Borrelia lusitaniae and Green Lizards (Lacerta viridis), Karst Region, Slovakia

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In Europe, spirochetes within the *Borrelia burgdorferi* sensu lato complex are transmitted by *Ixodes ricinus* ticks. Specific associations are described between reservoir hosts and individual genospecies. We focused on green lizard (*Lacerta viridis*) as a host for ticks and potential host for borreliae. In 2004 and 2005, a total of 146 green lizards infested by ticks were captured, and 469 *I. ricinus* ticks were removed. Borrelial infection was detected in 16.6% of ticks from lizards. Of 102 skin biopsy specimens collected from lizards, 18.6% tested positive. The most frequently detected genospecies was *B. lusitaniae* (77.9%–94.7%). More than 19% of questing *I. ricinus* ticks in areas where lizards were sampled tested positive for borreliae. *B. garinii* was the dominant species, and *B. lusitaniae* represented 11.1%. The presence of *B. lusitaniae* in skin biopsy specimens and in ticks that had fed on green lizards implicates this species in the transmission cycle of *B. lusitaniae*.

The causative agents of Lyme borreliosis, spirochetes of the *Borrelia burgdorferi* sensu lato complex, are maintained in natural foci by circulation between the vector ticks and reservoir hosts of various vertebrate taxa. The *B. burgdorferi* s.l. complex encompasses 12 species (1–3); 4 species have been clearly established as pathogenic to humans: *B. afzelii*, *B. garinii*, *B. burgdorferi* s. s., and *B. spielmanii* (4–6). *B. valaisiana* and *B. lusitaniae*, which were previously considered non-pathogenic, may cause disease as well (7,8). Different species are associated with distinct ecologic features, levels of pathogenicity, and clinical symptoms in patients.

In Europe, *I. ricinus* ticks infest a wide variety of vertebrate hosts, such as mammals, birds, and lizards. The vertebrate hosts are necessary to maintain the tick population and may also serve as reservoirs for the pathogen. Therefore, the identification of reservoir host species is essential to clarify the transmission patterns of *B. burgdorferi* s.l. in natural foci. The importance of rodents for maintaining *B. afzelii* (9), and of birds for *B. garinii* and *B. valaisiana* (10), in endemic regions of Slovakia is now indisputable. The National Park Slovak Karst is within the region in which *B. burgdorferi* s.l. in questing ticks and birds has been reported (V. Taragel’ová, unpub. data). In this area, 2 lizard species occur sympatrically, the common wall lizard (*Podarcis muralis*) and the green lizard (*Lacerta viridis*). The green lizard, the dominant species, is frequently infested by immature stages of *I. ricinus* ticks (11).

The importance of lizards in the maintenance cycles of *B. burgdorferi* s.l. spirochetes is still controversial. In Italy, *B. lusitaniae* was detected in blood and tissue samples of *P. muralis* (12). Furthermore, *Psammodromus algirus*, the most abundant lizard species in North Tunisia, was found to be the primary host for immature stages of *I. ricinus*. Thus, it could play a role in the circulation of *borreliae* (13). *B. burgdorferi* s.s., *B. andersonii*, and *B. bissetii* were detected in the blood of 9 lizard species in the southeastern United States (14). On the other hand, several other studies in the United States have shown that the lizards *Sceloporus occidentalis* and *Elgaria multicaudata* are reservoir-incompetent for borreliae because they possess borreliacidal factor in their blood (15,16). However, 2 lizard species, *Eumeces inexpectatus* and *Anolis carolinensis*, can sustain *B. burgdorferi s. s.* infection (17).

In the Slovak Karst (southeastern part of Slovakia), the green lizard is the major host for immature stages of *I. ricinus* ticks (11). Therefore, the main aim of this study was to find out whether green lizards can participate in the maintenance cycles of *B. burgdorferi* s.l. in natural foci and...
whether an association with specific borrelial genospecies exists.

Materials and Methods

Study Area

The study was conducted in the National Park Slovak Karst. This area represents a part of the Inner Carpathians in southeastern Slovakia (48°36′ N, 20°52′ E). The climate is warm with low humidity and average temperatures of −4°C in January and 18°C in July. The average rainfall is 700 mm/year.

Tick and Lizard Collection

This survey was conducted in 2004–2005, from May to September, when lizards and ticks are active. Questing I. ricinus nymphs and adults were collected by flagging the vegetation in areas where lizards were sampled. Ticks were immediately stored in 70% ethanol.

Green lizards were captured along hiking paths by hand or by noosing, in which a loop made from fishing nylon was attached to the end of a wooden stick and dangled in front of a lizard, which would be captured as it walked through the loop. Animals were characterized by sex and age (adult, subadult, juvenile) and examined for ticks. Ticks were removed with forceps immediately after capture and stored in 70% ethanol. Biopsy specimens (a 2-cm distal part of the tail and a 1-mm × 1.5-mm piece of skin from collar scales) were taken from each lizard with sterile scissors and put in separate vials with 70% ethanol. Skin from collar scales) were taken from each lizard with 70% ethanol.

Tick and lizard specimens were segregated by species and sex. Only sterile scissors and put in separate vials with 70% ethanol. Biopsy specimens (a 2-cm distal part of the tail and a 1-mm × 1.5-mm piece of skin from collar scales) were taken from each lizard with sterile scissors and put in separate vials with 70% ethanol. Tissue DNA from lizards’ tails and scales was extracted by using DNeasy tissue kit (Qiagen, Hilden, Germany). Extraction steps were conducted according to the manufacturer’s protocol. Genomic DNA from ticks was isolated by alkaline hydrolysis (18). Incubation time was extended from 5 to 30 min. Isolated DNA was stored at −20°C.

DNA Sequencing of PCR Products

Sequencing was performed at the Department of Molecular Biology (Faculty of Natural Sciences Comenius University, Bratislava, Slovak Republic). PCR of the 5S-23S rDNA intergenic spacer was conducted according to the protocol described above. For the fla gene amplicons, DNA strands were sequenced as described previously (21). PCR products were purified by using a QIAquick PCR purification kit (Qiagen). The complementary strands of each sequenced product were manually assembled. Sequences were compared with GenBank entries by Blast N2.2.13 (22). Homologous sequences were aligned by using the CLUSTAL W Multiple Sequence Alignment Program (version 1.81) (23). Sequence similarity among the sequences were calculated by EMBOSS Align, a pairwise alignment algorithm (http://www.ebi.ac.uk/emboss/align). The accession numbers of 5S-23S rDNA intergenic spacer sequences obtained in this study are DQ539339 and DQ539340. Accession numbers of flagellin sequences obtained in this study are DQ788618, DQ788619, and DQ788620.
**Data Analysis and Statistics**

To estimate the probability of a tick’s becoming infected after engorging on a green lizard and to measure the degree of infectiousness of infected animals, specific infectivity $I_s$ (24) and transmission coefficient $β_{H-T}$ (9) were calculated. Individual infectivity ($I_i$) is defined as the proportion of larvae derived from an individual lizard that are infected ($I_i = 1/l_h$, $l_h$ is the number of larvae that become infected, and $I_h$ is the total number of larvae derived from that host). The specific infectivity ($I_s$) of a reservoir host species is defined as the sum of individual infectivities and number of individual lizards sampled ($I_s = \sum I_i/n_s$, $n$ is the number of individual ticks captured). The host-to-tick transmission coefficient ($β_{H-T}$) is defined as the portion of the sum of individual infectivities and the number of lizards that infected $≥ 1$ larva ($β_{H-T} = \sum I_i/n_s$, $n$ is the number of individual hosts that gave rise to at least 1 infected tick). Differences in the prevalence of $B. burgdorferi$ s.l. in $I. ricinus$ were evaluated statistically with the 2-tailed $χ^2$ test (degrees of freedom $[df] = 1$). A value of $p≤0.05$ was considered statistically significant.

**Results**

**Lizards and Infestation with Ticks**

One hundred forty-six (84 male, 52 female, and 10 subadult) of 165 (89 male, 61 female, and 15 subadult) captured green lizards were infested by ticks during the study period. In total, 469 (199 larvae and 270 nymphs) ticks were removed and further identified as $I. ricinus$. Male lizards were infested with 363 ticks (131 larvae and 267 nymphs), from which the fragment of cytochrome $b$ gene was amplified. These ticks were further analyzed for the presence of $B. burgdorferi$ s.l. in $I. ricinus$ were evaluated statistically with the 2-tailed $χ^2$ test (degrees of freedom $[df] = 1$). A value of $p≤0.05$ was considered statistically significant.

**$B. burgdorferi$ Prevalence in Ticks Collected from Lizards**

DNA isolation was successful in 464 ticks (197 larvae and 267 nymphs), from which the fragment of cytochrome $b$ gene was amplified. These ticks were further analyzed for the presence of $B. burgdorferi$ s.l. In total, 77 (16.6%) ticks carried borreliae. The infection prevalence between nymphs (15.2%) and larvae (17.6%) did not differ significantly ($p = 0.49669$, $df = 1$) (Table 1). Twenty-nine percent of tick-infested lizards carried $≥ 1$ infected tick. Infected lizards yielded $2$ infected larvae per host.

**$B. burgdorferi$ Prevalence in Lizards**

Isolated genomic DNA from tails and skin biopsy specimens from collar scales was tested for the presence of $B. burgdorferi$ sensu lato. None of $53$ tested tail samples was positive. Of $102$ skin biopsy specimens collected from green lizards, $19$ ($18.6%$) tested positive. Differences in infection prevalence between sexes ($18.2%$ in males vs. $23.7%$ in females) were not significant. Of $9$ skin biopsy specimens from subadult individual lizards, $2$ ($22.2%$) were borrelia positive. The most frequently detected genospecies was $B. lusitaniae$ ($94.7%$), which was present in $18$ samples. One lizard was infected with $B. afzelii$.

**$B. burgdorferi$ Prevalence in Questing Ticks**

Cytochrome $b$ was amplified in $325$ of $331$ ($71$ female, $73$ male, and $187$ nymph) questing ticks. Therefore, only these $325$ ticks ($71$ female, $71$ male, and $183$ nymph) were analyzed further for the presence of $B. burgdorferi$ s.l. Sixty-three ($19.3%$) ticks tested positive. $B. burgdorferi$ s.l. were detected in $34$ ($69.2%$) nymphs,

### Table 1. Variability of Borrelia burgdorferi sensu lato in ticks collected on lizards

<table>
<thead>
<tr>
<th>Stage</th>
<th>No. ticks examined</th>
<th>No. positive ticks (%)</th>
<th>B. lusitaniae</th>
<th>B. afzelii</th>
<th>B. garinii</th>
<th>B. burgdorferi s.s.</th>
<th>B. valaisiana</th>
<th>B. lusitaniae + B. burgdorferi s.s.</th>
</tr>
</thead>
<tbody>
<tr>
<td>LARVAE</td>
<td>197</td>
<td>30 (15.2)</td>
<td>26 (86.7)</td>
<td>1 (3.3)</td>
<td>1 (3.3)</td>
<td>1 (3.3)</td>
<td>1 (3.3)</td>
<td>0</td>
</tr>
<tr>
<td>Nymphs</td>
<td>267</td>
<td>47 (17.6)</td>
<td>34 (74.5)</td>
<td>5 (10.6)</td>
<td>1 (2.1)</td>
<td>2 (4.3)</td>
<td>0</td>
<td>5 (10.6)</td>
</tr>
<tr>
<td>TOTAL</td>
<td>464</td>
<td>77 (16.6)</td>
<td>60 (77.9)</td>
<td>6 (7.8)</td>
<td>2 (2.6)</td>
<td>1 (1.3)</td>
<td>1 (1.3)</td>
<td>5 (6.5)</td>
</tr>
</tbody>
</table>
prevalence in female ticks was the same as in male ticks (22.5%), and it was lower in nymphs (16.9%).

RFLP analysis of the amplified products resulted in 5 distinct profiles. Of the 63 positive ticks, 21 (33.3%) were infected with *B. garinii*, 19 (30.2%) were infected with *B. afzelii*, 8 (12.7%) were infected with *B. burgdorferi* s.s., 7 (11.1%) were infected with *B. lusitaniae*, and 7 (11.1%) were infected with *B. valaisiana*. One nymph was infected simultaneously with *B. garinii* and *B. valaisiana* (Table 3).

Sequence Analysis
Representative samples of RFLP profiles that were different from the known profiles of positive controls were sequenced. The fragment of the 5S-23S rDNA intergenic spacer obtained from the *B. burgdorferi* s.l.–positive nymph (538N) from lizard belonged to *B. lusitaniae*. It was 100% identical to a Borrelia–positive skin biopsy specimen (277S) sampled from a lizard. Both obtained sequences were 100% identical with the Turkish *B. lusitaniae* strain Tr213 (AB 091802) and 98.9%, 98.4%, and 94.5% similar to PotiBL37 (AY 463167), PotiB2 (L30131), and PotiB3 (L30132) strains from Portugal, respectively. To better characterize *B. lusitaniae* circulating in ticks and lizards from Slovak Karst, the fla gene from a *B. burgdorferi* s.l.–positive questing adult tick and skin biopsy specimen from collar scale was amplified and sequenced. The flagellin sequence of *B. lusitaniae* detected in a skin biopsy specimen (277 S) was 100% identical and 99.6% similar to *B. lusitaniae* detected in questing adult ticks (43 ZLIF, 47 ZMLIM), respectively. Genotypes 277S and 43ZLIF were 100% identical with the Turkish *B. lusitaniae* strain Tr213 (AB091812) as well as with the Polish strain D23–04 (DQ 016623). Genotype 47ZLIM was 99.6%, 99.6%, and 99.4% similar to Tr213, D23–04, and PotiB2 (DQ111036), respectively.

Discussion
The role of lizard species in maintaining *B. burgdorferi* s.l. has not been clearly elucidated yet. In United States, some lizard species have sustained borrelial infection (14,17); however, other species are incompetent reservoir hosts (15,16). The reservoir competence of lizards seems to be species specific. Therefore the aim of our study was to establish whether a relationship exists between green lizards, the dominant lizard species in the Slovak Karst, and *B. burgdorferi* s.l., which circulates in this area.

Seventeen percent of ticks that fed on lizards were infected with *B. burgdorferi* s.l. Seventy-eight percent of all infected ticks were infected with *B. lusitaniae*. Moreover, 18.6% of skin biopsy specimens from lizards were positive for *B. burgdorferi* s.l., and almost all (94.7%) were infected with *B. lusitaniae*. Similarly, *B. lusitaniae* have been detected in blood and tissue samples of *Podarcis muralis* in Tuscany in Italy, where borreliae were detected in 2 of 14 tested whole tails from lizards (12). At the beginning of our study, we also collected the distal tip of a lizard’s tail because this method is minimally invasive and convenient for obtaining a tissue sample. The tissue at the tail, however, is squamous and keratinized, and none of the collected samples was borreliae positive. Therefore, we also obtained skin biopsy specimens from collar scales. These are elongated and extend from the skin on the ventral side, so collecting them is minimally invasive and perhaps more likely to detect infection with *B. burgdorferi* s.l. because most of the immature *I. ricinus* ticks parasitize at the dorsal area (pers. observation). Furthermore, collar scales were chosen to avoid detecting the borreliae that persist in the skin after feeding of the infected ticks, which may enable infection of ticks by “extended co-feeding” (25). In this manner, incompetent host species may contribute to the circulation of *B. burgdorferi* s.l. in nature. For example, in England,

<table>
<thead>
<tr>
<th>Genospecies</th>
<th>Males</th>
<th>Females</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\lambda$</td>
<td>$\beta_{M,T}$</td>
<td>$\lambda$</td>
</tr>
<tr>
<td><em>Borrelia burgdorferi</em> s.l.</td>
<td>0.0477</td>
<td>0.605</td>
<td>0.0221</td>
</tr>
<tr>
<td><em>B. lusitaniae</em></td>
<td>0.0377</td>
<td>0.518</td>
<td>–</td>
</tr>
<tr>
<td><em>B. garinii</em></td>
<td>0.0012</td>
<td>0.01</td>
<td>–</td>
</tr>
<tr>
<td><em>B. afzelii</em></td>
<td>0.0012</td>
<td>0.01</td>
<td>–</td>
</tr>
<tr>
<td><em>B. burgdorferi</em> s.s.</td>
<td>0.0012</td>
<td>0.01</td>
<td>–</td>
</tr>
</tbody>
</table>

Table 2. Specific infectivity ($\lambda$) and host-to-tick transmission coefficient ($\beta_{M,T}$)

<table>
<thead>
<tr>
<th>Stage</th>
<th>No. ticks examined</th>
<th>No. positive ticks (%)</th>
<th>B. afzelii</th>
<th>B. garinii</th>
<th>B. valaisiana</th>
<th>B. burgdorferi s.s.</th>
<th>B. lusitaniae</th>
<th>B. valaisiana + B. garinii</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nymphs</td>
<td>183</td>
<td>31 (19.6)</td>
<td>13 (41.9)</td>
<td>12 (38.7)</td>
<td>2 (6.4)</td>
<td>3 (9.6)</td>
<td>0 (0)</td>
<td>1 (3.2)</td>
</tr>
<tr>
<td>Females</td>
<td>71</td>
<td>16 (22.5)</td>
<td>4 (25)</td>
<td>4 (25)</td>
<td>3 (18.7)</td>
<td>3 (18.7)</td>
<td>2 (12.5)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Males</td>
<td>71</td>
<td>16 (22.5)</td>
<td>2 (12.5)</td>
<td>5 (31.3)</td>
<td>2 (12.5)</td>
<td>2 (12.5)</td>
<td>5 (31.3)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Total</td>
<td>325</td>
<td>63 (19.3)</td>
<td>19 (30.2)</td>
<td>21 (33.3)</td>
<td>7 (11.1)</td>
<td>8 (12.6)</td>
<td>7 (11.1)</td>
<td>1 (1.5)</td>
</tr>
</tbody>
</table>

Table 3. Variability of *Borrelia burgdorferi* sensu lato in questing ticks

1898 Emerging Infectious Diseases • www.cdc.gov/eid • Vol. 12, No. 12, December 2006
I. ricinus ticks cofeeding on sheep become infected with B. burgdorferi, although sheep themselves are refractory to infection (26). In Europe the principal importance of cofeeding to Lyme disease ecology has been suggested to be the extent of the range of vertebrate host species that contribute significantly to the maintenance of B. burgdorferi s.l. spirochetes in nature (27). Therefore, cofeeding transmission could also be responsible for B. afzelii, B. garinii, and B. burgdorferi s.s. infection in larvae that fed on lizards collected in our study, even though skin biopsy results yielded mostly B. lusitaniae. Another possible explanation for the presence of non–B. lusitaniae spirochetes is that these larvae may have been infected transovarially (28). Cofeeding transmission might explain why individual lizards with borreliae negative skin biopsy specimens carry borreliae-positive larvae. Because the quantity of borreliae is low in the vertebrate host and may lodge in deeper organs, detecting them in skin biopsy specimens may not always be possible (29). Thus, a negative skin biopsy result does not prove conclusively that the lizard is not infected.

Despite the fact that male lizards hosted >75% of all host-feeding ticks, as well as 79.2% of all infected ticks, the specific infectivity and host-to-tick transmission coefficient were almost the same for male and female lizards. The seasonal activity of green lizards and different patterns in male and female behavior were monitored in the Slovak Karst (I. Majlath, unpub. data). Larger numbers of ticks feeding on male lizards are associated with higher male activity in spring months, when tick activity peaks as well. Male lizards end hibernation first and are active when the air temperature reaches 10°C–12°C. They need to restock the energy that was depleted during winter and to gain energy for fighting other male lizards to compete for territory and females, for seeking female lizards, and for mating. Female activity increases in summer months when they are incubating eggs.

As determined by PCR, the overall prevalence of infection in our sample of questing ticks (19.3%) is consistent with 20.5% found in southern Czech Republic (20) but lower than that reported for a geographically close area in western Slovakia (40%–49%) (30). The total prevalence was higher in adults (22.5%) than in nymphs (19.6%), which is in agreement with the general pattern of increasing Borrelia prevalence through the life stages of ticks as their adults feed on a multiple hosts (31). The total prevalence of borreliae in male and female ticks was identical, but the distribution of genospecies was different. B. garinii was the predominant genospecies in this locality. B. garinii and B. valaisiana are the most commonly reported species in central Europe (32).

The high prevalence of B. lusitaniae in borrelia-positive larvae and nymphs as well as skin biopsy specimens from lizards suggests that green lizards are susceptible and transmission competent for B. lusitaniae. On the other hand, a lack or low prevalence of other genospecies in ticks that fed on lizards may suggest that these genospecies could be negatively selected against by green lizards. A similar suppressive effect of Madeiran wall lizard (Podarcis dugesii) on the transmission of spirochetes was observed (33). Borreliacidal activity against B. burgdorferi s.s. was observed in the lizards S. occidentalis and E. multicarinata in North America (15,16). These findings add to the growing support for the hypothesis that there are Borrelia species-specific associations with specific reservoir host species that result from Borrelia species–specific interactions with host serum complement (29).

Significant differences were found in B. lusitaniae prevalence in fed larvae compared with questing nymphs (p<0.001, df = 1); none of 183 examined nymphs was infected by this genospecies. This finding raises the question of whether borreliae are eliminated during molting and thus do not contribute to the transmission cycle or whether we were just unable to detect it. Significant differences were found in B. lusitaniae prevalence also in fed nymphs compared with questing adults (p<0.01, df = 1). The infection prevalence decreased from 74.5% in fed nymphs to 5% in questing adults. Reduction of infection prevalence has been observed in B. afzelii from 47% in nymphs engorged on the rodents to 7% in questing nymphs (9).

The occurrence of B. lusitaniae in ticks is frequent in some areas of the Iberian Peninsula and North Africa, where the organism often represents the only species of B. burgdorferi s.l. complex (13,34). In the rest of the Europe, it has been isolated or detected less frequently, with low prevalence in ticks (30,35,36). The prevalence of B. lusitaniae is the highest in southern Europe and can be exported to other areas by hosts such as birds (37). The 5S-23S rDNA and flagellin sequences of B. lusitaniae–positive ticks and skin biopsy specimens in our study were 100% identical to the B. lusitaniae strain Tr213 from a tick in Turkey (38). The distribution of this borrelial species may be associated with the distribution range of reservoir hosts, including lizards, that inhabit drier and warmer areas. These ecosystems are less abundant in central Europe than in the Mediterranean. Thus, lizards may influence the transmission cycle of borreliae in some localities in which they are the predominant host for ticks. In our study, we found B. lusitaniae in skin biopsy specimens and ticks that fed on green lizards. These findings implicate this species of lizard in the transmission cycle of B. lusitaniae. The competence of other lizard species that feed ticks should be also investigated. The low prevalence of B. lusitaniae in questing ticks, however, indicates that the ecology of
B. lusitaniae in endemic foci of central Europe is more complex. Further studies that analyze the circulation of B. burgdorferi s.l. among a broader spectrum of host species should be undertaken.

Acknowledgments
We thank Jean Tsao and Slavka Barlakova for their critical reading of the manuscript and helpful comments and Marcela Mirekova and Renata Ivanova for technical assistance.

This work was partly supported by the Slovak Agency of Research and Development, APVV-51-009205 project (M.D.), VEGA 2/6163/26 to (B.P.), and VEGA 1/1284/04 (I.M.).

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References

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