Prevalence and co-infection with tick-borne *Anaplasma phagocytophilum* and *Babesia* spp. in red deer (*Cervus elaphus*) and roe deer (*Capreolus capreolus*) in Southern Norway

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ABSTRACT

Anaplasma phagocytophilum and *Babesia* spp. are causative agents of tick-borne infections that are increasingly considered as a threat to animal and public health. To assess the role of cervids in the maintenance of zoonotic pathogens in Norway, we investigated the prevalence of *A. phagocytophilum* and *Babesia* spp. in free-ranging roe deer and red deer. Initial screening of spleen samples of 104 animals by multiplex real-time PCR targeting the major surface protein (*msp*2) gene and 18S rRNA revealed the presence of *A. phagocytophilum* infection in 81.1% red deer (*Cervus elaphus*) and 88.1% roe deer (*Capreolus capreolus*), and *Babesia* spp. parasites in 64.9% red deer and 83.6% roe deer, respectively. Co-infections were found in 62.2% red deer and 79.9% roe deer. Nested PCR and sequence analysis of partial *msp*4 and 18S rRNA genes were performed for molecular characterization of *A. phagocytophilum* strains and *Babesia* species. A total of eleven *A. phagocytophilum* *msp4* gene sequence variants were identified: five different variants were 100% identical to corresponding *A. phagocytophilum* sequences deposited in the GenBank database, while other six sequence variants had unique nucleotide polymorphisms. Sequence analysis of the 18S rRNA gene demonstrated the presence of multiple *Babesia* species, including *Babesia capreoli*, *Babesia divergens*, *Babesia venatorum* and *Babesia odocoilei/Babesia* cf. *odocoilei*. This study is the first report demonstrating the prevalence and molecular characterization of *A. phagocytophilum* strains and *Babesia* species in roe deer and red deer in Norway. The high infection and co-infection rates with *A. phagocytophilum* and *Babesia* spp. in red deer and roe deer suggest that these cervids may play an important role in the transmission of single and multiple pathogens.

1. Introduction

During the past two decades, the distribution and abundance of *Ixodes ricinus* ticks have increased in Europe due to climate changes (Medlock et al., 2013). *I. ricinus* is ubiquitous on the coastline of Southern Norway. Norway represents the northern limit in the geographical distribution of *I. ricinus*, and recently an expansion of the range further north and at higher elevations has been reported (the study was based on a questionnaire for veterinarians and occasional finding of adult ticks) (Jore et al., 2011). Soleng et al. (2018) reported that only a few ticks were collected in areas located at 66.1°N and no ticks were found further north up to 67.6°N using flagging method. Ticks occurring in new areas may increase the risk of tick-borne

infections, e.g., Lyme borreliosis, anaplasmosis and babesiosis. Hosts of the adult *I. ricinus* ticks are large vertebrates. Tick densities are likely to be strongly influenced by population density fluctuations in vertebrate host species and wildlife management. This may lead to the high tick densities in areas with free-ranging cervids (Wilson et al., 1988; Paul et al., 2016).

Tick-borne fever, caused by *Anaplasma phagcytophilum*, is a well-known disease of domestic animals in several countries in Europe, Asia, and America (Woldehiwet, 2010). The disease is characterized by infected neutrophils, high fever, neutropenia, reduced milk yield, abortion and reduced fertility in domestic sheep (Woldehiwet and Scott, 1993; Stuen et al., 2013b). In Europe, besides domestic ruminants, *A. phagocytophilum* has been identified in horses (*Equus caballus*)

(Silaghi et al., 2011b); in wild ruminants: roe deer (*Capreolus capreolus*) (Alberdi et al., 2000), red deer (*Cervus elaphus*) (Stuen et al., 2013a; Petrovec et al., 2002), moose (*Alces alces*) (Puraite et al., 2015a), fallow deer (*Dama dama*), sika deer (*Cervus nippon*) (Hapunik et al., 2011), chamois (*Rupicapra rupicapra*), Alpine ibex (*Capra ibex*), mouflon (*Ovis musimon*) (Silaghi et al., 2011a), European bison (*Bison bonasus*) (Scharf et al., 2011); in domestic animals: dogs (Egenvall et al., 1997), cats (Heikkilä et al., 2010); in small mammals (rodents and insectivores) (Liz et al., 2000); birds (de la Fuente et al., 2005) and humans (Petrovec et al., 1997; Strle, 2004).

Babesiosis is an emerging zoonotic disease and various wildlife species are reservoir hosts for zoonotic species of *Babesia* (Yabsley and Shock, 2012). Babesiosis signs vary in severity from asymptomatic infections to acute circulatory shock with anemia, dependent on immunity status, host age, *Babesia* species and parasite load (Zintl et al., 2003; Penzhorn, 2006). *Babesia* spp. have developed strategies to avoid the immune response, that enable them to survive inside the vertebrate host, with the production of antibodies beginning approximately 7 days after infection and persisting for several months (Chauvin et al., 2009). *B. divergens* is the primary agent of bovine babesiosis in northern Europe (Zintl et al., 2003) and is a reported human pathogen (Gorenflot et al., 1998). *B. divergens*, *B. capreoli*, *B. venatorum* have been described as occurring in wild European cervids (Duh et al., 2005; Bonnet et al., 2007; Malandrin et al., 2010; Zintl et al., 2011). *Babesia* antibodies have been found in pastured cows from southern Norway (Hasle et al., 2010).

The overall prevalence of *Babesia* spp. in questing *I. ricinus* ticks from various regions of Norway was 0.9% (Radzijevskaja et al., 2008; Øines et al., 2012), and included four *Babesiae* species, *B. venatorum*, *B. divergens*, *B. capreoli* and *Babesia* CH1-like strain. Another *Babesia* species *B. microti* has been detected in small rodents (Wiger, 1979). The occurrence of *B. capreoli* and *B. odocoilei*-like species in Norwegian moose has recently been reported (Puraite et al., 2015b). However, there continues to be a lack of data for the presence of *A. phagocytophilum* and *Babesia* spp. in roe deer and red deer in Norway.

The aim of the study was to investigate the prevalence of *A. phagocytophilum* and *Babesia* spp. in free ranging roe deer and red deer from Norway and to characterize the pathogens by sequence analysis of the partial *msp4* gene and 18S rRNA, respectively.

2. Materials and methods

Spleen samples were collected from 104 free ranging roe deer (67) and red deer (37) by hunters in the southern part of Norway during the hunting season in autumn 2013–2016. DNA was extracted from frozen spleens using Genomic DNA Purification Kit (Thermo Fisher Scientific) and stored at −20 °C until PCR analysis. The samples were screened for the presence of selected pathogen DNA using multiplex real time-PCR assay designed for this study to amplify a 98 bp product from *A. phagocytophilum* (*msp2* gene) and 214 bp product from *Babesia* spp. (18S rRNA) (Table 1). Multiple real time-PCR reaction was done in a total volume of 15 μl consisting 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.

Partial *msp4* gene of *A. phagocytophilum* (de la Fuente et al., 2005; Bown et al., 2007) and 18S rRNA of *Babesia* spp. (Armstrong et al., 1998; Rar et al., 2005) were amplified by nested PCR. As positive controls, isolates of *A. phagocytophilum* (**KT070846**) and *B. capreoli* (**KT279880**) from moose were used. Negative controls (sterile distilled water instead of DNA) were used in real time-PCR and nested PCR. PCR products were resolved through electrophoresis on a 1.5% agarose gel with ethidium bromide and visualized under UV light. The nested PCR amplification products of the *msp4* gene of *A. phagocytophilum* and 18S rRNA of *Babesia* spp. were extracted from the gel and purified using GeneJET Gel Extraction Kit (Thermo Fisher Scientific) following the manufacturer's instructions. Obtained sequences were compared with sequences available from the GenBank database with the BLASTn tool of the National Center for Biotechnology. Phylogenetic analysis was conducted using MEGA6 software (Tamura et al., 2013). Maximum-Likelihood tree with reliability tested using bootstrapping with 1000 replicates was constructed.

Sequences of *A. phagocytophilum* and *Babesia* spp. obtained in this study have been deposited in GenBank under the accession numbers **MH687349 - MH687360** and **MH697657 - MH697663**, respectively.

3. Results

Both *A. phagocytophilum* and *Babesia* spp. DNA was detected in examined red deer and roe deer spleen samples (Table 2). Based on real time PCR assays, 88.1% roe deer were positive for *A. phagocytophilum* and 83.6% were positive for *Babesia* spp. A total 81.1% red deer were positive for *A. phagocytophilum* and 64.9% were positive for *Babesia* spp. Co-infections were detected in 79.9% roe deer and 62.2% red deer (Table 2). Lower infection rates were observed using nested PCR. *A. phagocytophilum* DNA was detected in 52.2% roe deer and 51.4% red deer, while *Babesia* DNA was detected in 52.2% roe deer and 32.4% red deer. Co-infections were observed in 29.9% roe deer and 21.6% red deer (Table 2).

Sequencing of the partial (~380 bp) *msp4* gene of *A. phagocytophilum* showed 11 different sequence types among the 25 samples derived from roe deer (n = 15) and red deer (n = 10). Five different variants (two sequence variants were derived from red deer **MH687349**, **MH687350**, two other sequence variants derived from roe deer **MH687351**, **MH687352** and one sequence variant was common to both species, roe deer **MH687353** and red deer **MH687354**) with 100% identity to sequences deposited in the GenBank database, and six new sequence variants (**MH687355 - MH687360**) were identified (Table 3). Ambiguous nucleotides were observed in *msp4* sequences of *A. phagocytophilum* derived from 3 roe deer. In all cases ambiguous nucleotides were detected at positions where in other sequences either one of the two possible nucleotides was found, possibly indicating double infections.

A total of 31 sequences of the partial 18S rRNA gene (~300 bp) of

Table 1
Real time PCR primer sequences designed and used in this study.

Primer or probe	Sequence (5′-3′)	Target in assay
Primers		
*Anaplasma*_F	GGACAACATGCTTGTAGCTATGGAAGG	*A. phagocytophilum msp*2 gene
*Anaplasma*_R	CCTTGGTCTTGAAGCGGCTCGTA	
*Babesia*_F3a	GACTCCTTCAGCACCTTGAGA	*Babesia* spp. 18S rRNA
*Babesia*_F3b	GACCCCTTCAGGAGCTTGAGA	
*Babesia*_R3	CATGCACCACCACCCAWAGAATCA	
Probes		
*Anaplasma*_Zr	HEX-TCTCAAGCTCAACCCTGGCACCACCA-BHQ1	*A. phagocytophilum msp*2 gene
*Babesia*_Z	ROX-TGACGGAAGGGCACCACCAGGCGT-BHQ2	*Babesia* spp. 18S rRNA

Table 2
Detection of *Anaplasma phagocytophilum*, *Babesia* spp. in spleens of roe deer and red deer using nested PCR[a] and real time PCR[b].

Species	No. Total	No. positive (%)					
		Anaplasma phagocytophilum[a]	*Anaplasma phagocytophilum*[b]	*Babesia* spp.[a]	*Babesia* spp.[b]	Co-inf.[a+a]	Co-inf.[b+b]
Roe deer	**67**	35 (52.2%)	59 (88.1%)	35 (52.2%)	56 (83.6%)	20 (29.9%)	53 (79.9%)
Red deer	**37**	19 (51.4%)	30 (81.1%)	12 (32.4%)	24 (64.9%)	8 (21.6%)	23 (62.2%)

Babesia spp. were analyzed. Sequences derived from roe deer (n = 25) showed 99–100% similarity with *Babesia capreoli* (n = 18) (e.g. Genbank accession nos. **KT279880**, **KP15558**, **KU351824**), 100% nucleotide identity to *Babesia divergens* (n = 3) (e.g. Genbank accession nos. **HQ395757**, **GQ304525**, **KY242398**) and *Babesia venatorum* (n = 4) (e.g. Genbank accession nos. **KY945505**, **MG052939**, **KF773722**). Sequences derived from red deer (n = 6) were 100% identical to *B. divergens* (n = 1). Other sequences isolated in this study from red deer (n = 5) clustered with *Babesia odocoilei* and *B.* cf. *odocoilei* strains detected in cervids and *I. ricinus* ticks in Europe (**KM657254**, **KU550687**, **JX679176**, **KU351828**, **KT279886**, **JN543180**) and together with *B. odocoilei* sequences from the North America clade formed a separate group in phylogenetic tree. Phylogenetic analysis demonstrates genetic variability of *B. odocoilei*

strains from different geographic regions (Fig. 1).

4. Discussion

During the last two decades, the emergence of anaplasmosis has become more frequently diagnosed as the cause of human infections (Ismail et al., 2010). This may be partly influenced by increased numbers of animal reservoirs and tick vectors in areas now inhabited by humans, as well as increased public awareness of infection diseases. In western Norway, a part of the healthy adult population was tested by an indirect immunofluorescence assay (IFA) and 16.2% (49/301) of tested individuals were found exposed to *Anaplasma* spp. (Hjetland et al., 2015). Antibodies against different *Anaplasma* variants have shown to persist in sheep, horses, dogs, cattle, moose and red deer (Stuen et al.,

Table 3
Differences in the *msp4* nucleotide sequences among *A. phagocytophilum* strains. Strains detected in this study are bolded. Abbreviations: DE - Germany, FR - France, NO - Norway, PL - Poland, SK - Slovakia, SL - Slovenia, SP - Spain.

| Strain	15	18	24	25	26	28	36	39	54	67	69	72	78	87	123	132	138	141	150	154	159	165	172	174	177	181	195	204	216	222	234	237	240	246	249	261
EU857672 *Ovis aries* FR	G	C	T	G	C	A	T	G	A	A	C	C	T	A	C	A	T	C	T	T	G	T	A	T	C	G	C	T	G	C	G	T	T	A	A	T
EU857673 *Ovis aries* FR	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
EU180062 *Capreolus capreolus* SK	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
JN181108 *Ixodes ricinus* NO	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KM205420 *Rupicapra rupicapra* SL	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
MH687349 *Cervus elaphus*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KT070837 *Alces alces* NO	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	A	.	C	.	.	.
EU857665 *Bos taurus* FR	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	A	.	C	.	.	.
AY706389 *Bison bonasus* PL	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	A	.	C	.	.	.
MH687350 *Cervus elaphus*	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	A	.	C	.	.	.
AY829457 *Capreolus capreolus* SP	.	T	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	A	.	.	.	.	.	.	.
MH687351 *Capreolus capreolus*	.	T	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	A	.	.	.	.	.	.	.
EF067343 *Capreolus capreolus* SP	.	T	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	A	.	C	.	.	.	.	.
JX841250 *Rangifer tarandus* FR	.	T	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	A	.	C	.	.	.	.	.
KM205428 *Cervus elaphus* SL	.	T	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	A	.	C	.	.	.	.	.
KM205437 *Capreolus capreolus* SL	.	T	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	A	.	C	.	.	.	.	.
KM205439 *Ixodes ricinus* SL	.	T	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	A	.	C	.	.	.	.	.
KT070830 *Alces alces* NO	.	T	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	A	.	C	.	.	.	.	.
MH687352 *Capreolus capreolus*	.	T	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	A	.	C	.	.	.	.	.
AY706386 *Capreolus capreolus* DE	A	.	.	A	G	G	.	A	T	G	T	T	.	G	T	.	C	T	C	C	A	.	G	C	A	A	.	A	.	T	A	C	.	.	C	C
HQ661161 *Ixodes ricinus* SK	A	.	.	A	G	G	.	A	T	G	T	T	.	G	T	.	C	T	C	C	A	.	G	C	A	A	.	A	.	T	A	C	.	.	C	C
MH687353 *Capreolus capreolus*	A	.	.	A	G	G	.	A	T	G	T	T	.	G	T	.	C	T	C	C	A	.	G	C	A	A	.	A	.	T	A	C	.	.	C	C
MH687354 *Cervus elaphus*	A	.	.	A	G	G	.	A	T	G	T	T	.	G	T	.	C	T	C	C	A	.	G	C	A	A	.	A	.	T	A	C	.	.	C	C
MH687355 *Cervus elaphus*	.	T	C	.	.	.	.	A	G	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.
MH687356 *Cervus elaphus*	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.
MH687357 *Cervus elaphus*	.	T	C	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	A	.	C	.	.	.	.	.
MH687358 *Capreolus capreolus*	A	T	.	A	.	.	.	A	T	G	T	.	.	G	T	.	C	T	C	C	A	.	G	C	A	A	.	A	.	T	A	C	.	.	C	C
MH687359 *Capreolus capreolus*	A	.	.	A	.	G	.	A	T	G	T	T	.	G	T	.	C	T	C	C	A	.	G	C	A	A	.	A	.	T	A	C	.	.	C	C
MH687360 *Capreolus capreolus*	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	A	.	C	G	.	.	.	.
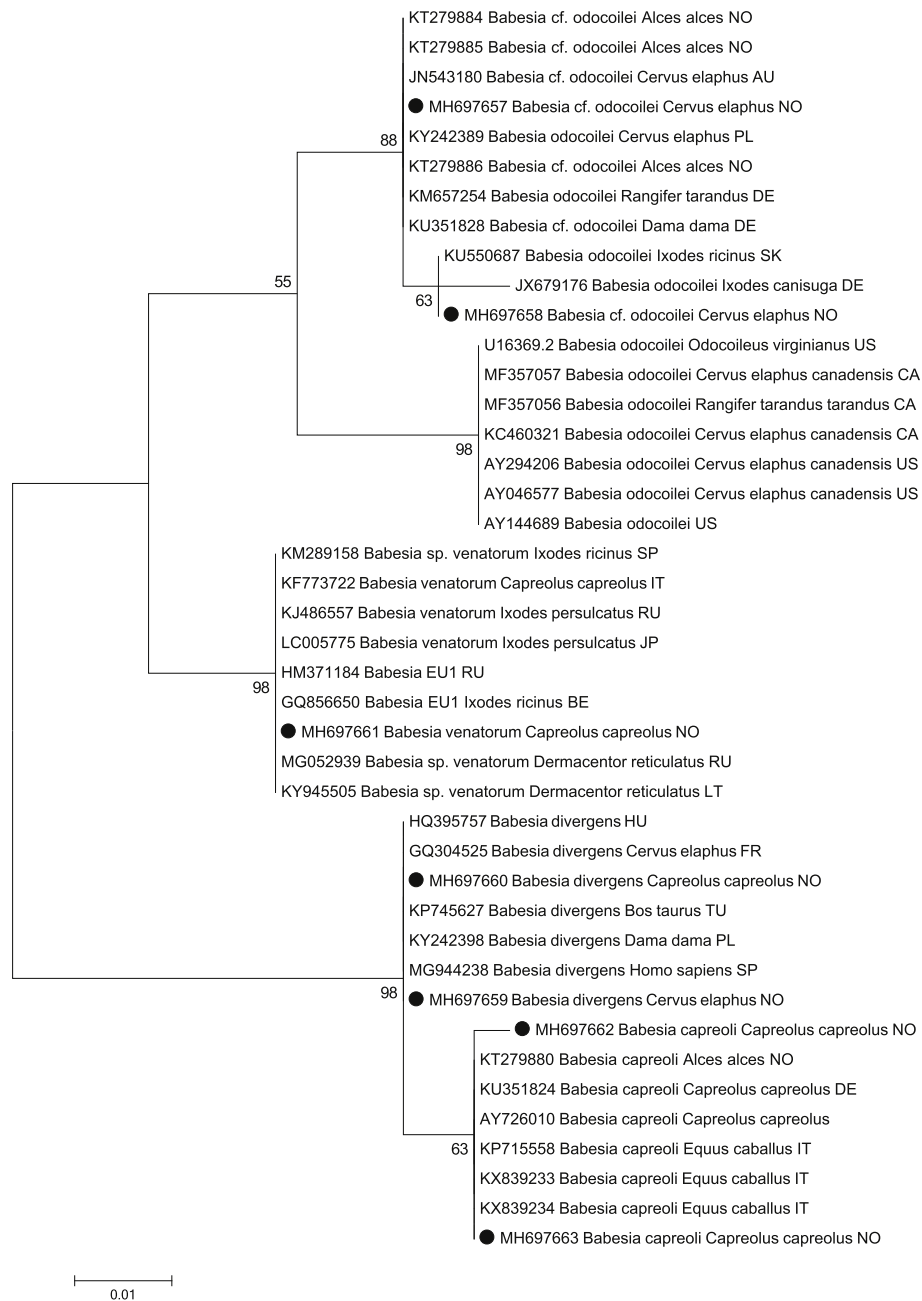
Fig. 1. Phylogenetic tree of *Babesia* isolates and samples of this study (●), based on fragments of 18S rRNA, generated using the Maximum-Likelihood clustering method in MEGA 6 software (1000 replicates; bootstrap values indicated at the nodes). Abbreviations: AU - Austria, BE - Belgium, CA - Canada, DE - Germany, FR - France, HU - Hungary, IT - Italy, JP - Japan, LT - Lithuania, NO - Norway, PL - Poland, RU - Russia, SK - Slovakia, SP - Spain, TU - Turkey, US - United States.

2013b; Woldehiwet, 2010). Stuen et al. (2002a) reported significantly higher seroprevalence for granulocytic *Ehrlichia* spp. found in roe deer (96%) than in moose (43%) and red deer (55%) from *I. ricinus* infested counties in Norway. A high prevalence of antibodies for *A. phagocytophilum* in moose serum (79%) from tick-infested areas in southern Norway has been detected by Milner and van Beest (2013).

A. phagocytophilum infections result in fevers that vary according to the age of animals, the variant of *A. phagocytophilum* involved, and immunological status of the host (Stuen et al., 2013b). In early studies it was observed that sheep and cattle with TBF displayed a range of clinical signs that were attributable to secondary infections (reviewed in Woldehiwet, 2008). Studies conducted in the UK and Scandinavia have clearly established that TBF variants of *A. phagocytophilum* are immunosuppressive, resulting in several disease syndromes including tick pyaemia, pasteurellosis and septicaemic listeriosis (Woldehiwet, 2008).

The epidemiology of *A. phagocytophilum* in wildlife is still poorly understood. Although clinical tick-borne fever among wild cervids is difficult to diagnose, it has been diagnosed in a moose calf, and in one roe deer calf from southern Norway. Both animals died from septicemic infections with bacteria regarded as opportunistic pathogens (Jenkins et al., 2001; Stuen et al., 2001a). A possible effect of *A. phagocytophilum* infections on the body weights of lamb and moose calves have been suggested (Grøva et al., 2011; Stuen et al., 2002b; Puraite et al., 2015a). Clinical and hematological reactions depend on the strains of the bacterium involved and the hosts (Stuen et al., 2013b). There is evidence from experimental study (Stuen et al., 2001b) that an ovine isolate of *A. phagocytophilum* causes a subclinical persistent infection in red deer, while a red deer isolate causes clinical response in lambs (Stuen et al., 2001b).

In the present study, we observed a high incidence of *A. phagocytophilum* in roe deer and red deer. However, the different results were obtained by nested and real-time PCR, the prevalence of bacteria detected by real-time PCR was higher compared with that by nested PCR. Different target genes were used for detection of *A. phagocytophilum* in real-time (*msp2* gene coding for major surface protein 2, MSP2) and nested PCR (*msp4* gene coding for major surface protein 4, MSP4) assays. The genome of *A. phagocytophilum* may contain > 80 copies of *msp2* paralogs, including full-length genes, while *msp4* gene present in a single copy (Cortney et al., 2004; Dunning Hotopp et al., 2006). Primers used in real-time PCR in this study were designed to amplify the conserved coding region of the *msp2* gene and the multiple copies of the gene in the *A. phagocytophilum* genome, thereby increasing the sensitivity of the assay (Table 1). *A. phagocytophilum* DNA was detected in 88.1% roe deer and 81.1% red deer by real-time *msp2* gene PCR, and in 52.2% roe deer and 51.4% red deer by nested *msp4* PCR. Thus, real-time *msp2* gene PCR had higher sensitivity compared with nested *msp4* PCR.

A previous study demonstrated the high incidence of *A. phagocytophilum* infections among moose (42.9%) in tick-infested area, whereas no infected animals were found in an area where ticks were absent or rare (Puraite et al., 2015a). In Norway, the infection rate of *A. phagocytophilum* in *I. ricinus* ranged from 2.1% to 20% (Rosef et al., 2009; Tveten, 2014; Henningsson et al., 2015). The highest prevalence of *A. phagocytophilum* in *I. ricinus* occurred in locations with the highest cervid density (Rosef et al., 2009; Mysterud et al., 2013). A great abundance of potential hosts (cervids) is an important factor for tick expansion and a potential for the spread of anaplasmosis (Gilbert, 2010).

A total of eleven *msp4* gene sequence variants of *A. phagocytophilum* were identified in this study. One variant was common in both species of cervids. The most frequent variant MH687352 (detected in 7 different individuals of roe deer) according to GenBank data was detected in roe deer, red deer, moose, reindeer and *I. ricinus* ticks from different European countries (e.g. France, Slovenia, Spain, and Norway) (de la Fuente et al., 2008; Strašek Smrdel et al., 2015). Three *msp4* sequence variants that share 100% identity to sequences deposited in the Gen-Bank database and three new (unique) sequences were detected in roe deer. Three *A. phagocytophilum msp4* sequence variants derived from red deer in this study had been previously described in chamois, sheep, roe deer and ticks in Slovenia (Strašek Smrdel et al., 2015), France (EU857673), Slovakia (Stefanidesova et al., 2008) and Norway (Paulauskas et al., 2012), while the other three *msp4* sequences had unique nucleotide polymorphisms and therefore differed from other *A. phagocytophilum* sequences previously submitted to GenBank. Two *A. phagocytophilum* genetic variants identified in this study in roe deer and red deer were identical to sequences derived from moose in Norway (Puraite et al., 2015a).

A. phagocytophilum strains detected in roe deer and red deer are heterogenic. However, based on gene targets used in this study, there were no correlations observed among host species and *A. phagocytophilum* genetic variants (Table 3).

Previous studies have suggested that *A. phagocytophilum* strains circulating in different ruminant species, possibly having distinct transmission cycles in nature, independent of each other and that there are biological, ecological, and pathological differences between different *A. phagocytophilum* genetic variants (Massung et al., 2002; Stuen et al., 2003, 2013a). Several 16S rDNA sequence types of *A. phagocytophilum* have been identified in sheep and red deer in Norway (Stuen et al., 2013a). High sequence heterogeneity is observed among *A. phagocytophilum* strains in *msp4* gene by comparing with 16S rDNA gene sequences: twenty-four different *msp4* sequence types were found circulating in a Norwegian sheep flock (Ladbury et al., 2008); high diversity of *msp4* sequences of *A. phagocytophilum* was detected among Austrian wild ungulates (Silaghi et al., 2011a). Bown et al. (2007) reported high variability of *msp4* sequences (11 variants) derived from 20

A. phagocytophilum strains found in different hosts from several European countries and the USA. Several *A. phagocytophilum msp4* gene variants were detected in red deer populations of Norway (Stuen et al., 2013a).

Sequence analysis of 16S rRNA and *ankA* gene demonstrated similarities of *A. phagocytophilum* strains detected in sympatric populations of red deer and sheep (Stuen et al., 2001b), and it was suggested, that red deer could act as the main wildlife reservoir of the *A. phagocytophilum* genetic variants that caused TBF in sheep (Stuen et al., 2013a). However, Stuen et al. (2013a) found that, based on *msp4* gene sequence analysis, *A. phagocytophilum* strains detected in roe deer were genetically distinct from those infected sheep. In the present study, two *msp4* sequence variants identified in red deer (MH687349, MH68735) were identical to those previously detected in sheep (EU857672, EU857673) and cows (EU857665, EU857666, EU85766). The red deer is currently expanding its distribution in Norway, which may lead to the increased risk of anaplasmosis in new regions (Mysterud et al., 2017).

Although *A. phagocytophilum* hronic infection has not been confirmed yet in any host, persistent infections have been found to occur in several mammalian species such as sheep, horse, dog, red deer, and cattle (Stuen et al., 2013b). The time of infection may differ according to *A. phagocytophilum* genetic variants and host species involved (Stuen et al., 2013b). In immune-competent sheep *A. phagocytophilum* is able to survive and propagate for several months or even years (Stuen et al., 2008; Granquist et al., 2010).

In this study, we used two PCR methods for detection of *Babesia* parasites in cervids: real-time PCR and nested PCR. Both PCR assays amplify the partial region of 18S rRNA gene and able to detect a broad-range of known and emerging *Babesia* spp. The results of this study showed, that real-time PCR was more sensitive in detection of *Babesia* DNA in spleen samples of cervids than nested PCR (Table 2): out of 80 *Babesia*-positive samples by real-time PCR, only 47 (58.7%) were positive by using nested PCR. Based on these findings, we hypothesized that difference in prevalence obtained using different PCR methods, could be explained by different level of parasitaemia in examined samples and higher sensitivity of real-time PCR allowing to detect low levels of parasites DNA.

The results from this study show high prevalence 80/104 (76.9%) of *Babesia* spp. in roe deer and red deer sampled from South Norway. The high prevalence of *Babesia* DNA obtained in spleen samples of roe deer and red deer (83.6% and 64.9%, respectively) indicated a high degree of exposure to *Babesia* parasites in the study area (Table 2). Previously reported incidence of *Babesia* spp. in cervids varied in different European regions: high incidence of *Babesia* spp. in roe deer was detected in Germany (62.8%; Kauffmann et al., 2017) and Sweden (52%; Andersson et al., 2016), while lower infection rates were reported in other European countries, including France (23%; Bonnet et al., 2007), Switzerland (26.1% and 23.9%; Hoby et al., 2009; Michel et al., 2014) and Slovenia (21.6%; Duh et al., 2005). Prevalence of *Babesia* spp. in red deer reported in Europe is ranged from 5.1% in Austria (Cézanne et al., 2017), 11.1% and 17.3% in Switzerland (Hoby et al., 2009 in Switzerland; Michel et al., 2014), to 26% in Ireland (Zintl et al., 2011). For detection of *Babesia* DNA, in these studies different PCR methods (conventional, nested or semi-nested) based on 18S rRNA gene were used.

Our findings demonstrated that roe deer and red deer harbored multiple *Babesia* species: sequences derived from red deer were similar with *Babesia* cf. *odocoilei, B. odocoilei* and *B. divergens,* while in roe deer *B. capreoli, B. divergens* and *B. venatorum* were detected. A previous study conducted in Norway reported these *Babesia* species in questing *I. ricinus* ticks, and the most prevalent was *B. venatorum* found in 71% of the positive ticks (Øines et al., 2012). All ticks infected with *B. venatorum* were found in the southern part of Norway, while the *B. divergens* positive ticks were found in the western and southern parts of the country (Øines et al., 2012). In Norway, *B. venatorum* has been also reported from ticks on migratory birds (Hasle et al., 2011). Roe deer has

been regarded as the main cervid host for *B. venatorum.* In the present study, we detected *B. venatorum* only in roe deer. In Europe, *B. divergens* and *B. venatorum* may result in a severe disease in humans, with most cases from spleenectomised patients in whom the disease has a high mortality (Herwaldt et al., 2003; Martinot et al., 2011), also some cases were attributed to *B. microti* which causes milder disease (Hildebrandt et al., 2007; Lempereur et al., 2015). Human babesiosis is a rare but potentially life-threatening parasitic disease. There are 39 published human cases in Europe which were clinically severe (ECDC). A severe case of human babesiosis due to *B. divergens* has only been diagnosed in one patient in Norway in 2007 (Mørch et al., 2014). A fatal case of human *B. divergens* infection was reported from Finland in 2010, and one severe case in was reported in Sweden in 1992 (reviewed in Mørch et al., 2014).

We detected *B. venatorum* and *B. divergens* in roe deer and *B. divergens* in red deer, which may be a potential reservoirs of these human pathogens. *Babesia* cf. *odocoilei* have been previously detected in moose (Puraite et al., 2015b) in Norway.

Fatal babesiosis, due to *B. divergens, B. capreoli* and *B. venatorum* infections were described in roe deer, reindeer and alpine chamois (Marco et al., 2000; Hoby et al., 2009; Kik et al., 2011; Michel et al., 2014). Severe infections due to *B. divergens* were observed in reindeer (Langton et al., 2003). However, clinical babesiosis in free-ranging wild ruminants appears to be rare, and asymptomatic babesiosis seems to be normal (Penzhorn, 2006). Babesiae may persist asymptomatically within its host for several years. In cattle, *B. bovis* infected calves show few, if any clinical signs of disease, and may become persistently infected (Chauvin et al., 2009). Several studies have showed, that during infection of cattle with *B. bovis*, *B. bigemina* or *B. divergens*; of reindeer and other splenectomized cervids with *B. divergens;* of sheep (splenectomized or intact) with B. *divergens* or with *B. ovis*, the parasites can persist for several months, even several years, at a very low level of parasitemia (reviewed in Chauvin et al., 2009; Zintl et al., 2003). These parasitemias, which are often undetectable, may serve as a source of parasites for the infection of new ticks feeding on these animals (Howell et al., 2007). Thus, persistently infected animals could potentially be involved in the maintenance of babesiae in nature and in spreading of the parasite to non-endemic areas where competent vectors are present.

Our study showed co-infections with *A. phagocytophilum* and *Babesia* spp. in both red deer and roe deer (Table 2). The high infection and co-infection rates with *A. phagocytophilum* and *Babesia* spp. in red deer and roe deer suggest that these cervids may play an important role in the transmission of single and multiple pathogens. There is a growing evidence that indicates that *A. phagocytophilum* infection results in a generalized immune suppression (which is most thoroughly described in sheep) by affecting the number of granulocytes and lymphocytes in peripheral blood and by affecting some of their functions (Woldehiwet, 2008; Brown and Barbet, 2016). Since *A. phagocytophilum* could in general weaken the immune system, it may influence the health status of co-infected animals.

Analysis based on long-term datasets (1995–2015) of the incidence of tick-borne diseases in humans and livestock in Norway demonstrated that exposure to ticks was an important factor influencing anaplasmosis and babesiosis incidences in livestock (cattle and sheep) in different Norwegian regions (Mysterud et al., 2017). The diseases occurred most frequently along the coast where ticks are present in high density, and tended to be declined towards the north, where the climate is colder. Climate changes over recent decades have probably led to a wider spatial distribution of vector ticks, and an extension in their periods of activity. The distribution of *I. ricinus* ticks continues to expand northwards in latitude and upwards in elevation in Europe and Norway (Jore et al., 2011; Medlock et al., 2013). As a consequence, chances for transmission of *A. phagocytophilum* and *Babesia* spp. are likely to be increased. The emergence of tick-borne diseases is also associated with the dynamics and distributions of various reservoir hosts of the pathogens. Babesiosis and anaplasmosis in cattle and anaplasmosis in

sheep had the highest incidences along the west coast region of Norway and the incidences of diseases were higher in areas with denser red deer populations (Mysterud et al., 2017). In Norway, the population of cervids has increased dramatically during the last decades (Stuen et al., 2013a). The red deer is currently expanding its distribution in Norway towards the south east and north (Mysterud et al., 2017). Higher densities of wild cervids and ticks resulting from warmer climate may enhance the risk of pathogens transmission, which may lead to the increased risk of tick-borne diseases in Norway.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgements

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