Original Article

Wien Klin Wochenschr (2007) 119/17–18: 534–537
DOI 10.1007/s00508-007-0851-y

WIENER KLINISCHE WOCHENSCHRIFT
The Middle European Journal of Medicine
Printed in Austria

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

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Accepted after revision June 13, 2007

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

Zusammenfassung. Lyme-Borreliose ist eine der häufigsten von Zecken vermittelten Erkrankungen, die in der Slowakei auftreten. In dieser Studie wurde *Borrelia burgdorferi* sensu lato in Wirt-suchenden Zecken, die aus drei Regionen der Slowakei gesammelt worden sind, nachgewiesen und kultiviert. Zur Identifizierung der Genospezies wurden zwei Methoden eingesetzt, die RFLP (restriction fragment length polymorphism) und der „reverse line blot". Ebenso wurde die Prävalenz von *B. burgdorferi* s.l. in den Zecken ermittelt, welche rund 32% betrug. Von den vier identifizierten Genospezies, *B. afzelii, B. burgdorferi* sensu stricto, *B. garinii* und *B. valaisiana*, wurde *B. garinii* am häufigsten nachgewiesen.

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. Martinský 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 pahorkatina 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. ricinus,* 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 recreational area; it is situated in central Slovakia near the town of Banská Štiavnica in the orographic 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. ricinus* 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 Piesman [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 halved 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 (*rrf*) and the 5'end of the 23S rRNA (*rrl*) 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* (NE11), *B. lusitaniae* (PotiB3), *B. afzelii* (NE632) and *B. valaisiana* (VS116) were used as positive controls. PCR products were digested overnight with Mse I endonuclease and then either stored at –20°C or immediately used for acrylamide-bisacrylamide gel electrophoresis [11].

For PCR followed by RLB, primers 23SBor and biotin-labeled 5SBor 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. ricinus*: 63 females, 22 males and 31 nymphs; six *D. marginatus*: 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. marginatus* 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. ricinus*: 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. ricinus* 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. ricinus* 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. ricinus* 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. ricinus* 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. ricinus* 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 1. Isolation of *B. burgdorferi* s. l. from questing *I. ricinus* ticks

Locality	No. of ticks	Number of isolates					
		BGA	BAF	BVS	BBss	BGA+BVS	BBss+BGA+BAF
Banská Štiavnica	19	3	1	0	1	1	1
Martinský les	62	9	2	3	0	0	0
Velký Lom	35	4	1	1	0	0	0
Total	116	16	4	4	1	1	1

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

Locality	No. of ticks	BGA	BAF	BVS	BBss	BGA + BVS	BAF + BVS	BBss + BGA + BAF
Banská Štiavnica	19	3 (15.8%)	1 (5.3%)	0 (0%)	1 (5.3%)	1 (5.3%)	0 (0%)	1 (5.3%)
Martinský les	62	11 (17.7%)	5 (8.1%)	4 (6.5%)	0 (0%)	0 (0%)	0 (0%)	0 (%)
Velký Lom	35	5 (14.3%)	1 (2.9%)	3 (8.6%)	0 (0%)	0 (0%)	1 (2.9%)	0 (0%)
Total	116	19 (16.4%)	7 (6%)	7 (6%)	1 (0.9%)	1 (0.9%)	1 (0.9%)	1 (0.9%)

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 France [20], Slovakia [8], Czech Republic [21] and Croatia [16]. However, *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* has been reported [26]. Here we were not able to detect any borrelia DNA in tick species other than *I. ricinus*. Since only a few *Dermacentor* and *Haemaphysalis* ticks were analyzed in the present study, further work is necessary, in particular to determine if these ticks have a role in the natural cycle of *B. burgdorferi* s.l. in the studied areas.

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 Martinský 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 Velký 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 Martinský 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.

Acknowledgement

The authors would like to thank Delphine Hügli, Francisca Morán and Yvan Kneubühler, and acknowledge the FEMS society for the fellowship. Collection of ticks was supported by grant 2/7020 from the Scientific Grant Agency of the Ministry of Education and the Slovak Academy of Sciences and by project APVV-51-009205 supported by the Slovak Research and Development Agency. Authors are grateful to Prof. G. Stanek for critical reviewing the manuscript.

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