Detection of Microbial Agents in Ticks Collected from Migratory Birds in Central Italy

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Abstract

Tick species characterization and molecular studies were performed within ornithological surveys conducted during 2010 and 2011 in the Lazio Region of central Italy. A total of 137 ticks were collected from 41 migratory birds belonging to 17 species (four partial migrants and 13 long-distance migrants). Most ticks were nymphs, with a predominance of *Hyalomma marginatum marginatum* and *H. m. rufipes*, and a small portion of *Ixodes* and *Amblyomma* species. All tick species analyzed were infected, and the molecular pathogen recognition revealed the presence of *Rickettsia aeschlimannii*, *Rickettsia africae*, *Erlichia* spp., *Coxiella burnetii*, *Borrelia burgdorferi* sensu lato group, and *Babesia microti*, whereas no genomic DNA of *Bartonella* spp. or *Francisella tularensis* was detected. The results of the survey show that *H. marginatum* ticks appear to be a vector of microbial agents that may affect human and animal health and that migratory birds may be an important carrier of these ticks. Additional studies are needed to better investigate the role of migratory birds in the epidemiology of these pathogens.

Key Words: Ticks—Migratory birds—*Rickettsia* spp.—*Erlichia* spp.—*Coxiella burnetii*—*Borrelia burgdorferi* sensu lato (s.l.)—*Babesia microti*—Co-infection.

Introduction

TICKS ARE COMPETENT VECTORS of viral, bacterial, and protozoan agents responsible for emerging diseases worldwide. The increase of human and animal mobility, along with global warming, represents a key factor for the introduction and spread of vector-borne diseases, including tick-borne diseases, in Europe (Khasnis and Nettleman 2005). Passive transport by migratory birds may also favor the spread of competent tick species across countries and continents (Hildebrandt et al. 2010). Migratory birds may be classified as local migrants, short-distance, long-distance, and vagrant and nomadic migrants (Georgopoulou and Tsiouris 2008). In this way, traveling over long distances and across geographical barriers, such as oceans and deserts, these avian species may be implicated in the transmission of zoonoses as biological and mechanical vectors and are hosts or carriers of infected ectoparasites, causing water-borne,

tick-borne, and insect-borne diseases (Georgopoulou and Tsiouris 2008). A study conducted in Norway to investigate the role of birds in microbial transport, allowed the detection of several genotypes of *Borrelia* spp. (i.e., *B. afzelii*, *B. garinii*, *B. valaisiana*, and *B. turdi*) in *Ixodes ricinus* ticks collected on various species of migratory birds (Kjelland et al. 2010, Hasle et al. 2011). In addition, the human pathogenic members of Anaplasmataceae, such as the spotted fever group (SFG) rickettsiae, *Coxiella burnetii*, and tick-borne encephalitis (TBE) virus were also detected in ticks collected from different species of migratory birds in Europe (Alekseev et al. 2001, Santos-Silva et al. 2006, Spitalska et al. 2006, Waldenstrom et al. 2007, Ioannou et al. 2009, Elfving et al. 2010). The identification of *Borrelia burgdorferi*, the etiologic agent of Lyme disease, found in Prince Edward Island, Atlantic Canada, was also associated with the introduction of *Ixodes dammini* through migratory birds (Artsob et al. 1992). Finally, migratory birds could also carry infected

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ticks potentially implicated in the Crimean–Congo hemorrhagic fever virus (CCHFV) spread. In this regard, the 2002 outbreak in Turkey was attributed to the birds' migration from the Balkans (Karti et al. 2004). In Italy, the role of resident or migratory birds in the maintenance and dispersal of tick-borne pathogens is still undefined, and the presence of pathogens implicated in tick-borne disease transmission cycles is unknown.

To fill this gap, we evaluated ticks parasitizing migratory birds and pathogens infecting these blood-sucking arthropods. We report here the results of tick species distribution and the molecular investigation of microbial agents found in ectoparasites collected from birds caught in seasonal bird ringing activities, in the Lazio Region, during the spring and autumn seasons of 2010 and 2011.

Material and Methods

Specimens collection

Fieldwork activities were conducted in 2010 and 2011, during the spring and autumn seasons (April to October), at different bird observatories located in the Lazio Region—Castel Di Guido, Acilia, and Paliano (near Rome), and Ventotene and Ponza Islands (central Tyrrhenian Sea). Ringing activities were carried out in Ventotene and Ponza from April, 2010, to May, 2011, when huge numbers of migrating birds stage on the small islands during their spring migration. Several species of migrant birds land at these stopover sites directly from the North African coast, after nonstop flights of up to 14–16 h over the sea (Pilaastro et al. 1995). In Paliano, the ringing activity was carried out in October, 2010, while in Castel Di Guido it was carried out in earlier September, 2010, during the postbreeding period, which coincides with the beginning of autumn migration. Birds were captured from professional ornithologists during regular ringing procedures. Each bird was identified by species and was banded. During ornithological studies, ticks were collected by opportunistic sampling on birds, consistent with the preliminary approach of the study. All collected ticks were analyzed morphologically (Manilla 1998) and then frozen separately at -80°C for further molecular analyses.

DNA extraction

Ticks collected from birds were individually dissected and homogenized under sterile conditions. Genomic DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to manufacturing protocol. DNA samples were stored at -20°C and later used as templates for PCR amplification.

Molecular tick identification

As is widely known, the morphological species identification of immature ticks belonging to *Hyalomma* genus is difficult, and the status of *H. marginatum* complex is still debated (Apanaskevich and Horak 2008). For this reason, molecular analysis was carried out on all *Hyalomma* specimens and extended to the further tick species, to confirm the morphological approach on all the specimens.

An approximately 340-bp fragment of the 12S rDNA sequence, corresponding to the fragment between positions 8123 and 8459 of the complete mitochondrial genome se-

quence of *Rhipicephalus sanguineus* (NC 002074), was amplified by primers T1B (5'-aaa cta gga tta gat acc ct-3') and T2A (5'-aaa gag tga cgg gcg ata tgt-3'), according to previously described procedures (Beati and Keirans 2001). Prior to sequencing, PCR products were purified by using Microcon-PCR devices (Merck Millipore, Germany), according to the manufacturer's instructions. Each strand of the amplified fragment was directly sequenced using primers T1B and T2A at MWG Biotech AG (Ebersberg, Germany). Sequences were compiled and analyzed by DS gene 1.5 (Accelrys Inc., Cambridge), and then submitted to GenBank (accession nos. KC817304–KC817421).

Molecular pathogen detection

Specific oligonucleotides (primers and probes) used in this study are listed in Table 1. Detection of *Rickettsia* spp. was performed by using primers RpCS.877p–RpCS.1258n, which amplified a 381-bp part of the *gltA* gene, and RR190.70F–RR190.701R, which amplified a 632-bp portion of *ompA* gene (Regnery et al. 1991, Roux et al. 1996). Positive *ompA* samples were purified using the Nucleo Spin Extract[®] kit (Macherey Nagel, Germany) according to the manufacturer's instructions.

The rickettsial *ompA* sequencing was performed by BioFab research (Italy; www.biofabresearch.it). DNA sequences were compared with available databases in GenBank using the Basic Local Alignment Search Tool (BLAST; blast.ncbi.nlm.nih.gov).

A classical PCR amplification was also performed for *Ehrlichia* spp. based on the 16S rRNA gene with PCR cycling conditions, as previously described (Parola et al. 2000). PCR products were resolved by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide.

The presence of *B. burgdorferi* s.l. group, *Bartonella* spp., *C. burnetii*, *Francisella tularensis tularensis* (type A), and *F. tularensis holarctica* (type B) in tick DNA extracts was tested by real-time PCR using specific primers and probes for each pathogen (Table 1). All real-time PCRs were performed in 20 μL (final volume) into glass capillary tubes (Roche Diagnostics GmbH, Mannheim, Germany) and were carried out in a LightCycler instrument (Roche Diagnostics), with protocols and PCR parameters as previously described (Pietilä et al. 2000, Ciervo et al. 2004, Klee et al. 2006, Kugeler et al. 2006). For *Babesia microti*, a PCR was performed with primers bab1 and bab4 (Persing et al. 1992), targeting a specific fragment (238 bp) from a gene encoding the nuclear small subunit ribosomal RNA (SS-rDNA).

The following pathogen genomic DNAs were used as positive controls in specific PCR analyses: *Rickettsia conorii*, *Rickettsia typhi*, *Anaplasma phagocytophila*, *B. burgdorferi* B31, *B. microti*, *Bartonella henselae* Houston-1, *C. burnetii*, and *F. tularensis* subsp. *tularensis*. Chi-square test was used to assess the association between pathogen occurrence and several biotic parameters. A p-value of < 0.05 was considered as statistically significant.

Results

Tick identification

DNA was successfully isolated from all of the tested ticks. The 137 tick specimens examined were morphologically

TABLE 1. PRIMERS AND PROBES USED FOR DETECTION OF PATHOGENS IN TICKS

Organism	Gene target	Primer/probe sequence (5' → 3')	Reference
<i>Rickettsia</i> spp	<i>gltA</i>	RpCS877: GGGGACCTGCTCACGGCGG RpCS1258n: ATTGCAAAAAGTACAGTGAACA	Regnery et al. (1991)
<i>Rickettsia</i> spp	<i>ompA</i>	Rp190.70F: ATGGCGAATATTTCTCCAAAA Rp190.701R: GTTCCGTTAATGGCAGCATCT	Roux et al. (1996)
<i>Ehrlichia</i> spp	16S <i>rRNA</i>	EHR16SD: GGTACCYACAGAAGAAGT EHR16SR: TAGCACTCATCGTTTACA	Parola et al. (2000)
<i>Borrelia burgdorferi</i> sensu lato	<i>recA</i>	nTM17.F: GTGGATCTATTGTATTAGATGAGGCTCTCG nTM17.R: GCCAAAGTTCTGCAACAT TAACACCTAAAG	Pietilä et al. (2000)
<i>Babesia microti</i>	<i>SS-rDNA</i>	Bab1: GTCTTAGTATAAGCTTTTATACAGCG Bab4: ATAGGTCAGAAACTTGAATGATACATCG	Persing et al. (1992)
<i>Bartonella</i> spp	<i>gltA</i>	BhCS.781p: GGGGACCAGCTCATGGTGG BhCS.1137n: AATGCAAAAAGAACAGTAAACA PAC1: GCAAAAAGATAAAAATGATTCTTTCCG-Fluorescein- PAC2: LCR640-CTTATGGGTTTTGGTCATCGAGT-Phosphate	Ciervo et al. (2004)
<i>Coxiella burnetii</i>	<i>icd</i>	icd-439F: CGTTATTTTACGGGTGTGCCA icd-514R: CAGAATTTTCGCGGAAAATCA icd-464TM: FAM-CATATTCACCTTTTCAGGCGTT TTGACCGT-TAMRA-T	Klee et al. (2006)
<i>Francisella tularensis</i> tularensis (type A)	<i>pdpD</i>	F: GAGACATCAATTAAGAAGCAATACCTT R: CCAAGAGTACTATTTCCGGTTGGT PDPD Taqman: FAM-AAATTTCTGCTCAGCAGGATTT TGATTTGGTT-TAMRA	Kugeler et al. (2006)
<i>Francisella tularensis</i> holarctica (type B)	<i>ISFtu2</i>	F: CTTGTACTTTTATTTGGCTACTGAGAACT R: CTTGCTTGGTTTGTAATATAGTGGAA ISFtu2Taqman: FAM-ACCTAGTTCAACCTCAAGACTT TTAGTAATGGGAATGTCA-TAMRA	Kugeler et al. (2006)

and/or molecularly identified as follows: *Hyalomma marginatum marginatum* ($n=38$; 27.7%), *H. marginatum rufipes* ($n=71$; 51.8%), *Hyalomma* spp. ($n=17$; 12.4%), *Amblyomma* spp. ($n=5$; 3.6%), *I. ricinus* ($n=1$; 0.7%), and *Ixodes* spp. ($n=5$; 3.6%). The majority of the ticks were nymphs (124), whereas only two larvae belonging to *Ixodes* spp. were identified. Nine specimens moulted in our laboratory generating five females and four males of *H. m. rufipes* and one female of *H. m. marginatum*.

All ticks were collected on 41 birds belonging to 17 species, randomly inspected during ringing activities. Among such bird species, four were partial migrants, such as the European Robin (*Erithacus rubecula*, Linnaeus 1758), the Common Blackbird (*Turdus merula*, Linnaeus 1758), the Common Buzzard (*Buteo buteo*, Linnaeus 1758), and the Blackcap (*Sylvia atricapilla*, Linnaeus 1758), whereas 13 were long-distance migrants, such as the Tree Pipit (*Anthus trivialis*, Linnaeus 1758), the Yellow Wagtail (*Motacilla flava*, Linnaeus 1758), the Common Redstart (*Phoenicurus phoenicurus*, Linnaeus 1758), the Whinchat (*Saxicola rubetra*, Linnaeus 1758), the Whitethroat (*Sylvia communis*, Latham, 1787), the European Pied Flycatcher (*Ficedula hypoleuca*, Pallas 1764), the Spotted Flycatcher (*Muscicapa striata*, Pallas 1764), the Black-Eared Wheatear (*Oenanthe hispanica*, Linnaeus 1758), the Willow Warbler (*Phylloscopus trochilus*, Linnaeus 1758), the Melodious Warbler (*Hippolais polyglotta*, Vieillot 1817), the Nightingale (*Luscinia megarhynchos*, Brehm 1831), the Woodchat Shrike (*Lanius senator*, Linnaeus 1758), and the European Honey Buzzard (*Pernis apivorus*, Linnaeus 1758). No bird species was caught in more than one collection site. Most of them were ringed in Ventotene Island and one (Willow Warbler) in

Ponza Island during spring, 2011; three birds (Blackcap, Blackbird, Nightingale) in Castel di Guido, during the late summer 2010; and one bird (Robin) in Paliano at the beginning of autumn. Two birds were accidentally found at the LIPU Wild Fauna Rescue Centre of Rome—a Common Buzzard collected in a northwestern area and a European Honey Buzzard, found in Acilia locality in the southern part of the city.

The molecular identification provided 12S sequence from 118 ticks, corresponding to the 86% of the whole sample. The 38 *H. m. marginatum* specimens, which were collected on Nightingale, Blackbird, and Blackcap at Castel di Guido and on the Common Buzzard in Rome, between July and September, generated a 339-bp fragment. No intraspecific variability was detected and 100% sequence identity resulted by comparison with the AF150034 sequence available in GenBank.

All *H. m. rufipes* specimens molecularly identified were collected between April and May—34 of them on nine bird species ringed on Ponza Islands (Ventotene and Ponza) and 37 on the hawk in Rome. Of the 71 specimens generating a 339-bp fragment, one found on a Common Whitethroat was characterized by a single variable site (T↔C transition at position 112) and three on the hawk, by another variable site (T↔C transition at position 277). Of the three haplotypes identified by the variable sites, the most common, shared by 67 specimens, showed 98% and 99% of identity with specimens from Morocco (AF150034) and Zimbabwe (AF150033), respectively. Out of 17 *Hyalomma* specimens identified only on at genus level, 11 were found on a European Honey Buzzard (and likely referred to *H. m. rufipes*) and the others were captured on four different birds.

Only one *I. ricinus*, detected on a Melodious Warbler, was found in Ventotene Island in May; the 343-bp sequence

TABLE 2. PATHOGENS IN TICKS PARASITIZING BIRDS

Bird species (no.) (common name)	No. of positive ticks/no. of tested (%)	Pathogens no.				
		Rickettsia	Erlichia	C. burnetii	Borrelia	B. microti
<i>Oenanthe hispanica</i> (1) (Black-Eared Wheatear)	1/1 (100)	0	0	0	1	0
<i>Phylloscopus trochilus</i> (1) (Willow Warbler)	1/1 (100)	0	0	0	1	0
<i>Luscinia megarhynchos</i> (7) (Nightingale)	24/25 (96)	3	0	8	24	0
<i>Turdus merula</i> (4) (Common Blackbird)	3/4 (75)	1	0	0	2	0
<i>Sylvia atricapilla</i> (2) (Blackcap)	1/2 (50)	0	0	1	1	0
<i>Erithacus rubecula</i> (2) (European Robin)	3/4 (75)	0	0	0	3	0
<i>Sylvia communis</i> (6) (Common Whitethroat)	12/15 (80)	4	10	3	3	0
<i>Saxicola rubetra</i> (5) (Whinchat)	14/17 (82)	13	10	2	0	1
<i>Anthus trivialis</i> (3) (Tree Pipit)	3/3 (100)	2	2	0	0	0
<i>Lanius senator</i> (1) (Woodchat Shrike)	1/1 (100)	0	1	0	0	0
<i>Ficedula hypoleuca</i> (2) (European Pied Flycatcher)	2/2 (100)	1	1	0	1	0
<i>Motacilla flava</i> (1) (Yellow Wagtail)	1/1 (100)	1	0	0	0	0
<i>Phoenicurus phoenicurus</i> (2) (Common Redstart)	2/2 (100)	1	2	0	1	0
<i>Muscicapa striata</i> (1) (Spotted Flycatcher)	1/1 (100)	0	0	0	1	0
<i>Pernis apivorus</i> (1) (European Honey Buzzard)	36/40 (90)	19	12	26	0	0
<i>Buteo buteo</i> (1) (Common Buzzard)	6/7 (86)	4	6	2	0	0
<i>Hippolais polyglotta</i> (1) (Melodious Warbler)	1/1 (100)	1	0	0	1	1
Total	112/127	50	44	42	39	2

shared 99% of identity with specimens from Italy (JN248424) and Switzerland (AF150029). In October, four *Ixodes* were collected on two Robins in the Paliano site; for two nymphs, it was impossible to obtain the 12S sequence, whereas 340- and 339-bp fragments were generated for two larvae, respectively. In these two haplotypes, nine transitions (G↔A) and four indels were detected and showed a low identity with the sequences available in GenBank (95% and 94% with two isolates of *Ixodes brunneus*, JQ319376 and JQ319374, respectively). The unique *Ixodes* specimen found on the hawk generated a different 12S haplotype and the 342-bp sequence shared only 90% and 89% sequence identity with *Ixodes pavlovskyi* (JQ85388) and *Ixodes persulcatus* (JQ85386), respectively. Five ticks belonging to *Amblyomma* genus were found on four bird species (Whinchat, European Pied Flycatcher, Spotted Flycatcher, Common Whitethroat) from Ventotene, between April and May. A 338-bp fragment was generated, resulting in two haplotypes due to a single transition (G↔A at position 104) in one specimen with respect the other four. The more common haplotype shared a low identity (91–92%) with several sequences of *Amblyomma variegatum* isolates available in GenBank (for example HQ856499 from Kenya).

Molecular pathogen detection

All captured birds were infested with ticks. Out of 139 ticks, only 127 were available for infectious agents analyses—116 *Hyalomma*, (37 *H. m. marginatum*, 71 *H. m. rufipes*, and eight *Hyalomma* spp.), 5 *Amblyomma* spp., and six *Ixodes* (one *I. ricinus* and five *Ixodes* spp.).

The number of ticks detected per infested bird was in the range of one to five ticks for most birds, but five birds were parasitized by more than six ticks. Specifically, a European Honey Buzzard, a Nightingale, a Whinchat, a Common Whitethroat, and a Common Buzzard, carried 40 (37 *H. m. rufipes*, two *Hyalomma* spp., and one *Ixodes* sp.), 16 (*H. m. marginatum*), 11 (10 *H. m. rufipes*, and one *Hyalomma* sp.),

nine (eight *H. m. rufipes*, 1 *Hyalomma* sp.), and seven (*H. m. marginatum*) ticks, respectively.

Rickettsia spp., *Erlichia* spp., *C. burnetii*, and *B. burgdorferi* s.l. group were detected in 50, 44, 42, and 39 ticks, respectively (Table 2), whereas genomic DNA of *Bartonella* and *Francisella* was never detected. All 50 rickettsiae detected belonged to the SFG. BLAST analysis of rickettsial *ompA* assigned sequences to human pathogenic *R. aeschlimannii* ($n=48$; 96%) and *R. africae* ($n=2$; 4%) with 98–100% sequence similarity.

The 48 *R. aeschlimannii* were found in *H. m. marginatum* ($n=8$; 17%), *H. m. rufipes*, ($n=36$; 75%), *Hyalomma* spp. ($n=3$; 6%), and *Amblyomma* sp. ($n=1$; 2%) from 10 species of birds—two partial migrants (one Common Blackbird and one Common Buzzard), and eight long-distance migrants (one Tree Pipit, one Yellow Wagtail, one Common Redstart, three Whinchat, two Whitethroat, one European Pied Flycatcher, three Nightingale, and one European Honey Buzzard). The *R. africae* was detected in *Hyalomma* spp. and *I. ricinus* ticks detached from two species of birds—the Tree Pipit and the Melodious Warbler.

The 44 *Erlichia* spp. were observed in *H. m. marginatum* ($n=6$; 14%), *H. m. rufipes*, ($n=35$; 79%), *Hyalomma* sp. ($n=1$; 2%), and *Amblyomma* spp. ($n=2$; 5%) from eight species of birds—one partial migrant (one Common Blackbird) and seven long-distance migrants (two Tree Pipit, two Common Redstart, three Whinchat, four Whitethroat, one European Pied Flycatcher, one Woodchat Shrike, and one European Honey Buzzard).

The 42 *C. burnetii* were found in *H. m. marginatum* ($n=10$; 24%), *H. m. rufipes* ($n=29$; 69%), *Hyalomma* spp. ($n=2$; 5%), and *Ixodes* sp. ($n=1$; 2%) from seven species of birds—two partial migrants (one Common Blackbird and one Blackcap) and five long-distance migrants (one Common Redstart, two Whinchat, two Whitethroat, two Nightingale, and one European Honey Buzzard).

The 39 *B. burgdorferi* s.l. group were detected in *H. m. marginatum* ($n=27$; 69%), *H. m. rufipes*, ($n=5$; 13%),

TABLE 3. BACTERIAL PATHOGEN CO-INFECTIONS IN TICKS PARASITIZING BIRDS

Bird species (no.) (common name)	Pathogens ^a no.								
	Ri/Er	Ri/Cb	Er/Cb	Er/Bo	Ri/Bo	Cb/Bo	Ri/Er/Cb	Ri/Er/Bo	Ri/Cb/Bo
<i>Luscinia megarhynchos</i> (7) (Nightingale)	0	0	0	0	3	7	0	0	0
<i>Sylvia atricapilla</i> (2) (Blackcap)	0	0	0	0	0	1	0	0	0
<i>Sylvia communis</i> (6) (Common whitethroat)	3	1	2	0	0	0	0	1	1
<i>Saxicola rubetra</i> (5) (Whinchat)	9	0	0	0	0	0	1	0	0
<i>Anthus trivialis</i> (3) (Tree pipit)	1	0	0	0	0	0	0	0	0
<i>Ficedula hypoleuca</i> (2) (European Pied flycatcher)	0	0	0	1	0	0	0	0	0
<i>Phoenicurus phoenicurus</i> (2) (Common redstart)	1	0	1	0	0	0	0	1	0
<i>Pernis apivorus</i> (1) (European Honey Buzzard)	5	8	2	0	0	0	3	0	0
<i>Buteo buteo</i> (1) (Common Buzzard)	2	0	0	0	0	0	2	0	0
<i>Hippolais polyglotta</i> (1) (Melodious Warbler)	0	0	0	0	1	0	0	0	0
Total	21	9	5	1	4	8	6	2	1

^aRi, *Rickettsia* spp; Er, *Ehrlichia* spp; Cb, *C. burnetii*; Bo, *Borrelia burgdorferi* sensu lato.

Amblyomma spp. ($n=3$; 8%), and *Ixodes* spp. ($n=4$; 10%) from 11 species of birds—three partial migrants (three European Robin, two Common Blackbird, and one Blackcap) and eight long-distance migrants (one Common Redstart, three Whitethroat, one European Pied flycatcher, one Spotted Flycatcher, one Black-eared wheatear, one Willow warbler, one Melodious Warbler, and seven Nightingale).

The *B. microti* was found in one *I. ricinus* and in one *Hyalomma* sp. from two species of birds (the Melodious Warbler and the Whinchat) found in Ventotene Island between April and May. Statistically, we found a positive association ($p=0.001$), between pathogen presence in tick specimens and host migrating habit, feeding and nesting behavior. No significant association was assessed between pathogen occurrence and tick species.

Co-infection analysis

Among the 127 samples analyzed, 112 (88.2%) were positive at least for one pathogen. Specifically, 49% (55/112) were infected with one pathogen, 43% (48/112) were co-infected with two pathogens, and 8% (9/112) carried three pathogens (Table 3). As shown in Table 3, the most frequent co-infections were found between *Rickettsia/Ehrlichia*, *Rickettsia/Coxiella*, *Borrelia/Coxiella*, *Ehrlichia/Coxiella*, *Rickettsia/Borrelia*, and among *Rickettsia/Ehrlichia/Coxiella*.

In particular, the European Honey Buzzard was the most infected bird, reporting 31.2% (15/48) and 33.3% (3/9) of ticks co-infected with two or three agents (Table 3). Co-infection was also found in the two ticks infected with *B. microti*. Actually, *I. ricinus* was co-infected with *R. africae* and *B. burgdorferi* s.l. group, and *Hyalomma* spp. with *R. aeschlimannii*.

Discussion

Ticks play an important role as vectors, but also as reservoirs, of microorganisms, and are able to transmit a greater variety of pathogens than any other arthropod. The impact of environmental factors, such as climatic changes, adds additional complexity to their role. However, climate affects tick survival mostly during the nonparasitic periods of their life cycle, because host-seeking activity is inhibited outside certain ranges of temperature and rainfall (Randolph 1997, Ogden et al. 2004). Tick species may adapt to a new area and

might be considered as an epidemiological marker for a number of infectious agents transmitted by them.

Because pathogens can spread easily through the movements of infested birds, we evaluated the relationships of different tick-borne pathogens and birds.

In our study area, entomological data showed nymphs of *H. marginatum* are the prevailing ticks collected on birds. In the majority of cases, the two subspecies, *H. m. marginatum* and *H. m. rufipes*, were detected with a noncasual distribution of birds. In fact, *H. m. marginatum* ticks were found on partial migratory birds such as the Common Blackbird, the Blackcap, the European Robin, the Tree Pipit, the Common Buzzard, and the Melodious Warbler, whereas *H. m. rufipes* together with *Amblyomma* spp. were picked up on long-distance migratory birds in spring migration. This observation appears to be consistent with the geographic distribution of the two taxa, since *H. m. marginatum* is fully expected in the European and Italian tick fauna, whereas *H. m. rufipes* is reported as the most widespread species of the genus in Africa. However, it has to be considered that, in the last decades, the presence of this species has been reported along the northern latitude of Hungary in central-eastern Europe and to southern Europe in Balkan countries (Feider et al. 1965, Omeragic 2011). Such species composition, including also *Amblyomma* spp. and *I. ricinus*, has been already reported although in a few specimens by previous studies of ticks of migratory birds (Waldenstrom et al. 2007, Elfving et al. 2010, Molin et al. 2011), confirming their role in transferring early stages of tick species along the migratory routes (Hornok and Horváth 2012).

Ticks belonging to the *Amblyomma* genus and *Hyalomma* spp., which are uncommon in Italy, are widespread in tropical and subtropical areas and parasitize a wide variety of domestic and wild mammalian hosts, including birds. Unlike *Amblyomma* spp., *H. m. marginatum* is one of the most significant species in the Mediterranean region (Estrada-Peña et al. 2004).

Obviously, further studies will be crucial to establish the exotic ticks and pathogens maintenance throughout the years and the endemic stability in central Italy. In fact, several characteristics of this area, such as the highly favorable climatic conditions and a large population of susceptible hosts, could be decisive for the management of new/emerging zoonoses with a high public health impact.

The main infectious agent found in our study was *R. aeschlimannii*, which was found in *H. m. marginatum* and *H. m.*

rufipes ticks, consistent with previous studies conducted in Sardinia (Italy), in southern Europe and Africa (Pretorius et al. 2002, Matsumoto et al. 2004, Mura et al. 2008). We also found *R. africanae* in the same vector, as already described in Sicily, Italy (Beninati et al. 2005). Moreover, we detected the presence of important zoonotic bacteria that are known to infect autochthonous ticks, such as *I. ricinus* or *R. sanguineus*. In this regard, our results suggest that *H. marginatum* ticks may be a natural carrier for *B. burgdorferi* s.l., *Ehrlichia* spp. and *C. burnetii*.

Some *Babesia* species can infect more than one genus of ticks, whereas *B. microti* was previously believed to infect ticks of the genus *Ixodes* (Homer et al. 2000). Surprisingly, in our study, *B. microti* was found both in *I. ricinus* and in *Hyalomma* spp. collected from two different birds.

Contrary to prior belief, our novel tick/pathogen association indicates that *Hyalomma* spp. can be infected with *B. microti*, although further study would need to confirm their ability to transmit this pathogen. In addition, the high percentage of *Hyalomma* species found in our study highlights how migratory birds may be important hosts for these ticks. Consequently, this finding suggests that some bird species may represent another reservoir for this pathogen.

In Europe, human cases of babesiosis reported over the past years have been traditionally attributed to *Babesia divergens* transmitted by *I. ricinus* (Herwaldt et al. 2003, Casati et al. 2006). However, an autochthonous human case of *B. microti* was described in Germany (Hildebrandt et al. 2007), and the same piroplasm was recovered in actively questing *I. ricinus* in recreational areas in Germany, with considerable public health implications (Silaghi et al. 2012).

Remarkably, in our investigation a high percentage of tick infections (81%) and co-infection with multiple pathogens was found (51%). This is probably due to the high number of pathogens investigated in this study. However, this finding may be explained by the fact that the European Honey Buzzard was found to be a carrier of 32% (36/112) of all infected ticks found in our study, carrying 38% (19/50) of all *Rickettsia* spp., 27% (12/44) of all *Ehrlichia* spp., and 62% (26/42) of all *C. burnetii*. Similarly, 21% (24/112) of infected ticks carrying 61% (24/39) of all *Borrelia* spp. were found in seven Nightingales. Therefore, the high percentage of infections and co-infections may be due to the detection of multiple infections in infected ticks collected on these specific bird species. The high proportion of co-infections found in our study can also be explained by nonspecific feeding tick habits on a wide range of vertebrate species that are reservoirs for multiple tick-borne agents. Because many ticks can harbor two or more infectious pathogens and transmit them simultaneously, cases of multiple infections with tick-borne pathogens are not a rare event in humans (Swanson et al. 2006).

In conclusion, our data confirm that migratory birds may disperse unusual ticks and pathogens of high medical significance with potential consequence for human and animal health. Further investigations are needed to better define the role of migratory birds in the introduction and circulation of tick-borne diseases in Italy and in other countries of the Mediterranean basin.

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