznie krętki *Borrelia burgdorferi* sensu lato. Określone patogenne genogatunki *Borrelia* wywołują różne symptomy chorobowe. *Borrelia* jest przenoszona na ludzi przez kleszcze.
Cel pracy. Ocena częstości występowania genogatunków *B. burgdorferi* s.l. w populacji kleszczy *Ixodes ricinus.*
Materiał i metody. Badania wykonano metodą PCR-RFLP na podstawie analizy genu flagelliny. Obszar badań obejmował tereny rekreacyjne województwa śląskiego (powiatu tarnogórskiego).
Wyniki. Zidentyfikowano 11 monoinfekcji oraz 2 infekcje podwójne. W analizowanych próbkach 6 (40,0%) z nich wykazało zakażenia *B. burgdorferi sensu stricto*, 5 (33,3%) zakażenie *B. garinii* oraz 4 (26,7%) *B. afzelii.* Analizowane kleszcze w dwóch przypadkach wykazały jednoczesne zakażenie *B. burgdorferi* s.s. oraz *B. garinii*.
Wnioski. Badania dotyczące określenia częstości występowania genogatunków *B. burgdorferi* s.l. w populacji kleszczy *Ixodes ricinus* na terenach rekreacyjnych są podstawą do oszacowania ryzyka zakażenia. Technika PCR-RFLP jest ponadto łatwą i szybką metodą genotypowania gatunków *Borrelii* (**Adv Clin Exp Med 2006, 15, 6, 1003–1008**).

Słowa kluczowe: *Borrelia burgdorferi,* genogatunki, gen flagelliny, PCR-RFLP, kleszcze.

Borreliosis is a tick-borne infection mainly transmitted by the tick *Ixodes ricinus* in Europe, *Ixodes persulcatus* in Eastern Europe, Russia, and East Asia, and *Ixodes pacificus* and *Ixodes scapularis* in North America. The etiologic agent is the microaerofilic Gram-negative spirochete *Borrelia burgdorferi*. The epidemiology and clinical symptoms of borreliosis have been well characterized and involve the skin, joints, nervous system, and heart. *Borrelia burgdorferi* sensu lato (s.l.) has been divided into 11 genospecies: *Borrelia burgdorferi* sensu stricto (s.s.), *Borrelia garinii, Borrelia afzelii*, *Borrelia valaisiana*, *Borrelia lusitaniae*, *Borrelia japonica*, *Borrelia andersonii*, *Borrelia tanukii, Borrelia turdi*, *Borrelia bissettii*, and *Borrelia sinica*. Different genospecies exhibit different pathogenicities. Furthermore, different aspects of pathogenicity were ascribed to genetic variations of *Borrelia burgdorferi*. *B. burgdorferi* s.s., *B. afzelii*, and *B. garinii* cause significant morbidity in humans. The pathogenic roles of *B. lusitaniae* and *B. valaisiana* are still undetermined. However, *B. valaisiana* and *B. lusitaniae* have been detected in patients with borreliosis symptoms. Moreover, DNA of *B. bissettii* and *B. sinica* have been isolated from patients with Lyme borreliosis [1, 2].

A geographic distribution of the genotypes is also observed. *B. burgdorferi* s.s has been detected in America and Europe. *B. garinii, B. afzelii*, and *B. valaisiana* are European and Asian isolates. *B. lusitaniae* are distributed throughout Europe. *B. andersonii* and *B. bissettii* were identified in America. *B. bissettii* was also detected in Europe. *B. japonica*, *B. tanukii, B. turdi*, and *B. sinica* are specific to Asia [1, 2].

One of the most widely used methods allowing the direct detection of the causative agent of Lyme borreliosis is PCR (Polymerase Chain Reaction). This molecular method is used in the detection of *B. burgdorferi* s.l. DNA in ticks [3, 4] and also in human specimens [5, 6]. There are several molecular methods allowing the typing of *B. burgdorferi*: species-specific PCR, RFLP (Restriction Fragment Length Polymorphism), plasmid fingerprinting, RAPD (Random Amplified Polymorphic DNA), PFGE (Pulsed-Field Gel Electrophoresis), PCR followed by Single-Strand Conformation Polymorphism (SSCP), hybridization, and nucleic acid sequence analysis [2].

Material and Methods

The study was conducted in recreational areas of the Silesian region of Poland in the Tarnowskie Góry administrative district (Krupski Młyn, Zielona, Lubliniec, Tarnowskie Góry, Świerklaniec, Tworóg, and Zbrosławice). A total of 85 ticks (*Ixodes ricinus*) were collected using the flagging method. A total of 40 females, 27 males, and 18 nymphs were examined. Total DNA was extracted from the ticks and the presence of spirochetes was confirmed by PCR assay using target sequences of the flagellin (*fla*) gene and agarose gel electrophoresis of DNA was performed as described previously [7]. The PCR product had a size of 442 base pairs (bp). All PCR-positive samples (14, 16.5%) were used in the further investigation.

The PCR product was excised and extracted from agarose gel using a Perfectprep Gel Cleanup Kit (Eppendorf, Germany) according to the manufacturer's recommendations. The extracted DNA was diluted (1 : 20) and amplified in a 25 µl (total volume) reaction mixture. Each reaction mixture contained 12.5 µl of a twofold concentration of PCR Master Mix (50 U/ml of *Taq* polymerase supplied in a proprietary reaction buffer, pH 8.5; 400 µM each of dATP, dGTP, dCTP, and dTTP; and 3 mM $MgCl_2$; Promega, USA), 2.5 µl of each primer (BFL1 and BFL2) at a concentration of 10 µM, 5 µl of cleaned DNA, and 2.5 µl molecular grade water (Eppendorf, Germany). A primer set was chosen in the *fla* gene sequence regions based on previously published sequences [3]: BFL1: 5'-GCT CAA TAT AAC CAA ATG CAC ATG -3' and BFL2: 5'-CAA GTC TAT TTT GGA AAG CAC CTA A- 3'. The primers were synthesized by the Institute of Biochemistry and Biophysics, Polish Academy of Science (Poland). PCR amplifications were performed in a Mastercycler Personal thermal cycler (Eppendorf, Germany). The PCR program used was: initial denaturation at 94°C for 2 min, denaturation at 94°C for 30 s, annealing at 60°C for 60 s, extension at 72°C for 60 s for 40 cycles, and final extension at 72°C for 60 s.

PCR-RFLP analysis was used to distinguish three genospecies of *B. burgdorferi* s.l. (*B. burgdorferi* s.s, *B. garinii*, and *B. afzelii*). Reference sequences of the *fla* gene of *B. burgdorferi* s.s, *B. garinii* and *B. afzelii* were downloaded from a database (GenBank accession numbers: AY083505, AY603344, and AY342021, respectively). An alignment of the *fla* gene sequences made it possible to distinguish SNPs differentiating the analyzed genospecies. The Jellyfish software was used to select *Tas*I restriction enzyme for PCR-RFLP analysis.

The amplified DNA was digested with *Tas*I. The restriction enzyme and buffer were obtained from MBI Fermentas (Lithuania). Twenty µl of PCR product was digested at 65°C for 3 hours on a Mastercycler Personal thermal cycler (Eppendorf, Germany) in a solution containing 1 µl of *TasI* (activity 10 U/µl), 4 µl of B^+ buffer (10 mM

Tris-HCl, pH 7.5; 10 mM $MgCl_2$; 0.1 mg/ml BSA), and 15 µl of molecular grade water (Eppendorf, Germany). The digestion products were analyzed on 3% agarose gels (Serva) in a Tris-Borate-EDTA (pH 8.3) buffer at 100 V for 1.5–2 hours (Sigma Maxi Horizontal Gel Electrophoresis, SHU 20 Sigma-Aldrich, Germany). The gel was stained with ethidium bromide (1%, Serva, Germany). GeneRuler™ 100bp DNA Ladder (MBI Fermentas, Lithuania) was used as a size marker. Blue/Orange 6x Loading Dye (Promega, USA) was used as a loading buffer. The gel was visualized under the UV light of a transilluminator (BTX-20M, CBS Scientific, USA).

The positive tick samples were analyzed in duplicate. In addition, each time the PCR-RFLP analysis was performed, negative (molecular grade water instead of DNA) and positive control samples (B31 strain *B. burgdorferi* DNA, DNA – Gdańsk, Poland) were included. All procedures were performed in a three-room environment (isolation, PCR, and electrophoresis suite) to minimize the risk of contamination. The *B. burgdorferi* genospecies detected were analyzed according to sex and the area of tick origin.

Results

The PCR method of DNA analysis showed that 14 out of 85 samples (16.5%) were positive for *B. burgdorferi* s.l. The numbers of infected females, males, and nymphs were 7, 6, and 1, respectively.

RFLP analysis of the amplified products resulted in characterization of four distinct profiles (Table 1). In this study, 11 monoinfections and 2 dual infections were detected. Of the 14 positive samples, 6 (40.0%) were infected with *B. burgdorferi* s.s., 5 (33,3%) with *B. garinii*, and 4 (26,7%) with *B. afzelii.* Two of the analyzed ticks were infected simultaneously with *B. burgdorferi* s.s. and *B. garinii*. In one positive sample, the genospecies remained undetermined (Table 2). We did not find differences in the prevalence of genospecies *B. burgdorferi* s.l. between male and female *I. ricinus*. We also found no differences in the occurrence of genotypes among the particular recreational areas.

Discussion

PCR-based approaches are powerful methods for the direct detection of *B. burgdorferi* s.l. DNA in

Table 1. Genospecies of *B. burgdorferi* s.l and their corresponding restriction patterns obtained by *Tas*I RFLP analysis of PCR products generated from *fla* gene fragment of *Borrelia* DNA

Tabela 1. Genogatunki *B. burgdorferi* s.l wraz z odpowiadającymi im wzorami restrykcyjnymi (produktami cięcia fragmentu genu *fla Borrelia* restryktazą *Tas*I)

Borrelia genospecies (Genogatunki *Borrelia*)	RFLP pattern (Wzór RFLP)	*Tas*I restriction fragment sizes (bp) (Wielkości fragmentów restrykcyjnych (pz) po cięciu *Tas*I)
B. burgdorferi s.s.	A	29-88-93-228-232
B. afzelii	B	29-81-83-93-151
B. garinii	C	29-93-281-320
B. burgdorferi s.s./*B. garinii*	A/C	29-88-93-228-232 / 29-93-281-320

Table 2. Results of genotyping of *B. burgdorferi* s.l. isolated from ticks *I. ricinus*

Tabela 2. Wyniki genotypowania *B. burgdorferi* s.l. wyizolowanej z kleszczy *I. ricinus*

Sex and stage (Płeć i stadia rozwojowe)	No. of positive ticks (Liczba zakażonych kleszczy)	No. of ticks positive for genospecies of *B. burgdorferi* s.l. (Liczba zakażonych kleszczy poszczególnymi genogatunkami *B. burgdorferi* s.l.)				
		B. burgdorferi s.s.	*B. garinii*	*B. afzelii*	*B. burgdorferi* s.s./ *B. garinii*	nontypeable (nieokreślony)
Nymph (Nimfy)	1	1				
Male (Samce)	6	1	2	2	1	
Female (Samice)	7	2	1	2	1	1
Total (Razem)	14	4	3	4	2	1

infected ticks and provide information important for epidemiology and vector ecology. Targets that have often been used for the amplification of *B. burgdorferi* DNA are outer surface protein A (*ospA*) [8], flagellin (*fla*) [9, 10], and ribosomal RNA genes [11]. In this study, a highly conserved region of the *fla* gene [12] was chosen to detect *B. burgdorferi* s.l. by PCR amplification. Several authors have shown adequate specificity and sensitivity of the *fla*-specific primers used in the presented study [3, 13].

For classifying *Borrelia* strains into genomic species, a rapid and sensitive PCR-RFLP analysis is widely used [9–11]. In general, the sequence of the *fla* gene is highly conserved among *B. burgdorferi* strains, although in the middle region of the gene some microheterogeneity exists [14]. In the present study, a 442-bp-long fragment of the *fla* gene covering the region of the highest variability was used to identify genotypes of *B. burgdorferi* s.l by the PCR-RFLP approach. The *Tas*I restriction enzyme operating on the chosen region allows distinguishing *B. burgdorferi* s.l. into three genotypes. Similarly, the advantage of using PCR-RFLP analysis on the considered fragment of the *fla* gene for *Borrelia* genotyping was exploited by Wodecka and Skotarczak [10], where the *Fsp*4H1 enzyme was applied.

Manifestations of Lyme borreliosis vary geographically, depending in part on the predominant genotypes. The results of this study show the presence of *B. burgdorferi* s.s., *B. garinii,* and *B. afzelii* in the studied region. In previous studies, the three genospecies of *B. burgdorferi* s.l. were found in Europe and in Poland [4, 10, 15]. However, the prevalence values of the genospecies of the spirochete in ticks are very variable throughout Europe and even in Poland.

The dominating genospecies in this study was *B. burgdorferi* s.s., followed by *B. garinii* and *B. afzelii.* Similar results were obtained in another part of Silesia (Katowice province) [15] and in northwest Poland [10]. These results are also in agreement with reports from Germany [16], eastern Slovakia [17], the Czech Republic [4], Italy [18], and in the Eindhoven region of Holland [19].

According to Stańczak et al. [15], *B. afzelii* moderately dominates in Poland, followed by *B. burgdorferi* s.s. and *B. garinii.* These proportions correspond to the results obtained in other European countries. *B. afzelii* is the predominant genospecies in the southern part of the Czech Republic [11] and in the Lyon region of France [9]. A different rate was published for southern Belgium [20], where *B. garinii* was the most prevalent, followed by *B. burgdorferi* s.s. and B. *afzelii.* Rijpkema et al. [21] described the genotyping of *B. burgdorferi* strains in Dutch *I. ricinus* ticks. *B. garinii* also had the highest rate. This rate corresponds to results from Spain [22] and Switzerland [23].

In the present study, dual infection with *B. burgdorferi s.s.* and *B. garinii* was detected in two cases (14.2%). Stańczak et al. [15] noted mixed infection of *B. burgdorferi* s.s./*B. garinii* in 15 samples (9.8%) of ticks collected in different Polish woodlands in 1996–1998. Part of this study conducted in Katowice province showed a higher percentage of these dual infections, i.e. 33.3%. Mixed infections were associated mainly with *B. burgdorferi* s.s. and *B. garinii* in the study of Misonne et al. [20] in southern Belgium and a high rate in the Czech Republic [4]. Mixed infections of *B. burgdorferi* s.l. species have been found in many other studies [9, 11, 15, 21]. Co-infections could be explained by transovarial transmission or by a tick feeding on different hosts [9]. In contrast, Wodecka and Skotarczak [10] did not find multiple infection in their analyzed samples. These varying results could depend on differences in study populations and the methods used.

The results presented in this paper show that in one positive sample the genospecies remained undetermined. Danielova et al. [4], Missone et al. [20], Stańczak et al. [15], and Quessada et al. [9] had similar results: the genotypes remained undetermined in several positive samples. This may be due to different genospecies not defined for the purpose of the research or to reasons of methodology [4].

We did not find differences in the prevalence of genospecies of *B. burgdorferi* s.l. in male and female *I. ricinus*. These results for ticks were in agreement with the results of Quessada et al. [9]. We also found no differences in the occurrence of genotypes among the sites of recreational areas. No differences in rates of prevalence between collection areas were also observed by others [9, 20].

Important for the evaluation of the risk of infection is the investigation of the prevalence of *B. burgdorferi* s.l. genospecies in tick populations of endemic and recreational areas. The heterogeneity among isolates of *B. burgdorferi* has important implications for understanding the epidemiology and the clinical diversity of borreliosis.

In conclusion, this study confirms that the recreational areas of the Silesian region are risk areas for contracting borreliosis. Furthermore, the PCR-RFLP technique is a reliable and rapid method for detecting borrelial DNA in ticks and differentiating the three genotypes of *Borrelia* commonly associated with borreliosis.

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