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Molecular characterization of *Anaplasma phagocytophilum* infection in the cervids and feeding ticks from Lithuania

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Anaplasma phagocytophilum is a bacterial pathogen, which is a major cause of zoonotic disease, anaplasmosis. The main vectors of *A. phagocytophilum* are ticks of the *Ixodes ricinus* complex. *A. phagocytophilum* has a broad geographic distribution and a high degree of biological and clinical diversity. Epidemiological studies in multiple countries have shown that the prevalence of *A. phagocytophilum* highly depends on the density of ticks and their potential hosts such as the cervids, which are one of the main sources of nutrition for *Ixodes* ticks. In Lithuania, the cervids are important game animals but their contribution as reservoirs for *A. phagocytophilum* remains unknown. The objectives of the study were to investigate the prevalence of *A. phagocytophilum* infections in the cervids and feeding ticks and to characterize the *A. phagocytophilum* strains obtained from the cervids and ticks based on sequence analysis of *msp4* gene. A total of 187 ticks were collected from 44 cervids (roe deer, red deer, and moose) harvested by professional hunters during the hunting seasons of 2010–2013 and 2016–2017 in Lithuania. Blood and spleen samples were collected from 29 animals (27 roe deer and two red deer). *A. phagocytophilum* DNA was identified in ten (37.04%) of the 27 roe deer. The overall prevalence of *A. phagocytophilum* in *I. ricinus* and *D. reticulatus* ticks was 39.3% (70/178) and 22.2% (2/9) respectively. The sequence analysis of the *msp4* gene of *A. phagocytophilum* revealed nine different sequence types: five *msp4* sequence types were detected in ticks and seven in roe deer.

Keywords: *Anaplasma phagocytophilum*, ticks, *Ixodes ricinus*, *Dermacentor reticulatus*, cervids

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INTRODUCTION

Anaplasma phagocytophilum is small gram-negative obligate intracellular bacterium, which is the main agent causing zoonotic diseases such as granulocytic anaplasmosis in humans (HGA) and animals (Nicholson et al., 2010; Hajdusek et al., 2013). *A. phagocytophilum* has a broad geographic distribution and a high degree of biological and clinical diversity. *A. phagocytophilum* was first recognized in Europe 80 years ago as a causative agent of tick-borne fever (TBF) in domestic ruminants. Ticks act as the main vector of *A. phagocytophilum. A. phagocytophilum* is mainly transmitted by ticks belonging to the *Ixodes ricinus* complex: *Ixodes ricinus* in Europe, *Ixodes persulcatus* in Eastern Europe and East Asia, and *Ixodes scapularis* and *Ixodes pacificus* in North America (Jaarsma et al., 2019). Infected ticks can transmit *A. phagocytophilum* to new hosts during the blood meal of its following stage. *Ixodes* ticks can be infected by *A. phagocytophilum* at each stage (except as eggs), but only nymphs and adult females can transmit this bacterium. *Anaplasma* bacteria infects a wide range of wild mammalian, domestic animals, rodents and humans. Two distinct *A. phagocytophilum* clades were detected based on four genes of bacteria genome: one clade contained *A. phagocytophilum* genotypes from questing *I. ricinus* and feeding *I. ricinus* from a broad array of hosts (humans, ungulates, birds and dogs), while the other clade comprised solely genotypes found in rodents and feeding *I. trianguliceps* (Blaňarová et al., 2014).

The role of wildlife species in the circulation of *A. phagocytophilum* is yet to be clearly determined, but several species of wild ruminants are thought to be important reservoirs. Several species of wild ruminant have been suggested to act as reservoir hosts, amongst them mainly the roe deer and the red deer (Woldehiwet 2010; Stuen et al., 2013; Dugat et al., 2015). In Lithuania, the cervids such as the roe deer, the red deer, and the moose are important game animals but their contribution as reservoirs for *A. phagocytophilum* remains unknown. Epidemiological studies in multiple countries have shown that the prevalence of *A. phagocytophilum* highly depends on the density of ticks and their potential hosts such as the cervids, which are one of the main sources of nutrition for *Ixodes* ticks. Previous studies have also suggested that *A. phagocytophilum* strains circulating in different ruminant species, possibly having distinct transmission cycles in nature, are independent of each other (Massung et al., 2002; Stuen et al., 2003, 2013).

The objectives of the study were to investigate the prevalence of *A. phagocytophilum* infections in the cervids and feeding ticks and to characterize the *A. phagocytophilum* strains obtained from the cervids and ticks based on sequence analysis of *msp4* gene.

MATERIALS AND METHODS

Ticks were sampled from 44 cervids representing three species: the roe deer *Capreolus capreolus*, the red deer *Cervus elaphus*, and the moose *Alces alces* (Table 1) harvested by professional hunters during the hunting seasons of 2010–2013 and 2016–2017 in nine districts of Lithuania (Biržai, Jonava, Jurbarkas, Kaunas, Rumšiškės, Tauragė, Ukmergė, Vievis, and Vilnius). Ticks were collected from hosts using tweezers and placed into 1.5 ml tubes with 70% ethanol. Blood or spleen samples were collected from 29 animals (27 roe deer and two red deer) hunted from 2010 to 2013.

Identification of tick species and determination of the life stage was done microscopically based on morphological characters (Estrada-Peña et al., 2004). From partially or fully engorged ticks DNA was extracted using Genomic DNA Purification Kit (Thermo Fisher Scientific, Lithuania), while from unfed ticks DNA was extracted using a modified procedure with the ammonium hydroxide solution (2.5%) (Stańczak et al., 1999). The lysates were stored at –20°C until PCR analysis. The samples were screened for the presence of *A. phagocytophilum* by nested PCR as described by de la Fuente et al. (2005) and Bown et al. (2007): partial *msp4* gene was amplified using primers

MSP4AP5/MSP4AP3 for the first PCR reaction and msp4f/msp4r for the second reaction. PCR was performed by using 2X PCR Master Mix (Thermo Fisher Scientific, Lithuania). Negative and positive controls were included in all runs. The nested PCR amplification products of the *A. phagocytophilum msp4* gene were extracted from the agarose gel and purified using GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Lithuania) following the manufacturer's instructions. The obtained sequences were analysed using MEGA X software and compared with the sequence data available from GenBank using the BLAST program. The phylogenetic tree was constructed using the Neighbor-joining method (NJ) with bootstrap analysis of 1000 replicates. The sequences obtained in our study received GenBank accession numbers MT886200–MT886214.

RESULTS AND DISCUSSION

The presence of *A. phagocytophilum* DNA in the analysed samples was confirmed by amplification of 381 bp fragments of *msp4* gene. *A. phagocytophilum* DNA was identified in ten (37.04%) of the 27 roe deer. Neither of two examined red deer was infected with *A. phagocytophilum*. A total of 187 ticks of different developmental stages and sexes were collected from *C. capreolus*, *C. elaphus*, and *A. alces* (Table 1). Nine ticks were identified as *Dermacentor reticulatus* (four females and five males) and 178 as *Ixodes ricinus* (89 females, 87 males and two nymphs). For the detection of *A. phagocytophilum*, each tick was analysed individually. Based on nested PCR assay, *A. phagocytophilum* DNA was detected in 58.6% (70/178) *I. ricinus* and 22.2% (2/9) *D. reticulatus* ticks. The bacterium was detected only in adult ticks. Both males and females of *I. ricinus* and *D. reticulatus* were infected with *A. phagocytophilum* (Table 1). The incidence of infection varied between different sexes of *I. ricinus*: females (48.3%; 43/89) were more frequently infected than males (31.0%; 27/87). Our findings showed that more frequently *A. phagocytophilum*-infected ticks were found on the roe deer and the moose (Table 1). From one moose 57 ticks of both species (52 *I. ricinus* and five *D. reticulatus*) were collected, of which 49.1% (28; 27 *I. ricinus* and one *D. reticulatus*) were infected with *A. phagocytophilum*. A total of 105 ticks (104 *I. ricinus*, and one *D. reticulatus*) were collected from 39 roe deer, and *A. phagocytophilum* infection was detected in

Table 1. **Prevalence of *Anaplasma phagocytophilum* in ticks collected from the cervids in Lithuania**

Cervids species	No. of cervids	Tick stage	Number of infected ticks/number of collected ticks (%)		
			Ixodes ricinus	*Dermacentor reticulatus*	Total:
Roe deer (*Capreolus capreolus*)	39	Female	37/69 (53.6)	0/1 (0)	37/70 (52.9)
		Male	5/33 (15.2)	–	5/33 (15.2)
		Nymph	0/2 (0)	–	0/2 (0)
		Total:	**42/104 (40.4)**	**0/1 (0)**	**42/105 (40.0)**
Moose (*Alces alces*)	1	Female	5/8 (62.5)	–	5/8 (62.5)
		Male	22/44 (50.0)	1/5 (20.0)	23/49 (46.9)
		Nymph	–	–	–
		Total:	**27/52 (51.9)**	**1/5 (20.0)**	**28/57 (49.1)**
Red deer (*Cervus elaphus*)	4	Female	1/12 (8.33)	1/3 (33.3)	2/15 (13.33)
		Male	0/10	–	0/10
		Nymph	–	–	–
			1/22 (4.5)	**1/3 (33.3)**	**2/25 (8.0)**
Total:	44		**70/151 (46.4)**	**2/9 (22.2)**	**72/187 (38.5)**

40.0% (42 *I. ricinus* ticks) (Table 1). From four red deer, 25 ticks (22 *I. ricinus* and 3 *D. reticulatus*) were collected, and *A. phagocytophilum* DNA was detected in two ticks (one *I. ricinus* and one *D. reticulatus* females) (Table 1).

In our previous studies conducted in Lithuania, the prevalence of *A. phagocytophilum* infection in questing *D. reticulatus* ticks reached 8% (Paulauskas et al., 2012). However, *A. phagocytophilum* is found rarely (and at low prevalence) in questing *D. reticulatus* ticks. In eastern Poland, the prevalence of *A. phagocytophilum* in questing *D. reticulatus* varied from 0.7% to 2.0%, depending on the tick collection area (Opalinska et al., 2016; Zajac et al., 2017). The results obtained in several studies suggest that this tick species is not a competent vector of *A. phagocytophilum* (Zygner et al., 2008). The ability of this tick species to transmit *A. phagocytophilum* has not been yet studied sufficiently. Furthermore, the presence of pathogens in ticks collected from the host does not provide information whether the source of infection is the blood of the host or whether the pathogen was present in the tick before feeding (Karbowiaka et al., 2014).

The prevalence of *A. phagocytophilum* in European *I. ricinus* tick populations varies. In Lithuania, the prevalence of *A. phagocytophilum* infection in questing *I. ricinus* ticks was found to be 2.9% (Paulauskas et al., 2012). In this study, the observed overall prevalence of *A. phagocytophilum* in *I. ricinus* feeding on the cervids was high (39.3%). In a similar study conducted in Poland, in total, 238 partially or fully engorged female and 63 non-engorged male *I. ricinus* ticks were obtained from 51 cervids (20 fallow deer, 18 roe deer, and 13 red deer) (Michalik et al., 2009). The overall prevalence of *A. phagocytophilum* was higher in females (22.7%) than in males (9.1%) of *I. ricinus* (Michalik et al., 2009). It was noticed that female and male ticks from the PCR-positive cervids were more frequently infected than ticks from the PCR-negative hosts (Michalik et al., 2009). In a study performed in Germany, 331 engorged *I. ricinus* ticks from 44 roe deer individuals were screened, and 86.1% of them were infected with *A. phagocytophilum* (Overzier et al., 2013) with the higher infection rate detected in females (99.0%) than in males (64.8%). It was also noticed that engorged adult ticks removed from the roe deer were significantly more often positive for *A. phagocytophilum* than questing adult ticks (Overzier et al., 2013).

Wild ruminants are among the major feeding hosts for ticks in Europe. A great abundance of potential hosts is an important factor for tick expansion and potentially for the spread of anaplasmosis. There is evidence that higher cervid population densities may influence higher ticks densities, suggesting a positive effect on the prevalence of *A. phagocytophilum*. A study conducted in Norway demonstrated that the highest prevalence of *A. phagocytophilum* in *I. ricinus* occurred in locations with the highest densities of the roe deer and the red deer (Rosef et al., 2009). In Europe, *A. phagocytophilum* has been detected in local wild ruminant species with different prevalence (reviewed in Stuen et al., 2013). The roe deer show *A. phagocytophilum* prevalence rates reaching up to 98.9%, and several genetic variants of *A. phagocytophilum* (both potentially pathogenic and non-pathogenic) have been found in the roe deer in Europe (Overzier et al., 2013). It has been suggested that the roe deer mainly acts as a reservoir of several *A. phagocytophilum* non-pathogenic variants for other animal species (Stuen et al., 2013). Other cervid species in Europe may also constitute efficient reservoir hosts of *A. phagocytophilum* as the pathogen has been detected in the red deer with up to 87% prevalence, in the fallow deer (*Dama dama*) with up to 72%, in the sika deer (*Cervus nippon*) with up to 50%, and in the moose with up to 42.9% (reviewed in Stuen et al., 2013; Pūraitė et al., 2015; Ražanskė et al., 2019).

The sequence analysis of the *msp4* gene among the 15 samples derived from the roe deer (n = 8) and *I. ricinus* (n = 6) and *D. reticulatus* (n = 1) ticks revealed ten different sequence types: five *msp4* sequence types were detected in ticks and seven in roe deer. Two sequence variants (1 and 2) were detected in both *I. ricinus* ticks and roe deer (Table 2).

Table 2. **Differences in the *msp4* nucleotide sequences among *A. phagocytophilum* strains detected in ticks and roe deer in Lithuania**

Strain/host	variant	Nucleotide positions																																			
																			1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2
					1	1	3	3	4	4	4	5	5	7	8	8	9	9	0	2	4	5	5	6	6	7	7	9	9	9	9	2	4	5	5	5	6	7
		1	5	6	0	3	3	6	3	4	6	6	7	2	5	7	0	6	5	9	1	6	9	5	8	2	7	0	2	5	9	2	0	2	5	8	7	9
MT886200 / *I. ricinus* from *A. alces*	1	G	A	T	A	A	G	T	G	C	A	C	G	A	A	C	C	C	A	T	C	T	C	T	T	T	G	A	C	C	G	T	C	A	T	C	A	T
MT886201 / *I. ricinus* from *C. capreolus*		·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
MT886203 / *I. ricinus* from *C. capreolus*		·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
MT886207 / *C. capreolus*		·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
MT886211 / *C. capreolus*	2	·	·	·	·	G	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
MT886204 / *I. ricinus* from *C. capreolus*		·	·	·	·	G	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
MT886205 / *I. ricinus* from *C. capreolus*	3	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·
MT886213 / *C. capreolus*	4	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	Y	·	·	·	·	·	·	·	·	·	·	·	·
MT886214 / *C. capreolus*	5	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	T		·	·	·	·	·	·	·	·	·	·	Y	·	·	·	·	·	·	·	·
MT886208 / *C. capreolus*	6	A	G	·	G	·	·	C	·	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·	·	T	·	·
MT886209 / *C. capreolus*	7	A	G	·	G	R	·	C	·	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·	·	Y	·	·
MT886210 / *C. capreolus*		A	G	·	G	R	·	C	·	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·	·	Y	·	·
MT886216 / *I. ricinus* from *C. elaphus*	8	A	G	·	G	G	·	C	·	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·	·	·	·	T	·	·	·	G	·	T	·	·
MT886202 / *D. reticulatus* from *C. elaphus*	9	A	G	·	G	G	·	C	·	·	·	T	·	·	·	·	·	T	·	C	·	·	·	C	·	·	·	·	·	T	·	·	·	G	·	T	·	·
MT886212 / *C. capreolus*	10	A	G	C	G	G	A	C	A	T	G	·	A	T	G	T	T	T	G	·	T	C	T	·	C	C	A	G	·	A	A	A	T	·	C	T	C	C

The analysed sequences showed 98–100% homology to each other (differing at one to 32 nucleotide positions) and to the other *msp4* gene sequences deposited in GenBank. Ambiguous nucleotides were observed in *msp4* sequences of *A. phagocytophilum* derived from four roe deer (variants 4, 5, 7). In all cases, ambiguous nucleotides were detected at positions where in other sequences either one of the two possible nucleotides were found, possibly indicating double infections (Table 2). Four *A. phagocytophilum* sequences (variant 1) derived from the roe deer (sample MT886207) and from *I. ricinus* ticks obtained from one moose (sample MT886200) and two roe deer individuals (samples MT886201 and MT886203) were 100% identical to each other and to other European sequences reported from *I. ricinus* (Slovenia, KM205439) and *D. reticulatus* (Lithuania, JN181091) ticks and the roe deer from Germany (KU712165) and Slovenia (KM205437) (Figure). Four *A. phagocytophilum msp4* sequence variants that were found in five roe deer (samples MT886214, MT886208, MT886209, MT886210, MT886212) had unique nucleotide composition (variants 5, 6, 7, 10) and therefore differed from other previously identified *A. phagocytophilum* sequences in the GenBank database (Table 2). *A. phagocytophilum msp4* sequence derived from *D. reticulatus* tick (sample MT886202) collected from the red deer (variant 9) differed from other *msp4* sequences detected in this study and was identical to the sequences derived from the roe deer in Slovakia (EU180060) and Hungary (MF974860) (Table 2, Figure).

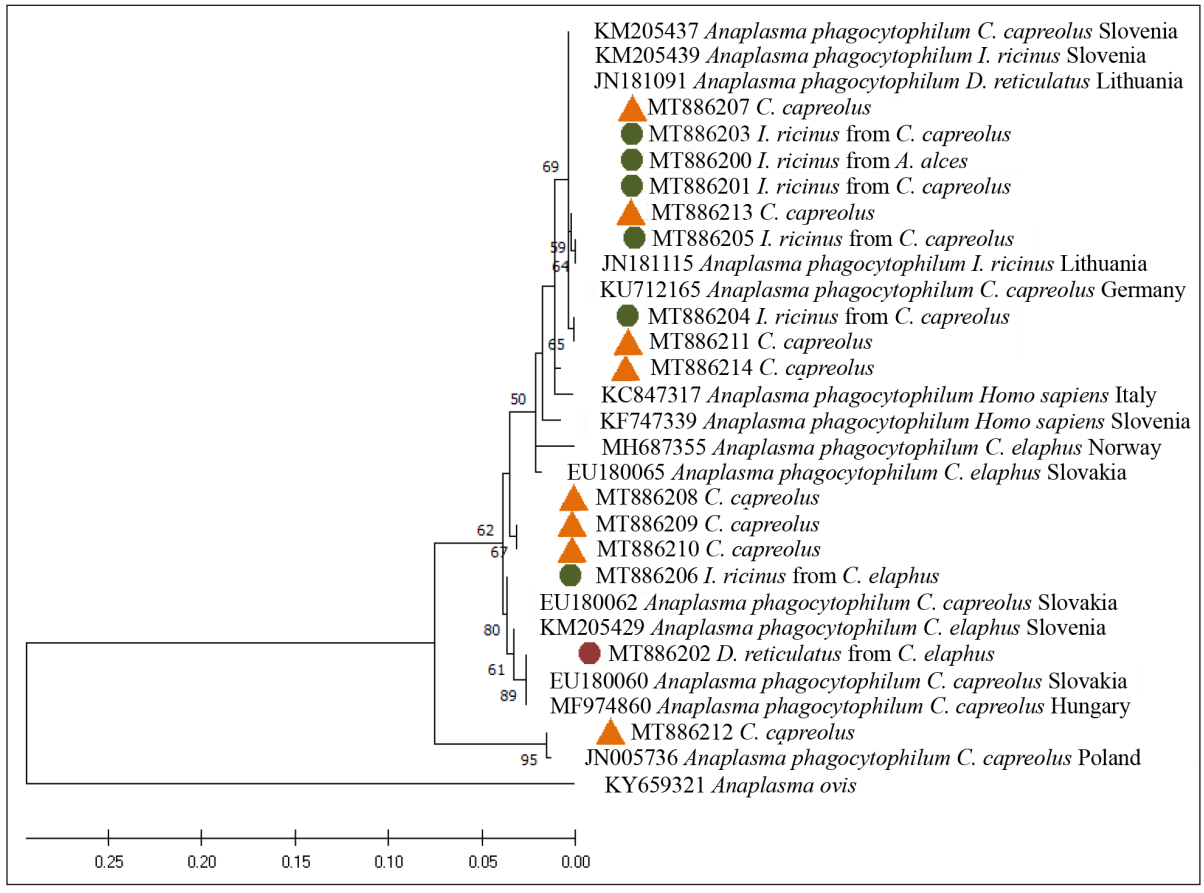

Figure. Phylogenetic tree of the *msp4* gene sequences of *A. phagocytophilum* created using the Neighbor–Joining method and bootstrap analysis of 1000 replicates. Sequences with accession numbers were taken from GenBank for comparison. Samples sequenced in the present study are marked: – *A. phagocytophilum* samples from roe deer; – samples from *I. ricinus* ticks; – sample from *D. reticulatus* tick

One roe deer (sample MT886212) harboured *A. phagocytophilum msp4* sequence (variant 10), which markedly differed from other sequences detected in ticks and roe deer in this study. The closely related *A. phagocytophilum msp4* sequence (differed at one nucleotide position) was identified in the roe deer in Poland (Figure).

The majority of the *A. phagocytophilum* strains circulating in Europe are associated with the infection of the ruminants (Stuen et al., 2013). Previous studies demonstrated high sequence heterogeneity among *A. phagocytophilum* strains in *msp4* gene isolated from different ruminant species. Bown et al. (2007) reported high variability of *A. phagocytophilum msp4* sequences (11 variants) derived from 20 different hosts from several European countries and the USA. Six different *A. phagocytophilum msp4* gene variants have been identified in the roe deer and six in the red deer in Norway (Ražanskė et al., 2019). In previous studies conducted in Lithuania, seven *msp4* gene variants of *A. phagocytophilum* were detected in *I. ricinus* and *Dermacentor reticulatus* ticks (Paulauskas et al., 2012). Most of *A. phagocytophilum* strains detected in *I. ricinus* and *D. reticulatus* ticks collected in Lithuania are identical to the strains derived from various cervid species (Paulauskas et al., 2012).

CONCLUSIONS

Our results provide new information on the prevalence and distribution of *A. phagocytophilum* strains in the roe deer and *I. ricinus* and *D. reticulatus* ticks collected from the roe deer, the red deer, and the moose in Lithuania. The results of the present study demonstrate high infection rates of *A. phagocytophilum* in ticks parasitizing different cervid species and the possible risk of transmitting these infections to their hosts. Five *msp4* gene variants of *A. phagocytophilum* were detected in ticks and seven in red deer. Ten *A. phagocytophilum msp4* sequences derived from ticks and the roe deer in this study had been previously described in ticks, the roe deer, the red deer, and the moose in other European countries, while five sequences derived from the roe deer differed from other previously identified *A. phagocytophilum msp4* gene sequences in the GenBank database.

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ANAPLASMA PHAGOCYTOPHILUM INFEKCIJOS, NUSTATYTOS LIETUVOJE ELNINIAMS GYVŪNAMS IR JUOS PARAZITUOJANČIOMS ERKĖMS, MOLEKULINĖ CHARAKTERISTIKA

Santrauka

Anaplasma phagocytophilum bakterija yra pagrindinis zoonotinės ligos – anaplazmozės – sukėlėjas. Pagrindiniai *A. phagocytophilum* pernešėjai yra *Ixodes ricinus* kompleksui priklausančios erkės. *A. phagocytophilum* turi platų geografinį pasiskirstymą ir didelę biologinę bei klinikinę įvairovę. Įvairiose šalyse atlikti epidemiologiniai tyrimai rodo, kad *A. phagocytophilum* paplitimas labai priklauso nuo erkių tankumo ir jų potencialių šeimininkų, tokių kaip elniniai gyvūnai, kurie yra vieni iš pagrindinių *Ixodes* genties erkių maitinimosi šaltinių. Lietuvoje elniniai gyvūnai yra svarbūs medžiojamieji gyvūnai, tačiau jų kaip *A. phagocytophilum* rezervuarų vaidmuo lieka nežinomas. Tyrimo tikslai buvo ištirti elninių gyvūnų ir juos parazituojančių erkių užsikrėtimą *A. phagocytophilum* bei apibūdinti *A. phagocytophilum* padermes, išskirtas iš elninių gyvūnų ir erkių, remiantis *msp4* geno sekų analize. Erkės buvo surinktos nuo 44 elninių gyvūnų (stirnų, tauriųjų elnių ir briedžių), sugautų 2010–2013 ir 2016–2017 m. medžioklės sezono metu. Iš viso nuo gyvūnų buvo surinktos 187 erkės. Buvo paimti 29 gyvūnų (27 stirnų ir 2 tauriųjų elnių) kraujo ir blužnies mėginiai. *A. phagocytophilium* DNR buvo nustatyta 10 (37,04 %) iš 27 stirnų. Bendras *I. ricinus* ir *D. reticulatus* erkių užsikrėtimas *A. phagocytophilum* atitinkamai buvo 39,3 % (70/178) ir 22,2 % (2/9). *A. phagocytophilum msp4* geno sekų analizė atskleidė devynis skirtingus sekų variantus: erkėse buvo aptikti penki *msp4* sekų variantai, o stirnose – septyni.

Raktažodžiai: *Anaplasma phagocytophilum*, erkės, *Ixodes ricinus*, *Dermacentor reticulatus*, elniniai gyvūnai