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Detection of Borrelia burgdorferi sensu lato in Lizards and Their Ticks from Hungary

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Abstract

To investigate the involvement of lizard species in the natural cycle of *Borrelia burgdorferi* sensu lato (s.l.) in Hungary, a total of 186 reptiles belonging to three species—126 green lizards (*Lacerta viridis*), 40 Balkan wall lizards (*Podarcis taurica*), and 20 sand lizards (*Lacerta agilis*)—were captured in 2007 and 2008. All ticks removed from the lizards were *Ixodes ricinus*, either larvae (324/472; 68.6%) or nymphs (148/472; 31.4%). More than half (66/126; 52.4%) of *L. viridis* individuals were infested, and the prevalence of tick infestation on both the other two species was 35% each. All 472 *I. ricinus* ticks and tissue samples collected from 134 collar scales and 62 toe clips of lizards were further analyzed for the presence of *B. burgdorferi* s.l. with polymerase chain reaction. The amplification of *B. burgdorferi* s.l. DNA was successful in 8% ($n = 92$) of *L. viridis*, 9% ($n = 32$) of *P. taurica*, and 10% ($n = 10$) of *L. agilis* tissue samples. Restriction fragment length polymorphism genotyping identified the species *Borrelia lusitaniae* in all tested lizard samples. Prevalence of *B. burgdorferi* s.l. in ticks collected from *L. viridis*, *P. taurica*, and *L. agilis* was 8%, 2%, and 0%, respectively. Most of the infected ticks carried *B. lusitaniae* (74% of genotyped positives); however, *Borrelia afzelii* (5%) and *B. burgdorferi* sensu stricto (21%) were detected in ticks removed from green lizards and Balkan wall lizards, respectively. We conclude that lizards, particularly *L. viridis*, can be important hosts for *I. ricinus* larvae and nymphs; thus, they can be regarded as reservoirs of these important pathogen vectors. The role of green lizards has been confirmed, and the implication of Balkan wall lizards is suggested in the natural cycle of *B. lusitaniae* at our study site.

Key Words: *Borrelia burgdorferi* sensu lato—*Borrelia lusitaniae*—*Lacerta agilis*—*Lacerta viridis*—Lizards—*Podarcis taurica*.

Introduction

Recently emerged and reemerging infectious diseases are dominated by zoonoses most of which originate in wildlife. Among these, vector-borne diseases (including tick-borne diseases) have shown a significant rise in the last decade (Jones et al. 2008). Lyme borreliosis (LB), for example, has become the most prevalent vector-borne human disease in the temperate zone of the Northern Hemisphere (Margos et al. 2008). In Europe, the disease is maintained through complex interactions between the pathogen, *Borrelia burgdorferi* sensu lato (s.l.), different tick species and a large number of vertebrate hosts. Over 230 animal species are implicated as hosts for *Ixodes ricinus* ticks, the most significant vector, and our knowledge concerning the reservoir potential of different vertebrate host species in LB epidemiology is far from complete (Gern 2008).

At present, LB spirochetes constitute a group of 14 named species (Rudenko et al. 2008a). Several of these, namely, *B. burgdorferi* sensu stricto (s.s.), *Borrelia afzelii, Borrelia garinii*, and *Borrelia spielmanii*, are associated with disease in humans (Margos et al. 2008). Although previously considered as nonpathogenic, *Borrelia bissettii* (Rudenko et al. 2008b), *Borrelia valaisiana* (Diza et al. 2004, Saito et al. 2007), and *Borrelia lusitaniae* (Collares-Pereira et al. 2004, da Franca et al. 2005, Lopes de Carvalho et al. 2008) have recently been reported to cause disease as well. Various studies in endemic areas throughout Europe reported special associations between some *Borrelia* species and particular groups of vertebrate hosts. Small mammals were found to be the key reservoirs for

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B. afzelii (Humair et al. 1999, Hanincová et al. 2003a), and *B. garinii* belonging to the OspA type 4 was associated with rodents (Postic et al. 1997). *B. spielmanii* was only found in the garden dormouse (*Eliomys quercinus*) and the hazel dormouse (*Muscardinus avellanarius*) in France (Richter et al. 2004) and the European hedgehog (*Erinaceus europaeus*) in Germany (Skuballa et al. 2007). Birds are reported to be associated with *B. garinii* and *B. valaisiana*, but *B. burgdorferi* s.s. seems to be maintained by both birds and rodents (Humair et al. 1998, Hanincová et al. 2003b, Duneau et al. 2008, Gern 2008). An explanation for these associations was given by Kurtenbach et al. (2002), who showed that the complement present in the blood of the host is the active component in the *Borrelia* host specificity. Although *in vitro* tests demonstrating the serum sensitivity of different *Borrelia* species matched the known reservoir status of many vertebrates, it seems that these associations are less rigid and exceptions can frequently be observed in natural cycles (Humair et al. 1999, Korenberg et al. 2002, Fomenko et al. 2008).

The recent recognition of lizards as hosts for LB spirochetes exemplifies how our understanding of the epidemiology of this disease is changing. Although known long ago as important hosts for ixodid ticks (Jellison 1934, Grulich et al. 1957, Bauwens et al. 1983), lizards had not previously been considered as potential reservoirs of LB spirochetes. Their role in the circulation of *B. burgdorferi* s.l. has been underestimated compared to that of small mammals and birds, since several lizard species have been shown to possess a complement with borreliacidal activity (Lane and Loye 1989, Wright et al. 1998). Matuschka et al. (1992) concluded in their study that sand lizards (*Lacerta agilis*) do not support infection by spirochetes; instead, they play a zooprophylactic role diverting larval ticks that might have become infected and absorb nymphs that previously had been infected. This supposed dilution effect of lizards was later refuted by the same authors (Richter and Matuschka 2006). They compared the prevalence and species of spirochetes in rodent- and lizard-associated ticks at a site in Germany and provided evidence that reptiles are involved in the natural cycle of *B. lusitaniae*. Those authors admit that spirochetes used in their previous experiment (Matuschka et al. 1992) were most probably *B. afzelii* or *B. burgdorferi* s.s. and that sand lizards failed to infect xenodiagnostic ticks for this reason.

In Hungary, LB was first reported in 1984 and has remained a frequently diagnosed disease in the country (Lakos et al. 1985, Lakos 1991, Bózsik 2004, Földvári et al. 2005). The aim of the present study was to investigate whether and how lizards are involved in the natural cycle of *B. burgdorferi* s.l. in Hungary.

Materials and Methods

Study area

Fieldwork was carried out near Gödöllő, central Hungary (N47°57′, E19°33′). The study area was a mosaic of meadows, arable fields, planted wood mostly black locust (*Robinia pseudacacia*), and scrubland. Scrublands consist of common hawthorn (*Crataegus monogyna*), blackthorn (*Prunus spinosa*), and dog rose (*Rosa canina*). Meadows are dominated by xerophilic grasses (e.g., *Festuca* spp., *Dactylis glomerata, Calamagrostis epigeios*, and *Stipa* spp.). Lizards were captured mostly on forest edges, in scrublands, and in a nearby sand pit. The climate is warm with low humidity and average temperatures of −2.2°C in January and 19.9°C in July. The average rainfall is 600 mm/year.

Lizard and tick collection

Lizards were collected from April to September in 2007 and 2008 during their activity period. They were captured by hand or noosing, where 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 upon walking through the loop. Animals were identified, sex and age (adult, subadult, juvenile) were determined according to body size, and morphological characters and the presence of ticks were examined. Ticks were removed with forceps immediately after capture and stored in 70% ethanol. A tissue sample from collar scales (1–1.5 mm) and/or toe clip was taken from each individual with sterile scissors and put in separate vials with 70% ethanol. Ticks were identified, sex was determined, and they were further examined for the presence of *B. burgdorferi* s.l.

DNA isolation

Immediately before DNA extraction, ticks and tissues were dried for 30 min to evaporate the ethanol. Each sample was cut individually with sterile scissors or a disposable sterile scalpel. Genomic DNA from lizard scales, toe clips, and ticks was isolated by either QIAamp DNA mini kit (Qiagen, Hilden, Germany) using an overnight Proteinase K digestion or alkaline hydrolysis (Guy and Stanek 1991) with 30 min of incubation. Isolated DNA was stored at −20°C.

Polymerase chain reaction

In 2007, primers BSL-F and BSL-R were used, which amplify an approximately 250 bp region of the *outer surface protein A* gene from all LB spirochetes (Demaerschalck et al. 1995, Földvári et al. 2005). Samples from this year could not be genotyped because of DNA impairment.

For samples collected in 2008, a portion of the 5S (rrfA)–23S (rrlB) rDNA intergenic spacer of *B. burgdorferi* s.l. was amplified (Derdáková et al. 2003). PCR amplification was performed in a total of 25 μL reaction mixture of a MasterTaq DNA polymerase kit (Eppendorf AG, Hamburg, Germany) containing 10.4 μL of deionized water, 5 μL of 5× TaqMaster PCR Enhancer, 2.5 μL of 10× Taq buffer (with 15 mM Mg^{2+}), 1.5 μL of a 25 mM solution of $Mg(OAc)_2$, 0.1 μL of Taq DNA polymerase (5 U/mL), 0.5 μL of dNTP-mix (10 mM) (Fermentas, Vilnius, Lithuania), 1.25 μL of each primer (10 pmol/μL) (Invitrogen, Paisley, Scotland), and 2.5 μL of DNA template. The PCR products were electrophoresed in a 1% agarose gel, stained with ethidium bromide, and viewed with a UV transilluminator.

Restriction fragment length polymorphism analysis

Positive PCR products from the 5S–23S rDNA intergenic spacer regions were further analyzed by restriction fragment length polymorphism. Previously extracted DNA of *B. afzelii, B. garinii, B. valaisiana, B. burgdorferi* s.s., and *B. lusitaniae* were used as positive controls. For each positive sample, 13 μL of amplified DNA was digested at 65°C overnight in a solution containing 5U of Tru1 I (300 U/mL) and 1× Buffer R (Fer-

mentas). Electrophoresis was carried out in 16% polyacrylamide gel at 150 V for 3 h. Gels were stained with SYBR Gold nucleic acid gel stain (Molecular Probes, Leiden, The Netherlands) for 20 min, and bands were viewed with a UV transilluminator (Derdáková et al. 2003).

All procedures (DNA isolation, PCR, and electrophoresis) were performed in separate rooms using different pipettes and racks, with separate coats and disposable gloves worn in each laboratory to prevent carry-over contamination and to avoid false-positive results. PCR mixtures were prepared in a sterile PCR box. All liquid handling procedures were performed using disposable sterile filter tips. In each DNA isolation and PCR, negative control (sterile deionized water) was included.

Statistics

For data analysis, R-environment (R Development Core Team 2006) and programme Quantitative Parasitology 3.0 (Rózsa et al. 2000) were used. Differences between tick infestation levels and prevalence of borreliae in ticks and tissue samples were evaluated statistically using Fisher's exact test. A value of $p \leq 0.05$ was considered significant.

Results

Tick infestations of lizards

During the study 186 lizards, belonging to three species, were captured: 126 (121 adult and 5 juvenile) green lizards (*Lacerta viridis*), 40 (39 adult and 1 juvenile) Balkan wall lizards (*Podarcis taurica*), and 20 (19 adult and 1 juvenile) sand lizards (*L. agilis*). All ticks found on the lizards were *I. ricinus.* More than half (66/126; 52.4%) of *L. viridis* individuals were infested with ticks (Table 1). Altogether 397 ticks (267 larvae and 130 nymphs) were collected from green lizards. The median intensity of infestation was 3.5 ticks per lizard. The highest number of ticks collected was 47 (from a male lizard). There was a significant difference ($p = 0.013$) in the prevalence of tick infestation between female (42.9%) and male (65.5%) green lizards. Only one juvenile *L. viridis* carried a tick, and none of the two juveniles of *L. agilis* and *P. taurica* were infested.

Fourteen (35%) of 40 *P. taurica* captured were infested with *I. ricinus* (Table 1). The total number of ticks was 55 (50 larvae and 5 nymphs), and the median intensity was two ticks per lizard. The difference in the prevalence of tick infestation between male (27%) and female (42%) Balkan wall lizards was not significant ($p = 0.496$).

Prevalence of tick infestation among sand lizards was also 35% (7/20) (Table 1). Altogether, 20 *I. ricinus* (7 larvae and 13 nymphs) were removed; the median intensity was two ticks per lizard. There was a significant difference ($p = 0.038$) in the prevalence of tick infestation between female (21%) and male (80.0%) *L. agilis* individuals.

Prevalence of B. burgdorferi *s.l. in lizards and ticks*

Altogether 472 *I. ricinus* ticks and 196 tissue samples (134 from collar scales and 62 from toe clips) collected from lizards were analyzed for the presence of *B. burgdorferi* s.l. Eleven of 134 collar scales (92 *L. viridis*, 32 *P. taurica*, and 10 *L. agilis*) were positive (Table 2); 7 *L. viridis* (3 males and 4 females), 3 *P. taurica* (1 male and 2 females), and 1 female *L. agilis*. There was no significant difference ($p = 0.775$) in the prevalence of borreliae among lizard species. Juvenile lizards were negative. Only 1 of the 62 toe clips tested positive originating from a female *L. viridis; B. lusitaniae* was confirmed with restriction fragment length polymorphism in all positive tissue samples tested.

Prevalence of *B. burgdorferi* s.l. in ticks collected from *L. viridis, P. taurica*, and *L. agilis* was 8%, 2%, and 0%, respectively. The overall prevalence of the LB spirochetes in nymphs (10.8%) was significantly higher ($p = 0.016$) than in larvae (4.6%) (Table 3). Of 397 ticks that had fed on *L. viridis* 30 ticks (14 larvae and 16 nymphs) were infected with *B. burgdorferi* s.l. Only samples obtained in 2008 were genotyped. Six nymphs and eight larvae were infected with *B. lusitaniae. B. burgdorferi* s.s. was detected in two nymphs and one larva and *B. afzelii* in a larva. Only a single larva out of 55 ticks (50 larvae and 5 nymphs) removed from *P. taurica* individuals was infected with *B. burgdorferi* s.s. None of the 20 ticks that were collected from *L. agilis* was positive for borreliae. *B. lusitaniae* was the most prevalent (74%) species among genotyped positive ticks followed by *B. burgdorferi* s.s. (21%) and *B. afzelii* (5%).

Discussion

At our Central European study site we found that lizards can be important hosts for *I. ricinus* immatures and specifically *L. viridis* and *P. taurica* may play a role in the circulation of *B. burgdorferi* s.l. Although tick infestation prevalences and intensities may be underestimated in this study because *I. ricinus* larvae that have recently attached to lizards are often under scales and not easily located, we observed high prevalences of tick infestation in the three lizard species captured. More than half of the green lizards carried *I. ricinus*, so this reptile may constitute a particularly important host for larvae and nymphs in this area. Various species of lizards have been described as tick hosts (Grulich et al. 1957, Bauwens et al.

TABLE 1. PREVALENCE AND MEDIAN INTENSITY OF *IXODES RICINUS* INFESTATION ON LIZARDS IN GÖDÖLLŐ, HUNGARY (2007–2008)

Species	No. of lizards		Prevalence (%)	Median intensity
	Total	Infested		
Lacerta viridis	126	66	52.4	3.5
Podarcis taurica	40	14	35.0	2.0
Lacerta agilis	20	7	35.0	2.0
Total	186	87	46.8	3.0

TABLE 2. PRESENCE OF *BORRELIA BURGDORFERI* SENSU LATO IN TISSUE SAMPLES COLLECTED FROM LIZARDS IN GÖDÖLLŐ, HUNGARY (2007–2008)

Species	No. of collar scales/toe clips	No. of positive collar scales/ toe clips	Prevalence (%) of Borrelia infection in collar scales/ toe clips
L. viridis	92/47	7/1	8/2
P. taurica	32/15	3/0	9/0
L. agilis	10/0	1/0	10.0/0
Total	134/62	11/1	8.2/2

Table 3. *Borrelia* Infections of *Ixodes ricinus* Ticks Collected from Lizards in Gödöllő, Hungary (2007–2008)

Stage	No. of ticks	No. of infected ticks (%)	No. of ticks positive (% of Borrelia-positive ticks)			
			B. burgdorferi *s.l.*[a]	Borrelia lusitaniae	B. burgdorferi *s.s.*	Borrelia afzelii
Larvae	324	15 (4.6)	6 (40)	6 (40)	2 (13)	1 (7)
Nymphs	148	16 (10.8)	6 (37)	8 (50)	2 (13)	0 (0)
Total	472	31 (6.6)	12 (39)	14 (45)	4 (13)	1 (3)

[a]*B. burgdorferi* s.l. (genospecies not determined for 2007 samples). s.l., sensu lato; s.s., sensu stricto.

1983, Scali et al. 2001, Dsouli et al. 2006, Majláthová et al. 2006, 2008), to our knowledge; however, this is the first record of *P. taurica* as host of *I. ricinus*.

Tick infestation intensity was higher on males than females for *L. viridis* and *L. agilis*. Male-biased tick infestation is in accordance with several other studies (Lane and Loye 1989, Dsouli et al. 2006, Majláthová et al. 2006). A possible cause is that male lizards are more active and have larger home ranges than females (Bauwens et al. 1983, Eisen et al. 2001). The higher infestation intensity observed in males also may be related to the difference in their testosterone concentration. Salvador et al. (1996) described that during the breeding season *Psammodromus algirus* males that were implanted with testosterone carried a higher number of *I. ricinus* larvae and nymphs than control lizards.

Nearly 70% of the ticks removed from the three lizard species were larvae, which is in contrast with the larva/nymph ratio reported previously on lizards in Tunisia (Dsouli et al. 2006), Slovakia (Majláthová et al. 2006, 2008), Romania (Majláthová et al. 2008), and Poland (Gryczynska-Siemiatkowska et al. 2007, Majláthová et al. 2008). Richter and Matuschka (2006) found that lizards were parasitized by up to eight times more nymphal ticks than were rodents at their study site in Germany; however, this may also be due to the ticks' slower feeding on ectothermic hosts. The causes for these differences can be various biotic (e.g., other tick hosts and tick activity) and abiotic (vegetation, humidity, temperature, etc.) conditions, and their elucidation requires further comparative studies.

The amplification of the partial rDNA intergenic spacer of *B. burgdorferi* s.l. was successful in tissue samples of all three lizard species. The potential reservoir role of green lizards (Majláthová et al. 2006) and sand lizards (Richter and Matuschka 2006, Majláthová et al. 2008) has been previously shown; however, this is the first evidence that Balkan wall lizards might also be involved in the epidemiology of LB spirochetes. *B. lusitaniae* was detected in every positive tissue sample representing the first focus of this species in Hungary. Evidence on the human pathogenicity of *B. lusitaniae* is being gathered (Collares-Pereira et al. 2004, da Franca et al. 2005, Lopes de Carvalho et al. 2008), although there is little information on the geographical distribution and reservoir hosts of this spirochete. The prevalence of *B. lusitaniae* in questing ticks varies in different parts of Europe. Previously, this species was considered to be restricted to the Mediterranean basin (as indicated in its name), where it represents the dominant or only species present in *I. ricinus* ticks (De Michelis et al. 2000, Younsi et al. 2001, Sarih et al. 2003, Amore et al. 2007). However, it seems to be widespread with several endemic foci reaching as far north as Poland (Postic et al. 1997, Gern et al. 1999, Wodecka and Skotarczak 2005, Poupon et al. 2006, Majláthová et al. 2008). The candidate reservoir hosts are lizards as reported in Germany (Richter and Matuschka 2006), Slovakia, Romania, Poland (Majláthová et al. 2006, 2008), Italy (Amore et al. 2007), and Hungary (present study), where this was the only *Borrelia* species detected in tissue samples of reptiles. Birds may also play a role in the transmission of *B. lusitaniae*, and they may serve as the means of transportation for this species to new areas (Poupon et al. 2006). In Slovakia and in Romania as high as 46% and 57% of lizards were found to carry this spirochete (Majláthová et al. 2008). Our study showed that 8% of green lizards, 9% of Balkan wall lizards, and 10% of sand lizards carried *B. lusitaniae*, indicating that other reservoirs might also be present at the area.

We found *B. burgdorferi* s.l. in 8% and 2% of ticks removed from *L. viridis* and *P. taurica*, respectively. Most of the genotyped infected ticks carried *B. lusitaniae*; however, *B. afzelii* was detected in a larva removed from a green lizard and *B. burgdorferi* s.s. was found in nymphs and a larva from green lizards and in a larva from a Balkan wall lizard. Infected nymphs could have been infected with spirochetes either from larval instars via transstadial infection or directly from the lizards. Since transovarial transmission is rare in the life cycle of LB spirochetes (Piesman et al. 1986), in the case of positive larvae, lizards were assumed to be the source of infection. Feeding larvae most probably acquired infection from systemically *B. lusitaniae*–infected reptiles as experimentally also shown by Dsouli et al. (2006). There is no similar evidence for *B. afzelii* or *B. burgdorferi* s.s., which may also infect lizards systemically; therefore, these species could have been taken up by larvae while cofeeding with infected nymphs (Gern and Rais 1996). This supports the hypothesis that the importance of the cofeeding mechanism to LB ecology can be the extent of the range of vertebrate hosts that contribute to the maintenance of *B. burgdorferi* s.l. in Europe (Randolph et al. 1996). In addition, lizards live longer than mice and voles; thus, with a possible systemic spirochetal infection they might serve as infective hosts for several tick generations.

We conclude that lizards, particularly *L. viridis*, are important hosts for *I. ricinus* larvae and nymphs and thus can be regarded as reservoirs of these important vectors. We confirmed the role of *L. viridis* and suggest the implication of *P. taurica* in the natural cycle of *B. lusitaniae* at our study site. *B. burgdorferi* s.s. and *B. afzelii* were also detected in feeding larvae and nymphs, which indicates either a systemic infection of the lizards with these spirochetes or cofeeding infectivity of these reptiles. Taken together, lizard species in Europe may serve as important hosts in the ecology of LB spirochetes. However, further field and experimental studies are necessary to unveil whether and how specific associations

between particular lizard species and *B. lusitaniae* or other spirochetes exist.

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Disclosure Statement

No competing financial interests exist.

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