Detection and identification of *Borrelia burgdorferi* sensu lato genospecies in ticks from three different regions in Slovakia

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Nachweis und Identifizierung von *Borrelia burgdorferi* sensu lato-Genospezies in Zecken aus drei verschiedenen Regionen der Slowakei


Summary. Lyme borreliosis is one of the most common tick-borne diseases that occur in Slovakia. In this study, *Borrelia burgdorferi* sensu lato was detected and cultivated from questing ticks collected in three areas of Slovakia. Two methods, restriction fragment length polymorphism and reverse line blot, were used for identification of isolates and determination of the prevalence of *B. burgdorferi* s.l. in the ticks. The prevalence of *B. burgdorferi* s.l. in *I. ricinus* detected by reverse line blot was 31.9%. Four genospecies, namely *B. garinii*, *B. valaisiana*, *B. afzelii* and *B. burgdorferi* sensu stricto were found. *B. garinii* was the most prevalent genospecies.

Key words: *Borrelia burgdorferi*, tick, *Ixodes ricinus*, Slovakia, reverse line blot.

Introduction

Lyme borreliosis is the most common tick-borne disease in the northern hemisphere. This disease, which may affect several organ systems, is caused by spirochetes of the genospecies complex *Borrelia burgdorferi* sensu lato (s.l.) [1]. *B. burgdorferi* s.l. comprises at least 12 named genospecies, seven of which are known to occur in Europe: *B. afzelii*, *B. garinii*, *B. valaisiana*, *B. lusitaniae*, *B. burgdorferi* sensu stricto (s.s.), *B. bissettii* and *B. spielmanii* [2, 3]. The tick *Ixodes ricinus* is the main vector of *B. burgdorferi* s.l. in Europe.

*B. garinii*, *B. afzelii* and *B. burgdorferi* s.s. are known to be associated with neurological, dermatological and arthritic symptoms, respectively [4], and recently *B. lusitaniae* has been isolated from a patient with chronic skin lesions in Portugal [5]. The most prevalent borrelia species in Europe are *B. afzelii*, *B. garinii*, *B. burgdorferi* s.s. and *B. valaisiana* [2]. *B. lusitaniae* occurs mainly in the western Mediterranean basin and in North Africa, where the highest prevalence of this species has been found in *I. ricinus* [6, 7]. In previous studies, six *B. burgdorferi* s.l. genospecies have been detected in Slovakia: *B. afzelii*, *B. garinii*, *B. valaisiana*, *B. burgdorferi* s.s., *B. lusitaniae* and *B. bissettii* [8–10].

The aim of the present study was to obtain isolates of *B. burgdorferi* s.l. from host-seeking ticks collected in one locality of western Slovakia and two localities of central Slovakia that differ in vegetation and climate. Genospecies were identified using restriction fragment length polymorphism (RFLP) [11] and reverse line blot (RLB) [12, 13].

Materials and methods

Ticks were collected at three different localities in spring 2005. Martinínský les (17°22′E, 48°15′30″N) represents a residual forest in a highly urbanized and agricultural region, situated in the orographic entity Trnavská páhorkatina hills in south-western Slovakia. The climate is warm. The vegetation type is Panonian oak-hornbeam woods with high biodiversity and occurrence of forest-steppe and Mediterranean plant species [14]. The dominant tick species is *I. ricinus*, but *Haemaphysalis concina* and *Dermacentor reticulatus* might be collected sporadically. *I. ricinus* in this locality is known to harbor various tick-borne pathogens [8]. The second collection site (19°20′E, 48°18′30″N) is situated in the orographic entity Krupinská planina. A moderately warm mountain climate and Carpathian oak-hornbeam woods are characteristic for this area [14]. Ticks were collected on pasture habitats near the village of Velký Lom. Three tick species, *Dermacentor mar-
ginanus, D. reticulatus and I. ricius, occur in this locality. The third locality (18°55'30"E, 48°25'30"N) is an arboretum created in 1900 and nowadays used as a recreational area; it is situated in central Slovakia near the town of Banská Štiavnica in the orography entity Štiavnické vrchy (mountain). The climate is moderately cool and vegetation types are beech and fir woods with forb-rich undergrowth [14]. More than 200 exotic tree species occur in this area. I. ricius is the only tick species previously found in this locality.

Ticks were collected by flagging low vegetation with a white cotton cloth and were then maintained at 4°C in tubes with wet filter paper until examination for borreliae.

Three different methods were used for detection and identification of borreliae, as previously described: isolation and dark-field microscopy [8], detection and characterization of B. burgdorferi s.l. using PCR/RFLP [11], and RLB [12, 13]. Ticks were rinsed in 70% ethanol, air dried and cut longitudinally into two pieces. One or both halves were transferred to culture tubes containing BSK II medium modified according to Sinsky and Piezman [15], and cultivated at 34°C as previously described [8]. The presence of spirochetes in cultures was detected by dark-field microscopy after seven days of incubation and then at weekly intervals for a month. Positive cultures were subcultivated to maintain the isolates in the laboratory.

Before polymerase chain reaction (PCR), all positive and negative primary cultures were processed as previously described [11]. The pellet from 1.2 ml of culture was washed twice, resuspended in 40 µl nanopure water and boiled for 10 min. In addition, direct DNA extracts were prepared from 23 half-ticks using ammonium hydroxide as described previously [16]. Briefly, ticks were boiled for 15 min at 100°C in 100 µl of 0.7 M ammonium hydroxide in a heating block to free the DNA. The tube was then cooled to room temperature and left open for 15 min at 100°C to evaporate the ammonia.

Primers selected to amplify the variable spacer region between the 3’end of the 5S rRNA (rfp) and the 5’end of the 23S rRNA (rfp) were used for PCR followed by RFLP [11]. A Whatman Biometra TGradient Thermocycler 96 (Göttingen, Germany) was used for DNA amplification. Isolates of B. burgdorferi s.s. (B31), B. garinii (NEI1), B. lusitaniae (Poti83), B. afzelii (NE632) and B. valaisiana (VS16) were used as positive controls. PCR products were digested overnight with Mse I endonuclease and then either stored at −20°C or immediately heated for acrylamide-bisacrylamide gel electrophoresis [11].

For PCR followed by RLB, primers 23Sbor and biotin-labeled 5SSbor were used to amplify the intergenic spacer region between 5S and 23S rRNA genes [12]. The PCR products were hybridized to seven oligonucleotide probes (75 pmol) [13] blotted in lines on an activated Biodyne C membrane (Pall Europe Ltd., Portsmouth, UK) using a Miniblotter 45 (Immunetic, Cambridge, MA, USA). Hybridization was visualized after exposing the membrane to X-ray film (Hyperfilm ECL; Amersham Biosciences, Otelfingen, Switzerland).

**Results**

In total, 123 ticks (116 I. ricius: 63 females, 22 males and 31 nymphs; six D. marginitatus: 4 females and 2 males; and one H. concinna female) were processed for isolation of B. burgdorferi s.l. in BSK II medium. Spirochetes were not observed in cultures prepared from D. marginitatus and H. concinna, and these cultures were also negative by PCR/RFLP and PCR/RLB.

Cultures positive by dark-field microscopy were obtained from 27/116 (23.3%) I. ricius: 18 females (18/63, 28.6%), 4 males (4/22, 18.2%) and 5 nymphs (5/31, 16.1%). Isolates were identified as B. garinii, B. valaisiana, B. afzelii and B. burgdorferi s.s. (Table 1) by both molecular methods. B. garinii was the most frequently isolated species. In addition, B. burgdorferi s.l. DNA was detected and characterized by PCR-RFLP in eight negative I. ricius primary cultures. Using RLB, borrelial DNA was detected in two additional cultures where no spirochetes were observed by dark-field microscopy and where PCR-RFLP results were negative. The overall prevalence of B. burgdorferi s.l. in I. ricius was 30.2% (35/116) using RFLP and 31.9% (37/116) with RLB. The most prevalent species, according to results obtained with RLB, was B. garinii (56.8%), followed by B. valaisiana (24.3%) and B. afzelii (24.3%). B. burgdorferi s.s. was rare (5.4%).

At the different study sites, the prevalence of B. burgdorferi s.l. detected by RLB in I. ricius was 32.3% (20/62) in Martinský les, 36.8% (7/19) in Banská Štiavnica and 28.6% (10/35) in Velký Lom. B. garinii, B. afzelii and B. valaisiana were present in ticks from all localities, whereas B. burgdorferi s.s. was detected only in ticks from Banská Štiavnica. The prevalence of the various B. burgdorferi s.l. genospecies in I. ricius at the studied localities is summarized in Table 2.

Single infections of individual ticks with only one borrelia species represented 92% of all infections. Three co-infections were detected by RLB: B. garinii with B. valaisiana in one tick from Banská Štiavnica; B. afzelii, B. garinii and B. burgdorferi s.s. in another tick from the same locality; and B. afzelii and B. valaisiana in one tick from Velký Lom.

Twenty-three I. ricius from Velký Lom were used for isolation of borreliae in BSK II medium as well as for direct borrelia DNA extraction from the tick. Results differed in two ticks: in one, B. garinii was detected in

<table>
<thead>
<tr>
<th>Locality</th>
<th>No. of ticks</th>
<th>Number of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BGA</td>
<td>BAF</td>
</tr>
<tr>
<td>Banská Štiavnica</td>
<td>19</td>
<td>3</td>
</tr>
<tr>
<td>Martinský les</td>
<td>62</td>
<td>9</td>
</tr>
<tr>
<td>Velký Lom</td>
<td>35</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>116</td>
<td>16</td>
</tr>
</tbody>
</table>

BGA Borrelia garinii; BAF Borrelia afzelii; BVS Borrelia valaisiana; BBss Borrelia burgdorferi ss.
Table 2. *B. burgdorferi* s. l. in *I. ricinus* ticks detected by reverse line blot

<table>
<thead>
<tr>
<th>Locality</th>
<th>No. of ticks</th>
<th>BGA</th>
<th>BAF</th>
<th>BVS</th>
<th>BBss</th>
<th>BGA+BVSS</th>
<th>BAF+BVS</th>
<th>BBss+BGA+BAF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Banská Štiavnica</td>
<td>19</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Martiníky les</td>
<td>62</td>
<td>11</td>
<td>5</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Vlký Lom</td>
<td>35</td>
<td>5</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>116</td>
<td>19</td>
<td>7</td>
<td>7</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

BGA Borrelia garinii; BAF Borrelia afzelii; BVS Borrelia valaisiana; BBss Borrelia burgdorferi ss.

Culture but the tick extract was negative; in the other, although co-infection with *B. valaisiana* and *B. afzelii* was found in culture, only *B. valaisiana* was detected in the tick extract.

**Discussion**

*B. afzelii, B. garinii, B. burgdorferi* s.s. and *B. valaisiana* are the most prevalent *B. burgdorferi* s.l. genospecies in Europe [2] and *B. afzelii* seems to be the predominant genospecies in questing *I. ricinus* in northern Europe [17–19]. *B. afzelii* was also the most prevalent according to some studies on ticks from Czech Republic [21] and Croatia [16]. *B. garinii* was detected as the most prevalent genospecies in host-seeking ticks from Belgium [22] and Switzerland [23], and *B. valaisiana* was found to be predominant in Ireland [24]. A high prevalence of *B. valaisiana* was also detected in England [25].

The occurrence of six borrelia genospecies, namely *B. afzelii, B. garinii, B. valaisiana, B. burgdorferi* s.s., *B. lusitaniae* and *B. bissettii*, has been reported in Slovakia [8–10]. In that study, four *B. burgdorferi* s.l. genospecies were isolated from *I. ricinus* collected in three areas in Slovakia. Isolates were identified by RFLP and RLB. In addition, borrelial DNA was detected in negative cultures. However, the isolation rate from *I. ricinus* (23.3%) was lower than previously described at one of the studied localities [8].

Ticks belonging to the *I. ricinus* complex are the most important European vectors of *B. burgdorferi* s.l., although detection of *B. garinii* and *B. afzelii* DNA in *D. reticulatus* was not detected in the present study. However, the isolation rate from *I. ricinus* (23.3%) was lower than previously described at one of the studied localities [8].

Two DNA extraction methods were compared on a small sample of ticks; detection of *B. burgdorferi* s.l. after DNA extraction of primary cultures and DNA extraction directly from the ticks. Results differed in two ticks and this should be continued on a larger number of ticks.

The overall prevalence of *B. burgdorferi* s.l. in *I. ricinus* was 30.2% using RFLP and 31.9% with RLB. Thirty-five ticks were positive with both methods, but two ticks were positive only by RLB. RLB was more effective in revealing co-infection, as two of three co-infections were detected only by RLB. The estimated prevalence rate in questing *I. ricinus* was lower than previously detected by immunofluorescence in Martiníky les [8]. Higher and similar prevalences were detected in ticks from Slovakia in two other studies using RLB [9, 10].

The most prevalent species was *B. garinii*, followed by *B. valaisiana* and *B. afzelii, B. burgdorferi* s.s. was rare. The high prevalence of *B. garinii* contrasts with previous studies describing *B. afzelii* as the dominant species in host-seeking ticks [8, 9]. *B. garinii* and *B. valaisiana* are often associated with ticks infesting birds [10, 13, 25] and the high prevalence of these species in questing *I. ricinus* may suggest the importance of birds as reservoirs in maintaining *B. burgdorferi* s.l. at the studied sites.

*B. burgdorferi* s.l. was detected in questing *I. ricinus* from localities with different altitudes, microclimate and vegetation. As far as we know, the prevalence of borreliae in ticks from the surroundings of Banská Štiavnica and Vlký Lom has not been studied before. Three *B. burgdorferi* genospecies were found at all the localities, whereas *B. burgdorferi* s.s. were found exclusively in Banská Štiavnica. This genospecies was also not detected in a previous study on ticks from Martiníky les [8]. Further work is necessary to determine the occurrence and seasonal distribution of *B. burgdorferi* s.l. genospecies in the three studied localities. The presence of several *B. burgdorferi* s.l. genospecies and their co-infections in ticks from the studied areas may represent a potential risk for visitors to these regions.

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**References**

sensu lato species by multilocus sequence analysis and confirmation of the delineation of *Borrelia spielmani* sp. nov. Int J Syst Evol Microbiol 56: 873–881


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