

# *Borrelia burgdorferi* sensu lato genospecies in *Ixodes ricinus* ticks feeding on passerine birds in southern Norway

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Birds are increasingly considered important in the global dispersal of tick-borne pathogens. The purpose of this study was to investigate the prevalence of *Borrelia burgdorferi* sensu lato genospecies in *Ixodes ricinus* ticks collected on passerine birds from two ornithological stations (Jomfruland and Lista) in southern Norway. In both sampling sites a total of 608 (109 larvae and 499 nymphs) of immature ticks were collected from 157 infested birds representing 26 species. The presence of *B. burgdorferi* s.l. DNA in ticks was tested using direct PCR amplification of the *ospA* gene, localized on lp-54 plasmid in *B. burgdorferi* s.l. genome. Twenty one out of 157 infested (13%) birds carried *B. burgdorferi* s.l. infected *Ixodes ricinus* ticks. *B. burgdorferi* s.l. DNA was detected in 19.7% of the tick samples. In total, 6.4% of larvae (7/109) and 10% of nymphs (50/499) were positive. The vast majority of infected ticks were found on blackbird (*Turdus merula*). The most frequent genospecies was *B. garinii* (85.5%), followed by *B. afzelii* (9.0%) and *B. valaisiana* (5.5%). Five different *B. garinii* genotypes were identified. Our findings showed that the blackbirds may support the circulation and spread of *B. garinii* and *B. valaisiana*. *B. garinii* was not detected in questing ticks from investigated locations. *B. afzelii* genospecies, however, dominated in questing ticks, rodents and ticks from rodents.

**Key words:** passerine birds, *Ixodes ricinus* ticks, *Borrelia burgdorferi* s.l. genospecies

## INTRODUCTION

Lyme borreliosis (LB) is the most frequent tick-borne disease in the northern hemisphere, including Norway. In Europe, nine *Borrelia* genospecies belonging to the complex *B. burgdorferi* s.l. (*B. burgdorferi* sensu stricto, *B. garinii*, *B. ba-*

*variensis*, *B. afzelii*, *B. valaisiana*, *B. lusitaniae*, *B. bissettii*, *B. spielmanii*, and *B. turdi*) have been detected in the tick vector *Ixodes ricinus* (Stanek, Reiter, 2011; Hasle et al., 2011). According to the concept of host-specific *Borrelia* association, based on differential properties of the hosts complement systems, various reservoir hosts harbor different *Borrelia* species (Kurtenbach et al., 1998; Kurtenbach et al., 2002a). *B. valaisiana* and most

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OspA types of the highly heterogeneous *B. garinii* species are associated with birds (Kurtenbach et al., 2002b; Hanincová et al., 2003). It is epidemiologically important that the different genomic groups of *B. burgdorferi* s.l. cause diverse clinical manifestations of LB in humans: *B. garinii* is associated with neuroborreliosis (e. g. meningoradiculoneuritis) while *B. afzelii* is usually predominant in the skin forms (acrodermatitis chronica atrophicans), and *B. burgdorferi* s.s. is the common agent of arthritis (Ruzic-Sabljić et al., 2008).

Birds are considered important in the global dispersal of tick-borne pathogens and establishing foci of tick-borne diseases (Olsen et al., 1995; Comstedt et al., 2006). The migratory routes of birds in Europe are diverse with both North-South and West-East directions. It is, however, difficult to determine the place of origin of ticks that parasitize on migratory birds. Ixodid ticks can travel hundreds of kilometers on birds along migration routes before they get de-attached. The ticks that are transported to Norway with migratory birds could be transferred from a quite large area, because different species of birds have different overwintering areas, different migratory routes, and migrate in different periods during spring and autumn (Main, 2002). The dominant direction of migration during spring in Northern Europe is from southwest towards northeast, and the opposite direction during autumn (Acheson, 1975).

Previous studies conducted in Europe have shown that different bird species differ in their competence in transmitting of *Borrelia* pathogens and infect ticks. In Europe, pheasants (*Phasianus colchicus*), some passerine bird species, like blackbirds (*Turdus merula*) and song thrushes (*Turdus philomelos*), and a few seabird species have been shown to be reservoir competent for *B. garinii* and *B. valaisiana* (Kipp et al., 2006; Kurtenbach et al., 1998; Taragel'ová et al., 2008). Because the proportion of particular clinical forms of Lyme borreliosis varies in European countries, it has been of interest to analyze the distribution of different genospecies of *B. burgdorferi* s.l. in ticks and reservoir hosts.

The aim of our study was to investigate the occurrence of *B. burgdorferi* s.l. genospecies in ticks from passerine birds in Norway and compare *Borrelia* infection in ticks from vegetation and small rodents.

## MATERIALS AND METHODS

### Sample collection

Passerine birds were captured at two ornithological stations in the southern coast of Norway Jomfruland (58°52'N, 09°36'E) and Lista (58°07'N, 06°40'E) during the spring migratory period (April) in 2006–2007 (Table 1). The birds were identified, examined for ticks and released back into the wild. Only tick-infested birds were included in this study. Ticks were collected from birds using tweezers and placed in microtubes with 70% ethanol and placed at 4 °C before DNA analysis. All ticks were identified to species level using appropriate taxonomic keys (Filippova, 1977; Hillyard, 1996) and molecular assays.

### DNA extraction

DNA extraction was performed after the ticks were removed from the birds and carried out by lysis in ammonium hydroxide (Rijpkema et al., 1996). Forty µl for larvae and 80 µl for nymph of 2.5% ammonia solution was added to the sample in a 0.5 ml microcentrifuge tube and heated at 99 °C for 25 min in a thermostat. After a brief centrifugation the tubes were opened and heated 10–15 min to evaporate ammonia. The lysates were stored at –20 °C.

DNA from engorged ticks was extracted using the Genomic DNA Purification Kit (*Thermo Fisher Scientific*, Lithuania) according to the protocol suggested by the manufacturer.

### Molecular identification of tick species

For accurate taxonomical identification of *Ixodes* ticks with damaged parts of gnathosoma we used species-specific PCR with Ixri-F/Ixri-R primers, as described previously (Paulauskas et al., 2009).



**Table 1.** (continued)

Bird species	No of tested ticks		n (%) of <i>Bbsl</i> infected ticks		<i>B. burgdorferi</i> s.l. genospecies, n (%) of <i>Bbsl</i> infected ticks										
	L	N	L	N	<i>Bg</i>		<i>Ba</i>		<i>Bss/Bg</i>		<i>Ba/Bg</i>		<i>Bv</i>		
					L	N	L	N	L	N	L	N	L	N	
<i>Lullula arborea</i>	2	2													
<i>Oenanthe oenanthe</i>		1		1		1									
<i>Saxicola rubetra</i>		3													
<i>Prunella modularis</i>	1	18													
<i>Anthus pratensis</i>		1													
<i>Phylloscopus trochilus</i>	1	2													
<i>Carduelis chloris</i>	1	2													
<i>Sylvia atricapilla</i>		3													
<i>Sylvia communis</i>	1	3													
<i>Sturnus vulgaris</i>	16	15		1(6.7)											1(6.7)
<i>Fringilla coelebs</i>	10	4													
<i>Coccyzus erythrophthalmus</i>		3													
<i>Luscinia svecica</i>		1													
Total	49	219		15(6.9)		11(5.0)		1(0.5)							3(1.4)

L – larvae, N – nymphs, *Bbsl* – *B. burgdorferi* s.l., *Ba* – *B. afzelii*, *Bg* – *B. garinii*, *Bss* – *B. burgdorferi* s.s., *Bv* – *B. valaisiana*

**Detection of *B. burgdorferi* s.l.**

The presence of *B. burgdorferi* s.l. DNA was tested by direct PCR amplification of the *ospA* gene, localized on lp-54 plasmid in *B. burgdorferi* s.l. genome. We used primers SL-F and SL-R designed by Demaerschalck et al. (1995) for amplifying 307 bp fragment of *ospA* gene. Reaction volume of 25 µl contained 12.5 µl 2x PCR Master Mix (*Thermo Fisher Scientific*, Lithuania), 20 pmol of each primer (SL-F and SL-R) (*Roth*, Germany), 6 µl double-distilled water, and 2.5 µl of the processed tick sample. In each PCR run positive (DNA of *Borrelia* positive ticks) and negative (double distilled

water) controls were used. PCR conditions were the following: 95 °C for 2 min, followed by 40 cycles of 94 °C for 20 sec; 62 °C for 20 sec; and 72 °C for 30 sec. The final extending was at 72 °C for 2 min. PCR amplification products were separated on 1.5% agarose gel and visualized by UV transillumination (*EASY Win32, Herolab*, Germany). The obtained specific products of 307 bp were considered a positive result.

**Identification of *B. burgdorferi* s.l. genospecies**

Multiplex PCR with species-specific primers (Demaerschalck et al., 1995) was performed to differentiate *B. burgdorferi* s.s., *B. garinii*, and

*B. afzelii* genospecies. Reaction mixture of 30 µl contained 15 µl Multiplex Master PCR Mix (Qiagen, USA), 20 µl of each primers (GI, GII and GIII) (Roth, Germany), and 3 µl DNA of the processed tick sample. The cycle consisted of initial denaturation at 95 °C – 15 min for HotStart Tag Polymerase activation, denaturation at 95 °C for 30 s, annealing at 65 °C for 90 s, and extension at 72 °C for 60 s. The procedure was repeated 40 times, and the final extension was done at 72 °C for 2 min. Positive and negative controls were used in each PCR run. PCR amplification products were resolved onto 2.0% agarose gel and visualized under UV light. The presence of *B. valaisiana* DNA in tick lysates was tested using species-specific primers (Liebisch et al., 1998) which amplified 549 bp fragment of 16S rRNA gene.

#### DNA sequencing and nucleotide sequences accession numbers

Nucleotide sequences of *Borrelia* DNA were confirmed by direct sequencing of amplified *B. garinii*, *B. valaisiana*, and *B. afzelii* PCR products. DNA fragments were purified with Nucleospin extract II Kit (Macherey-Nagel, GmbH & Co). For DNA sequencing reaction, BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) was used according to manufacturer recommendations. Sequencing was performed using ABI Prism 310xl Genetic Analyzer (Applied Biosystems Foster City, CA, USA). The obtained sequences were edited and aligned with *Borrelia* gene sequences registered in GenBank database using the Mega 5.05 software. Partial sequences of the *ospA* gene and 16S rRNA gene were submitted to the GenBank under the following accession numbers: HM623279-HM623295.

In order to investigate the heterogeneity of *B. garinii* strains found in ticks fed on the birds and rodents, sequences of 345 bp fragment of *ospA* gene were analyzed and compared with sequences of previously characterized strains submitted in the Gene Bank.

## RESULTS

In both sampling sites a total of 608 feeding (semi-engorged to almost fully engorged) immature ticks (109 larvae and 499 nymphs) were collected from 157 infested birds representing 26 species (Table 1). All ticks belonged to *I. ricinus* species.

Twenty one out of 157 infested (13%) birds carried *B. burgdorferi* s.l. infected *I. ricinus* ticks. The distribution of ticks infected with *B. burgdorferi* s.l. among different bird species are shown in Table 1. *B. burgdorferi* s.l. DNA was detected in 19.7% of the tick samples. In total, 6.4% (70/109) of larvae and 10% (50/499) of nymphs were infected with *B. burgdorferi* s.l. (Table 1). The observed difference in prevalence of infection was not statistically significant ( $\chi^2 = 1.36$ ;  $d = 1$ ;  $p = 0.2430$ ). The vast majority of infected ticks were found on blackbird *T. merula* (Table 1). In Jomfruland, 7 out of 33 *T. merula* (21.2%) and in Lista, 7 out of 32 (21.9%) carried *B. burgdorferi* s.l. infected ticks. *B. burgdorferi* s.l. infected larvae were found only on *T. merula* species. *Borrelia* infection was significantly more frequent in ticks collected from birds in Jomfruland (12.4%; 42/340) than from those in Lista (5.6%; 15/268) ( $\chi^2 = 8.05$ ;  $d = 1$ ;  $p = 0.0046$ ). No *Borrelia* infected larvae were found in Lista, but in Jomfruland 11.7% (7/60) of larvae were infected. The prevalence of *Borrelia* in nymphs collected from birds in Jomfruland was significantly higher compared with those in Lista (12.5% versus 6.9%;  $\chi^2 = 4.35$ ;  $d = 1$ ;  $p = 0.037$ ).

Sequence analysis of 15 PCR products confirmed *B. garinii* (HM623279-HM623290), *B. valaisiana* (HM623291, HM623292), and *B. afzelii* (HM623295) species.

The most frequent genospecies was *B. garinii* (85.5%), followed by *B. afzelii* (9.0%) and *B. valaisiana* (5.5%). *B. valaisiana* was detected only in bird feeding ticks from Lista (Table 1). Mixed infections with two genospe-

cies (*B. garinii*/*B. afzelii* and *B. garinii*/*B. burgdorferi* s.s.) were detected in two nymphs found in Jomfruland on *T. merula*.

We identified and compared *B. garinii* strains obtained in ticks feeding on the avian hosts. Among the 12 *ospA* sequences obtained from ticks that had fed on birds, five different genotypes were identified (Table 2). The sequences had one or two nucleotide differences at four positions and one deletion. Nine of obtained *B. garinii* sequences were identical to *ospA* sequences of OspA serotype 6 type previously characterized and submitted in the GenBank (the *ospA* sequence of reference strain N34 Y108794, G25 Z290806), whereas four genotypes were unique and have so far not been described (Table 2).

**DISCUSSION**

In Norway, several studies have been done to analyze migrating bird infestation with ticks and to

assess the role of different bird species in transporting of *Borrelia* infected ticks (Mehl et al., 1984; Paulauskas et al., 2009; Hasle et al., 2009; 2011; Kielland et al., 2010).

Mehl et al. (1984) showed that in Norway among migratory passerines birds, the most infested birds with *Ixodes ricinus* ticks were *Turdus* spp., *Erithacus rubecula*, *Phoenicurus phoenicurus*, *Prunella modularis*, *Anthus trivialis*, and *Luscinia svecica*. Hasle et al. (2009), who carried out the study in 2003–2005 from 4 bird observatories along the southern Norwegian coast, found the highest prevalence of tick infestation in thrushes and dunnock (*Prunella modularis*). Our study conducted during 2006–2007 showed that the most infested bird species found in Jonfruland was *T. merula*, and in Lista – starling (*Sturnus vulgaris*) (Paulauskas et al., 2009). According to Kielland et al. (2010), tree pipit (*Anthus trivialis*), chaffinch (*Fringilla coelebs*), and whitethroat (*Sylvia communis*) were found

**Table 2.** *B. garinii* genotypes observed among the *ospA* gene sequences (345 bp) in ticks from birds and rodents

Source of <i>B. garinii</i>	GenBank accession number	Variable sites				
		7	8	9	9	6
<i>Ixodes ricinus</i> from <i>Turdus merula</i>	HM623280	T	A	T	A	G
<i>Ixodes ricinus</i> from <i>Turdus merula</i>	HM623281	.	.	.	.	.
<i>Ixodes ricinus</i> from <i>Turdus merula</i>	HM623282	.	.	.	.	.
<i>Ixodes ricinus</i> from <i>Turdus merula</i>	HM623283	.	.	.	.	.
<i>Ixodes ricinus</i> from <i>Turdus merula</i>	HM623284	.	-	.	.	.
<i>Ixodes ricinus</i> from <i>Turdus merula</i>	HM623285	G	.	C	.	.
<i>Ixodes ricinus</i> from <i>Turdus merula</i>	HM623286	.	.	.	.	.
<i>Ixodes ricinus</i> from <i>Turdus merula</i>	HM623287	.	.	.	.	.
<i>Ixodes ricinus</i> . from <i>Turdus merula</i>	HM623288	.	.	.	.	.
<i>Ixodes ricinus</i> . from <i>Sturnus vulgaris</i>	HM623289	.	.	.	.	.
<i>Ixodes ricinus</i> . from <i>Oenanthe oenanthe</i>	HM623290	.	.	.	.	C
<i>Ixodes ricinus</i> from <i>Carduelis cannabina</i>	HM623279	.	.	.	C	.
<i>Ixodes ricinus</i> from <i>Apodemus flavicollis</i>	HM119231	.	.	.	.	.
	N34 Y108794	.	.	.	.	.
	G25 Z290806	.	.	.	.	.

“.” indicates the same base; “-” indicates the base deletion

the most commonly infested birds in Lista in 2008.

In the present study the highest infection rate was detected in ticks collected from *T. merula* in Jomfruland where birds of this species harbored 82% of *B. burgdorferi* s.l. infected ticks (Table 1). All infected larvae were carried by *T. merula* and harbored *B. garinii* genospecies. Since transovarial transmission is rare, we suggest that the larval ticks most probably have been infected by feeding on the blackbirds.

The dominance of *B. garinii* among ticks collected from birds in this study is in accordance with other studies in Norway and elsewhere (Kielland et al., 2010; Hasle et al., 2011; Hannicová et al., 2003; Taragelová et al., 2008; Kurtenbach et al., 2002b). Hasle et al. (2011) found that *B. garinii* was the dominant species in feeding ticks on birds at Jomfruland, while *B. valaisiana* and *B. afzelii* were confirmed with a much lower prevalence. However, *B. valaisiana* dominated in nymphs in Lista, while *B. garinii* was detected with two-times lower prevalence (Hasle et al., 2011). Kielland et al. (2010) reported that *B. garinii* was a more frequently found genospecies in bird feeding ticks during autumn migration in Lista.

In order to compare *Borrelia* genospecies in ticks engorged on migratory birds with genospecies occurred at the investigated localities, we included in analysis the data on prevalence of *Borrelia* genospecies in questing ticks in Jomfruland and Lista (Table 3; Rosef et al., 2009). However, we did not detect *B. garinii* species in questing ticks at these locations (Table 3). It is possible that ticks harbored *B. garinii* were transported

by migrating birds from wintering localities. Another possibility is that birds as reservoirs of *B. garinii* genospecies could be an infection source for local ticks. However, if captured birds were the members of the local population, they have spread *B. garinii* infected ticks to the vegetation.

In Lista, questing ticks were collected only in 2007, and 2.3% of them were found infected with *B. afzelii*. In Jomfruland, ticks from vegetation were collected at the same time as ticks from birds during 2006 and 2007, and one year later (2008) in the same period. Overall, 5.3% of questing ticks from Jomfruland were infected, and identified species were *B. afzelii*, *B. burgdorferi* s.s. and co-infection of *B. afzelii* and *B. burgdorferi* s.s (Table 3).

According to our previous studies, in Jomfruland *B. afzelii* genospecies was detected in yellow-necked mice *Apodemus flavicollis* (Paulauskas et al., 2008) and in *I. ricinus* ticks feeding on yellow-necked mice (Radzijeuskaja et al., 2013). The case of co-infection of *B. afzelii* and *B. garinii* in a nymph from *A. flavicollis* was also detected (Radzijeuskaja et al., 2013). *B. garinii* strain detected in ticks that fed on a rodent was identical to that obtained in most of analyzed ticks from birds, and represented OspA serotype 6 strains.

*B. afzelii* dominated in questing ticks (Table 3), rodents and ticks from rodents in investigated areas, and probably was eliminated in the ticks feeding on birds (Kurtenbach et al., 2002a). *B. afzelii* infected nymphs obtained on migratory birds in this study could be of local origin.

**Table 3.** Prevalence of *B. burgdorferi* s.l. genospecies in questing *I. ricinus* ticks in Lista and Jomfruland

Location	Female, N (n)	Male, N (n)	Nymph	Total N (n)	Bbsl (%)	Ba (%)	Bg (%)	Bss (%)	Bv	Ba/Bss (%)
Lista (2007)	43(3)	32(0)	54(0)	129(3)	2.3%	3(2.3)	–	–	–	–
Jomfruland <sup>a</sup> (2006/7/8)	67(6)	58(2)	118(5)	243(13)	5.4%	7(2.9)	–	5(2.1)	–	1(0.4)

N – number of tested ticks, n – number of infected ticks, Bbsl – *B. burgdorferi* s.l., Ba – *B. afzelii*, Bg – *B. garinii*, Bss – *B. burgdorferi* s.s., Bv – *B. valaisiana*

<sup>a</sup> Rosef et al., 2009

The occurrence of *B. burgdorferi* s.l. genospecies in ticks collected from blackbirds could be related to migration routes. According to the investigation of migration behavior of *T. merula* in Western Europe, the breeding range of the blackbird covers the entire region except for the extreme north of Fennoscandia. Britain and Ireland are important host areas for Fennoscandian blackbirds (Main, 2002). Thousands of blackbirds arrive in Norway from Great Britain at the end of March and early April. Studies conducted in the British Islands (Kurtenbach et al., 1998; Kurtenbach et al., 2001; Kurtenbach et al., 2002b) showed that the most prevalent genospecies in *I. ricinus* ticks was *B. garinii*, followed by *B. valaisiana* and *B. burgdorferi* s.s. with low prevalence (below 1%). *B. garinii* seems to be adapted to birds (Kurtenbach et al., 2002a) and that may influence infection of birds with *B. garinii* and consequently *B. garinii* infection in feeding ticks found on *T. merula* in the southern coast of Norway.

## CONCLUSIONS

Most of the infected ticks were found on blackbird *T. merula*. The most frequent genospecies was *B. garinii* (85.5%), followed by *B. afzelii* (9.0%) and *B. valaisiana* (5.5%). The *Turdus* species have a potential of hosting and spreading *I. ricinus* ticks and *Borrelia*. We did not detect *B. garinii* species in questing ticks at the investigated locations. However, *B. afzelii* genospecies dominated in questing ticks, in rodents and ticks from rodents, and probably was eliminated in the ticks feeding on birds.

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**IXODES RICINUS ERKIŲ, MINTANČIŲ ANT  
ŽVIRBLINIŲ PAUKŠČIŲ, BORRELIA BURG-  
DORFERI SENSU LATO GENOTIPAI PIETŲ  
NORVEGIJOJE**

*Santrauka*

Paukščiai yra svarbūs erkių pernešamų patogenų platintojai. Pagrindinis šio tyrimo tikslas buvo ištirti *Borrelia burgdorferi sensu lato* genotipų paplitimą tarp *Ixodes ricinus* erkių, surinktų nuo žvirblinių paukščių iš dviejų ornitologinių stočių (Jomfruland ir Lista) Pietų Norvegijoje. Tirtose vietose nuo 157 užkrėstų paukščių, atstovaujančių 26 rūšims, surinkta 608 (109 lervos ir 499 nimfos) nesubrendusios erkės. Siekiant nustatyti *B. burgdorferi s. l.*, erkėse gausinamas specifinis ospA genas, kuris aptinka-

mas lp-54 genominėje plazmidėje. Iš 157 užsikrėtusių paukščių 21 pernešė *I. ricinus* erkės, užkrėstas *B. burgdorferi s. l. B. burgdorferi s. l.* patogenų aptikta 19,7 % mėginių. Iš viso 6,4 % lervų (7/109) ir 10 % nimfų (50/499) mėginių buvo teigiami. Daugiausiai užsikrėtusių erkių nurinkta nuo juodojo strazdo (*Turdus merula*). Dažniausias genotipas buvo *B. garinii* (85,5 %), mažiau paplitę – *B. afzelii* (9 %) ir *B. valaisiana* (5,5 %). Identifikuoti penki skirtingi *B. garinii* tipai. Rezultatai rodo, kad juodasis strazdas gali dalyvauti *B. garinii* ir *B. valaisiana* bakterijų cirkuliacijoje ir paplitime. Iš tirtų vietų surinktose erkėse *B. garinii* neidentifikuota. Tarp erkių, graužikų ir erkių, surinktų nuo graužikų, dominavo *B. afzelii* genotipas.

**Raktažodžiai:** žvirbliniai paukščiai, *Ixodes ricinus* erkės, *Borrelia burgdorferi s. l.* genotipai