



Research Article

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Molecular screening for bacterial pathogens in ticks (*Ixodes ricinus*) collected on migratory birds captured in northern Italy

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Abstract: Migratory birds have an important role in transporting ticks and associated tick-borne pathogens over long distances. In this study, 2,793 migratory birds were captured by nets in a ringing station, located in northern Italy, and checked for the presence of ticks. Two-hundred and fifty-one ticks were identified as nymphs and larvae of *Ixodes ricinus* (Linnaeus, 1758) and they were PCR-screened for the presence of bacteria belonging to *Borrelia burgdorferi* sensu lato, *Rickettsia* spp., *Francisella tularensis* and *Coxiella burnetii*. Four species of *Borrelia* (*B. garinii*, *B. afzelii*, *B. valaisiana* and *B. lusitaniae*) and three species of *Rickettsia* (*R. monacensis*, *R. helvetica* and *Candidatus Rickettsia mendelii*) were detected in 74 (30%) and 25 (10%) respectively out of 251 ticks examined. Co-infection with *Borrelia* spp. and *Rickettsia* spp. in the same tick sample was encountered in 7 (7%) out of the 99 infected ticks. We report for the first time the presence of *Candidatus Rickettsia mendelii* in *I. ricinus* collected on birds in Italy. This study, besides confirming the role of birds in dispersal of *I. ricinus*, highlights an important route by which tick-borne pathogens might spread across different countries and from natural environments towards urbanised areas.

Keywords: Migratory birds, tick-borne pathogens, *Borrelia* spp., *Candidatus Rickettsia mendelii*, molecular characterisation

Migratory birds can carry ticks and associated tick-borne pathogens (TBPs) over long distances (e.g. Elvfang et al. 2010, Lindeborg et al. 2012, Paduraru et al. 2012). In addition, many avian species can be reservoirs of several TBPs (Gryczyńska and Welc-Falęciak 2016). The spread of ticks across geographical barriers was confirmed by molecular studies that found rather homogeneous genetic structure of tick populations (Porretta et al. 2013, Røed et al. 2016). This finding could be explained by the movement of tick hosts such as migratory birds and mammals across different environments (Galdikaitė et al. 2013, Porretta et al. 2013).

Over recent decades, climate and environmental changes have contributed to the range expansion of some tick species, e.g. *Ixodes ricinus* (Linnaeus, 1758), to northern latitudes and to higher altitudes (Dantas-Torres 2015). Ticks and related TBPs are also rapidly expanding from wild and natural areas to urban and peri-urban zones increasing human health risk (Corrain et al. 2012, Rizzo-

li et al. 2014, Biernat et al. 2014, Mehlhorn et al. 2015, Paul et al. 2016, Hansford et al. 2017).

For example, Lyme disease borreliae were detected in host-seeking *I. ricinus* collected in wild and suburban recreational areas close to Milan (Pistone et al. 2010, Olivieri et al. 2017) and the presence of various TBPs was reported in birds captured in urban areas of Tuscany (Ebani et al. 2016). These results are in agreement with a global trend, observed not only in the Palearctic region, but also in the Nearctic. As an example, in a suburban area of Chicago (in USA), the presence of *Borrelia* spp. was reported in different tick species collected on wild birds (Hamer et al. 2012).

The aim of this study was to investigate the presence of four groups of important zoonosis-causing bacterial agents (*Rickettsia* spp., *Borrelia burgdorferi* sensu lato, *Francisella tularensis* and *Coxiella burnetii*) in *I. ricinus* collected from migratory birds captured at the border of a highly urbanised and interconnected area in northern Italy.

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Fig. 1. Geographical location of the bird ringing station Fondazione Europea il Nibbio, Lombardy, Italy (in a highly urbanised area).

MATERIALS AND METHODS

Collection and identification of ticks

Ticks were collected from migratory birds trapped with nets at the ringing station Fondazione Europea il Nibbio–FEIN (Arosio, Como, Italy 45°43'54.87"N, 9°12'40.10"E, 353 m a.s.l.; Fig. 1) during their autumn migration from central Europe. Our survey was conducted from September 6th to October 29th 2010. We followed a strategy adopted in Hornok et al. (2014): infected birds were divided into two categories based on their feeding behaviour, those preferentially feeding at ground level and those feeding on trees and bushes. The tick prevalence was compared between the two different categories of bird species. All visible ticks were removed from each bird, and placed in single vials in absolute ethanol and stored at -20°C. Ticks were examined using stereomicroscope (Leica MS5, Microsystems GmbH, Wetzlar, Germany) and identified using standard taxonomic keys (Manilla 1998).

Statistical analysis

Exact confidence intervals (CIs) for the prevalence rates at the 95% level were calculated according to Sterne's method (Reiczigel 2003). Sample prevalence data were analysed using Fisher's exact test. Differences were considered statistically significant when $P < 0.05$. All the statistical analyses were performed using the open source environment R – version 3.4.1 (R Core team 2017).

DNA extraction, PCR protocols, sequence data analyses

For DNA extraction, ticks were washed with sterile, distilled water to remove ethanol. Homogenates were then prepared from each dried sample using sterile pestles. DNA was extracted using a commercial kit (DNeasy® Blood & Tissue kit, Qiagen) following manufacturer's instructions. Extracted DNAs were then

quantified with Nanodrop 1000 (Thermo Scientific, Wilmington, DE, USA). To confirm morphological identifications, all samples were subjected to a PCR protocol amplifying a fragment of gene coding for cytochrome oxidase I subunit (*COI*) following Lado et al. (2016). A subset of the obtained amplicons was then sequenced (i.e. those obtained from DNAs of morphologically unidentifiable ticks due to partial damage of the body and a random 40% of the remaining individuals).

All the DNAs that resulted in positive amplifications using PCR primers targeting *COI* were examined for the presence of the following zoonotic etiological agents: *Borrelia burgdorferi* sensu lato, *Rickettsia* spp., *Coxiella burnetii* and *Francisella tularensis*. Molecular screening for *B. burgdorferi* sensu lato was carried out by PCR targeting the ribosomal 16S rRNA gene (Marconi and Garon 1992). Positive samples were amplified and sequenced using a PCR for the 23S–5S rRNA Internal Transcribed Spacer (ITS) (Chu et al. 2008).

Detection of bacteria belonging to the genus *Rickettsia* was performed by targeting a fragment of the gene coding for the enzyme citrate synthase (*gltA*), following Labruna et al. (2004) and a fragment of the gene coding for the outer membrane protein (*ompA*), following Roux et al. (1996). Specific PCR protocols targeting the transposon-like genome region and a fragment of the 16S rRNA gene were used to test for the presence of *C. burnetii* (see Berri et al. 2000) and *F. tularensis* (see Forsman et al. 1994), respectively.

All PCR positive samples were bidirectionally sequenced by ABI technology (Applied Biosystems, Foster City, CA, USA). Forward and reverse electropherograms were manually corrected and merged into a consensus sequence using the BioEdit v7.1 sequence alignment editor (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>) and compared with those available in GenBank using BLASTn (www.ncbi.nlm.nih.gov/blast/Blast.cgi). Species assignment was confirmed when the sequence identity was $\geq 99\%$.

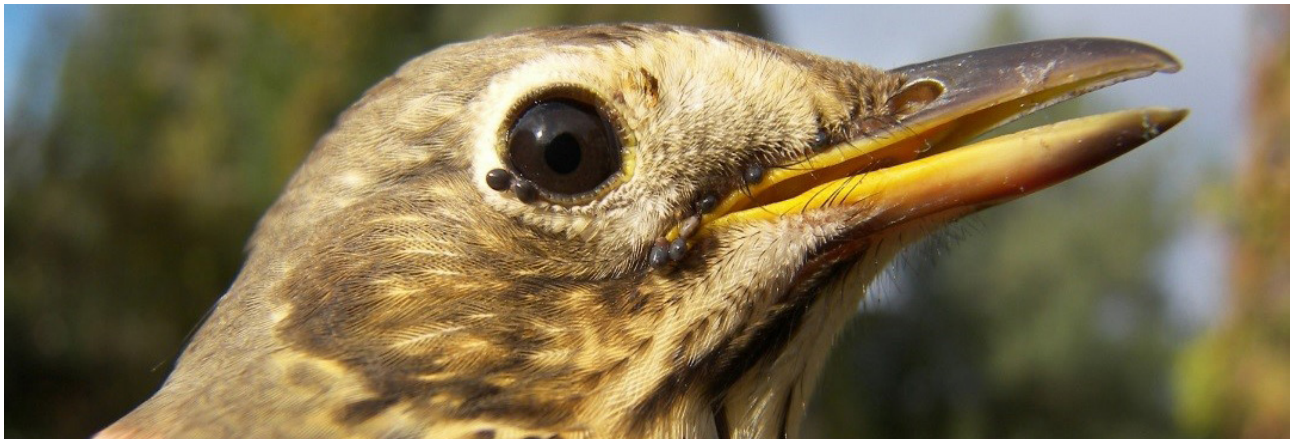


Fig. 2. Song thrush (*Turdus philomelos* Brehm) parasitised by larvae and nymphs of *Ixodes ricinus* (Linnaeus, 1758), showing an example of the co-feeding phenomena.

Table 1. Information on the captured birds and their ticks and tick-borne pathogens.

Bird species (%)	Feed. pref. (%)	Dist. migr. (%)	Ticks	Average	PB	PR	PBR
<i>Anthus trivialis</i> (Linnaeus) 10/108 (9)	GL 122/1713 (7)	long 10/108 (10)	10 (2; 8)	1.0	0	0	0
<i>Erithacus rubecula</i> (Linnaeus) 16/472 (3)		Short 112/1605 (7)	23 (13; 10)	1.4	0	2 (2; 0) [9%]	0
<i>Fringilla coelebs</i> Linnaeus 5/249 (2)			6 (3; 3)	1.2	0	1 (0; 1) [17%]	0
<i>Parus major</i> Linnaeus 3/99 (3)			3 (1; 2)	1.0	1 (1; 0) [33%]	0	0
<i>Turdus iliacus</i> Linnaeus 8/20 (40)			41 (14; 27)	5.1	8 (1; 7) [2%; 17%]	5 (0; 5) [12%]	(0;1)
<i>Turdus merula</i> Linnaeus 1/71 (1)			6 (4; 2)	6.0	0	1 (1; 0) [17%]	0
<i>Turdus philomelos</i> Brehm 79/694 (11)			183 (56; 127)	2.3	64 (8; 56) [4%; 31%]	16 (4; 12) [2%; 7%]	(1;5)
<i>Sylvia atricapilla</i> (Linnaeus) 1/442 (0.2)	AGL 2/517 (0.4)	Middle 1/442 (2)	1 (1; 0)	1.0	0	0	0
<i>Coccothraustes coccothraustes</i> (Linnaeus) 1/75 (1)		Short 1/75 (1)	1 (0; 1)	1.0	1 (0;1) [100%]	0	0

For every bird species is reported the ratio between the parasitised birds and the total number of screened birds (2,230 individuals belonging to the nine reported species), in brackets is reported the values expressed as percentages. GL – ground level; ABL – above ground level; TICKS – total number of collected ticks: in brackets are the values expressed for larva (first value) and nymph (second value) stages; AVERAGE – average number of ticks collected for each infected bird; PB – number of ticks infected with *Borrelia* spp.; within brackets are positive larvae and nymphs, in square brackets the percentage of prevalence; PR – number of ticks infected with *Rickettsia* spp; within brackets are positive larvae and nymphs, in square brackets the percentage of prevalence; PBR – number of ticks simultaneously infected with both pathogens.

RESULTS

During the sampling campaign, 2,793 birds belonging to 41 different species were captured and checked for the presence of ticks (Fig. 2). A total of 274 ticks were collected from 124 birds, belonging to nine different species: *Turdus philomelos* Brehm, *Turdus merula* Linnaeus, *Turdus iliacus* Linnaeus, *Erithacus rubecula* (Linnaeus), *Sylvia atricapilla* (Linnaeus), *Parus major* Linnaeus, *Fringilla coelebs* Linnaeus, *Anthus trivialis* (Linnaeus), *Coccothraustes coccothraustes* (Linnaeus).

Statistical analysis showed that birds preferentially feeding at ground level were more frequently infected by ticks than those preferentially feeding above ground level ($P < 0.0001$), with 122 out of 1713 (7% [CI: 6–8%]) and 2 out of 517 (0.4% [CI: 0.04–1%]) parasitised individuals, respectively. Of the 274 individual ticks, 94 were at the larval stage (34.3%) and 180 nymphs (65.7%). *COI* amplicons were obtained for 251 DNAs from the total 274 DNA

samples (88/94 larvae and 163/180 nymphs). BLASTn analyses on all the sequenced *COI* amplicons confirmed the morphological identification of the specimens (100% identity with *I. ricinus*). Pathogens screening was conducted on DNAs extracted from 88 larvae and 163 nymphs.

PCR positivity to *Borrelia* spp. was detected in 10 out of 88 larvae (11%) and 64 out of 163 nymphs (39%) and bacteria belonging to the genus *Rickettsia* were detected in 7 out of 88 larvae (8%) and 18 out of 163 nymphs (11%). Co-infection with both pathogens was encountered in 7 out of 99 (7.1%) infected ticks. *Francisella tularensis* and *C. burnetii* were not detected in any samples. (Table 1).

Four different genospecies of *Borrelia* spp. were identified: *Borrelia garinii* (19.5%), *Borrelia afzelii* (4.8%), *Borrelia valaisiana* (2.4%) and *Borrelia lusitaniae* (0.4%). Six amplicons did not produce high quality sequences, perhaps due to the presence of multiple strains or genospecies, impeding identification at species level (Table 2). The

Table 2. *Borrelia* spp. and *Rickettsia* spp. genotypes detected from *Ixodes ricinus* (Linnaeus, 1758) collected on migratory birds.

Species	N ^o sequences	Genomic region	GeneBank acc. No.
<i>B. afzelii</i>	3	ITS	LT907801
<i>B. afzelii</i>	4	ITS	LT907802
<i>B. afzelii</i>	1	ITS	LT907803
<i>B. afzelii</i>	4	ITS	LT907804
<i>B. garinii</i>	12	ITS	LT907805
<i>B. garinii</i>	8	ITS	LT907806
<i>B. garinii</i>	9	ITS	LT907807
<i>B. garinii</i>	3	ITS	LT907808
<i>B. garinii</i>	11	ITS	LT907809
<i>B. garinii</i>	6	ITS	LT907810
<i>B. lusitaniae</i>	1	ITS	LT907811
<i>B. valaisiana</i>	6	ITS	LT907812
<i>R. helvetica</i>	5	<i>gltA</i> gene part. seq.	LT907813
<i>R. mendelii</i>	3	<i>gltA</i> gene part. seq.	LT907814
<i>R. monacensis</i>	14	<i>gltA</i> gene part. seq.	LT964677
<i>R. monacensis</i>	16	<i>OmpA</i> gene part. seq.	LT907815
<i>Rickettsia</i> sp.IRS3	3	<i>OmpA</i> gene part. seq.	LT964678

22 electropherograms of *gltA* nucleotide sequences, after BLASTn search, allowed to identify *Rickettsia monacensis* (14 nucleotide sequences), *Rickettsia helvetica* (5 nucleotide sequences) and three sequences assigned to *Candidatus Rickettsia mendelii*. The remaining three electropherograms showed multiple double-peaks and therefore were not resolved in consensus sequences. Concerning the second marker, targeting a fragment of *ompA* gene, 20 amplicons were obtained and allowed to identify *R. monacensis* (16 nucleotide sequences) and a second haplotype (LT964678) with 100% identity with *Rickettsia* sp. IRS3 (three nucleotide sequences). Electropherograms of the remaining amplicon did not produce a high-quality consensus sequence. Furthermore, five DNAs of the tick samples positive to *R. helvetica* using primers targeting *gltA* were not amplified using primers targeting *ompA*. Notably, it is known that the primers used do not amplify the *ompA* gene for *R. helvetica* (Roux et al. 1996). Therefore, the results obtained using the two primer pairs are congruent. More details including GenBank accession numbers are presented in Tables 1 and 2.

DISCUSSION

As already highlighted by previous contributions, our study suggests an increasing risk for human health linked to the current dispersion trend of ticks and associated TBPs outside their elective wild-natural environments (Rizzoli et al. 2014, Kowalec et al. 2017). Here, migratory birds were captured by nets, in a woodland zone geographically located ~30 km north of Milan, one of the most populated and interconnected areas in Europe. Our results, in agreement

with a previous study, show that parasitism rate is connected with birds feeding behaviour (Hornok et al. 2014), indicating that birds are more easily infected when they feed at ground level than when feeding on trees and bushes (Table 1). Indeed, the most parasitised individuals belonged to the ground-level feeders *Turdus iliacus*, *T. philomelos* and *Anthus trivialis* (see Michalik et al. 2008).

Due to the different bird species that can serve as tick hosts and the highly variable routes followed by long and short-range migrators, our results are hardly comparable with other European studies (Toma et al. 2014, Wallménius et al. 2014, Klaus et al. 2016). We focused our molecular screening on pathogens in larvae and nymphs of *I. ricinus*, one of the tick species most commonly feeding on passerine birds in central Europe (Michalik et al. 2008, Biernat 2016, Klaus et al. 2016).

Four genospecies of Lyme-disease-causing borreliae (*B. afzelii*, *B. garinii*, *B. lusitaniae* and *B. valaisiana*) were detected in ticks collected from birds during autumn migration from Central Europe. *Borrelia garinii* was the most prevalent genospecies (20%), as reported in other studies focused on *I. ricinus* from birds (Michalik et al. 2008, Dubská et al. 2011). *B. afzelii*, known to be mostly associated with rodents, was found in 12 nymphs (5%). These findings are in agreement with other surveys in Europe (e.g. Margos et al. 2009).

In addition, we report the presence of *R. helvetica* and *R. monacensis*, which are well-known to be implicated in the development of human diseases (e.g. Nilsson et al. 1999, Parola et al. 2013, Nilsson et al. 2014). Remarkably, few ticks (three nymphs collected on two individuals of *T. iliacus*) were positive for *Candidatus Rickettsia mendelii*, a recently described non-spotted-fever-group rickettsia, previously detected in *I. ricinus* from the Czech Republic (Hajdusko-va et al. 2016).

Our results correspond to studies that indicate that birds may have a relevant role as reservoirs of TBPs (Michalik et al. 2008, Hornok et al. 2014). We found spirochetes of *B. burgdorferi* s.l. in 10 out of 88 larvae (11%), a relatively high prevalence of the bacterium considering an inefficient transovarial transmission route (Rollend et al. 2013). Therefore, larvae might have acquired spirochetes through ingestion of infected blood of birds (Voordouw 2015) since the co-feeding mechanism was recently demonstrated to be inefficient in the same avian hosts (Heylen et al. 2017).

In conclusion, our work presents further evidence for the role of birds in the dispersal of *I. ricinus* and pathogens transmitted by this species, emphasising the possibility of introduction or re-introduction of TBPs in non-endemic and highly populated areas.

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