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Molecular investigation of tick-borne pathogens in ticks collected on migratory birds in Lithuania

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Wild birds are increasingly considered to be important in the global dispersal of tick-borne pathogens as they are capable of transporting infected ticks over large distances. To define the role of migrating birds as hosts and disseminators of infected ticks in Lithuania we analysed immature stage of ticks feeding on different passerine bird species. During autumn 2009 and 2010 we screened 3 959 migrating birds at Ventės Ragas ornithological station and found 7.2% birds infested with ticks. The most infested bird species were *Erithacus rubecula* and *Prunela modularis*. We used PCR and sequence analyses for detection and identifying of pathogens in ticks collected from migrating birds. Forty eight tick pools (consisting of 487 *Ixodes ricinus* ticks) were screened for tick-borne pathogens. *Borrelia* spp. were detected in 9 tick pools, *Babesia* spp. in 6 tick pools and *Anaplasma phagocytophilum* in one tick pool. Three *Borrelia* species were identified: *B. garinii*, *B. afzelii* and *B. miyamotoi*. The results of the present study showed the pathway of introduction of *B. miyamotoi* in Lithuania and confirmed the impact of birds on spreading of non-native invasive pathogens in new areas. The present study is the first report of *Babesia microti* in Lithuania. We did not find tick-borne encephalitis virus (TBEV) in ticks collected from migrating birds. The bird species that carried the highest number of infected ticks were *Parus major* and *Erithacus rubecula*.

Key words: migratory birds, *Ixodes ricinus*, *Borrelia* spp., *Babesia microti*, *Anaplasma phagocytophilum*

INTRODUCTION

Ixodes ricinus tick is common and widespread in Lithuania [1–3]. This species acts as vector for a wide range of pathogens of humans and animals, including *Borrelia* spp., *Babesia* spp., *Anaplasma* spp., *Ehrlichia* spp., *Rickettsia* spp., *Francisella tularensis*, and arboviruses such as Louping ill virus (LIV) and tick-borne encephalitis virus (TBEV) [4, 5].

Ticks have very little mobility, but they may be transported over long distances by their vertebrate hosts during feeding, in particular, bird hosts may efficiently transport ticks across geographical barriers [6–9] and spread tick-borne pathogens. Birds, especially ground-feeding species, are at risk of tick infestation and are considered important in the global dispersal of ticks and tick-borne pathogens through their migration within and between continents [10, 11]. It is possible that migratory birds are involved in dispersing of infected ticks from Eastern and Central Europe to other countries where they could raise a public health risk.

The aim of our study was to collect ticks from migratory birds in Lithuania, to define species of birds involved in spreading of ticks and to examine those ticks for *Borrelia burgdorferi* sensu lato, *Babesia* spp., *Anaplasma phagocytophilum* and tick-borne encephalitis virus.

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MATERIALS AND METHODS

Birds were trapped in nets during regular ringing work at Ventės Ragas ornithological station in Lithuania (55°34'N, 21°19'E). Tick screening comprised rapid visual assessment for the presence of any ticks on bare body parts, especially around the eyes and beak of each bird. All ticks were removed with tweezers, placed separately into snap-lid tubes with RNA*later*® (Ambion) and stored according to the manufacturer's instructions.

All collected ticks were identified as *I. ricinus*, pooled (10 nymphs per pool and 20 larvae per pool) and each tick pool was crushed with the tip of a glass rod in liquid nitrogen. RNA and DNA extraction was performed using TriPure Isolation reagent (Roche Diagnostics).

The presence of pathogen RNA and DNA was tested using polymerase chain reactions. Sequences of PCR primers and fragment lengths of amplicons are given in Table 1.

Detection of *B. burgdorferi* s. l. was performed using the FL6 and FL7 primers targeting conserved regions of *fla* gene [12]. The *Osp*A gene located on the linear 49-kb plasmid was used as target in multiplex PCR for genotyping *B. burgdorferi* s. l. Genotyping of positive samples was done using genospecies-specific primers for *B. burgdorferi* sensu stricto (GI), *B. afzelii* (GIII) and *B. garinii* (GII) [13] and by direct sequencing of the 16S (*rrsA*) – 23S (*rrlA*) intergenic spacer (IGS), using the IGS1-F and IGS1-R primers for the first PCR and IGS2-F and IGS2-R primers for the nested PCR [14].

A. phagocytophilum DNA was detected amplifying a fragment of the *msp4* gene using MAP4AP5 and MSP4AP3 primers for the first PCR and *msp4f*, *msp4r* primers for the nested PCR as previously described [15, 16].

For the detection of *Babesia* spp., a part of the 18S rRNA gene was amplified according to Casati [17] using BJ1 and BN2 primers.

For the detection of TBEV specific RNA, F-TBE 1, R-TBE 1 primers and a probe (TBE-Probe-WT) of a quantitative real time RT-PCR protocol according to Schwaiger and Cassinoti [18] were used. According to this method 86 bp fragment of 3' non-coding region of all 3 TBE viral subtypes is amplified.

PCR products of borrelial 16S–23S intergenic spacer and partial 18S rRNA gene were bidirectionally sequenced using the ABI Prism 3130 genetic analyzer (Applied Bio-Systems, USA). The obtained sequences were edited using Mega 5.05 program and aligned with each other and with the previously published sequences in the GenBank using the ClustalW multiple alignment option. A phylogenetic tree was then constructed based on the sequence distance method using the Neighbor-Joining (NJ) algorithm of Mega 5.05 Software. Pairwise distances between the sequences were computed using the Kimura 2-parameter method. The obtained *Borrelia* and *Babesia* sequences were submitted to the GenBank.

Table 1. **Sequences of PCR primers and probe used in this study**

Pathogen	Primer	Sequence (5'-3')	Fragment length (bp)
B. burgdorferi s. l.	FL6	TTCAGGGTCTCAAGCTTGCACT	276
	FL7	GCATTTTCAATTTTAGCAAGTGATG	
B. burgdorferi s. s.	GI	AACAAAGACGGCAAGTACGATCTAATT	544
		TTACAGTAATTGTTAAAGTTGAAGTGCC	
B. garinii	GII	TGATAAAAACAACGGTTCTG GAAC	345
		GTAACTTTCAATGTTGTTTTGCCG	
B. afzelii	GIII	TAAAGACAAAACATCAACAGATGAAATG	189
		TTCCAATGTTACTTTATCATTAGCTACTT	
Borrelia spp.	IGS1-F	GTATGTTTAGTGAGGGGGGTG	400–1 000
	IGS1-R	GGATCATAGCTCAGGTGGTTAG	
	IGS2-F	AGGGGGGTGAAGTCGTAACAAG	
	IGS2-R	GTCTGATAAACCTGAGGTCGGA	
A. phagocytophilum	MAP4AP5	ATGAATTA-CAGAGAATTGCTTGTAGG	849
	MSP4AP3	TT-AATTGAAAGCAAATCTTGCTCCTATG	
	msp4f	CTATTGGYGGNGCYAGAGT	380
	msp4r	GTTCATCGAAAATTCCGTGGTA	
Babesia spp.	BJ1	GTCTTGTAATTGGAATGATGG	470
	BN2	TAGTTTATGGTTAGGACTACG	
TBEV	F-TBE 1	GGGCGGTTCTTGTTCTCC	68
	R-TBE 1	ACACATCACCTCCTTGTCAGACT	
	TBE-Probe-WT	FAM-TGAGCCACCATCACCCAGACACA-BHQ1	

RESULTS AND DISCUSSION

Prevalence and intensity of ticks on migrating birds

A total of 3 959 migrating passerine birds of 19 species were captured and examined for ticks at Ventės Ragas ornithological station in Lithuania during the autumn migrations in 2009 and 2010. Only *I. ricinus* ticks were found. A total of 297 (54%) nymphs and 258 (46%) larvae were collected, no adult ticks were found, confirming that subadult ticks predominate on birds [10].

From 287 infested passerine birds of 11 different bird species 555 immature *I. ricinus* ticks were collected, giving a prevalence of 7.2% (287 of 3 959 birds), a relative intensity of 0.14% tick per bird (555 ticks per 3 959 birds) and mean intensity 1.93 ticks per infested bird (555 ticks per 287 birds). The prevalence of infested birds was lower in 2009 autumn migration: 2% (39 of 2 102 birds) compared to 2010 autumn migration: 13% (248 of 1 857 birds). The relative intensity of tick infestation and the mean intensity of tick infestation were also lower during 2009 autumn migration compared to 2010 autumn migration with 0.03 tick per bird (68 ticks per 2 102 birds) and 0.26 tick per bird (487 ticks per 1 857 birds), respectively and with 1.7 ticks per infested bird (68 ticks per 39 birds) and 2 ticks per infested bird (487 ticks per 248 birds), respectively. The bird species most infested by ticks were European Robin (*Erithacus rubecula*) and Dunnock (*Prunela modularis*) (Table 2).

Our results confirm the results of other scientists that birds are infested with fewer ticks in comparison with other hosts [19–21, 11]. In this study we found that the relative intensity of ticks on birds was 0.14 ticks per bird (555 ticks per 3 959 birds), this is ≈99 times less than that we found on rodents in Lithuania, where relative intensity of ticks was 13.92 (11 430 ticks per 821 rodent) [1]. Our finding supported the opinion of Comstedt et al. [11] that migratory bird contribution in hosting and disseminating ticks may be at least as important as that of other hosts because of their high number of migrating populations. Ventės Ragas ornithological station is located in Ventės Ragas peninsula on the eastern coast of Curonian Lagoon on the White Sea–Baltic Sea migratory flyway. Millions of birds pass this place annually [22].

Tick-borne pathogens in ticks collected from migratory birds

Forty-eight tick pools consisting of 487 ticks (252 larvae and 235 nymphs) collected in autumn 2010 were screened for tick-borne pathogens.

B. burgdorferi s. l. infections were found in 19% tick pools (in nine tick pools consisting of 20 larvae and 63 nymphs). These pathogens were detected in ticks collected from Great Tit (*Parus major*), European Robin (*E. rubecula*), Winter Wren (*Troglodytes troglodytes*), Hawfinch (*Coccothraustes coccothraustes*) and Brambling (*Fringilla montifringilla*) bird species (Table 3).

We identified two *Borrelia* genospecies from *B. burgdorferi* s. l. group (*Borrelia* species that caused Lyme borreliosis): *B. afzelii* (in two pools of 20 nymphs) and *B. garinii* (in four tick pools consisting of 31 nymph). Sequencing of 470 bp fragments of 16S-23S IGS and BLAST alignment confirmed the identification of *B. miyamotoi* in one tick pool consisting of 10 nymphs (Fig. 1). *B. miyamotoi* belongs to a relapsing fever group, which is a genetically and ecologically different group of borreliae. *B. miyamotoi* genospecies was previously isolated only in Japan from *I. persulcatus* ticks.

Table 2. **Infestation of migratory birds by *Ixodes ricinus* ticks***

Bird species	2009 autumn				2010 autumn			
	No. birds	No. ticks	No. (%) birds infested	Mean no. ticks per infested bird	No. birds	No. ticks	No. (%) birds infested	Mean no. ticks per infested bird
Aegithalos caudatus	–	–	–	–	533	8	8 (1.5)	1
Coccothraustes coccothraustes	–	–	–	–	3	4	3 (100)	1.3
Troglodytes troglodytes	37	1	1 (2.7)	1	103	29	20 (19.4)	1.5
Prunela modularis	15	1	1 (6.6)	1	17	14	5 (29.4)	2.8
Erithacus rubecula	435	46	21 (4.8)	2.2	261	223	92 (35.2)	2.4
Turdus merula	1	3	1 (100)	1	1	13	1 (100)	13
Regulus regulus	65	1	1 (1.5)	1	56	3	3 (5.4)	1
Parus montanus	–	–	–	–	105	6	6 (5.7)	1
Parus caeruleus	747	1	1 (0.1)	1	173	3	3 (1.7)	1
Parus major	260	4	3 (1.1)	1.3	566	179	105 (18.6)	1.7
Fringilla coelebs	408	11	10 (2.4)	1.1	10	5	2 (20)	2.5
Total:	1 968	68	39 (2)	1.7	1 828	487	248 (13.6)	2

* Only bird species with at least one tick infested individual are included in the table

Table 3. **Prevalence of *Borrelia* spp., *Babesia* spp. and *Anaplasma phagocytophilum* in bird-fed *Ixodes ricinus* ticks collected at Ventės Ragas ornithological station in autumn of 2010**

Bird species	*Borrelia* species; positive tick pools (number of nymphs; larvae)				*Babesia* spp.	*A. phagocytophilum*
	Borrelia spp.	*B. afzelii*	*B. garinii*	*B. miyamotoi*		
Parus major	4 (32; 0)	1 (10; 0)	2 (22; 0)	1 (10; 0)	2 (22; 0)	1 (10; 0)
Parus caeruleus	–	–	–	–	–	–
Erithacus rubecula	2 (10; 20)	1 (10; 0)	–	–	4 (10; 58)	–
Troglodytes troglodytes	1 (2; 0)	–	–	–	–	–
Prunella modularis	–	–	–	–	–	–
Coccothraustes coccothraustes	1 (4; 0)	–	1 (4; 0)	–	–	–
Fringilla montifringilla	1 (5; 0)	–	1 (5; 0)	–	–	–
Total:	9 (53; 20)	2 (20; 0)	4 (31; 0)	1 (10; 0)	6 (32; 58)	1 (10; 0)

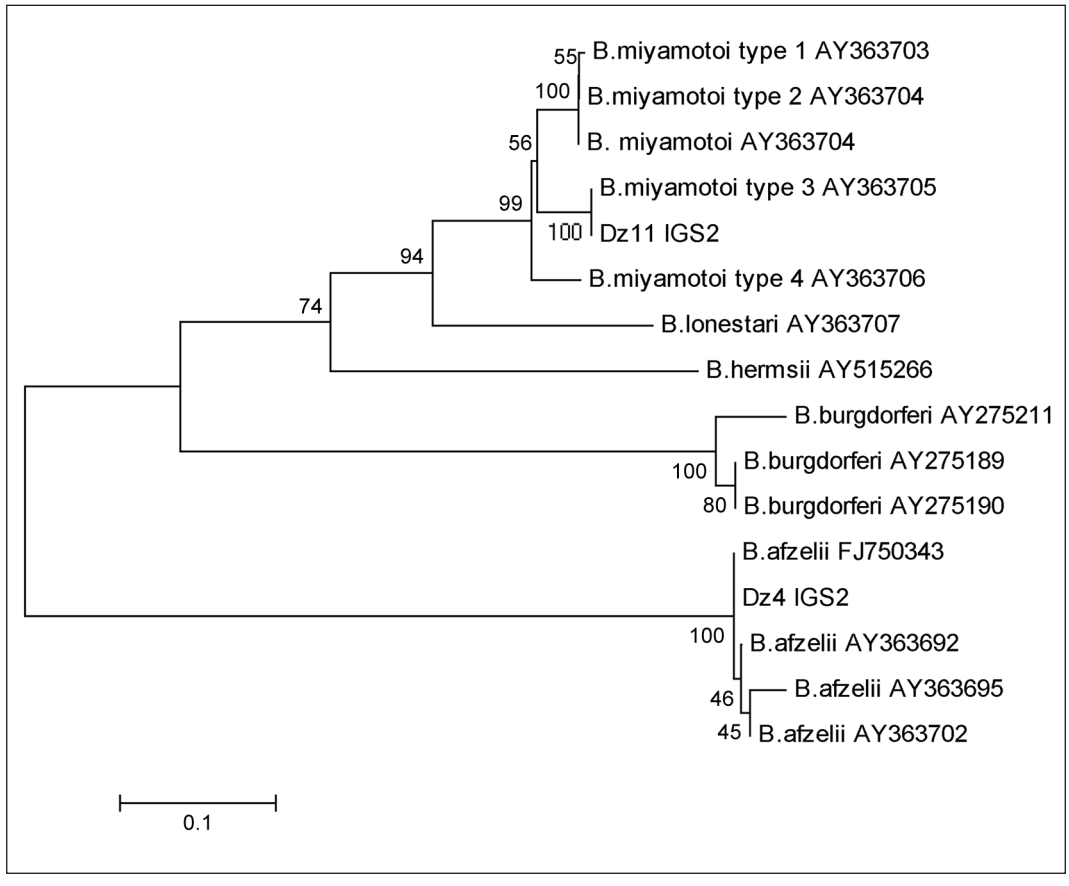

Fig. 1. Phylogenetic tree based on a comparison of 16S–23S ribosomal RNA gene intergenic spacer sequences of *Borrelia* species obtained by the Neighbor-Joining method and bootstrap analysis of 500 replicates. Strains with sequence accession number are given from the GenBank for comparison. Samples sequenced in the present study are indicated as Dz11 and Dz4. Abbreviations: Dz – ticks collected from *Parus major*. The scale bar indicates nucleotide substitutions per site

During the last two decades *B. miyamotoi* has been found at a low infection rate in *I. persulcatus* and *I. ricinus* [23–26] in Eurasia, and in *I. scapularis* and *I. pacificus* in North America [14, 27, 26]. In Europe, *B. miyamotoi* was first reported in Sweden in *I. ricinus* ticks. According to Ašoklienė [28], in Lithuania *B. miyamotoi* was detected in *I. ricinus* ticks collected from vegetation in 2005. The results of the present study showed the pathway of introduction of *B. miyamotoi* to Lithuania, and confirmed the impact of birds on spreading of non-native invasive pathogens in new areas. In the present study *B. miyamotoi* was detected in ticks feeding on Great Tit.

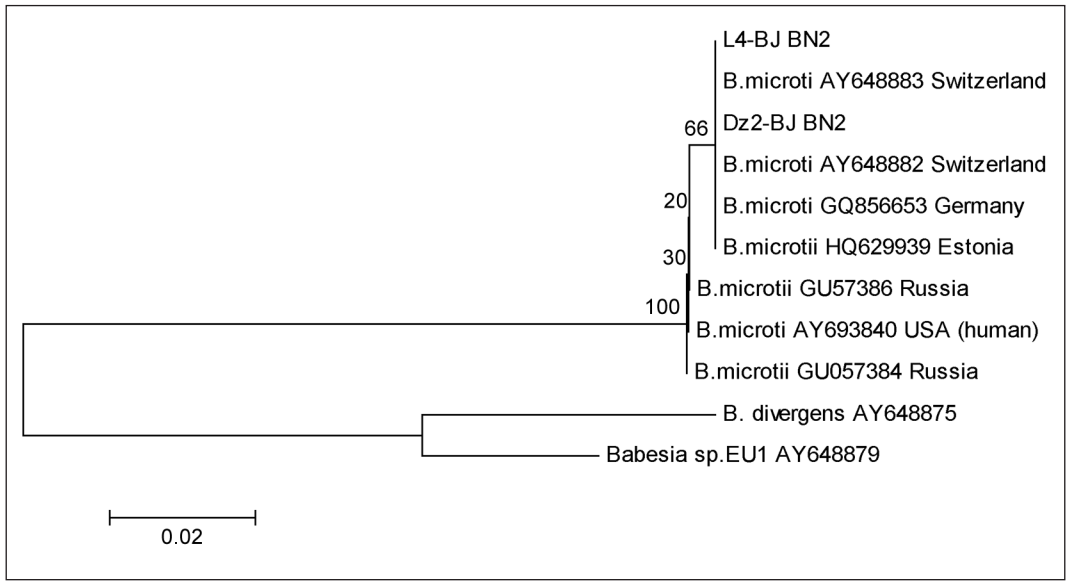

Fig. 2. Phylogenetic tree based on partial 18S r RNA gene sequences of *Babesia* species. The phylogenetic tree was constructed using the Neighbor-Joining method and bootstrap analysis of 500 replicates.
The GenBank accession numbers for the sequences used in comparison are given. The Lithuanian sequences from the present study are indicated as Dz2 and L4. Abbreviations: Dz – ticks collected from *Parus major*; L – ticks collected from *Erithacus rubecula*

In 2 tick pools (consisting of 2 nymphs and 20 larvae) *Borrelia* genospecies were not identified.

The previous studies showed that in Lithuania about 13% of questing *I. ricinus* ticks are infected with *B. burgdorferi* s. l. The most abundant genospecies in questing ticks is *B. afzelii* (9–76%), followed by *B. garinii* (2.5–10%) and *B. burgdorferi* s. s. (0.4–7%) [12, 1]. In the present study *B. garinii* genospecies has been detected in four out of 9 positive tick pools collected from migratory birds. The low prevalence of *B. afzelii* in ticks collected from birds compared to host-seeking ticks may be explained by the observed differences in sensitivity to host serum among the *B. burgdorferi* s. l. strains. During feeding ticks take up host-derived molecules as complement and other blood components. It has been proposed that genospecies *B. afzelii* is sensitive to avian complement, and that these spirochetes are eliminated in the tick midgut, whereas *B. garinii* survives such a blood meal and can be transmitted to the host [29, 30].

Babesia spp. were detected in 13% of tick pools (in six tick pools consisting of 58 larvae and 32 nymphs) removed from *P. major* and *E. rubecula* species (Table 3). For identification of *Babesia* species, PCR products (470 bp fragment of *18S rRNR* gene) of two positive pools were sequenced. Phylogenetic analysis indicated that sequences belonged to *Babesia* genus and were closely related to *B. microti* species (Fig. 2). The Lithuanian *B. microti* samples were found identical to the sequences previously found in *I. ricinus* ticks in Switzerland, Belgium and Germany and slightly differed (by two nucleotide substitutions) from the strain belonging to the zoonotic "US" type, which has been reported as pathogenic for human [31] (Fig. 2). Previous studies in Lithuania showed that about 2–3% of questing *I. ricinus* ticks harbored *B. divergens* [12, 32]. The present study is the first report of *Babesia microti* in Lithuania.

A. phagocytophilum was found in one tick pool consisting of 10 nymphs removed from *P. major* (Table 3). *A. phagocytophilum* in questing *I. ricinus* ticks in Lithuania have an infection rate of 3–4% [12, 32]. The low prevalence of *A. phagocytophilum* in bird-fed ticks corresponds to previous investigations suggesting that birds have no reservoir competence for HGA agents [33].

TBE is an emerging disease in Lithuania [33], but we did not find TBE virus in ticks collected from migratory birds. TBEV has been isolated or serologically indicated from several bird species, especially anatids and gallinaceous birds, and most often from Eastern Europe or Russia [34]. In 2007 a Swedish group collected 1 155 ticks from 447 (3.4%) of 13 260 screened birds and examined ticks for TBEV. Tick infestation with TBEV was low: they found TBEV in 2/529 larvae and 4/409 nymphs [35]. However, little is known about the capability of birds to function as reservoirs of TBEV, and small rodents remain the most important reservoirs of virus [35]. The role of birds in the epidemiology of TBE is still unknown.

CONCLUSION

In the present study we detected *Borrelia* spp., *Babesia* spp. and *Anaplasma phagocytophilum* in ticks collected from migratory birds. Three *Borrelia* genospecies were identified: *B. garinii*, *B. afzelii* and *B. miyamotoi*. The results of the present study for the first time demonstrated the presence of *B. microti* in *Ixodes* ticks in Lithuania.

Infected ticks were carried by five bird species: *P. major, E. rubecula, T. troglodytes, C. coccothraustes* and *F. montifringilla*. The bird species that carried the highest number of ticks infected with different pathogens were *P. major* and *E. rubecula*. These avian species are ground-feeding which puts these birds at risk of tick infestation.

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MIGRUOJANČIUS PAUKŠČIUS PARAZITUOJANČIŲ ERKIŲ IR JŲ PLATINAMŲ PATOGENŲ MOLEKULINIAI TYRIMAI LIETUVOJE

Santrauka

Laukiniai paukščiai yra svarbūs erkių pernešamų patogenų platintojai tolimais atstumais. Norint išsiaiškinti migruojančių paukščių vaidmenį erkių pernešamų patogenų paplitimui Lietuvoje, buvo ištirtos nesubrendusios erkės, rastos ant įvairių žvirblinių paukščių. 2009 ir 2010 m. rudenį Ventės ornitologinėje stotyje patikrinus 3 959 migruojančius paukščius nustatyta, kad 7,2 % jų yra užsikrėtę erkėmis. Labiausiai erkėmis užsikrėtę buvo *Erithacus rubecula* ir *Prunela modularis*. Patogenai erkėse nustatyti DNR PGR ir sekoskaitos analizės metodais. Siekiant nustatyti, ar neužsikrėtę erkių pernešamais patogenais, buvo patikrinti 48 erkių pulai (surinkti iš 487 *Ixodes ricinus* erkių). *Borrelia* spp. buvo nustatyta 9, *Babesia* spp. – 6, o *Anaplasma phagocytophilum* – viename erkių pule. Iš jų buvo išskirtos trys *Borrelia* rūšys: *B. garinii*, *B. afzelii* ir *B. miyamotoi*. Mūsų tyrimai rodo, kad *B. miyamotoi,* kaip naujas invazinis patogenas, į Lietuvą gali patekti su migruojančiais paukščiais. Šio tyrimo metu *Babesia microti* Lietuvoje buvo nustatyta pirmą kartą. Erkėse, rastose ant rudenį migruojančių paukščių, neaptikta erkinio encefalito viruso. Daugiausiai patogenais užsikrėtusių erkių rasta ant *Parus major* ir *Erithacus rubecula*.

Raktažodžiai: migruojantys paukščiai, *Ixodes ricinus*, *Borrelia* spp., *Babesia microti*, *Anaplasma phagocytophilum*