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VERSITA

Human pathogenic borreliae in *Ixodes ricinus* ticks in natural and urban ecosystem (Czech Republic)

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Abstract

A total of 1279 field-collected *Ixodes ricinus* ticks were screened for *Borrelia burgdorferi* sensu lato (s.l.) in a natural and an urban ecosystem of Ostrava city (Czech Republic) by using molecular methods. Minimal prevalence rate for *Borrelia burgdorferi* s.l. in ticks for the urban park Bělský les was found to be 13.8% (17.6% in males, 17.8% in females and 11.7% in nymphs), similarly for the natural site Proskovice was minimal prevalence 15% (12.5% in males, 20% in females and 14.9% in nymphs). Six proven human pathogenic genomic species have been recorded in the study: *B. afzelii*, *B. garinii*, *B. burgdorferi* s.s., *B. valaisiana*, *B. lusitaniae*, and *B. spielmanii*. Emerging *B. spielmanii* was detected for the first time in *Ixodes ricinus* ticks in the region. Our results highlight the need for surveillance of zoonotic tick-borne pathogens even in urban areas.

Keywords,

Ixodes ricinus, *Borrelia burgdorgeri* s.l., genomic species, ixodid ticks

Introduction

Ixodid ticks (in Central Europe mainly *Ixodes ricinus*) represent a significant health risk for humans and many other vertebrate species as vectors of multiple pathogens of which the most important are flaviviruses of tick-borne encephalitis complex (TBEV), *Borrelia burgdorferi* sensu lato (s.l.), *Francisella tularensis*, *Anaplasma phagocytophilum*, *Rickettsia helvetica*, *R. slovaca*, *Babesia microti*, *B. divergens* and *B. venatorum* (EU1) (Hubálek and Rudolf 2011).

Borrelia burgdorferi s.l. is a complex of gramnegative bacteria in the Order *Spirochaetales* and contains 18 genomospecies. Spirochaete *B. burgdorferi* is prevalent in ixodid ticks in Europe, USA, Asia and North Africa, with variation in geographic and genetic distribution. Pathogenic genomic species present in Europe are: *B. burgdorferi* s.s., *B. afzelii*, *B. garinii*, *B. lusitaniae*, *B. valaisiana*, *B. bissettii*, *B. spielmanii* and *B. bavariensis* (Stanek and Reiter 2011).

The aim of this study was to determine the minimum prevalence rate (MIR) of an important tick-borne pathogenic spirochaete *Borrelia burgdorferi* s.l. in nymphal and adult host-seeking *Ixodes ricinus* ticks in two different ecosystems: a natural (a deciduous mixed forest at Proskovice – a total of 1197 ticks were examined) and an urban (a municipal park Bělský les located in Ostrava- a total of 276 ticks were examined) by using molecular biology techniques (Real-time PCR and Reverse-line blotting) in order to assess public health risk of urban and natural site for acquiring Lyme borreliosis which is reportable disease in the Czech Republic (a total of 3304 human infections recorded in 2012 according to The National Institute of Public Health).

Materials and Methods

Study sites

Ixodes ricinus ticks were collected at two study sites: Ostrava city (49°47′N 18°14′E) and Proskovice (Ostrava surroundings, 49°44′N 18°12′E). The first study site is the urban park. Local fauna is represented by small mammals and birds, and vegetation by broadleaved deciduous trees and grass (grass cutting and long-term treatment of local trees is performed on irregular basis). The forest is surrounded by housing estates and used for leisure activities and dog-walking. The second study site is the natural ecosystem outside the town. This mixed forest with dominant broadleaved trees is rarely visited by people. The fauna consists of small and

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medium-sized mammals, roe deer, birds, and occasionally wild boars.

Ticks were collected by flagging low vegetation between April and September (a period of seasonal activity of *I. ricinus* in Central Europe) 2010. The sampled ticks were divided into test tubes according to sex and developmental stage and pooled (5 nymphs per tube, 3 adults per tube) before being frozen at –60°C.

Extraction of nucleic acids

Homogenization of ticks and genomic DNA isolation

All *I. ricinus* ticks were surface sterilized with 70% ethanol (PCR quality) and mechanically disrupted using the TissueLyser apparatus (Qiagen, Hilden, Germany) in 245 µl of PBS (Oxoid, England). The total genomic DNA was extracted from 100 µl of the tick homogenate with a QIAamp DNA Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Ticks were examined for the presence of *B. burgdorferi* s.l. using TaqMan Real-time PCR procedure and assigned to genomic species by using conventional PCR followed by Reverse line blotting.

Real-time PCR procedure

Real-time PCR for detection of *B. burgdorferi* s.l. was performed according to Courtney *et al*. (2004) using Bb23Sf/Bb23Sr primers (specific for the *B. burgdorferi* 23S rRNA gene) and Bb23Sp TaqMan probe. The fluorogenic labels at the 5´ and the 3´ ends of the probe were BHQ1 and FAM, respectively. The PCR reaction was carried out on the 7500 Real-Time PCR system (Applied Biosystems, USA) by using the QuantiTect Probe RT-PCR Kit (Qiagen, Hilden, Germany). Cycling conditions as well as other PCR steps were performed according to Courtney *et al*. (2004) by using the QuantiTect Probe PCR Kit (Qiagen, Hilden, Germany).

Touch-Down PCR, Reverse-line blotting (RLB)

Primers B-5SBor/23SBor were used for a PCR amplification of *B. burgdorferi* s.l. DNA. In addition, touchdown temperatures ranging from 60 to 52°C for *B. burgdorferi* s.l. were applied on samples (to avoid amplifying nonspecific sequences). The PCR conditions including specific concentrations of reaction mixture as well as complete RLB hybridization assay were determined according to Rijpkema *et al*. (1995) and Schouls *et al*. (1999). The RLB probes designed for *B. burgdorferi* s.l., *B. garinii*, *B. afzelii*, *B. burgdorferi* s.s., *B. valaisiana*, *B. lusitaniae*, *B. spielmanii*, and *B. bissettii* were used (Gern *et al*. 2010). DNA extraction, PCR set-up as well as post-PCR processing were done in separate rooms to avoid possible cross-contamination of the samples.

Statistical evaluation

Differences between the MIR were evaluated using contingency tables and chi-square test at 5% probability level.

Results

A total of 1279 ticks were examined in 320 pools for human pathogenic tick-borne borreliae in the natural (251 pools) and the urban (69 pools) ecosystems in the surroundings of Ostrava. Results are briefly summarized in Table 1 and 2.

There were 217 pooled samples positive for *B. burgdorferi* s.l. (38 pooled samples from the city park and 179 pooled samples from the natural ecosystem). The overall MIR for Bělský les (the city park) was 13.8% (males 17.6%, females 17.8%, nymphs 11.7%). There was no significant difference in prevalence between males and females (P = 0.99) or between adults and nymphs (P = 0.17). The overall MIR for Proskovice (the natural ecosystem) was found to be 15% (males 12.5%, females 20%, nymphs 14.9%). There was no significant difference in the MIR between males and females (P = 0.35) or between adults and nymphs (P = 0.85) either.

B. burgdorferi s.l. genomic species were identified using reverse line blot assay (see Table 2). Overall six proven pathogenic genomic species have been recorded in the study: *B. afzelii*, *B. garinii*, *B. burgdorferi* s.s., *B. valaisiana*, *B. lusitaniae* and *B. spielmanii*.

We were unable to identify pathogenic genomic species in 17 *B. burgdorferi* s.l. positive samples, probably due to low DNA concentration. The most abundant *Borrelia* genomic species was *B. afzelii* (157 samples), followed by *B. valaisiana* (36 samples),

Table I. Numbers of tested *I. ricinus* ticks and *B. burgdorferi* s.l. prevalence (MIR ,%)

	Males	Females	Nymphs	Ticks in total
Ostrava-Bělský les (urban site)	17/51[a] (17.6)	16/45 (17.8)	36/180 (11.7)	69/276 (13.8)
Proskovice (natural site)	16/48 (12.5)	12/35 (20.0)	223/1114 (14.9)	251/1197 (15.0)
Total	33/99	28/80	259/1294	320/1473

[a]Number of pools/number of ticks tested
Explanation: Minimum infection rate (MIR) was calculated from the number of total ticks examined under assumption that every positive pool has contained only one infected individual tick

Table II. Numbers of positive samples for *B. burgdorferi* genospecies

	B. garinii	*B. afzelii*	*B. burgdorferi* s.s.	*B. valaisiana*	*B. lusitaniae*	*B. spielmanii*
Bělský les (urban site)	5	16	5	10	2	1
Proskovice (natural site)	6	141	14	26	4	0
Total	11	157	19	36	6	1

B. burgdorferi s.s. (19 samples), *B. garinii* (11 samples), *B. lusitaniae* (6 samples) and *B. spielmanii* (1 sample).

Discussion

B. burgdorferi s.l. is a worldwide complex of spirochaetes containing several genomic species. Some are recognized as human pathogens and cause Lyme borreliosis (Stanek and Reiter 2011). Here we present the MIR for *B. burgdorferi* in *I. ricinus* ticks in the natural and the urban ecosystms in the Czech Republic in order to assess the risk of acquiring the disease.

Rauter and Hartung (2005) calculated the overall prevalence of *Borrelia* species in Europe to be 13.7%. The most common genospecies were *B. afzelii* (38%), *B. garinii* (33%), *B. burgdorferi* s.s. (18%), *B. valaisiana* (19%), and *B. lusitaniae* (7%). These figures were calculated from results extracted from 154 records published from 1984 till 2003. Several studies concerning *B. burgdorferi* prevalence in ticks in the Czech Republic have been published so far. Bašta *et al*. (1999) established the prevalence of *B. burgdorferi* in *I. ricinus* ticks, collected in Prague (the capital of the Czech Republic) between years 1995–1998, to be 2.8–9.2%. *B. garinii* and *B. afzelii* were the most common genomic species detected. In city of Brno 12.1% of *I. ricinus* ticks tested positive for *B. burgdorferi* s.l. (Pejchalová *et al*. 2007). We report the MIR for the city park to be 13.8%, higher than both figures mentioned above. This might indicate a marked increase in *B. burgdorferi* prevalence in urban ecosystems in the Czech Republic or simply using more specific and sensitive molecular methods in our study.

Studies conducted in Slovakia reported identical group of genomic species as in the Czech Republic. *B. burgdorferi* MIR established by PCR was 30.2% (Smetanová *et al*. 2007). Another molecular study of Slovak *I. ricinus* ticks from 2012 yielded the MIR 25% and the first detection of *B. miyamotoi* in Slovakia (Subramanian *et al.* 2012).

Some other European countries reported prevalence of *B. burgdorferi* over 20%: Serbia – 42.5% (Milutinović *et al*. 2008), Latvia – 28% (Etti *et al*. 2003), Belgium – 23%; (Misonne *et al*. 1998), or Bulgaria – 30.7% (Christova *et al*. 2001), The prevalence in Poland varied – 5.4%, 12.3%, 22.2% and 22%, respectively (Cisak *et al.* 2006; Lenčáková *et al.* 2006; Stańczak *et al*. 2000; Sytykiewicz *et al*. 2012). All ticks tested in studies mentioned above were collected at woodland areas and forests and were analyzed individually (adults) or in pools (nymphs).

Figures reported from countries situated mostly in Western and Northern Europe only rarely exceeded 20% prevalence: Germany – 15.8% (Vögerl *et al.* 2012), Austria – 14.5% (Blaschitz *et al.* 2008), Luxembourg – 11.3% (Reye *et al.* 2010), Norway – 16% (Jenkins *et al.* 2001), Lithuania – 13.3% (Paulauskas *et al.* 2008), Denmark – 11% (Skarphédinsson *et al.* 2007), the Netherlands – 7.6% (Wielinga *et al.* 2006), Switzerland – 17.4% (Gern *et al*. 2010). In Ireland prevalence ranged between 11.5% – 28.9% according to study site (Kirstein *et al.* 1997).

Most common human pathogenic *B. burgdorferi* genomic species in Europe are *B. garinii*, *B. afzelii*, *B. burgdorferi* s.s. and *B. valaisiana* (Rauter and Hartung 2005). The frequency of individual genomic species varies among countries. We reported here the presence of all genomic species mentioned above and the first time detection of *B. spielmanii* in the Moravian region. *B. spielmanii* has been detected rarely in Germany and Switzerland (Gern *et al.* 2010; Vögerl *et al.* 2012). Many studies fail to include *B. spielmanii* detection in their analysis therefore its prevalence in Europe might be in fact higher than suggest available data.

These results contribute to the surveillance of selected tick-borne pathogens in the surroundings of Ostrava city. Molecular survey represents scientific background for the comparison of prevalence data among other European countries and complements missing information concerning occurrence of *Borrelia burgdorferi* s.l. in the tick *I. ricinus* in urban ecosystem. Spirochaete *B. spielmanii* has been detected for the first time in *I. ricinus* ticks from urban locality, highlighting the need for surveillance of neglected tick-borne pathogens even in urban areas.

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