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Persistent vector-borne infections in free-ranging red deer (*Cervus elaphus*) during the winter season in Norway

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Red deer (*Cervus elaphus*) are maintenance hosts for different species of vector-borne pathogens. However, there are not many studies reporting the presence of pathogens and their co-infections in live red deer populations during winter season. Therefore, the aim of this study was to investigate the presence and prevalence of vector-borne infections in red deer during winter to determine the persistence of these infections. Blood samples were analysed from 38 free-ranging adult red deer that had been chemically immobilised during winter season in Norway. Thirty animals (78.9%) were infected with *Anaplasma phagocytophilum*, *Babesia* spp., or *Bartonella* spp. Seven animals were co-infected with two pathogens, and four animals had triple infections. Sequence analysis revealed the presence of *A. phagocytophilum*, *Babesia divergens*, *Babesia odocoilei*, *Babesia bovis*, and *Bartonella schoenbuchensis*. This study shows that free-ranging red deer can be considered as a potential host for zoonotic and nonzoonotic pathogens in Norway.

Keywords: *Anaplasma phagocytophilum*, *Babesia* spp., *Bartonella* spp., *Cervus elaphus*, Norway

INTRODUCTION

Free-ranging red deer (*Cervus elaphus*) are considered as potential hosts for vector-borne pathogens and may contribute to the persistence of different pathogen species (Johnson et al., 2021). Tick species such as *Ixodes ricinus* serve as a potential vector for a variety of zoonotic pathogens, including *Anaplasma phagocytophilum*, *Borrelia burgdorferi* sensu lato, and *Babesia* spp. In Norway, the distribution of ticks has expanded further north and to higher altitudes (Jore et al., 2011). A higher tick density in certain areas may be attributed to large established wild cervid populations (Gandy et al., 2021).

Anaplasma phagocytophilum replicates in the granulocytes of mammalian hosts, causing human granulocytic anaplasmosis (HGA) in humans as well as diseases in animals, including tick-borne fever of ruminants and granulocytic anaplasmosis in equines and canines (Woldehiwet, 2010; Stuen et al., 2013a). Some strains of *A. phagocytophilum* are pathogenic to both humans and animals, whereas others are associated

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solely with animal diseases. In Norway, DNA of *A. phagocytophilum* has been detected in *I. ricinus* ticks, sheep and wild ruminants, including roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*), and moose (*Alces alces*) (Stuen et al., 2013a, b; Puraite et al., 2015). Additionally, a few cases of human granulocytic anaplasmosis have been reported (Stuen at al., 2008).

Babesia species are intraerythrocytic protozoan parasites, causing tick-borne haemoprotozoan babesiosis disease worldwide. In Europe, babesiosis has been reported in humans as well as in various domestic and wild mammals (Herwaldt et al., 2003). Although human cases of babesiosis are rare, one case caused by *Babesia divergens* has been documented in Norway (Mørch et al., 2015). In contrast, *B. divergens* infection is widespread in cattle, particularly in the coastal regions of southern Norway (Hasle et al., 2010).

Bartonella species are gram-negative intracellular bacteria that cause long-lasting intraerythrocytic infections in mammals (Chomel et al., 2009). Blood-feeding arthropods, including various species of biting flies, fleas, lice, and hard ticks, have been confirmed or are suspected to be competent vectors for the transmission of *Bartonella* spp. (Tsai et al., 2011). The deer ked, *Lipoptena cervi*, which is the predominant *Lipoptena* species in Europe, parasitises free-ranging cervids such as roe deer, red deer, and moose (Mysterud et al., 2016). Over the past few decades, *L. cervi* has become widespread across the Nordic countries (Välimäki et al., 2010).

The objective of the present study was to investigate the presence and prevalence of vector-borne infections in live red deer during winter to determine the persistence of these infections.

MATERIALS AND METHODS

Study area and blood sampling. Blood samples were collected from 38 chemically immobilised free-ranging adult red deer, comprising 29 hinds and nine stags. The animals observed at the feeding stations showed no clinical symptoms. The sampling took place at nine different winter-feeding sites located in Agder and Telemark counties in Norway, in January and February 2014, 2015, and 2016 (Fig. 1). Two

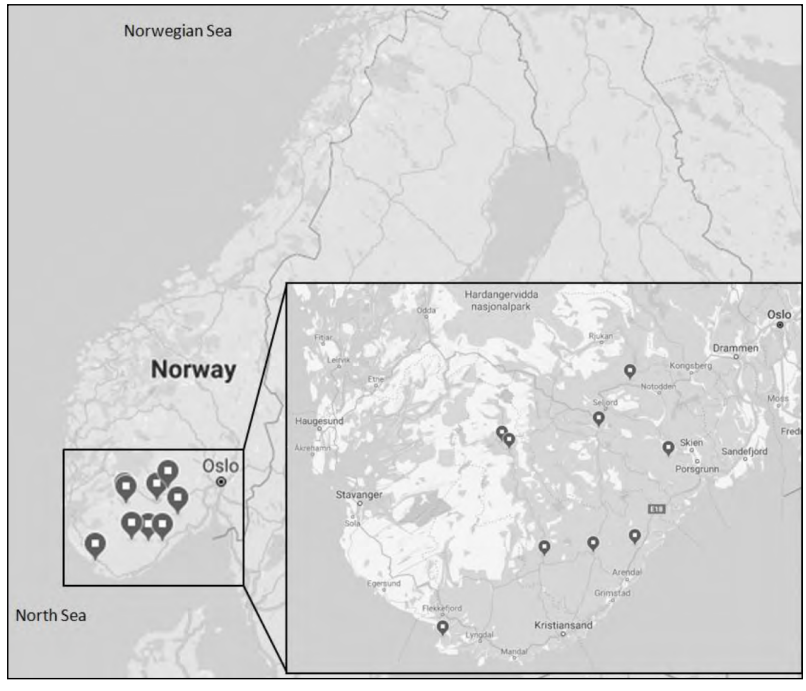

Fig. 1. Sampling locations in the counties of Telemark and Agder in Norway

coastal sites were snow-covered for shorter periods, ranging from two to three weeks, whereas the other seven sites were covered with snow for at least two months. The animals were darted and immobilised using a mixture of xylazine-tiletamin-zolazepam (Rompun® dry powder, Bayer AG, Leverkusen, Germany, and Zoletil forte® dry powder, Virbac International, Carros Cedex, France). Blood samples were obtained from the jugular vein and collected in 9 ml EDTA plastic tubes (Greiner Bio-One GmbH, Germany). The tubes were frozen at –20°C and stored until DNA extraction. The red deer were healthy before immobilisation. They were fitted with GPS collars and then released.

DNA extraction and PCR amplification. DNA was extracted from 200 µl of each blood sample using a commercial kit (GeneJET Whole Blood Genomic DNA Purification Mini Kit, Thermo Fisher Scientific, Lithuania), following the manufacturer's instructions.

The samples were tested for *A. phagocytophilum* and *Babesia* spp. DNA using a multi-plex real-time PCR assay targeting a 98 bp fragment of the *A. phagocytophilum msp2* gene and a 214 bp fragment of the *Babesia* spp. *18S rRNA* gene (Razanske et al., 2019). The multiplex real-time PCR reaction was performed in a total volume of 15 µl, consisting of 1x Sensi-Mix™ II Probe No-ROX (Bioline, UK), 1 µM of each primer, 0.5 µM of each probe, and 100 ng of extracted DNA. The PCR conditions included 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. Results were considered positive when repeats yielded cycle threshold (Ct) values <38.00. Amplification of a 381 bp fragment of the *A. phagocytophilum msp4* gene was accomplished using the primers MAP4AP5 and MSP4AP3 in the first step of PCR, followed by msp4f and msp4r in the nested PCR, as previously described (de la Fuente et al., 2005; Bown e al., 2007). A partial *18S rRNA* gene fragment (380 bp) of *Babesia* spp. were amplified by nested PCR using primers BS1/BS2 and PiroA/PiroC (Armstrong et al., 1998; Rar et al., 2005).

For the detection of *Bartonella* spp., a nested PCR was used to amplify a 0.9–1.6 kb fragment of the *16S-23S rRNA* intergenic spacer region (ITS) (Kaewmongkol et al., 2011).

Positive, previously tested samples (*A. phagocytophilum* – KT070846; *B. capreoli* – KT279880 and *Bartonella* sp. – MF491746), and negative reaction controls (distilled water without DNA) were used in all reactions.

DNA sequencing and sequence analysis. The obtained PCR products were visualised under UV illumination after electrophoresis on 1.5% agarose gels stained with ethidium bromide using a 100 bp DNA ladder as a molecular-weight size marker (Thermo Fisher Scientific, Lithuania). Amplicons were purified using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Lithuania) and sequenced.

MEGA11: Molecular Evolutionary Genetics Analysis version 11 (Tamura, Stecher, and Kumar 2021) was used in DNA sequences alignments and phylogenetic analysis. Phylogenetic trees were created using the Kimura-2 parameter model, neighbor joining (NJ) statistical method. Sequences of *A. phagocytophilum* strains, *Babesia* and *Bartonella* species were downloaded from GenBank database and implemented in the sequence alignment. The stability of inferred phylogenies was assessed by bootstrap analysis of 1000 randomly generated sample trees.

Nucleotide sequences were deposited in GenBank with the accession numbers MK626490-MK626495 (*msp4* gene of *A. phagocytophilum*), MK612774-MK612777 (*18S rRNA* gene of *Babesia* spp.), and MK620813-MK620817 (*ITS* region of *Bartonella* spp.).

RESULTS

Prevalence

A total of 30 (78.9%) blood samples from red deer were positive for at least one of the tested pathogens (*A. phagocytophilum*, *Babesia* spp. or *Bartonella* spp.) (Table 1). After performing a comparison of real-time PCR and nested PCR results, it was found that higher infection rates were observed using real-time PCR

Table 1. **Detection of *A. phagocytophilum*, *Babesia* spp. and *Bartonella* spp. in red deer (*Cervus elaphus*) using real-time and nested PCR**

Infection	Number of positive samples	Number of negative samples
A. phagocytophilum PCR[1]	12 (31.6%)	26
A. phagocytophilum PCR[2]	11 (28.9%)	27
Babesia spp. PCR[1]	18 (47.4%)	20
Babesia spp. PCR[2]	7 (18.4%)	31
Bartonella spp. PCR	21 (55.3%)	17
Double infection An + Bar PCR[1, 2]	5 (13.2%)	33
Double infection Bab + Bar PCR[1, 2]	2 (5.3%)	36
Triple infection An + Bab + Bar PCR[1, 2]	4 (10.5%)	34

[1] real-time PCR, [2] nested PCR, An – *A. phagocytophilum*, Bar – *Bartonella* spp., Bab – *Babesia* spp.

assay. A total of 12 (31.6%) of the tested animals were positive for *A. phagocytophilum* and eight (47.4%) for *Babesia* spp. meanwhile, the infections using nested PCR were 11 (28.9%) and seven (18.4%), respectively. *Bartonella* spp. DNA was detected in 55.3% of red deer using nested PCR. Seven animals were co-infected with two of the tested pathogens, while four were infected with all three.

Anaplasma phagocytophilum

Seven of the *A. phagocytophilum* positive samples of the partial *msp4* gene were subjected to phylogenetic analysis. Analysis of the obtained sequences revealed the presence of 14 polymorphic sites, which enabled six variants of the analysed fragment to be distinguished (Table 2). All six sequence variants were identical to sequences from the GenBank database (Fig. 2).

***Babesia* spp.**

Seven PCR products of partial *18S rRNA* gene of *Babesia* spp. were sequenced, and two *Babesia* species, *B. odocoilei* (n = 3) and *B. divergens* (n = 4), were identified (Fig. 3). Comparison with the data in the GenBank revealed three *B. odocoilei* variants in European countries with two polymorphic sites (Table 3). *Babesia odocoilei* sequences derived from countries in the American continent formed a separate group. *Babesia divergens* sequences obtained in this study were unique and showed two variants with one polymorphic site (Table 4). Additionally, five variable positions were detected by comparing the sequences with data from the GenBank.

***Bartonella* spp.**

Sequence analysis of the *16S-23S rRNA* ITS of *Bartonella* isolates demonstrated eight strains

Table 2. **Variable sites within the *A. phagocytophilum msp4* gene sequences described in this study**

Accession numbers	Nucleotide position													
	3	24	30	45	60	84	102	129	171	180	186	201	240	246
MK626490	A	T	T	G	A	T	C	C	T	T	C	T	G	T
MK626491	.	.	C	A	G	.	.	.	.	C	.	C	.	.
MK626492	.	.	C	.	.	.	.	.	C	.	.	C	.	.
MK626493	G	C	.	.	.	C	.	T	.	.	A	.	.	C
MK626494	.	C	.	.	.	.	.	.	C	.	.	C	A	C
MK626495	.	C	C	.	.	.	T	.	.	.	.	.	.	.

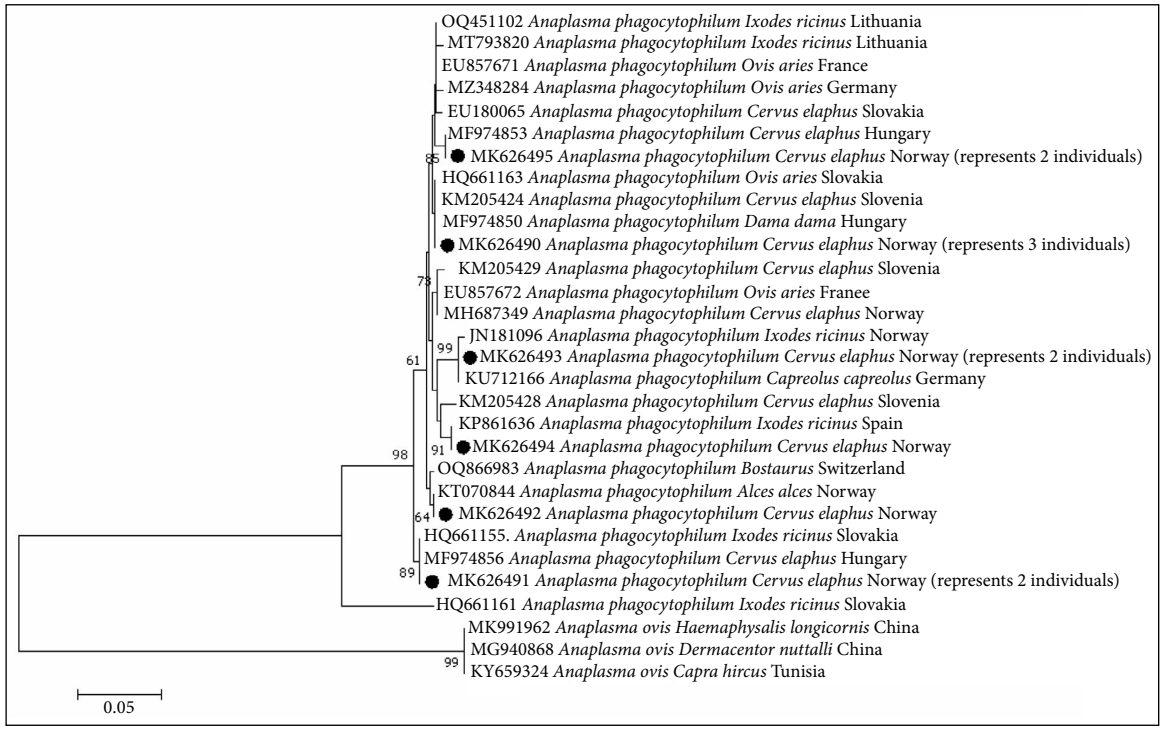

Fig. 2. Phylogenetic tree of *A. phagocytophilum msp4* gene sequences created using the neighbor-joining clustering method and Kimura 2-parameter model (1000 replicates; bootstrap values indicated at the nodes). Samples from this study are marked ● Sequence of *Anaplasma ovis* was used as outgroup

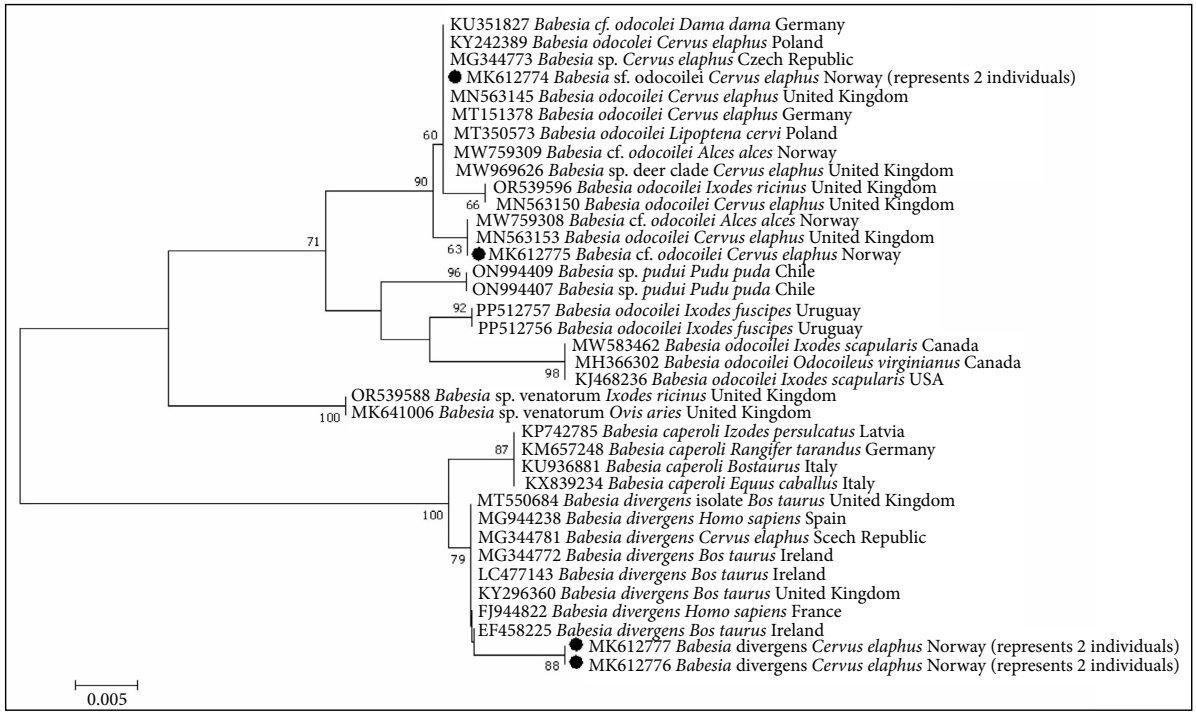

Fig. 3. Phylogenetic tree of *Babesia* spp. isolates based on *18S rRNA* gene, created using the NJ clustering method and Kimura-2 parameter model (1000 replicates; bootstrap values indicated at the nodes). Samples from this study are marked ●

Table 3. **Variable sites within the *B. odocoilei***

Variants	Accession numbers	Nucleotide position	
		174	204
1	MK612775[1], MW759308, MN563153	G	A
2	MK612774[1], MW759309, KU351827, MW969626, MG344773, KY242389, MN563145, MT151378, MT350573	T	.
3	OR539596, MN563150	T	C

[1] Sequences obtained in this study.

Table 4. **Variable sites within the *B. divergens***

Variants	Accession numbers	Nucleotide position				
		3	294	295	296	298
1	MK612776[1],	G	T	A	T	T
2	MK612777[1],	A	.	.	.	.
3	MG344772, LC477143	C	–	–	C	A

[1] Sequences obtained in this study.

97.7% similar to *B. bovis* and 13 strains 97.7% similar to *B. schoenbuchensis* (Fig. 4). The results of the comparison of 546 bp sequences obtained in this study revealed two strains of *B. bovis* with 15 variable sites (Table 5) and showed similarity to *B. bovis* sequences isolated from *C. capreolus* and *A. alces* from Norway. Meanwhile, *B. schoen-buchensis* sequences showed 42 variable sites (Table 6) and grouped with *B. schoenbuchensis* sequences isolated from different hosts (*C. elaphus*, *L. cervi*, *Lipoptena fortisetosa* and *Oeciacus hirundinis*) from European countries.

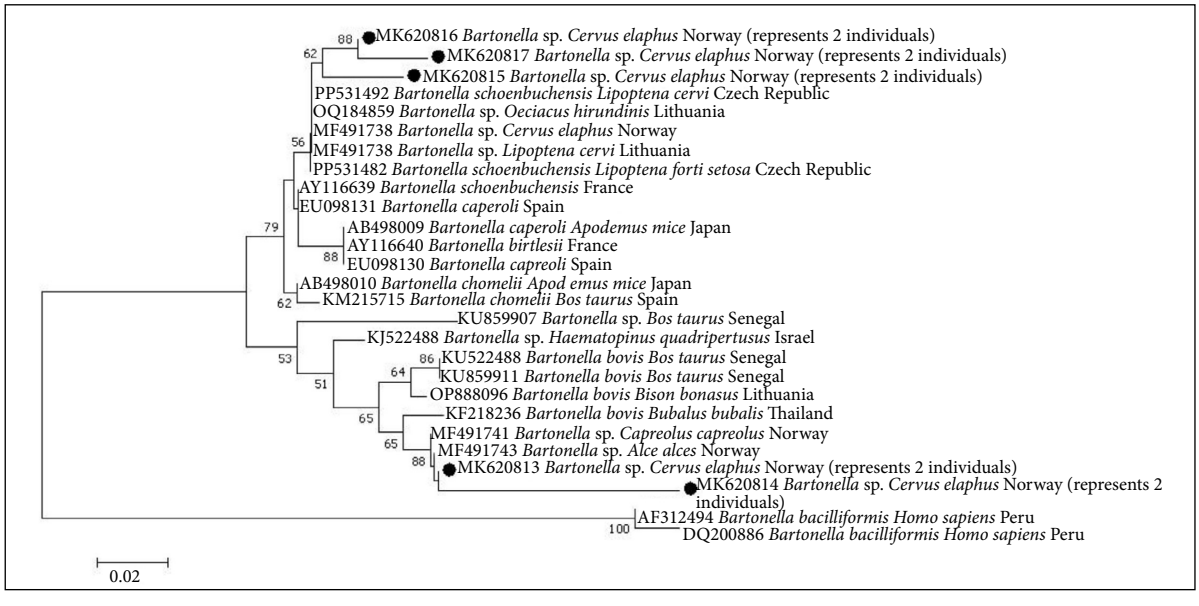

Fig. 4. Phylogenetic tree of *Bartonella* spp. isolates based on *16S-23S rRNA* ITS sequences, created using the NJ clustering method and Kimura-2 parameter model (1000 replicates; bootstrap values indicated at the nodes). Samples from this study are marked ●

Table 5. **Variable sites within *B. bovis 16S-23S rRNA* ITS sequences described in this study**

Accession numbers	Nucleotide position														
	7	22	74	82	83	110	119	120	133	134	136	152	153	164	173
MK620813	T	T	T	T	C	C	T	T	T	T	T	T	G	T	T
MK620814	A	C	A	A	T	T	C	C	C	C	C	C	C	C	C

Table 6. **Variable sites within *B. schoenbuchensis 16S-23S rRNA* ITS sequences described in this study**

Accession numbers	Nucleotide position																				
	3	15	27	34	55	58	69	93	99	110	127	130	156	178	225	231	236	252	340	342	350
MK620815	C	T	T	T	T	T	G	G	T	T	T	T	C	T	C	G	C	C	T	G	G
MK620816	T	.	G	.	.	G	.	C	A	C	.	G	T	.	G	T	T	.	A	A	C
MK620817	G	C	G	A	A	G	A	C	A	C	A	G	.	G	G	T	T	A	.	.	.

Accession numbers	Nucleotide position																				
	358	376	388	396	411	435	443	456	466	478	491	497	498	502	511	515	516	517	518	525	529
MK620815	C	T	A	G	G	T	T	T	G	G	A	G	C	A	A	T	G	T	T	C	T
MK620816	T	G	T	A	A	G	.	.	A	A	T	T	T	T	.	A	A	A	A	T	A
MK620817	G	.	.	.	.	.	G	G	.	A	.	A	A	.	G	A	A	A	A	.	A

DISCUSSION

In the present study, three pathogens including *A. phagocytophilum*, *Babesia* spp., and *Bartonella* spp. were detected in red deer during the winter season. The results indicated a significant presence of double and triple co-infections (28.9%) with *A. phagocytophilum*, *Babesia* spp., and *Bartonella* spp. (Table 1). Naturally existing co-infections in free-living red deer populations represent a potentially underestimated public health risk. Vectors such as *I. ricinus* ticks and *L. cervi* deer keds can carry several pathogens simultaneously, increasing the likelihood of co-transmission to animals (Nebbak et al., 2019; Jaenson et al., 2024). According to Mysterud et al. (2016), in Norway, adult deer keds emerge and seek hosts from late summer to the end of autumn, after which they spend the winter and the rest of their lives on the same host (Kaunisto et al., 2009). Additionally, a recent study reported winter activity and host-seeking behaviour of *I. ricinus* ticks in Scandinavia (Kjellander et al., 2023). Previous research also demonstrated

that pathogens such as *A. phagocytophilum* can alter the behaviour of *Ixodes* species ticks, like *I. scapularis*, increasing factors such as activity and cold resistance (Kjellander et al., 2023; Neelakanta et al., 2010).

Identification of *A. phagocytophilum* in the present study was obtained by real time and nested PCR (31.6% and 28.9%, respectively) (Table 1). The high infection rate detected during the winter period on apparently healthy red deer suggests that they have adapted to the infection and seems to be persistently infected without showing clinical symptoms. It is known that *A. phagocytophilum* may cause a chronic / systemic infection in sheep, horses, dogs, cattle, and red deer (Stuen et al., 2013a; Jahfari et al., 2014). However, the detection of *A. phagocytophilum* DNA in samples may also represent cases of reinfection (Hovius et al., 2018). Previous studies reported a higher prevalence of *A. phagocytophilum* (81.1% and 94%) in red deer collected in Norway during the autumn hunting season (Razanske et al., 2019; Stigum et al., 2019). In tick-infested locations (during the autumn hunting season), *A. phagocytophilum* was also detected in moose (41.4%) (Puraite et al., 2015) and in roe deer (88.1%) (Razanske et al., 2019). Additionally, *L. cervi* also can transmit *Anaplasma* species. As suggested by Vichova et al. (2011) and de Bruin et al. (2015) in the study from Slovakia and Hungary, deer ked may be mechanical vectors for the transmission of *A. phagocytophilum*.

In this study, the detection of *Babesia* DNA-was based on real-time PCR and nested PCR (47.4% and 18.4%, respectively) (Table 1). Previous studies revealed that red deer were infected with four *Babesia* species, including *B. capreoli*, *B. divergens*, *B. odocoilei*, and *B. venatorum* (Razanske et al., 2019; Welc-Falęciak e al., 2013). In the present study, zoonotic *B. divergens* and non-zoonotic *B. odocoilei* species were confirmed based on *18S rRNA* gene sequencing analysis. Clinical babesiosis in free-ranging wild ruminants appears to be rare, and silent babesiosis seems to be normal (Penzhorn, 2006). However, severe infections due to *B. divergens* and *B. odocoilei* were found in reindeer (Langton et al., 2003), but they normally live in tick-free areas (mountains) and are probably not adapted to the infection. In this study, apparently healthy adult red deer were studied, and the persistent infections seemed to cause asymptomatic babesiosis.

In the present study, a high *Bartonella* infection rate (55.3%) was detected in red deer during the winter period. Out of 21 sequences isolated, eight were identified as similar to *B. bovis*, while 13 were similar to *B. schoenbuchensis* clade (Fig. 4). The high prevalence of *Bartonella* observed in the blood of red deer during the winter period could indicate a persistent infection in these animals. However, as *L. cervi* adults could be found on cervid skin during wintertime, transmission of *Bartonella* to red deer from infected deer ked could occur during winter. Previously, *Bartonella* DNA was detected in 10.5% of roe deer, 35.1% of red deer, 36% of moose, 85% of adult wingless deer ked in Norway (Razanske et al., 2018; Halos et al., 2004). The detected *Bartonella* strains in red deer belonged to different lineages, one of which was closely related to *B. capreoli*, *B. chomelii*, and *B. schoenbuchensis* clade and the other to *B. bovis* (Halos et al., 2004). However, only *Bartonella* strains from the *Bartonella* clade associated with *B. capreoli*, *B. chomelii*, and *B. schoenbuchensis* were identified in deer keds (Razanske et al., 2018; Halos et al., 2004). *Bartonella* strains closely related to *B. schoenbuchensis* and *B. chomelii* were detected in other species of biting flies – louse fly (*Hippobosca equina*) and sheep ked (*Melophagus ovinus*) collected from wild and domestic ruminants in France and Romania (Chomel et al., 2009; Billeter et al., 2008; Skonhoft et al., 2013).

The results of this study provide evidence of multiple persistent vector-borne infections in red deer in Norway. The relatively high vector-borne infection rate detected in live, free-ranging red deer during winter indicates the role of red deer as a reservoir host. Additionally, the co-infection with three different pathogens (*A. phagocytophilum*, *Babesia* spp., and *Bartonella* spp.) highlights the need for further studies to fully understand the impact of the zoonotic pathogens.

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NUOLATINĖS VEKTORIŲ PLATINAMOS INFEKCIJOS ŽIEMĄ LAISVAI BESIGANANČIŲ TAURIŲJŲ ELNIŲ (*CERVUS ELAPHUS*) POPULIACIJOJE NORVEGIJOJE

Santrauka

Taurieji elniai (*Cervus elaphus*) yra įvairiais vektoriais pernešamus patogenus palaikantys šeimininkai. Nėra daug tyrimų apie tauriųjų elnių patogenus ir jų koinfekcijas žiemos sezoną, todėl šio tyrimo tikslas buvo ištirti vektorių pernešamas infekcijas, jų paplitimą ir išlikimą šioje populiacijoje. Išanalizuoti 38 laisvai gyvenančių suaugusių tauriųjų elnių kraujo mėginiai, surinkti po jų cheminio imobilizavimo žiemos sezono metu Norvegijoje. Trisdešimt gyvūnų (78,9 %) buvo užsikrėtę *Anaplasma phagocytophilum*, *Babesia* spp. arba *Bartonella* spp., septyni gyvūnai buvo koinfekuoti dviem patogenais, o keturiems nustatytos trys vienu metu pasireiškusios koinfekcijos. Sekoskaitos metu identifikuota *A. phagocytophilum*, *Babesia divergens*, *B. odocoilei*, *Bartonella bovis* ir *B. schoenbuchensis*. Šis tyrimas rodo, kad laisvai gyvenantys taurieji elniai Norvegijoje gali būti potencialūs zoonozinių ir nezoonozinių patogenų šeimininkai.

Raktažodžiai: *Anaplasma phagocytophilum*, *Babesia* spp., *Bartonella* spp., *Cervus elaphus*, Norvegija