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# Genetic diversity of *Borrelia burgdorferi* sensu lato isolates obtained from *Ixodes ricinus* ticks collected in Slovakia

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**Abstract.** In Europe, *Borrelia burgdorferi* sensu lato is diverse, including *B. burgdorferi* s.s., *B. garinii*, *B. afzelii*, *B. valaisiana* and *B. lusitaniae*. In this study, we focused on the distribution of the different *B. burgdorferi* species among *Ixodes ricinus* adult ticks collected in an endemic area within Slovakia. We compared results of prevalence of *B. burgdorferi* infection in ticks obtained by immunofluorescence (IF) and by isolation. Isolates were characterized by restriction fragment length polymorphism (RFLP) of the *rrf-rrl* intergenic spacer genes using *Mse*I. Using immunofluorescence we observed that 56/114 (49%) ticks were infected by *B. burgdorferi* s.l. Males were found to be more often infected (32/57, 56%) than females (24/57, 42%) but the difference was not significant ($p = 0.1895$). From the same 114 ticks a total of 37 isolates were obtained: 19 from males (33%) and 18 from females (32%). The RFLP identification revealed 25 *B. afzelii* (68%), 5 *B. garinii* (14%), 5 *B. valaisiana* (14%) and 2 *B. lusitaniae* (5%). The infection in ticks was more often detected by IF than by isolation ($p = 0.0153$) and isolation success was higher when the infection degree in ticks was high ($p = 0.0397$). The infection prevalence observed in this area is among the highest observed in Europe.

**Key words:** *Borrelia burgdorferi* s.l., Immunofluorescence, Isolation, *Ixodes ricinus*

## Introduction

Lyme borreliosis, the most prevalent tick-borne disease in the Northern hemisphere, is a multisystemic disorder caused by the spirochete *Borrelia burgdorferi* sensu lato (s.l.). In Europe, *B. burgdorferi* s.l. is diverse, including *B. burgdorferi* [1], *B. afzelii* [2], *B. garinii* [3], *B. valaisiana* [4] and *B. lusitaniae* [5]. The pathogeny of the last two *Borrelia* species for humans remains to be elucidated. *B. burgdorferi* s.s., *B. garinii* and *B. afzelii* have been frequently isolated from their tick vector *Ixodes ricinus* in Europe, whereas *B. valaisiana* and *B. lusitaniae* isolates are rather rare. Information concerning the prevalence of *Borreliae* as well as the prevalence of the different *Borrelia* species in tick populations are very important for the understanding of Lyme borreliosis epidemiology, as well as for prevention.

In this study we focused on the distribution of the different *B. burgdorferi* species among *I. ricinus* adult ticks collected in an endemic area within Slovakia. We compared results of the prevalence of *B. burgdorferi* infection in *I. ricinus* adult ticks obtained by immunofluorescence and by isolation. Isolates were characterized by restriction length polymorphism of the *rrf-rrl* intergenic spacer genes using *Mse*I [6] which allowed us to evaluate the heterogeneity of *B. burgdorferi* s.l. and the prevalence of different genospecies in ticks.

## Methods

*Collection of free-living ticks*

Host-seeking *I. ricinus* adults were collected by flagging the vegetation in a lowland habitat of Panonian oak-hornbeam woods with warm climate situated at 130 m altitude, in October 1998. The collection site (Martinsky les) is about 30 km north-east from Bratislava and is characterized by a high density of ticks and a high abundance of tick hosts. Ticks were identified to species, stage and sex, and were maintained in tubes containing grass until use for spirochete examination and isolation.

*Detection of spirochetes in ticks*

Free-living adults were briefly soaked in 70% ethanol and were then cut into two pieces. One half was used for immunofluorescence (IF) examination and the other half was used for isolation of spirochetes. For IF, halved ticks were smeared on glass slides using a pair of sterilized tweezers. Slides were treated as described previously [7]. Briefly, they were air dried and fixed in acetone for ten minutes. The tick tissues were overlaid with fluorescein isothiocyanate-conjugated antibodies [7] and incubated in humid chambers for 30 min at 33 °C. Slides were examined for *Borrelia* by Olympus epifluorescence microscope at 400×

magnification. Infection degree was based on an estimation of the number of spirochetes in halved ticks and was expressed as low, medium and high.

*Isolation of spirochetes*

Halved ticks were squashed with sterilized forceps in tubes containing BSKII medium modified according to Sinsky and Piesman [8]. Inoculated cultures were screened by dark-field microscopy for the presence of spirochetes after 10 days, and one month of incubation at 34 °C. Cultivable spirochetes were subcultured to allow maintenance of the isolates in the laboratory. Isolates were named SLNE874 to SLNE910.

*PCR and RFLP analysis*

Polymerase chain reaction (PCR) amplification and restriction fragment length polymorphism (RFLP) described by Postic et al. [6] were used for identification of *Borrelia* species. The variable intergenic spacer between repeated 23S (*rrl*)–5S (*rrf*) ribosomal genes of *B. burgdorferi* s.l. was used as a template for PCR and RFLP analysis using *Mse*I endonuclease. There is only one copy of *rrs* (16S) rRNA gene in *B. burgdorferi* s.l., whereas there are two copies tandemly duplicated of *rrl* (23S) rRNA gene and *rrf* (5S) rRNA gene. The use of primers chosen in conserved genes allows the amplification of the variable spacer between the two copies of these genes which is specific for *B. burgdorferi* s.l. [6]. The second step using digestion by *Mse*I allows a species specific characterization according to the restriction *Mse*I pattern of the *rrf-rrl* intergenic spacer [6]. Spirochetes in original culture tubes were identified by PCR/RFLP. *Borrelia* DNA was amplified by PCR directly from initial cultures to identify the genospecies present in the original materials and to detect mixed infections: 1 ml was used to test cultures containing spirochetes observed by dark-field microscopy. DNA detection was made in culture tubes negative by dark-field microscopy after 1 month incubation at 34 °C and containing ticks which were positive by IF.

*Statistical analysis*

To compare infection rates, infection degrees, and *Borrelia* species between males and females, the Fischer's exact test was used. The $\chi^2$-test was used to compare success of isolation and detection of *Borrelia* by immunofluorescence.

## Results

From 114 free-living *I. ricinus* ticks (57 females, 57 males) collected by flagging, 56 (49%) ticks were found infected by IF: 32 males (infection rate of male 56%) and 24 females (infection rate of females: 42%). There was no statistically significant difference in infection rate between males and females ($p = 0.1895$). The infection degree in the ticks was as followed: 36 (64%) had a low infection degree, 6 (11%) had a medium infection degree and 14 (25%) presented a high number of spirochetes. No difference in infection degree was observed between males and females ($p = 0.1162$).

A total of 37 isolates were obtained from the 114 ticks (33%): 19 from males (isolation rate from male: 33%) and 18 from females (isolation rate from females: 32%) (Table 1). The RFLP identification revealed 25 *B. afzelii* (68%) (from 15 males and 10 females), 5 *B. garinii* (14%) (from 2 males and 3 females), 5 *B. valaisiana* (14%) (from 2 males and 3 females) and 2 *B. lusitaniae* (5%) (from 2 females).

From the 56 ticks which were found infected by IF, 37 gave positive culture whereas 19 remained negative. DNA detection was made in these 19 culture

**Table 1.** *B. burgdorferi* s.l. isolation and infection degree in *I. ricinus* adults

| Isolate | Sex | *Borrelia* density | *Borrelia* sp. |
|---|---|---|---|
| SLNE874 | F | + | *B. afzelii* |
| SLNE875 | F | ++ | *B. garinii* |
| SLNE876 | F | ++ | *B. afzelii* |
| SLNE877 | M | + | *B. afzelii* |
| SLNE878 | F | + | *B. afzelii* |
| SLNE879 | M | +++ | *B. afzelii* |
| SLNE880 | M | + | *B. afzelii* |
| SLNE881 | M | + | *B. afzelii* |
| SLNE882 | M | + | *B. afzelii* |
| SLNE883 | M | +++ | *B. afzelii* |
| SLNE884 | M | + | *B. afzelii* |
| SLNE885 | F | + | *B. afzelii* |
| SLNE886 | F | +++ | *B. lusitaniae* |
| SLNE887 | F | +++ | *B. afzelii* |
| SLNE888 | F | ++ | *B. afzelii* |
| SLNE889 | F | + | *B. valaisiana* |
| SLNE890 | F | ++ | *B. lusitaniae* |
| SLNE891 | M | +++ | *B. afzelii* |
| SLNE892 | M | + | *B. afzelii* |
| SLNE893 | M | +++ | *B. afzelii* |
| SLNE894 | M | +++ | *B. afzelii* |
| SLNE895 | M | +++ | *B. garinii* |
| SLNE896 | F | + | *B. garinii* |
| SLNE897 | M | + | *B. valaisiana* |
| SLNE898 | F | +++ | *B. garinii* |
| SLNE899 | F | + | *B. afzelii* |
| SLNE900 | F | +++ | *B. afzelii* |
| SLNE901 | F | + | *B. valaisiana* |
| SLNE902 | M | + | *B. garinii* |
| SLNE903 | F | +++ | *B. afzelii* |
| SLNE904 | F | + | *B. afzelii* |
| SLNE905 | M | + | *B. valaisiana* |
| SLNE906 | M | +++ | *B. afzelii* |
| SLNE907 | M | + | *B. afzelii* |
| SLNE908 | M | ++ | *B. afzelii* |
| SLNE909 | M | + | *B. afzelii* |
| SLNE910 | F | + | *B. valaisiana* |

F: female; M: male; +: low; ++: medium; +++: high.

**Table 2.** Relation between infection degree and *Borrelia* species

| *Borrelia* species/ infection degree | Ba | Bg | Bv | Bl | ND | Total |
|---|---|---|---|---|---|---|
| + | 14 | 2 | 5 | 0 | 15 | 36 |
| ++ | 3 | 1 | 1 | 1 | 0 | 6 |
| +++ | 9 | 2 | 0 | 1 | 2 | 14 |
| Total | 26 | 5 | 6 | 2 | 17 | 56 |

Ba: *B. afzelii*; Bg: *B. garinii*; Bv: *B. valaisiana*; Bl: *B. lusitaniae*; ND: no isolate, *Borrelia* characterization not done; +: low infection; ++: medium infection; +++: high infection.

tubes and 2 were found to contain *Borrelia* DNA which was identified as *B. valaisiana* and *B. afzelii*.

Using IF for *Borrelia* detection in ticks gave a higher infection rate (49/114, 49%) than isolation from the same ticks (37/114, 33%). The difference was significative ($p = 0.0153$). Isolation was obtained from 21/36 (58%) infected ticks presenting a low number of spirochetes, from 6/6 (100%) infected ticks with a medium number of *Borreliae* and from 12/14 (86%) infected ticks with a high infection degree (Table 2). Success of isolation was related to degree of infection ($p = 0.0397$). Among ticks found infected by *B. afzelii* 14/26 (54%) presented a low infection degree, 3/26 (12%) a medium infection and 9/26 (35%) were highly infected. No statistical relation could be found between degree of infection and *Borrelia* species.

## Discussion

With the splitting of *B. burgdorferi* into different species, the occurrence of genospecies in field-collected *I. ricinus* ticks has only recently been studied in various endemic areas in Europe [9–17]. Since *rrf-rrl* restriction patterns were shown to be species specific [6], we used this method to characterize isolates obtained from free-living adult *I. ricinus* collected in a Lyme borreliosis endemic area in Slovakia. In this country, the first investigations of ticks for the presence of *Borreliae* were performed in 1985 already [18]. In a 5-year study, more than 10,000 ticks from different parts of the country (all districts of Slovakia) were investigated using dark field microscopy. An overall of 9.2% infection rate was detected ranging from 1 to 23% [19–21]. Some *B. burgdorferi* s.l. isolates were also obtained from ticks and feral rodents and characterized as *B. garinii*, *B. afzelii* [22, 23] as well as *B. burgdorferi* s.s. [17].

In the present study, the prevalence of *B. burgdorferi* s.l. in adult ticks (49% using IF) in the studied area is among the highest prevalences reported in other European Lyme borreliosis areas [24]. The infection rate obtained by *Borrelia* detection in ticks using IF gave a higher infection rate (49%) than isolation from the same tick (33%). One explanation for this might be that the infection is not uniformly distributed in the tick [25] and that using the same tick for IF and isolation reduced the chances for spirochetes to adapt to BSK medium by reducing the number of spirochetes which are introduced in BSK medium. In fact, from the 17 ticks found positive by IF and negative by culture, 16 presented a very low number of spirochetes. In Europe, *B. burgdorferi* s.s., *B. garinii* and *B. afzelii* have been obtained from various geographic areas. According to Saint Girons et al. [17], a greater frequency of *B. garinii* is observed in the west of Europe, followed by *B. afzelii*, *B. burgdorferi* s.s. and *B. valaisiana*. Among isolates obtained from the studied area, *B. burgdorferi* s.s. was absent although *B. burgdorferi* s.s. has been previously isolated in Slovakia [17], *B. afzelli* dominated (68%), followed by *B. garinii* and *B. valaisiana*. We believe that more isolates from ticks have to be obtained before a map of the differential distribution of the *Borrelia* species can be established in Europe.

*B. valaisiana*, which has been described recently [4], has been less frequently isolated except maybe in the Netherlands [4] and in Switzerland, where it has been isolated from birds and from bird feeding ticks [26]. In contrast, *B. valaisiana* DNA has been amplified from numerous ticks collected in Ireland [15]. This suggests that *B. valaisiana* spirochetes grow badly in BSK medium or that survival of *B. valaisiana* in ticks is difficult which makes isolation more difficult. Most ticks (5/6) which were infected by *B. valaisiana* in the present study presented very few spirochetes when examined by IF. This could be an explanation of the difficulty to isolate this *Borrelia* species. Although ticks which were infected by *B. afzelii* and from which we obtained most isolates also frequently presented a low infection (14/26) degree. This means that the number of spirochetes in a tick is not the only factor limiting their isolation in BSK medium.

Only rare *B. lusitaniae* isolates have been obtained till now, according to Le Fleche et al. [5] only 7 isolates have been reported. They have been isolated from *I. ricinus* ticks collected in Portugal [27], the Czech Republic, Moldavia and Ukraine [28]. Recently, 2 additional isolates have been obtained from *I. ricinus* ticks collected in North Africa (Tunisia) [29]. In the present study we obtained 2 additional isolates *B. lusitaniae*. If some of the reservoir hosts of *B. burgdorferi* s.s., *B. garinii*, *B. afzelii* and *B. valaisiana* have been identified [30], the animal species which act as reservoir hosts for *B. lusitaniae* have yet to be discovered.

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