



Original article

Coxiella burnetii in ticks and wild birds

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ABSTRACT

The study objective was to get more information on *C. burnetii* prevalence in wild birds and ticks feeding on them, and the potentialities of the pathogen dissemination over Europe by both.

Materials: Blood, blood sera, feces of wild birds and ticks removed from those birds or from vegetation were studied at two sites in Russia: the Curonian Spit (site KK), and the vicinity of St. Petersburg (site SPb), and at two sites in Bulgaria: the Atanasovsko Lake (site AL), and the vicinity of Sofia (site SR).

Methods: *C. burnetii* DNA was detected in blood, feces, and ticks by PCR (polymerase chain reaction). All positive results were confirmed by Sanger's sequencing of 16SrRNA gene target fragments. The antibodies to *C. burnetii* in sera were detected by CFR (complement fixation reaction).

Results: Eleven of 55 bird species captured at KK site hosted *Ixodes ricinus*.

C. burnetii DNA was detected in three *I. ricinus* nymphs removed from one bird (*Erithacus rubecula*), and in adult ticks flagged from vegetation: 0.7% *I. persulcatus* (site SPb), 0.9% *I. ricinus* (site KK), 1.0% *D. reticulatus* (AL site). *C. burnetii* DNA was also detected in 1.4% of bird blood samples at SPb site, and in 0.5% of those at AL site. Antibodies to *C. burnetii* were found in 8.1% of bird sera (site SPb). *C. burnetii* DNA was revealed in feces of birds: 0.6% at AL site, and 13.7% at SR site.

Conclusions: Both molecular-genetic and immunological methods were applied to confirm the role of birds as a natural reservoir of *C. burnetii*. The places of wild bird stopover in Russia (Baltic region) and in Bulgaria (Atanasovsko Lake and Sofia region) proved to be natural foci of *C. burnetii* infection. Migratory birds are likely to act as efficient "vehicles" in dispersal of *C. burnetii*-infested ixodid ticks.

1. Introduction

Much research is dedicated to the role of birds in the dispersal of ixodid ticks and tick-borne pathogens, e.g., tick-borne encephalitis virus (Waldenstrom et al., 2007; Movila et al., 2012; Geller et al., 2013), *Borrelia burgdorferi* sensu lato (Franke et al., 2010; Hasle et al., 2010; Kjelland et al., 2010; Socolovschi et al., 2012), *Anaplasma phagocytophilum* (Alekseev et al., 2001; Ogden et al., 2008; Geller et al., 2013), and *Babesia venatorum* (Hasle et al., 2011; Toma et al., 2014). However, the studies on the distribution of *Coxiella burnetii* by birds are not numerous, and only a few researchers applied advanced detection techniques (Ioannou et al., 2009; Socolovschi et al., 2012; Toma et al., 2014; Berthová et al., 2016).

C. burnetii refers to the genus *Coxiella*, phylum Proteobacteria, class Gammaproteobacteria, order of Legionellales, Coxiellaceae family (Seshadri et al., 2003; Arricau-Bouvery and Rodolakis, 2005; Thompson et al., 2013), it belongs to category B bioterrorism agents (Rotz et al., 2002; Kagawa et al., 2003; Madariaga et al., 2003). Q fever caused by *C. burnetii* presents a severe public health problem, and awareness of the disease must be promoted (Porter et al., 2011). Q fever is reported worldwide except in New Zealand, the reservoirs are extensive and include mammals and other animals (Angelakis and Raoult, 2010), and large outbreaks are recorded, e.g. in Russia, Bulgaria, France, Germany, and Holland (Tokarevich, 2007; Georgiev et al., 2013; Eldin et al., 2017), sheep and goats being the main source. The dissemination of Q fever pathogen by birds had never been studied in the Baltic region.

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Table 1
Biological materials: type, number of samples, place of collection.

Materials	Site KK	Site SPb	Site AL	Site SR	Total
Birds	601	416	874	51	1942
Blood samples	78	416	874	0	1368
Sera samples	0	74	0	0	74
Feces samples	0	0	175	51	226
Ticks removed from birds	143	0	0	0	143
Ticks removed from vegetation	226	418	99	0	743

Our objective was to get new information on the probability of *C. burnetii* dissemination in some regions of Europe by wild birds, including those migratory, and by ixodid ticks feeding on them.

2. Materials and methods

All materials (see Table 1) were collected in 2011–2012, in springtime, at migratory bird stopovers. Two sites are in Russia: the Biological Station Rybachy (55°09'N 20°49' E) on the Curonian Spit in the Kaliningrad region (hereinafter: KK site), and a suburb of St. Petersburg (59°59'N and 30°04'E) (hereinafter: SPb site), and other two are situated in Bulgaria: the Atanasovsko Lake (42°34'N, 27°28'E) (hereinafter: AL site), and Sofia region (42°50'N, 23°09'E) (hereinafter: SR site).

The territory under study belongs to the Palearctic biogeographical zone. The climate of SPb site is moderate and humid, transitional from continental to marine. Frequent change of air masses, caused largely by cyclonic activity, is typical for this region. At KK site maritime climate dominates with typically mild winters, but rather cool summers. In Bulgaria the climate is continental (SR site) but mitigated by the influence of the Black Sea (AL site). All those sites are used for stopovers by migratory birds in their seasonal flights to and from Africa, Asia and Europe, and Ixodidae ticks inhabit them, therefore the pathogen exchange between birds and ticks may easily occur.

The field works in Russia were performed in accordance with the Methodical Instructions MU 3.1.1029-01 “Epidemiology. Prevention of infectious diseases, capture, accounting and forecast of numbers of small mammals and birds in natural foci of infections” issued by the Russian Health Ministry. In Bulgaria all sampling procedures were done under Approval # 294/09.03.2011 of the Ministry of Environment and Water of Bulgaria. All birds (for species and numbers see Tables 2–5) were captured with nylon nets, and released into the wild after species identification and biological material sampling. To minimize stress all procedures were carried out as carefully and quickly as possible. At AL, KK and SPb sites blood was sampled from birds' wing vein (v. cutaneaulnaris) into individual microtubes, and transported to laboratory in a portable refrigerator at 4 °C. For DNA detection blood (0.1 ml) was transferred into a microtube filled with 1.5% sodium citrate solution (blood: sodium citrate = 5: 1v:v). At SPb site, if the blood sample was large enough, an aliquot was transferred into a microtube without sodium citrate, to prepare serum afterwards.

At AL and SR sites feces was sampled from all netted birds.

Ticks were collected from birds at KK site.

Ticks were flagged from vegetation at KK, SPb and AL sites with a flannel fabric 1.0 x 0.6 m, carefully removed every 5 min with surgical forceps. Each tick was disinfected by immersion into 75% ethanol for 5 s, rinsed with sterile phosphate-buffer saline, dried in the air, and placed in a clean dry microtube.

The tick species and stage of development (see Table 6) was identified by its morphological character (Filippova, 1977). All samples (blood, serum, feces, ticks) were kept at minus 20 °C. Each tick was homogenized individually in 0.3 ml of sterile sodium-phosphate buffer for subsequent DNA isolation. Blood, feces and ticks were tested for the presence of *C. burnetii* DNA, and blood sera for the antibodies to *C. burnetii*.

2.1. DNA isolation

DNA was extracted from homogenized ticks, blood samples (0.1 ml), and feces (0.1 g) using Diatom DNA Prepkrit (Izogen, Moscow) and following the manufacturer's recommendations. Diatom DNA Prepkrit is made as standard DNA extraction using guanidine-izothiocyanate lysis and sorption on the glass milk after ethanolic washing. The initial sample volume was 0.1 ml, and the final elution volume was 0.1 ml.

2.2. PCR

C. burnetii was detected by polymerase chain reaction (PCR) followed by sequencing of the amplicons. The *com1* and 16SrRNA genes were used for targets. If the PCR with 16S rRNA gene primer was positive, the same sample was tested with *com1* primer. Specific amplification of fragments of length 470 bp. of the gene *com1* and 457 bp 16S rRNA gene was carried out in standard PCR, using the MyCycler thermocycler (Bio-Rad, USA) and the primers of the following sequences: 16S rRNAF, 5' – GAAGTTCACCTTCTTAGTAG – 3', 16S rRNAR 5'– GCA GTTTCTGGGATTAGC – 3', *com1*F, 5'-AGTAGAAGCATCCCAAGCATT – 3', *com1*R, 5' – TAATTGGAAGTTATCACGCAGTT – 3'. The annealing temperature was 54 °C for 16S rRNA and 56 °C for *com1*.

Twenty-five µl of the reaction mixture contained 5 µl of a universal five-fold Screen Mix (Eurogen, Russia) comprising a mixture of dNTP, MgCl₂, reaction buffer and Taq DNA polymerase, 15 µl of sterile deionized water, each primer in final concentration 300 mkMand 3 µl of the DNA template. The amplification program involved 10 min at 94 °C, the next 40 cycles: 30 s at 94 °C, 30 s at annealing temperature and 1 min at 72 °C.

2.3. Positive and negative controls

For positive controls we used DNA isolated from Henzerling and M-44 *C. burnetii* strains (collection of St. Petersburg Pasteur Institute). In all amplification reactions sterile de-ionized water was used for negative controls.

2.4. Sequencing

Direct sequencing of amplified fragments was carried out with ABI capillary sequencer 3130 (Applied Biosystems, Foster City, CA, USA) using BigDye Terminator v3.1 reaction mix (Applied Biosystems). The resulting sequences were analyzed and aligned using Geneious software; nucleotide numbering and mutation nomenclature were based on a reference sequence RSA 493 strain (GenBank Accession Number [NC_002971.3](#), from NCBI).

2.5. Identification of haplotypes

Ugene 1.31.1.2 with MUSCLE algorithm was used to align the gene sequences for haplotype identification within 16S rRNA and *com1* (Okonechnikov et al., 2012). The gene sequence of *C. burnetii* Henzerling RSA 331 strain was used for reference.

2.6. Detection of antibodies

To detect the antibodies to *C. burnetii*, the standard complement fixation test was used (Lennette, 1974). *C. burnetii* II phase cultivated in chick embryos was used for antigen, after cleaning from tissue impurities by differential centrifugation and inactivation by formalin. The reaction was conducted at + 4 °C for 24 h. The result was considered positive if antibodies to *C. burnetii* were detected after at least 1:40 serum dilution.

Table 2
Site KK. Tick infestation in birds, and *C.burnetii* DNA in blood samples. NT = not tested.

#	Bird species	P_{tick} P_{blood}	Region of bird migration (Payevsky, 1973)
1	<i>Acanthis flammea</i> , Common redpoll	0/15 0/15	Boreal species
2	<i>Acrocephalus arundinaceus</i> , Great reed warbler	0/6 NT	Africa
3	<i>Acrocephalus schoenobaenus</i> , Sedge warbler	0/4 NT	Africa
4	<i>Acrocephalus scirpaceus</i> , Reed warbler	0/10 NT	Africa
5	<i>Anthus pratensis</i> , Meadow pipit	0/1 NT	Africa
6	<i>Anthus trivialis</i> , Tree pipit	1/5 NT	Africa
7	<i>Bombycilla garrulus</i> , Bohemian waxwing	0/10 0/10	Boreal species
8	<i>Carduelis cannabina</i> , Linnet	0/1 NT	Europe
9	<i>Carduelis carduelis</i> , European goldfinch	0/1 NT	Europe
10	<i>Carpodacus erythrinus</i> , Common rosefinch	0/1 NT	India
11	<i>Chloris chloris</i> , European greenfinch	0/7 NT	Europe
12	<i>Coccothraustes coccothraustes</i> , Hawfinch	1/6 NT	Europe
13	<i>Emberiza citrinella</i> , Yellow hammer	0/4 NT	Europe
14	<i>Emberiza schoeniclus</i> , Reed bunting	0/7 NT	Europe
15	<i>Ficedula hypoleuca</i> , Pied flycatcher	0/47 NT	Africa
16	<i>Ficedula parva</i> , Red-breasted flycatcher	0/2 NT	India
17	<i>Fringilla coelebs</i> , Common chaffinch	5/22 NT	Europe
18	<i>Fringilla montifringilla</i> , Brambling	0/5 NT	Europe
19	<i>Erithacus rubecula</i> , European robin	14/54 0/14	Europe
20	<i>Garrulus glandarius</i> , Eurasian jay	0/3 NT	Europe
21	<i>Hippolais icterina</i> , Icterine warbler	0/6 NT	Africa
22	<i>Hirundo rustica</i> , Barn swallow	0/9 NT	Africa
23	<i>Locustella naevia</i> , Grasshopper warbler	0/3 NT	Africa
24	<i>Loxia curvirostra</i> , Red crossbill	0/10 NT	Boreal species
25	<i>Luscinia luscinia</i> , Thrush nightingale	0/6 NT	Africa
26	<i>Luscinia svecica</i> , Bluethroat	0/1 NT	India
27	<i>Motacilla alba</i> , White wagtail	0/4 NT	Europe
28	<i>Motacilla flava</i> , Western yellow wagtail	0/4 NT	Africa
29	<i>Muscicapa striata</i> , Spotted flycatcher	0/7 NT	Africa
30	<i>Parus ater</i> , Coal tit	0/1 NT	Europe
31	<i>Parus caeruleus</i> , Blue tit	0/1 NT	Europe
32	<i>Parus major</i> , Great tit	1/10 0/9	Europe
33	<i>Parusmontanus</i> , Willow tit	0/2 NT	Boreal species
34	<i>Parus palustris</i> , Marsh tit	0/1 NT	Resident species
35	<i>Passer domesticus</i> , House sparrow	0/1 NT	Resident species
36	<i>Passer montanus</i> , Tree sparrow	0/1 NT	Resident species
37	<i>Phoenicurus ochruros</i> , Black redstart	0/3 NT	Europe
38	<i>Phoenicurus phoenicurus</i> , Common redstart	2/34 NT	Africa
39	<i>Phylloscopus collybita</i> , Chiffchaff	1/19 NT	Europe
40	<i>Phylloscopus sibilatrix</i> , Wood warbler	0/29 NT	Africa
41	<i>Phylloscopus trochilus</i> , Willow warbler	0/79 NT	Africa
42	<i>Prunella modularis</i> , Dunnock	0/3 NT	Europe
43	<i>Regulus regulus</i> , Goldcrest	0/5 0/5	Europe
44	<i>Saxicola rubetra</i> , Whinchat	0/4 NT	Africa
45	<i>Spinus spinus</i> , Eurasian siskin	0/14 NT	Europe
46	<i>Sturnus vulgaris</i> , Starling	1/3 NT	Europe
47	<i>Sylvia atricapilla</i> , Blackcap	0/39 NT	Africa
48	<i>Sylvia borin</i> , Garden warbler	0/4 NT	Africa
49	<i>Sylvia communis</i> , Greater whitethroat	0/9 NT	Africa
50	<i>Sylvia curruca</i> , Lesser whitethroat	0/16 NT	Africa
51	<i>Troglodytes troglodytes</i> , Wren	6/20 NT	Europe
52	<i>Turdus iliacus</i> , Redwing	0/13 0/13	Europe
53	<i>Turdus merula</i> , Blackbird	2/13 0/8	Europe
54	<i>Turdus philomelos</i> , Song thrush	4/13 0/4	Europe
55	<i>Turdus pilaris</i> , Fieldfare	0/3 NT	Europe
	TOTAL	38/601 0/78	

* P_{tick} = tick-infested birds/all examined birds.

** P_{blood} = positive blood samples/all tested.

2.7. Statistical analysis

We estimated: tick infestation prevalence (P), intensity of parasitization (I), mean density (D), standard deviation (Sd), and median (M). Tick infestation prevalence is the proportion between the number of tick- parasitized birds and that of all examined birds, intensity of parasitization is the number of ticks per parasitized bird, and mean density is the number of collected ticks/examined bird (Kahl et al., 2002).

3. Results

3.1. KK site

We netted 601 birds belonging to 55 species (see Table 2). Blood was sampled from 78 birds. All blood samples were negative for *C. burnetii* DNA.

All birds were examined, and 143 *Ixodes ricinus* (76 larvae, 67 nymphs) were removed from 38 birds belonging to 11 species (all Passeriformes) (Table 2 and 3). The mean density was 0.7 ticks per bird (0.03–2.8) (Table 3). The most frequently parasitized species were: *Turdus philomelos* (30.8%), *Troglodytes troglodytes* (30.0%), *Erithacus*

Table 3
Site KK. Tick-infested bird species: prevalence, number of ticks per bird, mean density, intensity of parasitization.

#	Birdspecies	Prevalence (P): tick-infested birds/ examined (%)	Number of ticks removed from all birds (larvae:nymphs)	Mean density (D), number of ticks per examined bird (larvae:nymphs)	Intensity of parasitization (I), number of ticks per tick-infested bird (larvae:nymphs)
1	<i>Anthus trivialis</i>	1/5 (20.0)	1 (0:1)	0.2 (0.0:0.2)	1 (0:1)
2	<i>Coccothraustes coccothraustes</i>	1/6 (16.7)	13 (9:4)	2.2 (1.5:0.7)	13 (9:4)
3	<i>Erithacus rubecula</i>	14/54 (25.5)	44 (13:31)	0.8 (0.2:0.6)	3 (0.9:2.1)
4	<i>Fringilla coelebs</i>	5/22 (22.7)	11 (7:4)	0.5 (0.3:0.2)	2.2 (1.4:0.8)
5	<i>Parus major</i>	1/10 (10.0)	4 (2:2)	0.4 (0.2:0.2)	4 (2:2)
6	<i>Phoenicurus phoenicurus</i>	2/34 (5.9)	1 (1:0)	0.03 (0.03:0)	0.5 (0.5:0)
7	<i>Phylloscopus collybita</i>	1/19 (5.3)	1 (1:0)	0.05 (0.05:0)	1 (1:0)
8	<i>Sturnus vulgaris</i>	1/3 (33.3)	6 (5:1)	2.0 (1.7:0.3)	6 (5:1)
9	<i>Troglodytes troglodytes</i>	6/20 (30.0)	16 (3:13)	0.8 (0.15:0.65)	2.7 (0.5:2.2)
10	<i>Turdus merula</i>	2/13 (15.4)	37 (32:5)	2.8 (2.5:0.3)	18.5 (16:2.5)
11	<i>Turdus philomelos</i>	4/13 (30.8)	7 (1:6)	0.5 (0.08:0.42)	1.8 (0.3:1.5)
	TOTAL	38/199 (19.0)	143 (76:67)	0.7 (0.4:0.3)	3.8 (2:1.8)

rubecula (25.5%), *Fringilla coelebs* (22.7%), *Turdus merula* (15.4%), and *Coccothraustes coccothraustes* (16.7%). Total tick infestation prevalence (P ± Sd) on birds was 19.0 ± 2.4%, with the highest value of 30.0% (6/20) on wrens. The average intensity of parasitization (I) was 3.8(0.5–18.5) ticks per bird (M = 2.7), 2.0 larvae/bird (M = 2) and 1.8 nymphs/bird (M = 1). *C. burnetii* DNA was found only in 3 *I. ricinus* nymphs removed from one *Erithacus rubecula*. Therefore, in this collection the *C. burnetii* prevalence was 0% in *I. ricinus* larvae and 4.5% in nymphs.

We also collected 226 *I. ricinus* (59 adults, 168 nymphs) from the vegetation. *C. burnetii* DNA was detected in 2 adults (Table 6). Therefore, in this collection the *C. burnetii* prevalence was 0% in *I. ricinus*

nymphs and 3.4% in adults.

3.2. SPb site

We captured 416 birds belonging to 34 species, and blood was sampled from all of them (Table 4). *C. burnetii* DNA was present in 6 samples (1.4%): *Coturnix coturnix* (1/10), *Larus ridibundus* (1/25), *Motacilla alba* (2/52), *Passer domesticus* (1/6), *Sturnus vulgaris* (1/16).

We prepared 74 sera, and antibodies to *C. burnetii* were found in 6 samples (8.1%): *Anas platyrhynchos* (1/7), *Corvus cornix* (1/7), *Turdus merula* (1/6), *Turdus pilaris* (3/21).

We collected 418 ticks from vegetation (see Table 6): 383 *I.*

Table 4
Site SPb. Examination of birds: *C. burnetii* DNA in blood, and antibodies to *C. burnetii* in sera: positive/tested. NT = not tested.

#	Bird species	<i>C. burnetii</i> DNA Antibodies to in blood: <i>C. burnetii</i> in sera: positive/tested positive/tested
1	<i>Acanthis flammea</i> , Common redpoll	0/1 NT
2	<i>Accipiter nisus</i> , Eurasian sparrowhawk	0/2 NT
3	<i>Anas crecca</i> , Eurasian teal	0/2 NT
4	<i>Anas platyrhynchos</i> , Mallard	0/14 1/7
5	<i>Anthus pratensis</i> , Meadow pipit	0/1 NT
6	<i>Anthus trivialis</i> , Tree pipit	0/2 NT
7	<i>Bombycilla garrulus</i> , Bohemian waxwing	0/1 NT
8	<i>Buteo buteo</i> , Common buzzard	0/1 NT
9	<i>Carpodacus erythrinus</i> , Common rosefinch	0/1 NT
10	<i>Columba livia</i> , Rock dove	0/13 0/6
11	<i>Corvus cornix</i> , Hooded crow	0/13 1/7
12	<i>Corvus monedula</i> , Western jackdaw	0/6 NT
13	<i>Coturnix coturnix</i> , Common quail	1/10 NT
14	<i>Dendrocopos major</i> , Great spotted woodpecker	0/5 0/3
15	<i>Erithacus rubecula</i> , European robin	0/2 NT
16	<i>Fulica atra</i> , Eurasian coot	0/1 NT
17	<i>Hirundo rustica</i> , Barnswallow	0/15 0/2
18	<i>Larus argentatus</i> , European herring gull	0/13 0/6
19	<i>Larus ridibundus</i> , Black-headed gull	1/25 0/4
20	<i>Luscinia megarhynchos</i> , Common nightingale	0/1 NT
21	<i>Motacilla alba</i> , White wagtail	2/52 0/2
22	<i>Muscica pastrata</i> , Spotted flycatcher	0/19 NT
23	<i>Oenanthe oenanthe</i> , Northern wheatear	0/26 0/1
24	<i>Passer domesticus</i> , House sparrow	1/6 NT
25	<i>Passer montanus</i> , Eurasian tree sparrow	0/44 0/5
26	<i>Pica pica</i> , Eurasian magpie	0/15 0/4
27	<i>Riparia riparia</i> , Sand martin	0/6 NT
28	<i>Scolopax rusticola</i> , Eurasian woodcock	0/1 NT
29	<i>Spinus spinus</i> , Eurasian siskin	0/2 NT
30	<i>Sturnus vulgaris</i> , Starling	1/16 NT
31	<i>Sylvia curruca</i> , Lesser whitethroat	0/2 NT
32	<i>Tetrao urogallus</i> , Western capercaillie	0/1 NT
33	<i>Turdus merula</i> , Blackbird	0/15 1/6
34	<i>Turdus pilaris</i> , Fieldfare	0/82 3/21
	TOTAL:	6/416 (1.4%) 6/74 (8.1%), (Sd = 17, M = 6) (Sd = 5, M = 5)

Table 5
Sites AL, SR. Examination of birds: *C. burnetii* DNA in blood and feces: positive/all tested. NT = not tested.

No	Bird species	Site AL		Site SR
		Blood positive/tested	Feces positive/tested	Feces positive/tested
1	<i>Acrocephalus arundinaceus</i> , Great reed warbler	0/57	0/8	NT
2	<i>Acrocephalus melanopogon</i> , Moustached warbler	0/1	0/1	NT
3	<i>Acrocephalus palustris</i> , Marsh warbler	0/20	0/8	NT
4	<i>Acrocephalus schoenobaenus</i> , Sedge warbler	0/35	0/8	NT
5	<i>Acrocephalus scirpaceus</i> , European reed warbler	0/3	0/3	3/16
6	<i>Alcedo atthis</i> , Common kingfisher	0/11	0/4	NT
7	<i>Anthus trivialis</i> , Tree pipit	0/8	0/2	NT
8	<i>Cecropis daurica</i> , Red-rumped swallow	0/3	0/1	NT
9	<i>Cettia cetti</i> , Cetti's warbler	0/8	0/3	NT
10	<i>Cyanistes caeruleus</i> , Blue tit	0/5	0/2	NT
11	<i>Delichon urbicum</i> , Common house martin	0/81	0/6	NT
12	<i>Emberiza schoeniclus</i> , Eurasian reed bunting	0/2	0/2	NT
13	<i>Ficedula parva</i> , Red-breasted flycatcher	0/2	0/2	NT
14	<i>Hippola isicterina</i> , Icterine warbler	0/1	0/1	NT
15	<i>Hirundo rustica</i> , Barn swallow	0/312	0/8	NT
16	<i>Jynx torquilla</i> , Eurasian wryneck	0/1	0/1	NT
17	<i>Lanius collurio</i> , Red-backed shrike	0/8	0/6	NT
18	<i>Locustella fluviatilis</i> , Riverwarbler	0/2	0/1	NT
19	<i>Locustella luscinioides</i> , Savi's warbler	0/7	0/4	NT
20	<i>Luscinia megarhynchos</i> , Common nightingale	3/3	0/1	NT
21	<i>Merops apiaster</i> , European bee-eater	0/12	0/6	NT
22	<i>Motacilla flava</i> , Yellow wagtail	0/74	0/8	1/7
23	<i>Muscica pastrata</i> , Spotted flycatcher	0/3	0/1	NT
24	<i>Passer hispaniolensis</i> , Spanish sparrow	0/33	0/6	NT
25	<i>Pelecanus onocrotalus</i> , Great white pelican.	0/30	1/30	1/18
26	<i>Phylloscopus collybita</i> , Chiffchaff	0/2	0/2	NT
27	<i>Phylloscopus trochilus</i> , Willow warbler	1/14	0/8	NT
28	<i>Riparia riparia</i> , Sand martin	0/30	0/8	NT
29	<i>Sylvia curruca</i> , Lesser whitethroat	0/20	0/8	NT
30	<i>Sylvia communis</i> , Common whitethroat	0/14	0/6	NT
31	<i>Sylvia borin</i> , Garden warbler	0/9	0/6	NT
32	<i>Sylvia atricapilla</i> , Blackcap	0/76	0/8	NT
33	<i>Turdus merula</i> , Common blackbird	0/4	0/3	1/3
34	<i>Tringa glareola</i> , Wood sandpiper	0/7	0/7	1/7
TOTAL		4/874 (0.5%) (Sd = 54, M = 8)	1/175 (0.6%) (Sd = 5, M = 5)	7/51 (13.7%) (Sd = 6, M = 7)

Table 6
Sites KK, SPb, AL. *C. burnetii* DNA in ticks removed from birds and from vegetation. NF = not found.

	Positive/tested (%)	Positive/tested (%)			
		Russia		Bulgaria	
		Site KK		Site SPb	
		Birds	Vegetation		
<i>Ixodes persulcatus</i>	adults	NF	NF	3/366 (0.8%)	NF
	nymphs	NF	NF	0/17 (0%)	NF
<i>Ixodes ricinus</i>	adults	NF	2/59 (3.4%)	0/31 (0%)	0/10 (0%)
	nymphs	3/67 (4.5%)	0/168 (0%)	0/4 (0%)	NF
	larvae	0/76 (0%)	NF	NF	NF
<i>Rhipicephalus</i> spp.	adults	NF	NF	NF	0/10 (0%)
<i>R. sanguineus</i> s.l.	adults	NF	NF	NF	0/12 (0%)
<i>R. bursa</i>	adults	NF	NF	NF	0/2 (0%)
<i>Hyalomma</i> spp.	adults	NF	NF	NF	0/3 (0%)
<i>Haemaphysalis</i> sp.	adults	NF	NF	NF	0/1 (0%)
<i>Dermacentor</i> sp.	adults	NF	NF	NF	0/7 (0%)
<i>D. reticulatus</i>	adults	NF	NF	NF	1/54 (1.9%)
TOTAL		3/143 (2.1%)	2/226 (0.9%)	3/418 (0.7%)	1/99 (1.0%)

persulcatus (366 adults, 17 nymphs) and 35 *I. ricinus* (31 adults, 4 nymphs). *C. burnetii* DNA was found in 3 (0.8%) *I. persulcatus* adults.

3.3. AL site

We examined 874 birds of 34 species (see Table 5). Blood was sampled from all of them, and *C. burnetii* DNA was found in 4 samples (0.5%): *Luscinia luscinia* (3/3), *Phylloscopus trochilus* (1/4). Feces were sampled from 175 birds, and *C. burnetii* DNA was revealed in one sample (0.6%): *Pelecanus onocrotalus* (1/30).

From the vegetation we flagged 99 ticks of 8 species, all adults. *C. burnetii* DNA was detected in 1 adult *D. reticulatus* (Table 6).

3.4. SR site

We captured 51 birds belonging to 5 species (see Table 5). Feces were sampled from all of them, and *C. burnetii* DNA was present in 7 samples (13.7%): *Acrocephalus scirpaceus* (3/16), *Motacilla flava* (1/7), *Pelecanus onocrotalus* (1/18), *Turdus merula* (1/3), *Tringa glareola* (1/7) (Table 5).

In all samples of biological materials that contained *C. burnetii* DNA the target fragments of 16 SrRNA gene were sequenced, and its identity with the sequences of the relevant *C. burnetii* gene deposited in the GenBank of the National Center for Biotechnological Information (USA) was confirmed. All obtained nucleotide sequences were deposited in the same database under ACC numbers JX154095.1, JX154094.1, MG6400362.1-640364.1, MG722699.1-722703.1. All *com1* sequences demonstrated 100% identity to the corresponding *com1* gene of *C. burnetii* (MH703036.1-703044.1).

Table 7
Characteristics of *C. burnetii* DNA-positive samples, and 16S rRNA haplotypes.

#	Sample	Geographic location	Host	Biological material	Haplotype of 16S gene
1	IxP-LO83	Russia, SPb site	<i>Ixodes persulcatus</i>	tick	902 G, 1036T
2	IxP-LO116	Russia, SPb site	<i>Ixodes persulcatus</i>	tick	902 G, 1036T
3	IxP-LO201	Russia, SPb site	<i>Ixodes persulcatus</i>	tick	902 G, 1036T
4	IxR91	Russia, KK site	<i>Ixodes ricinus</i>	tick	902 T, 1036C
5	IxR92	Russia, KK site	<i>Ixodes ricinus</i>	tick	902 T, 1036C
6	Bulg-Phtr	Bulgaria, AL site	<i>Phylloscopus trochilus</i>	blood	902 G, 1036T
7	Bulg-Lume1	Bulgaria, AL site	<i>Luscinia megarhynchos</i>	blood	902 G, 1036C
8	Bulg-Lume2	Bulgaria, AL site	<i>Luscinia megarhynchos</i>	blood	902 G, 1036C
9	Bulg-Lume3	Bulgaria, AL site	<i>Luscinia megarhynchos</i>	blood	902 G, 1036T
10	Bulg-Dermret	Bulgaria, AL site	<i>Dermacentor reticulatus</i>	tick	902 G, 1036T

Our analysis of nucleotide sequences has shown that the investigated *C. burnetii* isolates were presented by three haplotypes of 16S rRNA gene fragment, and one haplotype of *com1* gene. For the sample characteristics see Table 7. The numbering of oligonucleotide polymorphisms was carried out in comparison with Henzlerling (reference) strain. From the comparison of SNP-genotypes identified within 16S rRNA gene one may conclude that SNP 1036C > T is not associated with the type of host or geographic location, but evolves independently in *C. burnetii* population.

4. Discussion

Our study differs significantly from those earlier devoted to the role of birds in transporting of ixodid ticks and tick-borne pathogens: the materials were collected in two countries (Russia and Bulgaria), at the sites that differ much in their climatic and natural conditions and inhabited by different tick species; not only ticks, but also blood and feces of birds were sampled and tested for the pathogen presence; both molecular genetic and serological methods were used in the study, and each biological sample was examined individually; we studied the contribution of birds to the spread of *C. burnetii*. The earlier publications on the subject (see e.g., To et al., 1995; Ioannou et al., 2009; Socolovschi et al., 2012; Das et al., 2013, etc.) are not numerous, and probably this is a pioneer study on this problem in the Baltic region, and in Russia this is the first research where molecular genetic methods were applied to the pathogen detection in birds.

Ixodid ticks (Acari: Ixodidae) are known to be the primary vectors of many pathogens, *C. burnetii* including, that cause diseases in humans and animals (Walker, 2014). Those ticks are common ectoparasites in Europe, and birds often host subadults.

Migratory birds flying over long distances contribute to the global dissemination of dangerous pathogens including tick-borne pathogens (Georgopoulou and Tsiouris, 2008; Hasle, 2013). However, the role of migratory birds in the spread of *C. burnetii* is not studied sufficiently.

It has been established that *coxiella* persists in some bird species without obvious signs of the disease or cause polyorganous lesions, leading to death (Shivaprasad et al., 2008; Vapniarsky et al., 2012; Ebani et al., 2016).

The results of our studies on the parasitization of migratory birds at KK site give excellent agreement with those earlier obtained on the Curonian Spit (Movila et al., 2012, and in Norway (Hasle et al., 2009; Kjelland et al., 2010). Mostly the same bird species proved to be parasitized by ticks. In our collection 9 of 11 tick-hosting species, with the maximal parasitization rates, were migrants from Western or Central Europe (Payevsky, 1973). Taking into account that such stopover length is 1–14 days (Chernetsov, 2012), while the feeding time of *I. ricinus* larvae and nymphs is 2–3 days (Balashov, 2012), one may assume that those ticks could be brought by birds from wintering area or acquired at stopover, e.g. in Bulgaria.

If a bird had been infected or hosted some infected ticks, then “local” ticks could acquire the pathogens delivered from distant places

through co-feeding.

In our collection most of ticks were removed from ground-feeding birds that inhabit the lower storey where the exposure to ticks is more probable. Besides, the territory of the Kaliningrad region (KK site including), as well as the whole north-west of Russia, may be considered as the nesting place of those main tick-hosts rather than a simple stopover (Naumov, 1985). Therefore, it appears reasonable to assume stable parasitization of those birds with *I. ricinus* larvae and nymphs during the entire tick activity season, from April to October.

According to earlier findings in the Baltic region the prevalence of pathogens in Ixodes ticks hosted by migratory birds is rather low, with the exception of *Rickettsia helvetica*. For example, in *I. ricinus* collected from migratory birds in the Kaliningrad Region the pathogen prevalence rates are as follows: 0.7% (TBE virus), 2.2% (*Borrelia garinii*), 2.2% (*Borrelia afzelii*), 1.5% (*Borrelia valaisiana*). 1.5% (*Anaplasma phagocytophilum*), 0.7% (*Candidatus Neohelminthia mikurensis*), 11.8% (*Rickettsia helvetica*), 0.7% (*Babesia venatorum*) (Movila et al., 2012).

In the western Estonia in *I. ricinus* removed from migratory birds the results are similar: 0.4% (TBE virus), 4.4% (*B. burgdorferi* s.l.) and 0.4% (*Anaplasma phagocytophilum*) (Geller et al., 2013).

In our study *C. burnetii* prevalence in ixodid ticks removed from migratory birds was also rather small (2.1%), and did not come into conflict with findings of other studies. For example, *C. burnetii* DNA is not detected in Ixodidae collected from birds neither in the southern France (Socolovschi et al., 2012), nor in the northern Spain (Astobiza et al., 2011). However, in Cyprus *C. burnetii* is detected in 3 of 15 examined *Ixodes ventralis* ticks removed from *Alectoris chukar* (Ioannou et al., 2009).

In Italy *C. burnetii* is detected in 42 of 137 ticks (*Hyalomma marginatum* (n = 10), *Hyalomma rufipes* (n = 29), *Hyalomma* spp. (n = 2), and *Ixodes* spp. (n = 1)) removed from *Luscinia megarhynchos*, *Sylvia atricapilla*, *Sylvia communis*, *Saxicola rubetra*, *Anthus trivialis*, *Pernis ptilorhynchus*, and *Buteo buteo* (Toma et al., 2014).

The presence of *C. burnetii* DNA in three *I. ricinus* nymphs removed from the same *Erithacus rubecula* suggested the pathogen transmission from a female to sibs, or through co-feeding on a bird, as it happens due to co-feeding on mammals (Randolph et al., 1996).

Those pathways of transmission are probably valid for some other pathogens as well (Hasle, 2013; Geller et al., 2013).

At KK site we examined not only migrants, but also residents (0.5%, 3 species, 3 birds) and some boreal birds (6%, 4 species, 37 birds). *C. burnetii* DNA was revealed in none of them. However, those birds are known to be Ixodidae feeders (Babenko et al., 1985) and belong to the local basic ornithofauna, and therefore one must assume their participation in *coxiella* transmission. Our sampling being very limited we failed to prove this assumption, but further investigation of indigenous species, along with those migratory, is still among topical issues.

C. burnetii prevalence in ticks collected from vegetation differs significantly depending on the country. Thus, *C. burnetii* is found in none of 1891 questing ticks collected in the Netherlands (Sprong et al., 2012), or 887 ticks collected in Sweden (Wallménus et al., 2012).

However, *C. burnetii* is detected in questing ticks in Slovakia (less than 3%) (Rehacek et al., 1991), in Hungary (2.6%) (Spitalska and Kocianova, 2003), in Germany (1.9%) (Hildebrandt et al., 2010), in Spain (0.1%) (Barandika et al., 2008), in Russia (up to 4% in *I. persulcatus*) (Tokarevich, 2007), and in some other countries. In Austria 298 female *I. ricinus* are studied, and two *C. burnetii* strains are isolated (Rehacek et al., 1994).

The above data testify that in many European countries various tick species are infected with *C. burnetii*. However, absolute comparison of those figures produced in various places by various methods would present a difficulty. Moreover, even within the same territory the *C. burnetii* prevalence in ticks may vary considerably depending on the season and on the test method applied.

We also detected *C. burnetii* DNA in questing ticks collected from vegetation within bird habitats surveyed. Although the prevalence was low (0.7% in *I. persulcatus* at SPb site, 0.9% in *I. ricinus* at KK site, and 1.0% in *D. reticulatus* at AL site) it testified to the presence of natural Q fever foci. The activity of those foci in the vicinity of St. Petersburg, close to the migratory bird stopover site, is confirmed also by *C. burnetii* prevalence in small wild mammals caught at the same place (Freilikhman et al., 2010).

The role of ticks in direct infection of humans with *C. burnetii* is small, while it is crucial in *C. burnetii* life cycle, since in natural biocenoses ticks and vertebrates perform as equal hosts of the pathogen (Maurin and Raoult, 1999).

C. burnetii undergoes multiplication in the gut cells of ticks. Ticks release a large number of bacteria with feces and saliva and can be a source of *C. burnetii* infection in wild and domestic animals (Angelakis and Raoult, 2010).

In our study PCR was applied to *C. burnetii* DNA detection in ticks. The method does not involve the pathogen isolation, and does not grant the presence of living bacteria in the material under study. However, experimental infection of *I. ricinus* with *C. burnetii* (Daiter, 1963; Balashov, 2009) proves *C. burnetii* persistence in *I. ricinus* for 572 days within one generation, and for 737 days in the filial generations due to transphalal and transovarial transmission. Therefore, it is very probable that *C. burnetii* DNA in ticks removed from birds in our study proves that birds are able to carry living pathogens in their blood-sucking parasites.

It is likely that infection of wild birds with *C. burnetii* was firstly reported in 1952 (Babudieri and Moscovici, 1952). Later, *C. burnetii* was isolated from cocks (brain and liver) in Turkmenia (Zhmayeva and Pchelkina, 1957), in Kazakhstan *C. burnetii* prevalence in wild birds was proved with the complement fixation reaction (Borisov et al., 1959), and in Russia (in Dagestan and in the Stavropol Territory) it was revealed in rooks by the method of biological samples on guinea pigs (Basova et al., 1960). After that the study of *C. burnetii*-contaminated biological materials was restricted for safety reasons, and no testing of birds for the presence of this pathogen using molecular genetic methods was carried out in Russia for many years.

In Bulgaria, the complement fixation reaction was used to detect the antibodies to *C. burnetii* in ducks (5.8%), geese (4.1%), hens (4.2%), and *C. burnetii* was isolated from pigeons, turtle doves, crows, ravens, and pheasants (Martinov, 2007).

In Japan, *C. burnetii* was serologically detected in domestic (2%) and wild birds (19%), and mostly re-confirmed by PCR, and by the method of biological tests in mice (To et al. 1995). In the Basque Country (northern Spain) PCR survey of 167 birds revealed *C. burnetii* in *Milvus migrans* (1 of 7), and in *Gyps fulvus* (1 of 9) (Astobiza et al., 2011). In Slovakia, *C. burnetii* was detected in blood of 0.9% of birds (Berthová et al., 2016). It seems that for birds the most high infection rate is reported in Cyprus (Ioannou et al., 2009), but PCR tests being done in pools, one cannot conclude about the real prevalence rate.

We made individual blood tests, and *C. burnetii* DNA was detected in 1.4% of birds examined in Russia (SPb site), and in 0.5% of those in Bulgaria (AL site). Our results confirm the conclusion of our

predecessors about relatively low *C. burnetii* DNA incidence in birds blood, may be due to their biological peculiarities, e.g., high body temperature (Bicudo et al., 2010). One cannot exclude the inhibitory effects of blood and feces on the PCR results. However, the PCR with 16S rRNA gene primer had been tested in whole blood samples and proved its sensitivity to be at least 1×10^3 genomic equivalents per 1 ml (Panferova et al., 2016). Therefore, those inhibitory effects on our PCR results are rather small.

Other researchers had also shown some distinctive features of *C. burnetii* infection in birds. For example, the birds are known to be susceptible to *C. burnetii*, but reproduction of clinically pronounced Q fever is not always possible even when infected with a large dose of the pathogen (Balashov and Daiter, 1973). *C. burnetii* disappears from the blood of experimentally infected birds less than in 24 h (Tarasevich and Kulagin, 1961). However, sometimes it persists, e.g., in quails, after experimental infection, *C. burnetii* was detected in liver and kidney after 8 days, and agglutinating antibodies to *C. burnetii* after at least 66 days (end of the experiment) (Schmatz et al., 1977).

C. burnetii was detected in kidney of experimentally infected doves during 40 days, and up to 25 days in sparrows (Babudieri and Moscovici, 1952). In our study antibodies to *C. burnetii* were detected in 8.1% of blood sera samples at SPb site (see Table 4), while *C. burnetii* DNA was found in 0.6% (AL site) and 13.7% (SR site) of feces samples (see Table 5), and it means that *C. burnetii* probably caused infectious process in all those birds. In India similar results (one of 21 tested samples) are reported for PCR detection of *C. burnetii* in feces of wild birds using primers to com 1 (Das et al., 2013).

C. burnetii can be spread by contaminated droppings, feces or dust from bird nests, thus providing a source of infection in humans and animals (Stein and Raoult, 1999; Lin et al., 2008), and infected animals (goats and sheep) are the main source of *C. burnetii* in humans. The pathogen survives for a long time in the environment (Evstigneeva et al., 2005), e.g., in the USA, 24% of the investigated soil samples proved to be infected with *C. burnetii* (Kersh et al., 2010).

Millions of migratory birds, some of which carry ticks infected with *C. burnetii*, transfer multitude of pathogens, both to locations with natural foci of the infection, and to virgin territories.

The successful conquest of new territories by ticks and the formation of new natural foci depend on a number of drivers: socio-economic activity of people, climate change, vegetation, hosts, etc. (Khasnis and Nettleman, 2005; Korenberg, 2016). Within recent 20 years, a marked northward spread of ixodid ticks occurred in the European North of Russia (Glushkova and Galimov, 2011; Tokarevich et al., 2011; Revich et al., 2012; Tokarevich et al., 2017). Birds, especially migratory, transfer ticks infected with different pathogens to new territories much faster than any other hosts (Hasle, 2013), while climate changes and anthropogenic transformation of landscapes makes those territories suitable for their habitats establishment (Bespyatova et al., 2009). Birds can also contribute to the spread of infected ticks in urban parks (Hornok et al., 2014; Rizzoli et al., 2014), thereby exposing the urban population, children including, to the risk of contracting dangerous infections.

One may assume that migratory birds acquire or exchange pathogens in places of mass stopovers during long flights. In our study the birds examined in KK site were captured on their way from Central and Western Europe (43.4% of migrants), from India (6%), and from Africa (39%) (see Table 2). However, to confirm such assumptions, additional molecular and genetic studies on the pathogen from those regions are necessary.

In conclusion, our findings contribute to better understanding of the role of migratory birds in the distribution of *C. burnetii*. Birds act as an efficient “transport” for ixodid ticks infected with this pathogen. Considerable number of birds proved to be parasitized by *I. ricinus*, and *C. burnetii* DNA was detected in three *I. ricinus* nymphs removed from *Erithacus rubecula*.

Furthermore, *C. burnetii* DNA in bird blood and feces, and antibodies

to *C. burnetii* in bird sera might be considered as indirect evidence that birds perform as a natural reservoir of the pathogen.

We conclude that stopovers of migratory birds in Russia (the Baltic Sea Region) and in Bulgaria (Atanasovsko Lake and Sofia region) are natural foci of *C. burnetii* infection. There is good reason to involve those territories into zoonotic safety programs for monitoring the pathogen prevalence in local ticks and birds.

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