

# Identification of *Borrelia burgdorferi* genospecies isolated from *Ixodes ricinus* ticks in the South Moravian region of the Czech Republic

Ondřej Bonczek<sup>1,2</sup>, Alena Žáková<sup>3</sup>, Lýdia Vargová<sup>1</sup>, Omar Šerý<sup>1,2</sup>

<sup>1</sup> Laboratory of DNA diagnostics, Department of Biochemistry, Faculty of Science, Masaryk University, Brno, Czech Republic

<sup>2</sup> Laboratory of Animal Embryology, Institute of Animal Physiology and Genetics, The Academy of Sciences of The Czech Republic, Brno, Czech Republic

<sup>3</sup> Department of Comparative Animal Physiology and General Zoology, Faculty of Science, Masaryk University, Brno, Czech Republic

Bonczek O, Žáková A, Vargová L, Šerý O. Identification of *Borrelia burgdorferi* genospecies isolated from *Ixodes ricinus* ticks in the South Moravian region of the Czech Republic. *Ann Agric Environ Med.* 2015; 22(4): 637–641. doi: 10.5604/12321966.1185766

## Abstract

**Introduction.** During 2008–2012, a total of 466 ticks *Ixodes ricinus* removed from humans were collected and tested for the presence of *Borrelia burgdorferi* sensu lato (Bbsl). Ticks were collected in all districts of the South Moravian region of the Czech Republic (CZ).

**Objective.** The aim of this study was to determine the infestation of Bbsl in ticks *Ixodes ricinus* and the identification of genospecies of Bbsl group by DNA sequencing.

**Material and methods.** DNA isolation from homogenates was performed by UltraClean BloodSpin DNA kit (MoBio) and by automated instrument Prepito (Perkin-Elmer). Detection of spirochetes was carried out by RealTime PCR kit EliGene *Borrelia* LC (Elisabeth Pharmacon). Finally, all the positive samples were sequenced on an ABI 3130 Genetic Analyzer (Life Technologies) and identified in the BLAST (NCBI) database.

**Results.** A total positivity of the samples was 26%. For the first time in the Czech Republic, 5 of the isolated strains were genotyped as *Borrelia spielmanii* (7.1%). Other representatives of Bbsl were also observed: *B. afzelii* (70.0%), *B. garinii* (10.0%), *B. valaisiana* (8.6%), and *B. burgdorferi* s. s. (4.3%).

**Conclusion.** A general view of the spreading of Bbsl in the South Moravian region was demonstrated. The most interesting result of the study is the finding of *B. spielmanii* for the first time in this region.

## Key words

*Borrelia burgdorferi* s. l., *Borrelia spielmanii*, *Ixodes ricinus*, genotyping, RealTime PCR, DNA sequencing

## INTRODUCTION

The *Ixodes ricinus* tick is the most commonly mentioned transmitter of spirochetes belonging to the *Borrelia burgdorferi* sensu lato (Bbsl) group. Spirochetes of the Bbsl complex have been known since 1982 as the etiologic agent of Lyme disease (LD) transferred by *Ixodid* ticks [1, 2]. Evidence of the Bbsl, however, has been detected to-date not only in ticks but also in mosquitoes (imago and larvae) and other arthropods (Mesostigmata, Siphonaptera), [3, 4, 5, 6]. When only a low percentage of pathogenic borreliae DNA is present in mosquitoes, even in mosquitoes' larvae, only the mechanical way of transmission to humans is anticipated [7]. Only a tick bite is considered as a source of LD in humans. Ventral hypostome and chelicerae of the ticks are adapted to penetrate the skin and also to enable attachment for a longer time period [8]. Most ticks are removed from people 24–48 hours after the beginning of attachment. For infection, it is necessary to contaminate feeding sites with infected salivary secretions or regurgitated midgut contents of the *I. ricinus* [9]. Researchers predict that current knowledge about the time

required for Bbsl transmission from tick to mammals are not final and will be re-evaluated. Recently, papers describing the transmission of pathogenic Bbsl earlier than 24 hours after attachment have been published, e.g., Hynote et al. [10] and Crippa et al. [11], who suppose that the genospecies *B. afzelii* and *B. garinii* is transferred to the host rather than the American genospecies *Borrelia burgdorferi* sensu stricto (Bbss), and *I. ricinus* is suitable vector for *B. afzelii* rather than Bbss.

There are many studies of the rate of infection of ticks with *Borrelia*. Analysis of data from 155 records of studies conducted in Europe showed that the overall mean infection rate was 13.6% [12]. According to metaanalysis performed by Rauter et al. [12], the regions with the highest infection rates are located in central Europe (Austria, Czech Republic, southern Germany, Switzerland, Slovakia, and Slovenia).

The Bbsl complex currently contains at least 18 genospecies associated with particular reservoir hosts, 8 of which are distributed throughout Europe [13]; 5 are considered to be species with pathogenic potential: *B. afzelii*, *B. garinii*, *B. valaisiana*, *B. burgdorferi* sensu stricto and *B. lusitaniae*. Analyses of data from 53 records revealed a distinct pattern of *Borrelia* species distribution in Europe: *B. afzelii* and *B. garinii* are the most common species, followed by *B. valaisiana* and *B. burgdorferi* sensu stricto [12].

Address for correspondence: Omar Šerý, Laboratory of DNA diagnostics, Department of Biochemistry, Faculty of Science, Masaryk University, Kamenice 5, 625 00 Brno, Czech Republic.  
E-mail: bonczek@mail.muni.cz

Received: 18 July 2014; accepted: 25 March 2015



The region of South Moravia represents the optimal nature condition for the spreading of LD. Hubálek et al. [14] reported that 16.8 – 20.4 % of ticks collected in the region were positive for Bbsl. The aim of the presented study was to analyze the presence of Bbsl by RealTime PCR method in all ticks delivered to our laboratory from South Moravia, and collected from human skin. All RealTime positive samples were subsequently analyzed by DNA sequencing method to reveal Bbsl genospecies.

## MATERIALS AND METHOD

*Ixodes ricinus* ticks were collected from the skin of humans living in the South Moravian region during a 5 year period – 2008–2012. Persons participating in the study were attacked by ticks in suburban and rural areas, including forests, meadows, parks and gardens. The South Moravian region is located in the south-eastern part of the Czech Republic and shares a border with Austria and Slovakia. The highest point of the region is located about 842 meters above sea level, and the point with the lowest elevation is situated 150 meters above sea level.

The long-term normal precipitation is about 543 mm/year, and the average air temperature in 2008–2012 was about 9.4°C. According to information from the Czech Hydrometeorological Institute, the long-term normal air temperature is 8.3°C in the South Moravian Region [15].

*Ixodes ricinus* ticks feeding on people with no preference of point of life cycle were sampled in the South Moravian region of the Czech Republic. A total of 466 samples during the period April 2008 – December 2012 were obtained. *Borrelia*-specific DNA was isolated from homogenates using two methods. In 2008 – 2011, UltraClean BloodSpin DNA kit (MoBio, USA), and in 2012, automatic isolation by chemagic Prepito instrument (Perkin Elmer, Germany) with NA Body Fluid kit was used. DNA was stored in a refrigerator at 4°C. According to our experience, storage of borrelian DNA at a lower temperature than 4°C causes the reduction of successfully amplifiable DNA.

RealTime PCR assay based on the specific flagellin sequence amplification for the detection of *B. burgdorferi* s.l. was performed by EliGene Borrelia LC (Elisabeth Pharmacon, Czech Republic). The kit is used in clinical laboratories in the European Union as an *in vitro* diagnostic medical device. It has a high sensitivity that allows the detection of 1–10 *Borrelia* genomes in 5 µl of a DNA sample. Internal control was used according to the instruction manual of the kit during isolation of DNA and PCR assays to control process of amplification, and to avoid PCR inhibited samples and false negative results.

RealTime positive samples were subsequently DNA sequenced. The pre-analytical step of DNA sequencing included DNA amplification by two-steps nested PCR with primers specific for 5S-23S ribosomal RNA intergenic spacer (IGS). Both steps of nested PCR were carried out by KAPA2G Fast HotStart ReadyMix (Kappa Biosystems, USA). The 25 µl mixture contained 5 µM of each primer – B03 (5'-TCC ATT CGG TAA TCT TGG GA-3'), B04 (5'-CCG TGT CTT TGG CCA TAT TT-3') for the first step of nested PCR and B01 (5'-CTT ACC ACG ACC TTC TTC GC-3'), and B02 (5'-CCT GTT ATC ATT CCG AAC ACA G-3') for the second step. 5 µl of DNA isolate (in the case of first step of nested PCR)

was added to the mixture, and 1 µl of PCR product from the first step of nested PCR for the second step of nested PCR.

The conditions for the first step of nested PCR were 95°C for 2.5 min activation/pre-melt step, followed by 45 cycles of 95°C for 15 s melt, 56°C for 30 s anneal, 72°C for 10 s, with a final extension of 7 min.

The amplification programme for the second step of nested PCR was 95°C for 2.5 min activation/pre-melt step, followed by 45 cycles of 95°C for 15 s melt, 60°C for 15 s anneal, 72°C for 20 s, with a final extension of 7 min. The resulting products of amplification were separated on 1.5% agarose gel containing the visualization colour MidoriGreen (Nippon Genetics, Germany). The first step PCR product's length was about 500 bp and the second about 330 bp.

Amplicons were purified by using ExoI-FastAP (Fermentas, Latvia). The mixtures were incubated at 37°C for 15 min and 85°C for 15 min to inactivate the enzymes, followed by sequencing with BigDye<sup>®</sup> Terminator v.3.1 (Life Technologies, USA). Sequencing reactions were purified by using EDTA/ethanol precipitation, re-suspended in 10 µl Hi-Di Formamide (Life Technologies), and sequenced on an automated ABI 3130 Genetic Analyzer (Life Technologies). The BLAST algorithm (<http://www.ncbi.nlm.nih.gov/>) was used for identification of genospecies of the Bbsl. The following reference sequences from GenBank were used for alignment: CP002933.1 (*B. afzelii*), CP003151.1 (*B. garinii*), DQ393310.1 + AF497987.1 (*B. valaisiana*), AF497994.1 (*B. spielmanii*) and CP002228.1 (*B. burgdorferi* s. s.).

## RESULTS

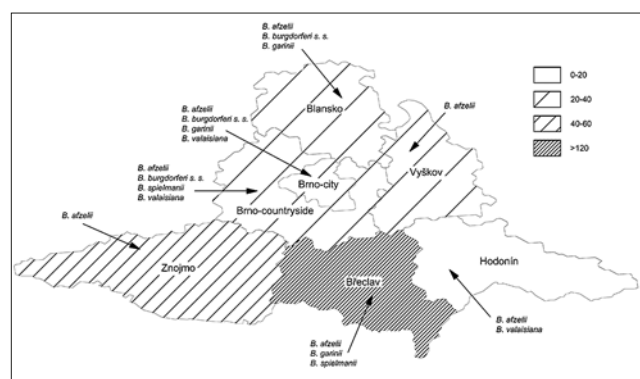
The annual positivity of the removed ticks ranged from 16 – 33%. A total positivity of the 466 collected samples was 26%. More than a half of the positive samples were sequenced and aligned (Tab. 1).

**Table 1.** Bbsl positive ticks *Ixodes ricinus* removed from human skin in the South Moravian region

Year of Collection	RealTime PCR			Sequenced (of Bbsl positive samples)	
	Examined	Positive		n	%
	n	n	%	n	%
2008	72	12	16.7	0	0.0
2009	75	35	33.3	19	54.3
2010	85	27	31.8	13	48.1
2011	104	24	23.1	18	75.0
2012	130	23	17.7	20	86.9
Total	466	121	26.0	70	58.7

The most frequently occurring genospecies was *B. afzelii* 70.0% (49), followed by *B. garinii* 10.0% (7), *B. valaisiana* 8.6% (6), *B. spielmanii* 7.1% (5), and finally, *B. burgdorferi* s. s. 4.3% (3). *B. afzelii* was detected in all districts of the South Moravian region. A complete review of the genospecies found and epidemiologic data about LD is shown in Figure 1.





**Figure 1.** Lyme disease incidence per 100 000 inhabitants in 2012 (data from the Institute of Health Information and Statistics of the Czech Republic, 2014) and Bbsl genospecies found in our study in the 7 districts of the South Moravian region (2008–2012)

## DISCUSSION

The South Moravian region has ideal climate conditions for the spreading of Bbsl. The average positivity of ticks corresponds with other results from that region and ranges from 20–30%, depending on specific study and locality [14, 16]. There are at least two studies in Central Europe testing for the presence of Bbsl in *I. ricinus* feeding on people. In the first study, published by Hubálek et al. [17], a total of 298 ticks were examined by dark field microscopy, from which 11.1% of the whole ticks collection were positive for the borreliae. A German study focusing on the federal state of Thuringia revealed only 6.1% prevalence of Bbsl in 618 collected samples analyzed by nested PCR and RFLP targeting a fragment of the *ospA* gene [18].

In the presented study, the lower number of successfully sequenced samples, especially in 2008 (28.6%), could have been caused by the small average G+C bases content in Bbsl genome. A small G+C/A+T ratio in Bbsl genomic DNA may have an effect on DNA structure, and may influence its stability necessary for the storage of Bbsl DNA.

Rauter et al. [12] reported, that the highest infection rates in Europe are found in the central regions; namely, in Austria, the Czech Republic, Germany, Switzerland, Slovakia and Slovenia. The Czech Republic is endemic in terms of the occurrence of LD, and the number of patients there is highest among European countries. Over 120 persons with Lyme disease, the highest number per 100,000 inhabitants in the Czech Republic, were reported in Břeclav district (a part of South Moravian region involved in the current study) in 2012, according to data from the Institute of Health Information and Statistics of the Czech Republic. The LD prevalence was 36.7/100 000 (in 2012) in the whole South Moravian region where the presented study was performed [19].

The number of patients diagnosed as having LD is increasing every year in the Czech Republic. From last 10 years survey (2003 – 2012), the total count of patients with LD is 3,844 cases per year. The total count of patients with LD in the years of the presented study (2008–2012) is higher – 3,988 LD cases per year [20]. The main reason could be the better education of physicians and increasing introduction of more specific diagnostic methods in clinical laboratories. An important cause for the higher incidence of LD in recent years can be observed in the higher abundance of positive ticks in the Czech Republic. For example, the

average number of ticks collected in a one hour interval was 36 [21], and 6 years later, the number of ticks collected by same method increased to 65 ticks [22]. There is no doubt that the total count of patients with Lyme borreliosis in the Czech Republic in the year 2011 was the highest (4,835) detected in the history of monitoring of patients in that country. To protect the human population against both the high invasion and infectivity of ticks, people should have knowledge and awareness about general tick biology, activity depending on the weather, and about the possibility of the application of appropriate insect repellents as an essential part of visits to nature [22, 23].

Humans in Europe are commonly infected by *B. burgdorferi* s.s., *B. garinii*, and *B. afzelii*. However, *B. bavariensis* (closely related to *B. garinii*) and *B. spielmanii* are also pathogenic for humans, and the pathogenicity of other genospecies (*B. lusitanae*, *B. valaisiana*, and *B. bissetii*) cannot be excluded [24]. In the presented results, the higher occurrence of *B. afzelii* (70%) was expected according to previous studies from the Czech Republic. *B. afzelii* with *B. garinii* (10%) are the most abundant genospecies in the Czech Republic [25]. In the current results, genospecies *B. valaisiana* (8.6%), *B. spielmanii* (7.1%) and *B. burgdorferi* s. s. (4.3%) were also identified. The local predominance of *B. garinii* detected in unfed *I. ricinus* ticks from the Prague region was reported by Bašta et al. [26]. Different results in genospecies variety have been obtained in neighbouring Poland where 25 patients with erythema migrans from Western Pomerania were examined. In 40% of them, DNA of *B. garinii*, in 20% DNA of *B. afzelii* and in 16% DNA of Bbss were present [27]. From the Lublin region of eastern Poland, a total of 2,251 unfed *I. ricinus* ticks were collected of which 5.4 % were PCR-positive, with a representation of 62.8% of Bbss, 39.8% of *B. afzelii* and 17.8% – *B. garinii* [1]. On the contrary, a high percentage of prevalence of Bbss (55.3%) DNA was detected in 11.5% of Bbsl infected samples of a total 406 examined *I. ricinus* ticks in a study from south-eastern Poland [2]. In this study, *B. afzelii* was confirmed in 38.3% of total positive ticks.

Besides the above-mentioned, well-known genospecies of Bbsl which are spread in the Czech Republic, *B. spielmanii* was detected in the presented study for the first time. A similar genotype, I-77 – A14S (the name of strain for *B. spielmanii* before delineation), was published by Derdákova et al. [28]. The single sample was collected in the South Bohemian region. This quite new genospecies was delineated by Richter et al. in 2004 [29, 30]. In Europe, there are several studies focusing on *B. spielmanii* as a potential cause of LD resulting from the analysis of isolation from patient's skin lesions [31, 32].

The second genospecies with no frequent occurrence in the studied region, based on previous studies, is *B. valaisiana*. The first occurrence was reported in 2003 from samples near to České Budějovice (South Bohemian region), [28] and its successful laboratory cultivation and detection in South Moravia was proved in 2007 [33]. The whole-genome sequences of both *B. spielmanii* and *B. valaisiana* have been known since 2011. This is important for the study of their pathogenesis and understanding of *B. burgdorferi* sensu lato diversity, etc. [34].

The occurrence of *Borrelia burgdorferi* s. s. was recorded in only 3 samples (4.3%). In a previous study carried out in the Czech Republic, this genospecies was noticed also in a tiny quantity [35]. Danielová et al. [36] described a high frequency of Bbss that exceeded the reported

occurrence of this genospecies in the Czech Republic. In other areas close to the Czech Republic, *Borrelia burgdorferi* s. s. was also observed in various frequencies: Slovakia [37], Poland [38], Austria [39], and Germany [40]. This may support the idea that *B. burgdorferi* s. l. genospecies are distributed locally, depending on climate, vectors, environment, etc. The methodological mistakes and/or differences could also be the reason for different inter-laboratory results.

Most of the previously described studies focused on identification of Bbsl genospecies relied on identification based on PCR-RFLP analysis, mixed dark field microscopy, alternative cultivation and PCR-RFLP. In the presented study, a different method was used for the analysis. For the first time, RealTime PCR was carried out for checking the positivity/negativity of samples, and all the positive samples were sequenced. The identified genospecies were relatively highly specific, and therefore RealTime PCR may be recommended for the routine detection of Bbsl in clinical samples, because all RealTime PCR-positive samples were sequenced trouble-free. It also confirmed its specificity and sensitivity.

## CONCLUSION

The presented observations contribute to the knowledge about the prevalence of Bbsl in the South Moravian region. The most important finding is the first identification of *B. spielmanii* in this region.

## Acknowledgements

This work was supported by a specific research project of the Masaryk University in Brno and by the Ministry of Industry and Trade of the Czech Republic, Project No. FR-TI1/478. Authors declare that they have no conflict of interest regarding this paper.

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