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Prevalence and co-infection of mosquito- and tick-borne pathogens in domestic dogs suspected for canine babesiosis in Lithuania

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During the past decade, vector-borne diseases (VBDs) have been continuously spreading in Europe, including Lithuania. VBDs are caused by bacteria, parasites, or viruses transmitted by the bite of hematophagous arthropods (mainly ticks and mosquitoes). Canine vector-borne diseases (CVBDs) are a growing global threat. Since the majority of these diseases have a zoonotic potential, their management requires a multidisciplinary approach. Global warming and simplified rules for travelling with animal companions provide ideal conditions for the circulation and spreading of vector-borne pathogens in non-endemic geographical regions. Information on CVBD agents at the local and regional levels allows veterinarians to better recognize the pathogens that can affect dogs, thus facilitating diagnosis and treatment. The aim of the present study was to investigate the prevalence and co-infection of mosquito- and tick-borne pathogens in domestic dogs using molecular DNA analysis methods. Blood samples were collected from dogs presented at different veterinary clinics in six regions of Lithuania. A total of 100 blood samples from dogs suspected for canine babesiosis were screened for the presence of tick-borne pathogens *Anaplasma phagocytophilum*, *Borrelia* spp., *Babesia canis* and mosquito-borne pathogens *Dirofilaria* spp. Results of real-time PCR analysis demonstrated the presence of *D. repens* in 23.0%, *A. phagocytophilum* in 35.0%, *Babesia* spp. in 81.0%, and *Borrelia* spp. in 19.0% of examined dogs. Double, triple, or even quadruple co-infections were detected. The present study is the first investigation of multiple vector-borne pathogens in dogs from Lithuania using molecular detection methods. Our findings demonstrate a high infection rate of vector-borne pathogens in dogs and suggest that co-infections with anaplasmosis, borreliosis, babesiosis, and dirofilariosis in dogs are expected in Lithuania.

Keywords: domestic dogs, *Babesia canis*, *Dirofilaria repens*, *Anaplasma phagocytophilum*, *Borrelia burgdorferi* sensu lato, Lithuania

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INTRODUCTION

In humans and animals, vector-borne diseases (VBDs) are caused by bacteria, parasites or viruses transmitted by the bite of hematophagous arthropods (mainly ticks and mosquitoes) (Beugnet, Marié, 2009). Canine vector-borne diseases (CVBDs) are a growing global threat. The majority of these diseases have a zoonotic potential therefore their management requires a multidisciplinary approach (Parola et al., 2005). Climatic changes, together with an increase in the movement of domestic dogs across Europe, have caused an increase in the geographical range of several vector-borne parasites (Cringoli et al., 2001).

Dirofilariosis is an emerging vector-borne parasitic zoonotic infection caused by nematodes of the genus *Dirofilaria* and transmitted by mosquitoes (Simón et al., 2012). The majority of cases in humans and animals are caused by two *Dirofilaria* species, *Dirofilaria repens* and *Dirofilaria immitis*, (McCall et al., 2008). The definitive mammalian hosts for *Dirofilaria* pathogens are primarily domestic dogs and wild canids. Adult nematodes of *D. repens* most often are found in subcutaneous tissues, whereas *D. immitis* is the causal agent of canine and feline cardiopulmonary dirofilariosis (Genchi et al., 2005). The microfilariae are found in peripheral blood and waiting to be picked up by a mosquito. Most of *D. repens*-infected dogs are asymptomatic. In some dogs, the infection induces localized dermatitis, skin nodules, pruritus, thinning, and asthenia (Genchi et al., 2009). In Lithuania, the first case of canine subcutaneous dirofilariosis was recorded in 2010. A recent study conducted in Lithuania reported 2.7% overall prevalence of *D. repens* infection in pet and shelter dogs (Sabūnas et al., 2019). Accurate identification of *D. repens* species in dogs is clinically important because of the zoonotic concerns and therapeutic implications in veterinary clinics.

Tick-borne infection canine babesiosis caused by *Babesia canis* is an emerging infectious disease in Europe (Irwin, 2009). Although previously uncommon, canine babesiosis has become quite frequent in Lithuania during the past decade. Expansion of *B. canis* in Lithuania, as in other European countries, is directly related to the expanding range of the main vector – *Dermacentor reticulatus* tick (Paulauskas et al., 2015). Tick-borne infection caused by bacterium *Anaplasma phagcytophilum* is a well-known disease in Europe and the USA. Due to the spread of Ixodid ticks, the geographical distribution of *A. phagocytophilum* is expanding to the regions of Northern Europe (Carrade et al., 2009). *A. phagocytophilum* has been detected in blood samples from a wide range of wild and domestic animals. Most dogs naturally infected with *A. phagocytophilum* probably remain healthy, as indicated by the high number of healthy seropositive dogs relative to dogs with the clinical disease (Kohn et al., 2011).

Lyme borreliosis (LB) is a zoonotic disease caused by the spirochete *Borrelia burgdorferi* sensu lato (Littman et al., 2006). The disease is transmitted primarily by ticks feeding on mammals and birds, with the most common vectors in Europe being *Ixodes ricinus* and *Ixodes persulcatus.* Much less is known about LB in animals than about the disease in humans (Goossens et al., 2001). The most common symptom of LB in dogs is migratory arthritis; carditis, glomerulonephritis, and neuritis are less common. In Europe, antibodies to *B. burgdorferi* s.l. and clinical symptoms of LB in dogs have been reported in several studies (reviewed by Goossens et al., 2001). If Lyme borreliosis is diagnosed early and treated correctly, the outcomes are generally considered to be excellent and dogs recover quickly. PCR tests for *B. burgdorferi* s.l. from blood can detect active infection sooner than serologic tests.

Infection with *A. phagocytophilum* and *Borrelia* spp. in dogs is mostly asymptomatic or characterized by nonspecific clinical signs, therefore it is especially important to use appropriate methods for early diagnosis of pathogens.

Co-infections with different VBDs are common, because some species are transmitted in the same arthropod vector (Víchová et al., 2014). Most vector-borne diseases have the

special feature of causing similar clinical signs and abnormal laboratory findings in dogs. Co-infected cases are complicated for practitioners and may cause failures in diagnosis, treatment, and prognosis (Cardoso et al., 2010; Gaunt et al., 2010; De Tommasi et al., 2013).

The aim of the present study was to investigate the prevalence and co-infection of mosquito- and tick-borne pathogens in domestic dogs using molecular DNA analysis methods.

MATERIALS AND METHODS

Blood samples from dogs of different breeds and age groups suspected for canine babesiosis were collected by veterinary practitioners from seven Lithuanian veterinary clinics (in Marijampolė, Vilnius, Klaipėda, Panevėžys, Kėdainiai, and two veterinary clinics in Kaunas) from 2016 to 2019 (Table 1).

A total of 100 blood samples were analyzed for the presence of different vector-borne pathogens using molecular detection methods (Table 1). DNA was isolated from 200-µl aliquots of EDTA blood using the GeneJet Whole Blood Genomic DNA purification kit (Thermo Fisher Scientific, Lithuania) as per manufacturer's instructions.

Partial internal transcribed spacer region 2 (ITS2) of the ribosomal RNA and cytochrome c oxidase subunit I (*cox1*) gene were used as targets in PCR for identification of *Dirofilaria* microfilariae species (Rishniw et al., 2006). PCR results were evaluated by agarose gel electrophoresis.

The samples were screened for the presence of tick-borne pathogens *A. phagocytophilum*, *Babesia* spp. and *Borrelia* spp. using multiplex real time-PCR assay designed by Maksim Bratchikov (Sakalauskas et al., 2019) to amplify a 98 bp fragment of *msp2* gene from *A. phagocytophilum*, a 214 bp fragment of *18S rRNR* gene from *Babesia* spp., and a 77 bp fragment of *23S rRNR* gene from *Borrelia* spp. Multiplex TaqMan real time-PCR was performed in a total volume of 15 µl consisting of 100 ng of extracted DNA, (1x) SensiMix™ II Probe No-ROX (Bioline), 1 µM of each primer, and 0.5 µM of each probe. The following PCR conditions were used: an initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 20 s, and annealing-extension at 60°C for 1 min. For all real-time PCR reactions, samples were considered positive if they had a cycle threshold (CT) value <40. Positive samples from real-time PCR were used in further amplifications in order to obtain PCR products for sequence analysis. Partial *msp4* gene of *A. phagocytophilum* and *16S (rrs)-23S (rrlA)* intergenic spacer (ITS region) of *Borrelia* spp. were amplified by nested PCRs. The amplification of *B. canis* DNA through conventional PCR was performed using primers BAB GF2 and BAB GR2, which amplify a 559 bp region of the *18S rRNA* gene of *B. canis* (Adaszek et al., 2009).

Representative positive PCR products were extracted from the agarose gel and purified using the GeneJet PCR purification kit (Thermo Fisher Scientific, Lithuania) as per manufacturer's instructions and further subjected to sequence analysis. The obtained sequences were analyzed using the Mega software package, version X, and compared with the sequence data available from GenBank using the BLAST program. Phylogenetic trees were constructed on the basis of the sequence distance method using the Neighbor joining and Maximum Likelihood algorithms.

RESULTS AND DISCUSSION

Detection of *D. repens*

The identification *of D. repens* was performed on the basis of 484 bp fragments of ITS-2 region. Blood samples positive for microfilaria were then verified with a *D. repens*-specific primer set based on partial (209 bp) amplification of *cox1* gene, as described by Rishniw et al. (2006). Based on both PCR assays, *D. repens* was identified in 23.0% of examined dogs (Figs. 1, 2; Table 1). The sequence analysis of the partial *cox1* gene showed that two *D. repens* sequences were 99–100% identical to the corresponding *D. repens* sequences deposited in GenBank. Two *cox1* gene sequences

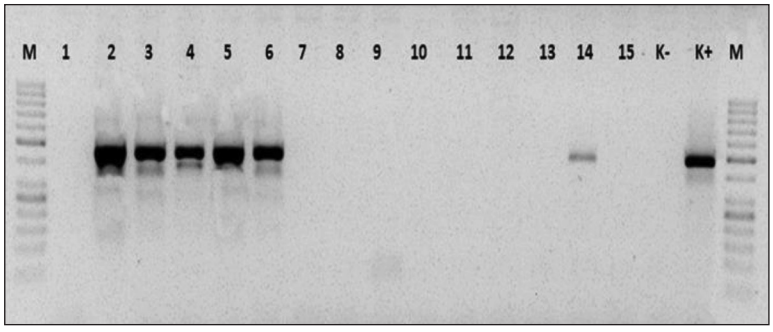

Fig. 1. PCR amplification of partial ITS-2 region of filarial species visualized by electrophoresis in a 1.5% agarose gel: 2–6, 14 tracks *D. repens*-positive samples; M – molecular weight marker 50 bp; K – negative control; K+ positive control

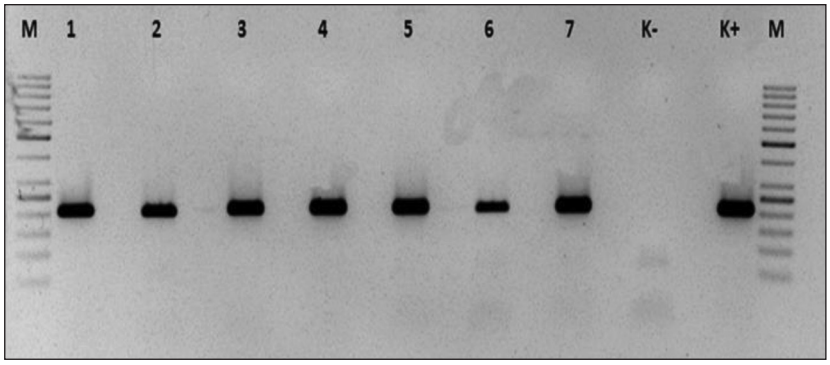

Fig. 2. PCR amplification of partial *cox1* gene of *D. repens* visualized by electrophoresis in a 1.5% agarose gel. 1–7 tracks *D. repens*-positive samples; M – molecular weight marker 50 bp; K – negative control; K+ positive control

Table 1. **Vector-borne pathogens detected in dogs in different veterinary clinics in Lithuania**

Location	*D. trepens* n/N	Real time PCR			Nested PCR
		A. phagocytophilum	*Borrelia* spp.	*Babesia* spp.	*A. phagocytophilum*
Kėdainiai	1/1	1/1	0/1	1/1	0/1
Vilnius	0/5	5/5	5/5	5/5	2/5
Panevėžys	1/8	4/8	3/8	8/8	1/4
Marijampolė	1/4	3/4	2/4	4/4	0/3
Klaipėda	0/5	2/5	1/5	5/5	0/2
Kaunas	20/77	20/77	8/77	58/77	2/20
Total	23/100	35/100	19/100	81/100	5/35

(17 Kr, 1Mar; Table 2) were 100% identical to corresponding sequences of *D. repens* previously detected in dogs from Lithuania (Sabūnas et al., 2019), while three other *cox1* sequences of *D. repens* were distinguished from the sequences available in GenBank based on one nucleotide substitutions (T/C) at the positions 146 nt in the analysed sequence (Table 2). Three *cox1* gene sequences of *D. repens* were deposited in GenBank under accession numbers MT345562 (1Ked), MT345563 (10Sn), MT345564 (41Sn). Phylogenetic relationship among *cox1* gene sequences of filarioid nematodes are presented in Fig. 3. Results of this study demonstrated that at least two *cox1* gene haplotypes of *D. repens* circulate in Lithuania.

Detection of *A. phagocytophilum, Borrelia* spp. and *Babesia* spp.

Results of real-time PCR analysis demonstrated the presence of DNA of *Babesia* spp. in 81.0% (81/100), *A. phagocytophilum* in 35.0% (35/100), and *Borrelia* spp. in 19.0% (19/100) of

Table 2. **Variable nucleotides detected in *cox1* gene sequence (209 bp) of *D. repens* isolates from Lithuania and other countries**

Samples	Nucleotide positions	
	146	155
	C	G
10Sn *Dirofilaria repens* Lithuania	·	·
41Sn *Dirofilaria repens* Lithuania	·	·
1Ked *Dirofilaria repens* Lithuania	·	·
17Kr *Dirofilaria repens* Lithuania	T	·
1Mar *Dirofilaria repens* Lithuania	T	·
MH469227 *Dirofilaria repens* Lithuania	T	·
MG787424 *Dirofilaria repens* Slovakia	T	·
AJ271614 *Dirofilaria repens* Italy	T	·
MH469229 *Dirofilaria repens* Lithuania	T	·
KC142193 *Dirofilaria repens* Slovakia	T	·
MF695085 *Dirofilaria repens* Austria	T	A

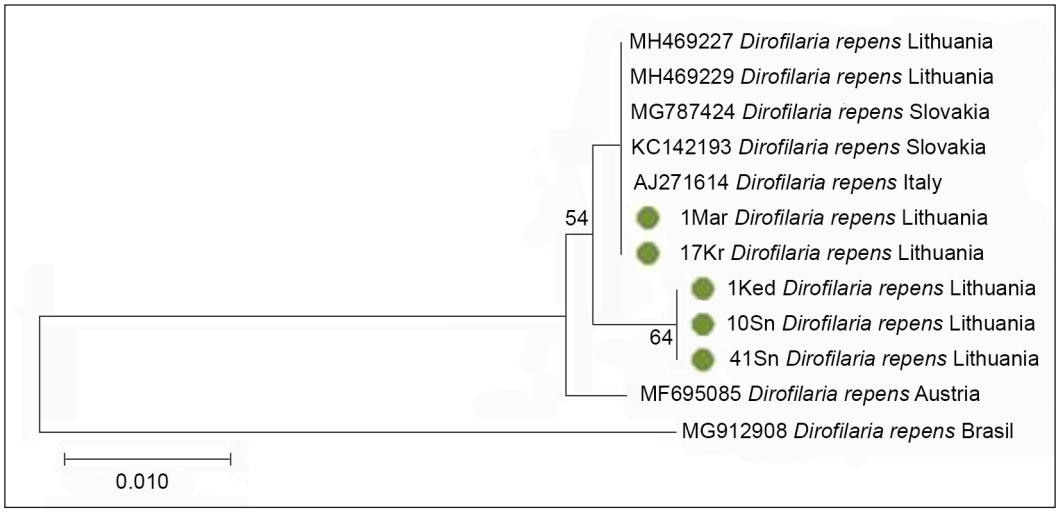

Fig. 3. Phylogenetic tree of the *cox1* gene sequences of *D. repens* created using the Neighbor-Joining method and bootstrap analysis of 1000 replicates. Sequences with accession numbers were taken from GenBank for comparison. Samples sequenced in the present study are marked

examined dogs (Table 1). For positive *A. phagocytophilum* samples, CT values varied from 14 to 39 cycles. For positive *Babesia* samples, CT values varied from 18 to 36 cycles. For *Borrelia* spp. positive samples, CT values ranged between 33 and 38 cycles, which demonstrated low bacteremia in the analysed samples.

In all positive samples, *B. canis* was identified based on amplifications of 559 bp fragments of 18S rRNA in species-specific PCR. *A. phagocytophilum msp4* gene was successfully amplified in five out of 35 (14.3%) samples positive for this pathogen by real-time PCR. Sequence analysis of the partial (381 bp) *msp4* gene of *A. phagocytophilum* showed that all three sequences were 100% identical to each other and to the corresponding sequences deposited in the GenBank database (Fig. 4).

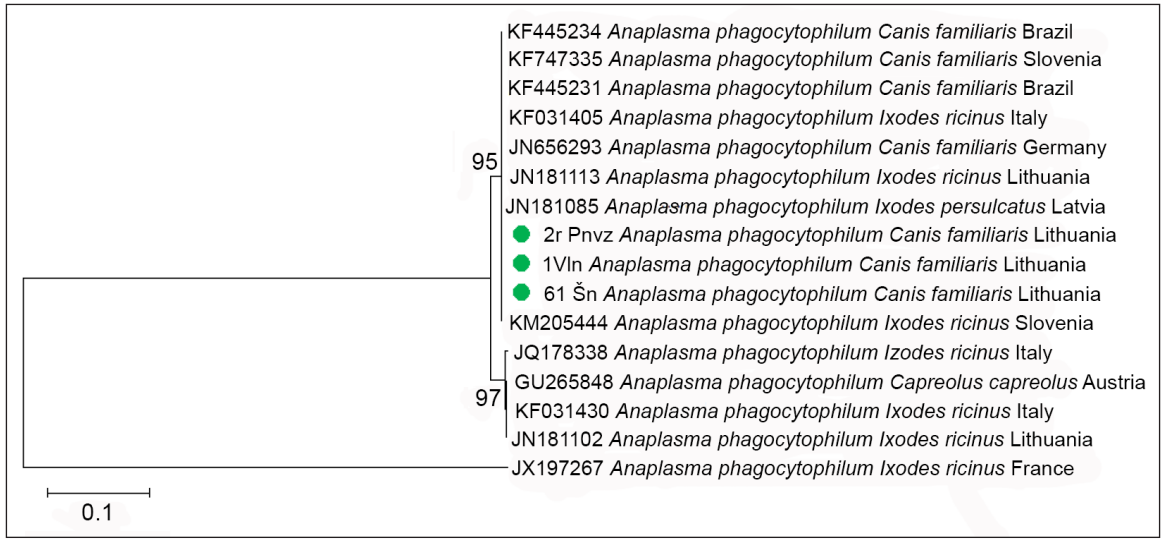

Fig. 4. Phylogenetic tree of the *msp4* gene sequences of *A. phagocytophilum* created using the Maximum Likelihood method and bootstrap analysis of 1000 replicates. Sequences with accession numbers were taken from GenBank for comparison. Samples sequenced in the present study are marked

Amplification of partial ITS region (varied from 450 to 1500 bp depending on *Borrelia* species) of *Borrelia* spp. by nested PCR were not successful, most likely because of a low load of bacteria in examined samples. Real-time PCR method was more effective in identifying of *Borrelia* DNA.

Vector-borne infections are increasingly important to the health of people and other animals worldwide. Tick-borne diseases are of great medical importance worldwide and affect dogs' health through the transmission of pathogens by blood sucking Ixodidae ticks. The geographic distribution of infected ticks has expanded because of bird migration as well as environmental and climatic changes. Climate change has already affected a wide range of vector-borne diseases in Europe. Ticks and mosquitoes, the diseases they transmit have a geographical range restricted by host movement and climatic factors. The increased mobility of domestic dogs has resulted in rapid extension of the geographical ranges for their ectoparasites and carried pathogens (Gray et al., 2008). All these factors may increase the risk of babesiosis, dirofilariosis, anaplasmosis, and Lyme borreliosis for dogs in Lithuania.

Co-infections

A. phagocytophylum spp. and *Borrelia* spp. co-infection was detected in nine samples (9%). *Borrelia* spp. and *Babesia* spp. co-infection was found in three samples (3%). In six samples (6%), *A. phagocytophylum* and *B. canis* co-infection was detected. Triple infections with *A. phagocytophilum*, *B. canis*, and *Borrelia* spp. were detected in seven samples. The high number of co-infections is due to the fact that ticks may be co-infected with several pathogens, with a subsequent high likelihood of co-transmission to animals. Out of 23 *D. repens*-infected dogs, 14 (60.9%) were co-infected with different tick-borne pathogens: four (28.6%) samples were co-infected with *B. canis*; three (21.4%) samples were co-infected with *A. phagocytophilum* and *Borrelia* spp.; two (14.2%) samples were co-infected with *A. phagocytophilum* and *B. canis*; other two (14.2%) samples were co-infected with *A. phagocytophilum*; one sample (7.1%) was infected with *Borrelia* spp. Quadruple co-infection with *D. repens*, *A. phagocytophilum*, *Borrelia* spp., and *B. canis* was detected in two samples (14.2%). The highest co-infection rate (28.6%) was detected between *D. repens* and *B. canis* pathogens. In warm climate countries,

where a large spectrum of different vectors is found, co-infections in dogs with *Ehrlichia*, *Anaplasma*, *Babesia*, *Borrelia*, *Bartonella*, *Rickettsia*, and *Dirofilaria* are detected (Beall et al., 2008; Sainz et al., 2015; Capelli et al., 2018). Findings of the present study suggest that co-infections with anaplasmosis, babesiosis, and dirofilariosis in dogs are expected in Lithuania. Co-infection cases are complicated for practitioners and may cause failures in diagnosis, treatment, and prognosis (Cardoso et al., 2010; De Tommasi et al., 2013). The results of this study may be useful in developing molecular diagnostic kits for infectious diseases using a real-time multiplex PCR method, which can detect multiple pathogens simultaneously (Courtney et al., 2004; Hojgaard et al., 2014). Diagnostic kits for different vector-borne diseases could be based on epidemiological data of different countries (De Tommasi et al., 2013).

CONCLUSIONS

The present study is the first investigation of multiple vector-borne pathogens in dogs from six different locations in Lithuania using molecular detection methods. Our study demonstrated a high prevalence of mosquito- and tick-borne infections in Lithuanian dogs and suggested that co-infections with anaplasmosis, borreliosis, babesiosis, and dirofilariosis are expected. Double, triple, or even quadruple co-infections were detected in the present study. Annual testing for mosquito- and tick-borne infections using modern molecular diagnostic methods is recommended for veterinarians.

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TARP NAMINIŲ ŠUNŲ BABEZIOZĖS UŽKRATĄ PLATINANČIŲ UODŲ IR ERKIŲ PATOGENŲ BEI KOINFEKCIJŲ ATVEJŲ NUSTATYMAS LIETUVOJE

Santrauka

Pastaraisiais metais stebimas vis didesnis vektorių pernešamų ligų plitimas į naujas geografines teritorijas Europoje, taip pat Lietuvoje. Vektorinės kilmės ligas sukelia bakterijos, parazitai ar virusai, kuriuos platina nariuotakojai kraujasiurbiai, dažniausiai – erkės ir uodai. Iš šunų vektorių pernešamų ligų labiausiai pasaulyje paplitusios erkių pernešamos ligos. Šių infekcijų kontrolė yra svarbi dėl patogenų zoonotinio potencialo. Skirtingi patogenai gali sukelti panašius ligos simptomus ir tai apsunkina ligų diagnozavimą. Vektorių plitimas ne endeminiuose geografiniuose regionuose siejamas su globaliu atšilimu, žemės ūkio veiklos pokyčiais bei žmonių kelionėmis su savo augintiniais į naujus regionus. Nuosekli informacija apie *šunų vektorių pernešamus* sukėlėjus naujose vietovėse ar regionuose leidžia veterinarijos gydytojams tiksliau ir greičiau nustatyti patogenus, galinčius sukelti šunų ligas, palengvina ligų diagnozavimą ir gydymą. Šio tyrimo tikslas – molekuliniais DNR analizės metodais įvertinti naminių šunų užsikrėtimą uodų ir erkių platinamais patogenais bei nustatyti koinfekcijų paplitimą. Šunų kraujo mėginiai buvo surinkti iš skirtingų Lietuvos veterinarijos klinikų šešiuose Lietuvos regionuose. Iš viso 100 šunų kraujo mėginių buvo patikrinta dėl erkių platinamų patogenų *Anaplasma phagocytophilum*, *Borrelia* spp., *Babesia canis* ir uodų pernešamų patogenų *Dirofilaria* spp. PGR analizė atskleidė, kad 23 % šunų užsikrėtė *D. repens*, 35 % – *A. phagocytophilum*, 19 % – *Borrelia* spp. ir 81 % – *Babesia* spp. Šunų kraujo mėginiuose buvo aptiktos dvigubos, trigubos ar net keturgubos koinfekcijos. Šiame tyrime šiuolaikiniais molekuliniais tyrimo metodais pirmą kartą buvo įvertintas naminių šunų užsikrėtimas skirtingais vektorių pernešamais patogenais Lietuvoje bei nustatytos anaplazmozės, boreliozės, babeziozės ir dirofiliariozės sukėlėjų koinfekcijos.

Raktažodžiai: naminiai šunys, *Babesia canis*, *Dirofilaria repens, Anaplasma phagocytophilum, Borrelia burgdorferi sensu lato*, Lietuva