PŮVODNÍ PRÁCE

The occurrence of *Ixodes ricinus* ticks and important tick-borne pathogens in areas with high tick-borne encephalitis prevalence in different altitudinal levels of the Czech Republic Part II. *Ixodes ricinus* ticks and genospecies of *Borrelia burgdorferi* sensu lato complex

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ABSTRACT

Study objective: Three years long research study (2011–2013) on population density of *Ixodes ricinus* and the infection rate of the pathogens that they transmit was conducted in four topographically distant areas in the Czech Republic. In the previous decade (2001–2010) thirteen loci with increased incidence of tick borne encephalitis cases were defined, suggesting the permanent interaction of human population with ticks and indicating the landmarks for study of the presence of other tick borne pathogens. The work program included the identification of existing spectrum of spirochetes from *Borrelia burgdorferi* sensu lato complex and the conditions of their occurrence and distribution.
Material and methods: In the areas of the Ústí nad Labem Region, Olomouc Region, South Bohemian Region, and Highlands Region, 600 m^2 plots were selected in the local optimal *I. ricinus* habitats where tick flagging was performed every year in the spring-summer and autumn seasons of the tick questing activity. Collected adult ticks (1369 males and 1404 females) were individually screened for *B. burgdorferi* s. l. spirochets.
Results: Spirochetes from *B. burgdorferi* s.l. complex were detected in all 13 studies sites in all altitudes from 280 to 1030 meters a. s. l. The total rate of infection was determined as 11.4% (males 10.4%, females 12.4%) with range limits from 1.4% (Ústí nad Labem in 2011) to 19.7% (South Bohemian Region, 2012).
Genospecies were detected in various proportions and in different combinations: *Borrelia afzelii, B. garinii, B. burgdorferi s. s., B. bavariensis, B. bissettii, B. valaisiana, B. spielmanii* and *B. lusitaniae*. The three-year observation justifies the assumption that the regional differences in infectivity of *I. ricinus* are based on the character of the local biocenosis of the respective region. The dynamics of its seasonal changes, conditioned by climatic factors, determines the annual differences.
Conclusion: Three of the medically most important *Borrelia* species formed a core group among all detected genospecies. *B. afzelii* was a dominated one (115 detections), followed by *B. garinii* (100) and by *B. burgdorferi* s.s. (19). Other genospecies were detected sporadically. However, the detection of *B. bissettii* should be emphasized due to the recently proven pathogenic effects of this genospecies and yet little-known sporadic expansion in the Czech Republic. The medical importance and distribution of other sporadically occurred genospecies is also discussed.

KEYWORDS:

***Ixodes ricinus* – *Borrelia afzelii* – *B. garinii* – *B. burgdorferi* s. s. – *B. bavariensis* – *B. valaisiana* – *B. spielmanii* – *B. lusitaniae* – *B. bissettii* – distribution – altitude – season – medical importance**

SOUHRN

Daniel M., Rudenko N., Golovchenko M., Danielová V., Fialová A., Kříž B., Malý M.: Výskyt klíštěte obecného *Ixodes ricinus* a významných patogenů jím přenášených ve vybraných oblastech se zvýšeným počtem onemocnění klíšťovou encefalitidou v různých nadmořských výškách v České republice
Část II. Klíště obecné *Ixodes ricinus* a genospecie komplexu *Borrelia burgdorferi* sensu lato

Cíl práce: Ve čtyřech topograficky odlehlých oblastech České republiky byl proveden tříletý výzkum (2011–2013) populační denzity klíštěte *Ixodes ricinus* a jeho infikovanosti jím přenášených hlavních patogenů. Bylo vybráno 13 lokalit se zvýšenou incidencí onemocnění klíšťovou encefalitidou v předchozí dekádě (2001–2010), indikující tím pravidelný kontakt obyvatelstva s *I. ricinus*, a tak i významná místa pro studium výskytu dalších patogenů. Do pracovního programu bylo zařazeno zjištění druhového spektra spirochét komplexu *Borrelia burgdorferi* sensu lato a podmínek jejich výskytu.

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Materiál a metodika: Ve vybraných lokalitách krajů Ústeckého, Olomouckého, Jihočeského a kraje Vysočina byly stanoveny fixní plochy (každá 600 m^2) v optimálních místních habitatech *I. ricinus*, na nichž byl prováděn každoroční kontrolní sběr v jaroletní a podzimní sezoně aktivity klíšťat. Sebraná dospělá klíšťata *I. ricinus* (1 396 samců a 1 404 samic) byla individuálně vyšetřena na přítomnost genospecií komplexu *B. burgdorferi* s. l.
Výsledky: Spirochéty *B. burgdorferi* s.l. byly detekovány v klíšťatech ve všech 13 zkoumaných lokalitách a v celém rozsahu nadmořské výšky 280–1030 m. Celková pozitivita dosáhla 11,4 % (samci 10,4 %, samice 12,4 %) v rozmezí hraničních hodnot 1,3 % (Ústí nad Labem v roce 2011) a 19,7 % (Jihočeský kraj, 2012). V různém poměru a kombinacích byly detekovány: *Borrelia afzelii, B. garinii, B. burgdorferi* s. s., *B. bavariensis*, *B. bissettii, B. valaisiana, B. spielmanii* a *B. lusitaniae*. Tříletá pozorování ukazují, že regionální rozdíly v infikovanosti *I. ricinus* jsou dány charakterem lokální biocenózy jednotlivých oblastí. Dynamika sezonních změn, podmíněná klimatickými faktory, určuje jejich meziroční rozdíly.
Závěry: Tři zdravotně nejvýznamnější genospecie tvoří hlavní část detekovaných borélií.
Převládá *B. afzelii* (115 detekcí), následována *B. garinii* (100) a *B. burgdorferi* s. s. (19).
Ostatní genospecie byly detekovány ojediněle. Je třeba zdůraznit význam zjištěného výskytu *B. bissettii*, vzhledem k závažnosti patogenního působení prokázaného v současnosti a dosud jen sporadickým znalostem o rozšíření této borélie na území České republiky. O významu v humánním lékařství se diskutuje také u ostatních sporadicky se vyskytujících genospecií.

KLÍČOVÁ SLOVA
klíště obecné *Ixodes ricinus* – *Borrelia afzelii* – *B. garinii* – *B. burgdorferi* s. s. – *B. bavariensis* – *B. bissettii* – *B. valaisiana* – *B. spielmanii* – *B. lusitaniae* – regionální výskyt – nadmořská výška – sezonnost – zdravotní význam

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INTRODUCTION

The presented work follows the publication of Daniel et al. [1] that evaluated and compared the occurrence and dynamic of *Ixodes ricinus* population and the infection rate of tick borne encephalitis (TBE) virus in them, defined during the three years long research study (2011–2013) in four topographically distant regions of the Czech Republic. The study sites that had the same common denominator such as the permanent contact of human population with tick *I. ricinus* and that revealed in previous decade (2001–2010) the increased number of cases of infection with tick borne encephalitis virus, were selected in those 4 regions that differ in the type of landscape [2]. Mentioned way of selection of the study sites allowed the suggestion that those sites will be suitable for research on the other pathogens transmitted by *I. ricinus*. That lead to identification of existing spectrum of spirochetes from *Borrelia burgdorferi* sensu lato complex and the conditions of their occurrence. Detailed criteria for selection and the method of determining the study sites are presented in Methods. To be able to compare and generalize the results from each area the design of the study was based on the three years of its duration which then included the entire course of development of at least one generation of *I. ricinus*. Another main concern of this study was the strict compliance of the work in general, and the technique of sample collection in particular, meaning that sample collection should be done by the same well trained and experienced researchers in the same well characterized loci. The main goal of our study was to compare the occurrence of three majorly distributed species from *B. burgdorferi* s. l. complex, (*B. afzelii, B. garinii* and *B. burgdorferi* s.s.) in tick *I. ricinus* infection rate in the monitored area, and its annual changes. Another task was to define the spectrum of less distributed or known species of *B. burgdorferi* s. l. complex, their occurrence in our natural environment and highlight their importance in human pathology.

MATERIAL AND METHODS

1. Selection of areas

In the areas with a high incidence of TBE reported in 2001–2010 [3], superior to the whole-country average, the study plots were selected in northern Bohemia (Ústí nad Labem Region), northern Moravia (Olomouc Region), southern Bohemia (South Bohemian Region), and in the Bohemian-Moravian Highlands (Highlands Region) according to the following key: in each area, two high prevalence municipalities (three municipalities in the South Bohemian Region) were identified. In the cadastres of these municipalities, the monitoring plots representing optimal habitats of *I. ricinus* ticks were selected in the closest vicinity of the built-up area. In addition, in each area, a plot located at a higher altitude than the surrounding landscape and providing a suitable habitat for *I. ricinus* ticks was selected. The middle of each plot was determined by the geographic coordinates. Large urban units (regional cities) were not included among the selected municipalities because the direct contact of TBE cases with the surrounding green spaces is not commonplace.

2. Localities

South Bohemian Region
Stožec Mt. 48°52´22˝ N, 13°49´41˝ E; 910 – 920 m a.s.l.
Strakonice – Starý Dražejov 49°16´31˝N, 13°52´46˝ E; 480 – 490 m a.s.l.
Netolice – 49°02´30˝ N, 14°10´51˝ E; 460 – 470 m a.s.l.
Zliv – 49°04´24˝ N, 14°22´11˝ E; 405 – 410 m a.s.l.
Ústí nad Labem Region
Povrly – 50°40´38˝ N, 14°08´31˝ E; 280 – 300 m a.s.l.
Benešov nad Ploučnicí – 50°43´56˝ N, 14°18´58˝ E; 280 – 300 m a.s.l.
Děčínský Sněžník – Medvědí louka – 50°48´03˝ N, 14°05´50 ˝ E; 550 – 570 m a.s.l.
Olomouc Region
Červenohorské sedlo – 50°07´20˝ N, 17°09´08˝ E; 1020 – 1030 m a.s.l.

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Jeseník – Křížový vrch – 50°13´37˝ N, 17°13´08˝ E; 530 – 550 m a.s.l.
Šumperk – Holubí vrch – 49°58´49˝ N, 16°59´08˝ E; 410 – 420 m a.s.l.
Highlands Region
Baliny (Velké Meziříčí) – 49°20´19˝ N, 15°58´04˝ E; 450 – 470 m a.s.l.
Bystřice nad Pernštejnem – 49°32´41˝ N, 16°17´23˝E; 400 – 450 m a.s.l.
Nedvědice – 49°27´59˝ N, 16°20´01˝ E; 330 – 370 m a.s.l.
The detail characteristic of study habitats is given in [1].

3. Collection of *Ixodes ricinus* ticks

Ticks were collected by the standard flagging technique [4] on the defined plots. The flag (50 x 70 cm) was made of white fabric with a slight nap (flannel). The ticks' collection was carried out by one person for three hours. Based on long-term experience, three hours of work performed by a skilled person at the average abundance of ticks in the area correspond to 600 m^2 of the area monitored. This approach makes it possible to focus in detail on the habitat and micro-relief of the area where ticks are actually present. Collected ticks were transported to the laboratory, identified to the species level, and stored at -80 °C until further processed. The presence of larvae was recorded only.

4. Tick DNA isolation

The collected ticks were separated by developmental stage, gender, collection area, and collection date. All male and female ticks were analyzed individually. Ticks were homogenized in 100 µl of PBS using an automatic homogenizer (TissueLyzer II (Qiagen). Pools were made from adult ticks by mixing of 10 µl of 10 adult tick samples originated from the same locality. Such pooled samples were used for RNA isolation and further detection of TBE virus [1]. The rest of the samples were used for further experiments. Isolation of genomic DNA was conducted using DNeasy® Blood & Tissue Kit (Qiagen) according to the manufacturer's protocol. After adding proteinase K the samples were left for overnight incubation at 56 °C. The DNA was eluted from the column by 50 µl of H_2O.

5. DNA purification, PCR amplification, and sequencing

Tick DNA samples were selectively controlled (10 samples out of each 100) for the efficiency of tick DNA isolation using PCR primers Ixri-F (GGAAATCCCGTCGCACG) and Ixri-R (CAAACGCGCCAACGAAC) that target a 150 bp fragment of 5.8S rRNA gene [5].
Detection of *B. burgdorferi* s.l. infection was performed using total tick DNA as template. The MasterTaq kit (Eppendorf, Germany) was used for amplification of fragment of chromosome localized *flagellin* gene using the gene-specific primers (Fla out F-5′-AARGAATTGGCAGTTCAATC-3′ and Fla out R-5′-GCATTTTCWATTTTAGCAAGTGATG-3′ [6] that produce specific 496 nt fragment. *B. burgdorferi* s.s. DNA was used as the positive control, and double-distilled water was used as the negative control in each PCR run.
The PCR products were separated by electrophoresis on 1.5% agarose gel and visualized under UV light. PCR products of the expected size were cut off the gel, purified, and submitted for direct sequencing. Sequences were identified using NCBI BLAST similarity search.
The presence of multiple infection was detected by multiplex PCR using GI, GII and GIII primers [7] designed on a basis of *ospA* sequences of *B. burgdorferi* s.s. (GI primer set), *B. garinii* (GII primer set) and *B. afzelii* (GIII primer set) that produced the 544 bp, 345 bp and 189 bp fragments, respectively. Positive samples that revealed the presence of other *Borrelia* species were double checked with previously described *ospC* primers F-5′-AAAGAATACATTAAGTGCGATATT-3′ and R-5′--GGGCTTGTAAGCTCTTTAACTG-3′ [8]. Amplified fragments were sequenced to detect possible co-infection.

6. Statistical analysis

The prevalence of infection *Borrelia burgdorferi* s.l. in ticks was calculated. To test the significance of differences in prevalence Pearson's Chi-squared test or Fisher's exact test were used. The statistical significance level was set to 0.05. The data were processed by the R software (R Core Team, 2014, version 3.1.2).

RESULTS

1. The detection of *Borrelia burgdorferi* sensu lato in adult *Ixodes ricinus* ticks

Two thousands seven hundreds and seventy three (2,773) adult *Ixodes ricinus* ticks were individually analyzed for the presence of *Borrelia burgdorferi* s.l. with positive finding in 11.4% (316 out of 2,773). Both tick genders in the examined group were represented about equally, and corresponded to a similar positive findings (F- 12.4%, M -10.4%). The overall result has threshold values, contingent to both ticks region of origin and year of ticks' collection, from 1.4% (Ústí nad Labem, 2011) to 19.7% (South Bohemian Region, 2012) of positive ticks.
Results of *B. burgdorferi* s.l. detection in ticks from various regions are shown in Table 1. High average positivity observed in the South Bohemian Region (15.5%) corresponds

Table 1. Prevalence of *Borrelia burgdorferi* sensu lato in adult *Ixodes ricinus* ticks in individual regions (2011–2013)

Region	Number of ticks					
	Males		Females		Males + Females	
	Analyzed	Positive	Analyzed	Positive	Analyzed	Positive
Ústí nad Labem	290	30	257	32	547	62
Olomouc	179	15	165	16	344	31
South Bohemian	460	69	490	78	950	147
Highlands	440	28	492	48	932	76
Total	**1369**	**142**	**1404**	**174**	**2773**	**316**

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Table 2. Prevalence of *Borrelia burgdorferi* sensu lato in adult *Ixodes ricinus* ticks in individual years 2011–2013

Year	Number of ticks					
	Males		Females		Males + Females	
	Analyzed	Positive	Analyzed	Positive	Analyzed	Positive
2011	408	33	450	42	858	75
2012	524	60	530	64	1054	124
2013	437	49	424	68	861	117
Total	**1369**	**142**	**1404**	**174**	**2773**	**316**

to a relatively uniform high positivity observed here in different years (2011–2013, i.e. 11.9%, 19.7%, 13.2%), unlike other regions with some year to year fluctuation. This was most clearly reflected in the Ústí nad Labem Region. The annual changes are documented in Table 2. The finding of an upward trend in overall positivity observed in different years (2011 – 8.7%, 2012 – 11.8%, 2013 – 13.6%) is supported by the fact that during all three compared years approximately equal numbers of adult *I. ricinus* ticks were examined.

2. Detection of individual genospecies of *Borrelia burgdorferi* sensu lato complex in adult *Ixodes ricinus* ticks

The ratios of genospecies found varied with individual locations and habitats. The following single genospecies were detected in various proportions and in different combinations: *B. afzelii, B. garinii, B. burgdorferi* sensu stricto, *B. bavariensis, B. bissettii, B. valaisiana, B. spielmanii* and *B. lusitaniae* (for details see Tables 3a-d). The presence of single genospecies was detected in 244 cases; double infection was found in 33 ticks. In one case, the presence of three spirochete species was determined. *Borrelia* species in 38 samples could not be identified.

Three of the medically most important *Borrelia* species formed a core group among all detected genospecies. *B. afzelii* was a dominated one (115 detections), followed by *B. garinii* (100) and with a substantial gap – by *B. burgdorferi* s.s. (19). These three genospecies were also most often observed in dual infection. The most common co-infection was *B. afzelii* + *B. garinii* (22x), followed by *B. afzelii* + *B. burgdorferi* s.s. (6x), and *B. garinii* + *B. burgdorferi* s.s. (2x). All other combinations were found rarely (*B. afzelii* + *B. lusitaniae* (2x), *B. afzelii* + unidentified species (1x). One case represented the triple infection of *B. afzelii* + *B. garinii* + *B. burgdorferi* s. s.

B. valaisiana, B. spielmanii, B. lusitaniae, B. bissettii and *B. bavariensis* were detected sporadically. However, the detection of *B. bissettii* should be emphasized due to the recently proven pathogenic effects of this genospecies [9, 10, 11, 12] and yet little-known sporadic expansion in the Czech Republic. *B. bavariensis* was not primarily identified as a separate species (detected twice as a result of sequence analysis) and thus was included among *B. garinii*. Significant is also the quadruple detection of *B. spielmanii*. Details about individual genospecies findings are documented in Tables 3a-d. Both qualitative and quantitative regional differences in the spectrum of the detected pathogenic spirochete genospecies were found. This result is even more evident due to the fact that in all cases a similar number of adult I. ricinus ticks were examined.

Concerning the relationship of tick density and *B. burgdorferi* s.l. prevalence, the direct correlation was observed. In both indicators such as the number of single positive infections and the number of 4 different double and triple combinations South Bohemia Region clearly dominates. On the opposite side of these results is Olomouc Region. The lowest total counts and only 2 genospecies, i.e. *B. afzelii* (8 detections) and *B. garinii* (16 detections) were detected in that region. Two cases of double infection with *B. afzelii* +*B. garinii* were identified as well. These findings correlate with the results of nymph and adult ticks' density and their mutual ratio in the same regions [1].

3. Statistical analyses

Prevalence of *Borrelia burgdorferi* s.l. complex in individual regions reached 11.3 %, in Ústí nad Labem Region, 9.0 %, in Olomouc Region, 15.5 % in South Bohemian Region, and 8.2 % in Highlands Region, and differed significantly among regions ($p < 0.001$). Differences among regions are also demonstrable from the perspective of most frequently occurring genospecies, *B. afzelii*, whose prevalence varies between 2.1% (Highlands Region) and 6.2% (South Bohemia Region) ($p < 0.001$), and *B. garinii*, whose prevalence varies between 2.1% (Highlands Region) and 5.1% (South Bohemia Region) ($p = 0.004$).

The prevalence of infection with *B. burgdorferi* s. l. differs in individual years significantly ($p = 0.006$). Infection rates are also significantly different depending on altitude of capture areas ($p < 0.001$). At a height below 400 m a.s.l. prevalence is 9.2 %, at an altitude of 400–600 m a.s.l. prevalence is 13.0 %, and at an altitude above 600 m a.s.l., where only 3 positive ticks from a total of 77 ticks were, prevalence has reached only 3.9 %.

There were no statistically significant differences in the incidence of co-infection among regions, years or altitudes.

DISCUSSION

The detection of tick-borne pathogens in the vast collection of *I. ricinus* accumulated over a wide area and within the three-year time period brought the results confirming the importance of the volume of the examined materials for determining the real total infection rate of the vector in the surveyed area. In the extensive set of the analyzed material the relatively low average values in comparison with frequently published data were determined. For example, Gern and Humair in their review [13] refer to 17.4% as European average for the infection of adult *I. ricinus* ticks, which in our case was achieved only exceptionally, and in the assessment of the

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Table 3a. Individual genospecies of *Borrelia burgdorferi* sensu lato detected in adult *Ixodes ricinus* ticks in Ústí nad Labem Region

							Single infection									Co-infection	
Locality	**Altitude m a.s.l.**	**Date**	**M (posit)**	**F (posit)**	**M+F (posit)**	**% posit**	**B.afz**	**B.gar**	**B.bav**	**B.ss**	**B.biss**	**B.val**	**B.spi**	**B.lus**	**Undet.**	**Number**	**Combination**
Year 2011																	
Povrly	280–300	19.7.	9 (0)	6 (0)	15 (0)	0	0	0	0	0	0	0	0	0	0	0	
		1.10.	12 (0)	5 (0)	17 (0)	0	0	0	0	0	0	0	0	0	0	0	
Benešov n. Ploučnicí	280–300	19.7.	3 (0)	1 (0)	4 (0)	0	0	0	0	0	0	0	0	0	0	0	
		2.10.	4 (0)	4 (0)	8 (0)	0	0	0	0	0	0	0	0	0	0	0	
Děčínský Sněžník	550–570	19.7.	3 (0)	7 (0)	10 (0)	0	0	0	0	0	0	0	0	0	0	0	
		1.10.	10 (0)	6 (1)	16 (1)	6.3	0	0	0	0	0	0	0	0	**1**	0	
Total			**41 (0)**	**29 (1)**	**70 (1)**	**1.4**	0	0	0	0	0	0	0	0	**1**	0	
% positive			0	3.5	1.4												
Year 2012																	
Povrly	280–300	16.7.	43 (2)	33 (4)	76 (6)	7.9	**2**	**2**	0	0	0	0	**1**	0	**1**	0	
		15.9.	15 (2)	19 (3)	34 (5)	14.7	**3**	**1**	0	0	0	0	0	0	0	**1**	1 B. afz+ss
Benešov n. Ploučnicí	280–300	17.7.	42 (0)	41 (1)	83 (1)	1.2	0	0	**1**	0	0	0	0	0	0	0	
		15.9.	6 (3)	4 (0)	10 (3)	30.0	0	**1**	0	**1**	0	0	0	0	**1**	0	
Děčínský Sněžník	550–570	17.7.	10 (0)	18 (0)	28 (0)	0	0	0	0	0	0	0	0	0	0	0	
		16.9.	6 (2)	1 (0)	7 (2)	28.6	**2**	0	0	0	0	0	0	0	0	0	
Total			**122 (9)**	**116 (8)**	**238 (17)**	**7.1**	**7**	**4**	**1**	**1**	0	0	**1**	0	**2**	**1**	
% positive			7.4	6.9	7.1												
Year 2013																	
Povrly	280–300	1.7.	47 (7)	38 (5)	85 (12)	14.1	**3**	**3**	0	**1**	0	0	**2**	0	**2**	**1**	1 B. afz+gar
		22.9.	22 (5)	26 (7)	48 (12)	25.0	**9**	**2**	0	0	0	**1**	0	0	0	0	
Benešov n. Ploučnicí	280–300	30.6.	23 (2)	20 (5)	43 (7)	16.3	**3**	**3**	0	0	0	0	0	0	0	**1**	1 B. afz+ss
		20.9.	8 (0)	7 (1)	15 (1)	6.7	**1**	0	0	0	0	0	0	0	0	0	
Děčínský Sněžník	550–570	1.7.	24 (7)	16 (4)	40 (11)	27.5	**5**	**4**	0	**1**	0	0	0	0	**1**	0	
		22.8.	3 (0)	5 (1)	8 (1)	12.5	0	0	0	0	0	0	0	0	0	**1**	1 B. afz+ss
Total			**127 (21)**	**112 (23)**	**239 (44)**	**18.4**	**21**	**12**	0	**2**	0	**1**	**2**	0	**3**	**3**	
% positive			16.5	20.5	18.4												

Table 3b. Individual genospecies of *Borrelia burgdorferi* sensu lato detected in adult *Ixodes ricinus* ticks in Olomouc Region

							Single infection									Co-infection	
Locality	**Altitude m a.s.l.**	**Date**	**M (posit)**	**F (posit)**	**M+F (posit)**	**% posit**	**B.afz**	**B.gar**	**B.bav**	**B.ss**	**B.biss**	**B.val**	**B.spi**	**B.lus**	**Undet.**	**Number**	**Combination**
Year 2011																	
Šumperk	410–420	6.7.	14 (0)	11 (1)	25 (1)	4.0	0	0	0	0	0	0	0	0	**1**	0	
		10.9.	11 (1)	7 (0)	18 (1)	5.6	0	0	0	0	0	0	0	0	**1**	0	
Červenohorské sedlo	1020–1030	6.7.	5 (0)	4 (0)	9 (0)	0	0	0	0	0	0	0	0	0	0	0	
		2.10.	1 (0)	1 (0)	2 (0)	0	0	0	0	0	0	0	0	0	0	0	
Jeseník	530–550	5.7.	9 (0)	12 (1)	21 (1)	4.8	0	**1**	0	0	0	0	0	0	0	0	
		11.9.	5 (1)	5 (0)	10 (1)	10.0	0	0	0	0	0	0	0	0	**1**	0	
Total			**45 (2)**	**40 (2)**	**85 (4)**	**4.7**	0	**1**	0	0	0	0	0	0	**3**	0	
% positive			4.4	5.0	4.7												
Year 2012																	
Šumperk	410–420	5.7.	11 (2)	12 (1)	23 (3)	13.0	**1**	**2**	0	0	0	0	0	0	0	0	
		29.9.	0	1 (0)	1 (0)	0	0	0	0	0	0	0	0	0	0	0	
Červenohorské sedlo	1020–1030	6.7.	6 (0)	6 (0)	12 (0)	0	0	0	0	0	0	0	0	0	0	0	
		28.9.	1 (0)	1 (0)	2 (0)	0	0	0	0	0	0	0	0	0	0	0	
Jeseník	530–550	9.7.	29 (2)	19 (2)	48 (4)	8.3	**1**	**2**	0	0	0	0	0	0	0	**1**	1 B. afz+gar
		28.9.	1 (1)	4 (2)	5 (3)	60.0	**1**	**2**	0	0	0	0	0	0	0	0	
Total			**48 (5)**	**43 (5)**	**91 (10)**	**11.0**	**3**	**6**	0	0	0	0	0	0	0	**1**	
% positive			10.4	11.6	11.0												
Year 2013																	
Šumperk	410–420	9.7.	29 (3)	20 (4)	49 (7)	14.3	**2**	**4**	0	0	0	0	0	0	**1**	0	
		28.9.	9 (1)	6 (1)	15 (2)	13.3	0	**2**	0	0	0	0	0	0	0	0	
Červenohorské sedlo	1020–1030	9.7.	10 (1)	13 (1)	23 (2)	8.7	0	**1**	**1**	0	0	0	0	0	0	0	
		28.9.	2 (0)	2 (0)	4 (0)	0	0	0	0	0	0	0	0	0	0	0	
Jeseník	530–550	8.7.	21 (2)	22 (1)	43 (3)	7.0	**1**	**2**	0	0	0	0	0	0	0	0	
		27.9.	15 (1)	19 (2)	34 (3)	8.8	**2**	0	0	0	0	0	0	0	0	**1**	1 B. afz+gar
Total			**86 (8)**	**82 (9)**	**168 (17)**	**10.1**	**5**	**9**	**1**	0	0	0	0	0	**1**	**1**	
% positive			9.3	11.0	10.1												

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Table 3c. Individual genospecies of *Borrelia burgdorferi* sensu lato detected in adult *Ixodes ricinus* ticks in South Bohemian Region

							Single infection									Co-infection	
Locality	**Altitude m a.s.l.**	**Date**	**M (posit)**	**F (posit)**	**M+F (posit)**	**% posit**	**B.afz**	**B.gar**	**B.bav**	**B.ss**	**B.biss**	**B.val**	**B.spi**	**B.lus**	**Undet.**	**Number**	**Combination**
Year 2011																	
Stožec	910–920	26.6.	1 (0)	3 (0)	4 (0)	0	0	0	0	0	0	0	0	0	0	0	
		29.9.	0	0	0		0	0	0	0	0	0	0	0	0	0	
Strakonice	480–490	10.7.	14 (2)	20 (3)	34 (5)	14.7	0	**3**	0	**1**	0	0	0	0	**1**	0	
		1.10.	3 (1)	3 (1)	6 (2)	3.3	**1**	**1**	0	0	0	0	0	0	0	0	
Zliv	405–410	12.7.	21 (2)	22 (3)	43 (5)	11.6	**3**	**2**	0	0	0	0	0	0	0	0	
		15.9.	30 (5)	52 (8)	82 (13)	15.9	**9**	**3**	0	0	0	**1**	0	0	0	0	
Netolice	460–470	15.7.	30 (0)	35 (1)	65 (1)	1.5	0	0	0	0	0	0	0	0	0	**1**	1 B afz+gar
		22.9.	29 (5)	22 (3)	51 (8)	15.7	0	**3**	0	0	0	0	0	**1**	**1**	**3**	2 B afz+lus 1 Bb ss+gar
Total			**128 (15)**	**157 (19)**	**285 (34)**	**11.9**	**13**	**12**	0	**1**	0	**1**	0	**1**	**2**	**4**	
% positive			11.7	12.1	11.9												
Year 2012																	
Stožec	910–920	26.6.	6 (0)	3 (0)	9 (0)	0	0	0	0	0	0	0	0	0	0	0	
		18.9.	0	3 (0)	3 (0)	0	0	0	0	0	0	0	0	0	0	0	
Strakonice	480–490	24.6.	17 (2)	16 (6)	33 (8)	24.2	**4**	**2**	0	0	0	0	0	0	**1**	**1**	1 B. afz+gar
		16.9.	10 (5)	12 (5)	22 (10)	45.5	**3**	**4**	0	**2**	0	0	0	0	0	**1**	1 B. afz+gar
Zliv	405–410	15.6.	84 (9)	76 (7)	160 (16)	10.0	**6**	**5**	0	**2**	**1**	0	0	0	**1**	**1**	1 B. afz + ss
		15.9.	35 (9)	26 (7)	61 (16)	26.2	**6**	**4**	0	**2**	0	0	0	0	**2**	**2**	2 B. afz+gar
Netolice	460–470	29.5	37 (13)	35 (8)	72 (21)	29.2	**10**	**8**	0	**1**	0	0	0	0	0	**2**	2 B. afz+gar
		12.9.	12 (1)	13 (4)	25 (5)	20.0	**3**	**1**	0	0	0	0	0	0	0	**1**	1 B. afz+gar+ss
Total			**201 (39)**	**184 (37)**	**385 (76)**	**19.7**	**32**	**24**	0	**7**	**1**	0	0	0	**4**	**8**	
% positive			19.4	20.1	19.7												
Year 2013																	
Stožec	910–920	5.9.	5 (1)	4 (0)	9 (1)	11.1	**1**	0	0	0	0	0	0	0	0	0	
Strakonice	480–490	5.7.	5 (0)	4 (1)	9 (1)	11.1	0	0	0	**1**	0	0	0	0	0	0	
		29.9.	2 (0)	2 (1)	4 (1)	25.0	0	**1**	0	0	0	0	0	0	0	0	
Zliv	405–410	3.7.	26 (3)	25 (2)	51 (5)	9.8	**1**	**2**	0	0	0	0	0	0	**1**	**1**	1 B. afz+gar
		3.9.	38 (4)	44 (8)	82 (12)	14.6	**5**	**4**	0	0	0	0	0	0	**1**	**2**	2 B. afz+gar
Netolice	460–470	21.6.	34 (6)	47 (6)	81 (12)	14.8	**3**	**5**	0	**1**	0	0	0	0	**3**	0	
		24.9.	21 (1)	23 (4)	44 (5)	11.4	**4**	0	0	0	0	0	**1**	0	0	0	
Total			**131 (15)**	**149 (22)**	**280 (37)**	**13.2**	**14**	**12**	0	**2**	0	0	**1**	0	**5**	**3**	
% positive			11.5	14.8	13.2												

% positive			11.5	14.8	13.2													

Table 3d. Individual genospecies of *Borrelia burgdorferi* sensu lato detected in adult *Ixodes ricinus* ticks in Highlands Region

							Single infection									Co-infection	
Locality	**Altitude m a.s.l.**	**Date**	**M (posit)**	**F (posit)**	**M+F (posit)**	**% posit**	**B.afz**	**B.gar**	**B.bav**	**B.ss**	**B.biss**	**B.val**	**B.spi**	**B.lus**	**Undet.**	**Number**	**Combination**
Year 2011																	
Baliny	450–470	2.7.	4 (0)	7 (1)	11 (1)	9.1	0	0	0	**1**	0	0	0	0	0	0	
		10.9.	10 (1)	12 (2)	22 (3)	13.6	**1**	**1**	0	**1**	0	0	0	0	0	0	
Bystřice	400–450	28.6.	10 (1)	8 (0)	18 (1)	5.6	0	0	0	0	0	0	0	0	0	**1**	1 B. afz+ss
		17.9.	10 (2)	14 (2)	24 (4)	16.7	**1**	0	0	0	0	0	0	0	**3**	0	
Nedvědice	330–370	6.7.	83 (8)	94 (10)	177 (18)	10.2	**4**	**3**	0	**2**	0	0	0	0	**4**	**5**	5 B. afz+gar
		17.9.	77 (4)	89 (5)	166 (9)	5.4	**1**	**2**	0	0	0	0	0	0	**6**	0	
Total			**194 (16)**	**224 (20)**	**418 (36)**	**8.6**	**7**	**6**	0	**4**	0	0	0	0	**13**	**6**	
% positive			8.3	8.9	8.6												
Year 2012																	
Baliny	450–470	3.6.	25 (0)	31 (1)	56 (1)	1.8	0	0	0	**1**	0	0	0	0	0	0	
		28.8.	12 (1)	11 (1)	23 (2)	8.7	0	**1**	0	0	0	0	0	0	**1**	0	
Bystřice	400–450	16.6.	36 (2)	39 (4)	75 (6)	8.0	**2**	**2**	0	0	0	0	0	0	0	**2**	1 B. afz+ss 1 B. afz+?
		24.8.	16 (2)	28 (2)	44 (4)	9.1	**2**	**1**	0	0	0	0	0	0	0	**1**	1 B. afz+gar
Nedvědice	330–370	16.6.	61 (2)	66 (5)	127 (7)	5.5	**1**	**2**	0	0	0	0	0	0	**2**	**2**	2 B. afz+gar
		24.8.	3 (0)	12 (1)	15 (1)	6.7	**1**	0	0	0	0	0	0	0	0	0	
Total			**153 (7)**	**187 (14)**	**340 (21)**	**6.2**	**6**	**6**	0	**1**	0	0	0	0	**3**	**5**	
% positive			4.6	7.5	6.2												
Year 2013																	
Baliny	450–470	15.6.	24 (3)	18 (3)	42 (6)	14.3	**2**	**3**	0	0	0	0	0	0	0	**1**	1 B. afz+gar
		12.9.	4 (0)	6 (1)	10 (1)	10.0	0	**1**	0	0	0	0	0	0	0	0	
Bystřice	400–450	8.6.	18 (0)	20 (3)	38 (3)	7.9	**2**	**1**	0	0	0	0	0	0	0	0	1 B. afz+ss
		8.9.	14 (0)	12 (1)	26 (1)	3.9	0	**1**	0	0	0	0	0	0	0	0	
Nedvědice	330–370	9.6.	22 (2)	13 (4)	35 (6)	17.1	**3**	0	0	**1**	0	0	0	0	**1**	**1**	
		12.9.	11 (0)	12 (2)	23 (2)	8.7	0	**2**	0	0	0	0	0	0	0	0	1 B. afz+ss
Total			**93 (5)**	**81 (14)**	**174 (19)**	**10.9**	**7**	**8**	0	**1**	0	0	0	0	**1**	**2**	
% positive			5.4	17.3	10.9												

Abbreviations
M (posit) – number of males analyzed(positive) ;F (posit) – number of females analyzed(positive); M + F (posit) – number of males+females analyzed (positive); B.afz – *Borrelia afzelii*; B. gar – *Borrelia garinii*; B. bav – *Borrelia bavariensis*; B. ss – *Borrelia burgdorferi* sensu stricto; B. biss – *Borrelia bissettii*; B. val – *Borrelia valaisiana*; B. spi – *Borrelia spielmanii*; B. lus – *Borrelia lusitaniae*; Undet. – *Borrelia burgdorferi* s. l. genospecies not identified

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situation in one locality and within time-limited period. However, analysis of 11,182 ticks from South Bohemia [14], determined 8.5% of total prevalence rate in adult ticks. This finding supports our results and simultaneously underlines the conclusion that the volume of the analyzed material and a sufficiently long period of time of its assembly is an essential criterion for assessing the general validity of the conclusions reached.

The data obtained displayed also high variability among collection sites and the year of tick collection, reaching the highest value of 19.7% (South Bohemian Region, 2012) of positive ticks and extremely low value 1.4% (Ústí nad Labem, 2011). A three-year observation justifies the assumption that the detected regional differences in infectivity of *I. ricinus* are based on the character of the local biocenosis of the respective region. The dynamics of its seasonal changes, conditioned by climatic factors, determines the annual differences, including differences in epidemiology of diseases transmitted by ticks.

The positive correlation in the relationship of tick density and *B. burgdorferi* prevalence was found. When combining the number of incidence of both nymphs and adult ticks infected with tick-borne encephalitis virus [1], the Region of South Bohemia clearly dominated. The same region is a predominant in the number of *B. burgdorferi* s.l. infection. This observation is in coordination with the results of Hönig et al. [14], where the same correlation was found for adult ticks.

The work presents the results of the detection of *B. burgdorferi* s.l. DNA as well as the presence of different *Borrelia* genospecies separately for each gender of *I. ricinus*. Different meanings of these findings should be emphasized based on the fact that male *I. ricinus* does not suck the blood of the host animal. His role in the development cycle is to fertilize female (which is also a precondition for her full engorgement). To fulfill this function, the power supply obtained in nymphal stage is sufficient. Therefore the male tick has no direct importance both in the epidemiology of human infections and in the cycle of zoonotic pathogens. However, there is speculation about the possibility of transmission of the spirochete from infected males to uninfected female. The act of fertilization in ticks is carried out by inserting a spermatophore using oral appendages of male to female's genital opening using secreted of saliva as an effective lubricant [15]. This could be an effective method of transmitting the infection. Almost identical ratio of the infection in male and female ticks supports this hypothesis.

The difference in the genospecies ratios was observed in various locations and habitats. This can relate to the different character of the habitats. A difference in the ecosystems in which borreliae circulate influences the composition of hosts for different developmental tick stages [16] and therefore leads to the representing of different *Borrelia* genospecies which are host-dependent. The total predominance of *B. afzelii* followed by *B. garinii* is concordant with the data on the occurrence of *B. burgdorferi* s.l. genospecies in Europe [17]. However, in two Regions the slight prevalence or equality of *B. garinii* were indicated. This can be explained by several reasons. Firstly, the fact that *B. bavariensis* was not primarily identified as a separate species and thus was included among *B. garinii* [18]. Secondly, this finding can be also supported by the fact that only adult ticks were checked for the *Borrelia* presence in this study. Margos et al. [19] indicated that the prevalence of *B. garinii* and *B. valaisiana* in adult ticks exceeded significantly the prevalence of these genospecies in nymphal ticks. Moreover, the predominance of *B. garinii* over *B. afzelii* in higher altitudes was already demonstrated in our previous study suggesting that small passerine birds moving on the ground are responsible for permanent local populations of *I. ricinus* in mountain localities with low numbers of small terrestrial mammals [20]. The prevalence of *B. garinii* over *B. afzelii* in *I. ricinus* ticks was also reported from Poland [21]. A higher prevalence of *B. afzelii* on the other hand may be caused by the increased involvement of rodents as *B. afzelii* specific hosts in the lower regions [22]. The prevalence of *B. burgdorferi* s.s. was rather low that is in agreement with other ecological studies from Europe [17, 23]. However, this fact is in discrepancy with our former findings from the different experimental plots of South Bohemia [16] where the frequency of the occurrence of *B. burgdorferi* s.s. was distinctly high. However, the prevalence of *B. burgdorferi* s.s. in this particular region is the highest among all the areas under investigation of this study.

Presence of multiple *Borrelia* genospecies within single tick samples was recorded. The presence of two *Borrelia* species in one tick was identified in 10.4% out of positive ticks. The most common co-infection was *B. afzelii* + *B. garinii*. However, as we did not distinguish *B. garinii* and *B. bavariensis* we may hypothesize, that some of the frequent *B. afzelii*-*B. garinii* co-infections are in fact *B. afzelii* and *B. bavariensis*. Such co-infection would be more likely, because these two genospecies share rodents as the main host species [18]. Interestingly, one female tick was co-infected with three genospecies (0.3%), indicating either co-infection of a single host by all of these genospecies, by sequential acquisition of *Borrelia* by immature stages or, rather disputable, from different hosts due to interrupted feeding.

Except the three traditional genospecies of *Borrelia* (*B. afzelii, B. garinii* and *B. burgdorferi* s.s.) other genospecies (*B. valaisiana, B. spielmanii, B. lusitaniae, B. bissettii* and *B. bavariensis*) were detected. It is worth to mention that in our previous studies the ticks were examined for the presence of *Borrelia*, targeting predominantly 3 main genospecies of interest. That is why the data confirming the presence of other species are of great interest.

The presence of *B. spielmanii* in the South Bohemian Region was previously detected [14, 24]. In this study the above mentioned genospecies was confirmed not only in the South Bohemia Region but in the North of the Czech Republic. These results correspond to those obtained from Germany where *B. spielmanii* was detected in 10.9% of the infected ticks [25]. Földvari and colleagues [26] reported the presence of *B. spielmanii* in skin biopsy from the patients with erythema migrans (EM) in Hungary. *B. spielmanii* was repeatedly reported in patients with EM in the Netherlands, Germany, Hungary and Slovenia [27]. Together with previous publications [28, 29], this finding suggests that *B. spielmanii* has a pathogenic role in human Lyme borreliosis (LB). Although *B. spielmanii* is distributed more focally than other species of the *B. burgdorferi* s.l. complex [30], it occurs all over Europe from the Netherlands through Germany and Czech Republic to Hungary [24, 28, 29, 30].

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B. valaisiana and *B. lusitaniae* were previously reported both as single infections or in the combination with other genospecies from the Czech Republic [14] [20] as well as from the other European countries, such as Slovakia [22, 32, 33], Greece [34], Germany [25], Sweden [35], Portugal [36], Switzerland [37]. The presence of these genospecies in ticks from the Czech Republic is of importance as both *B. valaisiana* and *B. lusitaniae* were already detected in human samples: *B. valaisiana* was detected in patients with LB symptoms in Switzerland and Greece [34, 38]. The first isolation of *B. lusitaniae* from human sample was reported in Portugal [39] followed by other reports that confirmed the association of human LB with *B. lusitaniae* [40, 41], indicating the potential involvement of these *Borrelia* genospecies in human LB.

Of particular interest could be the identification of *B. bissettii* in tick from the South Bohemia Region. Although widely distributed in the United States, this *Borrelia* genospecies is of limited sporadic expansion in the Old World. Previously detected in human patients with LB from the Czech Republic [9, 10] those results provide strong evidence of involved of *B. bissettii* in human LB in Europe. Hovewer, *B. bissettii* has never been reported in ticks in this highly endemic region. Only recently *B. bissettii* was detected in ticks in Europe. A single *I. ricinus* tick from Slovakia was found to be reactive with probes specific for *B. bissettii* [22]. However, the fact than this tick was also reactive with probes for two other genospecies of *B. burgdorferi* s.l. complex complicated the specific identification of the spirochetes. Later, *B. bissettii*-like DNA was identified in tick from the Czech Republic [42]. But already in 2014 Tappe and colleagues [25] while checking *B. burgdorferi* s.l. infections in *I. ricinus* in the city of Hanover (Germany) detected *B. bissettii* as a single infection already in ten ticks with the total prevalence of 2.1%. They also detected *B. bissettii* in 7 ticks in the co-infection with another *Borrelia*. Our finding of *B. bissettii* DNA in a female tick from South Bohemia confirms the fact that this *Borrelia* species is becoming more „popular" in the European samples both from hosts and vectors and the integration of this species into the complex of traditionally recognized European *Borrelia* is only the question of time. History has shown that when a pathogen is introduced into a new region and new ecosystem, one should expect the unexpected. New vectors may be involved in the transmission cycle and diseases in vertebrates with which the pathogen did not evolve, may be more severe than occurs in endemic regions. This could be the reason why in the last few years researchers and physicians have reported more unusual characteristics of LB [11, 12].

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