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Borrelia lusitaniae in Immature *Ixodes ricinus* (Acari: Ixodidae) Feeding on Common Wall Lizards in Tuscany, Central Italy

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ABSTRACT Lizards and small rodents were live captured in Tuscany, central Italy, from May through August 2005. Prevalence of infestation by larval *Ixodes ricinus* L. (Acari: Ixodidae) and mean numbers of larvae per host were not significantly different for common wall lizards, *Podarcis muralis* Laurenti, and *Apodemus* spp. mice, whereas infestation levels by nymphs were significantly greater on lizards. *Borrelia lusitaniae*, which was previously shown to be dominant in host-seeking *I. ricinus* in the same study area, was detected by polymerase chain reaction (PCR) in 19.8% (95% confidence interval: 14.4, 26.0) of larval ticks and in 52.9% (27.8, 77.0) of nymphs that were collected from lizards. Moreover, 18.8% (7.2, 36.4) and 25.0 (3.2, 65.1) of lizards' tail biopsies and blood samples, respectively, were positive for *B. lusitaniae*. Conversely, attached ticks and ear biopsies from *Apodemus* spp. mice were PCR negative. Passerine birds belonging to 10 species were live captured in March 2005, and *Borrelia valaisiana* was detected in 57.1% (18.4, 90.1) of *I. ricinus* nymphs feeding on Eurasian blackbirds, *Turdus merula* L. Results of this study suggest that lizards play an important role as reservoirs for *B. lusitanae* and may affect the dominance of this genospecies in the Mediterranean area.

KEY WORDS *Borrelia lusitaniae*, *Ixodes ricinus*, *Podarcis muralis*, Italy

In western Europe and the Mediterranean area, *Borrelia burgdorferi* s.l., including the causative agents of Lyme borrelsiosis, is maintained in transmission cycles involving the tick vector *Ixodes ricinus* L. and several species of vertebrate reservoir hosts (Gern and Humair 1998, Gern et al. 1998). Birds are major reservoirs for *Borrelia garinii* and *Borrelia valaisiana*, whereas mammals, such as small rodents and squirrels, are most important for *B. afzelii* and *B. burgdorferi* s.s. (Kurtenbach et al. 1998, 2002). Recent studies, however, suggested that the host specificity of *B. burgdorferi* s.l. genospecies is not absolute and may vary in different geographic areas (Pichon et al. 2003).

Borrelia lusitaniae was recently recognized as a potential agent of Lyme borreliosis, and it was the most prevalent genospecies of *B. burgdorferi* s.l. in host-seeking ticks in locations of southern Europe and the Mediterranean area (Sarih et al. 2003, Collares-Pereira et al. 2004, Younsi et al. 2005). Conversely, this genospecies was sporadic in central and northern Europe (Güner et al. 2003, Jouda et al. 2004, Wodecka and Skotarczak 2005, Lencakova et al. 2006).

Borrelia lusitaniae-infected *I. ricinus* larvae were collected from migratory birds in Switzerland (Poupon et al. 2006). A study in southern Germany, however, showed that immature ticks acquired *B. lusitaniae* through feeding on common wall lizards, *Podarcis muralis* Laurenti, and sand lizards, *Lacerta agilis* L. (Richter and Matuschka 2006). Moreover, the reservoir competence of lizard *Psammodromus algirus* L. for *B. lusitaniae* in Tunisia was demonstrated by xenodiagnosis (Dsouli et al. 2006). It was therefore suggested that birds become susceptible to *B. lusitaniae* due to the stress of migration and are responsible for its geographic diffusion. Lizards, however, may be responsible for perpetuating *B. lusitaniae* at foci where they are a major component of the population of vertebrate hosts for *I. ricinus* (Richter and Matuschka 2006).

In Italy, *B. lusitaniae* was the dominant genospecies of *B. burgdorferi* s.l. in host-seeking *I. ricinus* in an area in Tuscany (Le Cerbaie Hills, in the province of Pisa) characterized by the coexistence of relatively dry uplands, covered by Mediterranean vegetation, and humid bottomlands covered by deciduous wood (Bertolotti et al. 2006). In the same location, a case of Lyme borreliosis was reported in a forestry worker. In this study, we estimated the prevalence of infection by *B. burgdorferi* s.l. in feeding immature *I. ricinus* and tissues from lizards, small rodents, and a small sample

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of passerine birds in Le Cerbaie to generate hypotheses on the relative role of common reservoirs of infection.

Materials and Methods

Animal Capture. Lizards were captured by a noose affixed to a stick, at different sites in bottomlands and uplands of Le Cerbaie, during four sampling sessions in spring and summer 2005: May (4 d), June (2 d), July (4 d), and August (7 d). Lizards were identified by species, and attached ticks were removed with forceps and stored in ethanol 70%. A small amount of blood was obtained from a sample of lizards via tail fracture (a natural escape mechanism in lizards) and stocked in LongMire buffer. Tail tissues, including muscle and skin, were stored in 70% ethanol. The Commission for Bioethics and Animal Welfare of the Faculty of Veterinary Medicine of the University of Turin judged protocols that we used in animal capture and tissue sampling as appropriate from the point of view of animal welfare and justified by the scientific objectives of the study.

Small rodents were captured using 90 Sherman live traps (7.5 by 7.5 by 25 cm., Sherman Live Traps Co., Tallahassee, FL) baited with cereals. Traps were set 7 m apart in a 9 by 10 grid in bottomland habitat during trapping session (May–August 2005) for a total of 1,530 trap-nights. Captured mice were anesthetized with an intramuscular injection of a mixture of 2.5 μg of medetomidine HCl (Domitor, Pfizer Animal Health, Rome, Italy) and 1 mg of ketamine HCl (Ketavet 100, Intervet Italia, Milan, Italy). Biopsies were taken by cutting a triangular fragment from an ear's outer margin (also used as part of mouse individual identification) and stored in 70% ethanol. Attached ticks were removed and stored in 70% ethanol. After examination, anesthetized mice were injected with 5 μg of atipamezole HCl (Antisedan, Pfizer Animal Health) to reverse the effects of medetomidine, and they were released at the capture site after they completely recovered from anesthesia.

Passerine birds that were live captured by mist net in March 2005 were examined for ticks, and a small amount of blood was taken from ulnar vein by using a capillary tube. Birds were subsequently released at the site of capture.

Ticks were identified by species by using keys from Manilla (1998). Tick engorgement index (TEI) was evaluated as the ratio of body length to scutum width (Yeh et al. 1995).

Detection of *B. burgdorferi* s.l. DNA was extracted from ticks (previously homogenized with a pestle in microcentrifuge tubes) and from tails and ear biopsies from lizards and mice, respectively, by using a QIAGEN DNeasy tissue kit (QIAGEN GmbH, Hilden, Germany). According to the manufacturer's recommendations, tissue weight did not exceed 25 mg. DNA was suspended in nuclease-free water (50 μl for ticks and 100 μl for tissues) and used (5 μl per reaction) in PCR. Negative controls (distilled water) were added to verify the potential contaminations of samples during this phase. DNA was extracted from bird blood by using a Wizard Genomic DNA purification kit protocol for tissue extractions (Promega, Madison, WI), partially modified, whereas for lizard blood a QIAGEN DNeasy blood mini kit (QIAGEN GmbH) was used.

The infection by *B. burgdorferi* s.l. in ticks and tissues was investigated using a PCR protocol as described in Mannelli et al. (2005). The primer pair amplified a 225-bp DNA fragment in the intergenic spacer region included between genes codifying for the subunits 5S and 23S of ribosomal RNA (Rijpkema et al. 1995). We tested ticks with TEI > 2 to partially control for the potential effect of engorgement status in comparing infection prevalence among ticks collected from different animal species.

To standardize positive controls in PCR, we cloned amplified DNA of *B. afzelii* (Nancy strains) into the pDrive cloning vector by using standards protocols (QIAGEN GmbH). Plasmidic DNA was purified and quantified by fluorimetric method. We subsequently introduced in each PCR run, as a positive control, a dilution of plasmid preparation corresponding to 48 target copies that was consistently positive in preliminary testing. Distilled water and DNA extracted from *Escherichia coli* were used as negative controls. Amplified products were analyzed by electrophoresis in 2.5% agarose gel and visualized by staining with 0.1% ethidium bromide. The efficiency of extraction protocol was verified, for PCR-negative ticks only, by using a 16S rDNA PCR specific for mitochondrial sequences of hard and soft ticks (d'Oliveira et al. 1997).

Amplicons were purified using a QIAquick PCR purification Kit (QIAGEN GmbH). Sequencing of PCR products containing fluorescent dye terminators was performed on an ABI PRISM 310 genetic analyzer (Applied Biosystems, Milan, Italy), by using PCR-derived primers. Sequences were analyzed by Chromas 2.0 software (Technelysium, Helensvale, Australia) and submitted to BLAST to identify similarities to known sequences, and therefore to classify *B. burgdorferi* s.l.-positive samples by genospecies (Altschul et al. 1997).

Statistical Analysis. Prevalence and 95% exact binomial confidence intervals (CIs) of infestation by immature *I. ricinus* were calculated by vertebrate species (BINOMIAL option, PROC FREQ, SAS Institute 1999). Data from recapture of the same rodents during one trapping session were excluded from the analysis. Prevalence of infestation by *I. ricinus* larvae and nymphs, in lizards and mice, was compared by Fisher exact test (EXACT option, PROC FREQ, SAS Institute 1999). A two-tailed significance level $\alpha = 0.05$ was adopted. The degree of concurrent infestation by at least one *I. ricinus* larva and nymph on the same individual hosts was tested by Kappa statistics (AGREE option, PROC FREQ, SAS Institute 1999). Kappa is commonly used as a measure of agreement between categorical classification criteria, and a value not significantly different from zero indicates no agreement beyond chance (Fleiss 1981).

Table 1. Infestation of lizards and small rodents by immature *I. ricinus*, in Tuscany, Italy, from May to August 2005

Tick stage	Host species (*n* of hosts)			
	Larvae		Nymphs	
	Podarcis muralis (97)	*Apodemus* spp. (53)	*Podarcis muralis* (97)	*Apodemus* spp. (53)
% prevalence of infestation (95% CI)	74.2 (64.3, 82.6)	79.2 (65.9, 89.2)	26.8 (18.3, 36.7)	5.7 (1.2, 15.7)
Mean *n* of ticks/captured host (95% CI)	4.2 (3.3, 5.5)	3.5 (2.6, 4.7)	0.44 (0.29, 0.67)	ND
k (95% CI)	0.70 (0.49, 1.0)	1.1 (0.67, 2.0)	0.50 (0.22, 1.5)	ND

ND, not determined.

Mean numbers of ticks per host and 95% confidence intervals as well as negative binomial dispersion parameters were obtained using intercept-only generalized linear models (GLM) with PROC GENMOD in the SAS system. Negative binomial error (log link) was used to take into account aggregated distribution of ticks among hosts. Subsequently, GLMs were used to compare mean larval infestation among host species. Model checking was accomplished by goodness-of-fit statistics (deviance and Pearson chi-square) and by plotting deviance and Pearson residuals against adjusted predicted values (Littell et al. 2002).

Prevalence of PCR-positive results was calculated by host species and tick stage. The proportions of lizards and mice harboring *B. burgdorferi* s.l.-infected larvae were compared by Fisher exact test.

Results

In total, 409 *I. ricinus* larvae and 43 nymphs were collected from 97 *P. muralis* lizards; 187 larvae and three nymphs were collected from 53 rodents that, due to problems in the field discrimination of the woodmouse, *Apodemus sylvaticus* L., and the yellow-necked fieldmouse, *Apodemus flavicollis* Melchior, on the basis of external characters, were classified as *Apodemus* spp. No significant difference was found between prevalence of infestation by larvae on lizards and mice (Fisher exact test; $P = 0.55$) (Table 1).

Based on goodness-of-fit statistics and residuals plots, the GLMs with negative binomial error were considered as appropriate for the analysis of counts of larvae and nymphs on lizards and of larvae on mice. No significant difference was found between the mean numbers of larvae on these two host species ($P = 0.39$) (Table 1). The smallest negative binomial parameter *k*, however, indicated a greatest degree of aggregation of the distribution of larvae on lizards. Indeed, the maximum number of attached larvae was 28 for lizards and 20 for mice. TEI was approximately the same for larvae feeding on lizards, median (first and third quartiles) = 2.1 (1.7, 2.9) and on mice (2.1, 1.7, 2.6).

Prevalence of infestation by nymphs was much greater on lizards than on mice (Fisher exact test; $P < 0.005$) (Table 1). Up to four nymphs were found on two lizards, whereas only three of 53 mice that were examined were infested by one nymph. Kappa, as a measure of coinfestation of lizards by immature *I. ricinus*, was −0.01 (95% CI, −0.14, 0.12) and indicated no evidence of cofeeding of larvae and nymphs on the same individual hosts.

Prevalence of infection by *B. burgdorferi* s.l. in immature *I. ricinus* that were collected from lizards was significantly greater than prevalence of infection in ticks collected from mice. Moreover, all feeding ticks that were tested from 34 mice were PCR negative (Table 2). Tail tissues from 32 lizards carrying *B. burgdorferi* s.l.-infected larvae were tested by PCR and six specimens yielded positive results (prevalence 18.8%; 95% CI, 7.2, 36.4). The infection also was found in two of four blood samples from lizards carrying infected larvae, whereas negative results were obtained in blood from two lizards carrying PCR-negative larvae and from two lizards with no larvae (overall prevalence of *B. burgdorferi* s.l. in blood from lizards: 25.0%; 95% CI, 3.2, 65.1). Ear biopsies from 48 mice were PCR negative (0.0%; 95% CI, 0.0, 7.4). All *B. burgdorferi* s.l. amplicons that were identified in immature ticks and tissues collected from lizards belonged to *B. lusitaniae.* Two amplicons from larvae were not identified.

Twenty-five passerine birds belonging to 10 species were captured. One *I. ricinus* larva and seven nymphs were collected from two Eurasian blackbirds, *Turdus merula* L., three nymphs were collected from two

Table 2. Results of PCR for the detection of *B. burgdorferi* s.l. in immature *I. ricinus* collected from lizards and small rodents in Tuscany, Italy, from May to August 2005

Tick stage	Host species (*n* of hosts, *n* of tested ticks)			
	Larvae		Nymphs	
	Podarcis muralis (58, 202)	*Apodemus* spp. (34, 86)	*Podarcis muralis* (14, 17)	*Apodemus* spp. (3, 3)
% of hosts that carried *B. burgdorferi* s.l. infected ticks (95% CI)	46.5 (33.3, 60.1)	0.0 (0.0, 4.2)	50.0 (23.0, 77.0)	0.0 (0.0, 70.8)
% prevalence of *B. burgdorferi* s.l. in feeding ticks (95% CI)	19.8 (14.4, 26.0)	0.0 (0.0, 10.3)	52.9 (27.8, 77.0)	0.0 (0.0, 70.8)

n of hosts is number of hosts of each species that carried ticks that were tested by PCR.

dunnocks, *Prunella modularis* L., and from 11 house sparrows, *Passer domesticus italiae* Vieillot; one nymph was collected from one European robin, *Erithacus rubecula* L. Four of seven (57.1%; 95% CI, 18.4, 90.1) nymphs from blackbirds yielded PCR-positive results, and amplicons were classified as *B. valaisiana.* Nymphs from other bird species and all of the 21 blood samples from birds were negative to PCR (0.0%; 95% CI, 0.0, 16.1).

Discussion

B. lusitaniae was found in immature *I. ricinus* and tissues collected from common wall lizards and not from mice and passerine birds. These results, together with the relatively high levels of infestation by *I. ricinus* larvae and nymphs that we found on lizards, suggest a major role of these reptiles in the maintenance of *B. lusitaniae* in Tuscany, and our findings are in agreement with studies carried out in Germany and Tunisia (Dsouli et al. 2006, Richter and Matuschka 2006). The infection of lizard tissues, and the low levels of coinfestation by immature stages of *I. ricinus* (as shown by the Kappa statistics not significantly different from zero) suggest that the transmission of *B. lusitaniae* by lizards to larval ticks occurs through systemic infection rather than through the passage of spirochetes from nymphs to larvae cofeeding in proximity on the same host (Randolph et al. 1999). The relatively low prevalence of *B. lusitaniae* that we found in tail tissues from lizards carrying infected larvae could be attributed to the kinetics of spirochetes in reptile reservoir hosts that deserves further studies. Conversely, vertical transmission of spirochetes in ticks was unlikely as shown by negative PCR results in larvae that were collected from mice.

Prevalence of infection by *B. lusitaniae* in larvae feeding on lizards at our study area (19.8%) (Table 2) was comparable with prevalence that was obtained in Tunisia by xenodiagnosis (17.0%), but it was lower than prevalence in larvae feeding on lizards in Germany (31.7%). Moreover, in Germany, a relatively high prevalence of infection by *B. afzelii* (ranging from 26.0 to 41.8%) was found in larvae that fed on wild rodents, whereas in Tuscany, ticks and tissues from rodents were negative for *B. burgdorferi* s.l. Such a difference might be explained by a generally greater level of *B. burgdorferi* s.l. transmission in Germany and by geographic variations in the relative abundance of vertebrate hosts. Moreover, in Mediterranean areas, including our study location, lizards might be more abundant, relative to rodents, than in central Europe and may play a zooprophylactic role for *B. burgdorferi* genospecies other than *B. lusitaniae* (Richter and Matuschka 2006). Accordingly, in Tuscany, *B. lusitaniae* accounted for 82.9% of PCR-positive, host-seeking ticks, whereas mammal-associated genospecies, such as *B. afzelii* and *B. burgdorferi* s.s., accounted for 2.4% only (Bertolotti et al. 2006). The relatively high level of infection by *B. lusitaniae* that we found in nymphs feeding on lizards (although with a wide 95% CI due to a small sample of tested nymphs) (Table 2) was similar to prevalence of infection in nymphs feeding on *P. muralis* and *L. agilis* in Germany (Richter and Matuschka 2006).

Further investigation should be carried out at our study area to verify whether year-to-year fluctuations of mice populations might affect the transmission dynamics of different *B. burgdorferi* s.l. genospecies. Larger samples of passerine birds also should be examined during a longer sampling period to verify their hypothesized role in the diffusion of *B. lusitaniae,* in addition to bird-associated genospecies such as *B. valaisiana* that we found in this study.

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