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Bartonella spp. detection in ticks, *Culicoides* biting midges and wild cervids from Norway

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Abstract

Bartonella spp. are fastidious, gram-negative, aerobic, facultative intracellular bacteria that infect humans, and domestic and wild animals. In Norway, *Bartonella* spp. have been detected in cervids, mainly within the distribution area of the arthropod vector deer ked (*Lipoptena cervi*). We used PCR to survey the prevalence of *Bartonella* spp. in blood samples from 141 cervids living outside the deer ked distribution area (moose [*Alces alces*, *n* = 65], red deer [*Cervus elaphus*, *n* = 41] and reindeer [*Rangifer tarandus*, *n* = 35]), in 44 pool samples of sheep tick (*Ixodes ricinus*, 27 pools collected from 74 red deer and 17 from 45 moose) and in biting midges of the genus *Culicoides* (Diptera: Ceratopogonidae, 120 pools of 6,710 specimens). *Bartonella* DNA was amplified in moose (75.4%, 49/65) and in red deer (4.9%, 2/41) blood samples. All reindeer were negative. There were significant differences in *Bartonella* prevalence among the cervid species. Additionally, *Bartonella* was amplified in two of 17 tick pools collected from moose and in 3 of 120 biting midge pool samples. The *Bartonella* sequences amplified in moose, red deer and ticks were highly similar to *B. bovis*, previously identified in cervids. The sequence obtained from biting midges was only 81.7% similar to the closest *Bartonella* spp. We demonstrate that *Bartonella* is present in moose across Norway and present the first data on northern Norway specimens. The high prevalence of *Bartonella* infection suggests that moose could be the reservoir for this bacterium. This is the first report of bacteria from the *Bartonella* genus in ticks from Fennoscandia and in Culicoides biting midges worldwide.

KEYWORDS

biting midges, *Ixodes ricinus*, moose, One Health, red deer, reindeer

1 | INTRODUCTION

Bartonella spp. are included in the genus *Bartonella* (single member of family Bartonellaceae, order Rhizobiales), that comprises fastidious, gram-negative, aerobic, facultative intracellular, haemotropic bacilli, classified within the α-proteobacteria (Jacomo, Kelly, & Raoult, 2002; La Scola, Zeaiter, Khamis, & Raoult, 2003). This diverse genus includes 37 validated species and three subspecies as of 5 May 2020 (http://

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www.bacterio.net/bartonella.html). The Bartonellaceae family likely originated as environmental and commensal bacteria (in the guts of ectoparasitic and non-ectoparasitic insect species), before invading mammals, approximately 79 million years ago (Frank, Boyd, & Hadly, 2018). The finding of Bartonellaceae species in honeybees (Kekerova, Moritz, & Engel, 2016) and ants (Russell et al., 2009), among other arthropods, supports this hypothesis. In mammals, Bartonellaceae infect host endothelial cells, and are seed into the bloodstream, colonizing and persisting in the erythrocytes, where they are usually found (Okaro, Addisu, Casanas, & Andersona, 2017; Seubert, Schulein, & Dehio, 2002). Aside from mammalians, *Bartonella* spp. have also been found in loggerhead sea turtles (*Caretta caretta*; Valentine et al., 2007).

Bartonella is considered a blood-borne pathogen; the transmission of *Bartonella* usually occurs via haematophagous insects, for example body lice (Phthiraptera: Anoplura), fleas (Siphonaptera), biting flies (phlebotomine sandflies such as *Lutzomyia verrucarum*, Diptera: Nematocera) and deer keds (*Lipoptena cervi*, Diptera: Brachycera), as well as by bites or scratches by reservoir mammals (Caceres, 1993; Jacomo et al., 2002; Korhonen et al., 2015). Vertical transmission has also been suggested (Kosoy et al., 1998). The detection of *Bartonella* sp. in strictly aquatic species (i.e. cetaceans) suggests the presence of other possible transmission mechanisms (Harms et al., 2008).

Bartonella bacteremia in natural hosts is commonly asymptomatic (Jacomo et al., 2002). Nevertheless, some species are responsible for disease in humans, and domestic and wild mammals, being recognized as emerging anthropozoonoses (Breitschwerdt, 2014). Some of the *Bartonella* and *Candidatus* Bartonella known to infect humans are considered pathogenic (Okaro et al., 2017), such as *B. henselae* (cat scratch disease), *B. quintana* (trench fever), *B. bacilliformis* (Carrion's disease, also known as Oroya fever and verruga peruana), *B. ancashensis* (verruga peruana) and *Candidatus* Bartonella tamiae (febrile illness in humans in Asia; Karem, Paddock, & Regnery, 2000; Kosoy et al., 2008; Mullins et al., 2013). Clinical signs of bartonellosis include fever, lymphadenopathy, bacteremia, endocarditis, bacillary angiomatosis and peliosis hepatitis (Angelakis & Raoult, 2014). Humans are the only known reservoir for *B. quintana* and *B. bacilliformis* (Jacomo et al., 2002).

Ruminants are mainly infected by ruminant-associated species, classified in lineage II: *B. schoenbuchensis*, *B. bovis*, *B. capreoli*, *B. chomelii* and *B. melophagi* (Engel et al., 2011; Frank et al., 2018). Nevertheless, the infection by the human pathogenic species *B. henselae* (lineage IV, Engel et al., 2011) has been reported in cattle (*Bos taurus*) co-infected with *B. bovis* (Cherry, Maggi, Cannedy, & Breitschwerdt, 2009). In domestic cattle, *B. bovis* is associated with endocarditis, while *B. henselae* causes systemic reactive angioendotheliomatosis (Beerlage et al., 2012; Erol et al., 2013; Maillard et al., 2007). In spite of that, the possible pathogenesis of *Bartonella* spp. in other ungulate species, for example cervids, remains unclear. Interestingly, the zoonotic infections caused by the ruminant-associated species *B. schoenbuchensis* and *B. melophagi* have been diagnosed in ill humans (Maggi, Kosoy, Mintzer, & Breitschwerdt, 2009; Vayssier-Taussat et al., 2016). Some of the ruminant-associated *Bartonella* spp. have been described in cervids: *B. schoenbuchensis* was detected in European roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*) and moose (*Alces alces*; Adamska, 2008; Dehio et al., 2001; Guy et al., 2013; Regier et al., 2018; Welc-Falęciak et al., 2013); *B. bovis* was detected in European roe and red deer, elk (*Cervus canadensis*) and moose (Adamska, 2008; Guy et al., 2013; Maillard et al., 2004); and *B. capreoli* was identified in European roe deer, elk and Japanese Sika deer (*Cervus nippon*; Bai, Cross, Malania, & Kosoy, 2011; Bermond et al., 2002; Regier et al., 2018; Sato et al., 2012; Welc-Falęciak et al., 2013). Aside from *B. capreoli*, unclassified *Bartonella* species were observed in Sika deer (Maillard et al., 2004; Sato et al., 2012). *Bartonella* sp. and sequences highly similar to *Bartonella grahamii* were obtained in Korean water deer (*Hydropotes inermis argyropus*; Ko et al., 2013), while *Bartonella* sp. was also found in Persian fallow deer (*Dama mesopotamica*; Maillard et al., 2004). Additionally, different *Bartonella* nucleotide sequences were obtained from elk and mule deer (*Odocoileus hemionus*), some of them highly similar to *B. schoenbuchensis*, *B. bovis* or *B. capreoli* (Chang, 2000).

In free-ranging cervids from Fennoscandia (Norway, Sweden and Finland), bartonellae species have mainly been studied in moose, but have also been described in European roe deer and red deer (Duodu et al., 2013; Guy et al., 2013; Korhonen et al., 2015; Pérez Vera, Aaltonen, Spillmann, Vapalahti, & Sironen, 2016; Razanske et al., 2018). Two main lineages have been described, one highly similar to *B. chomelii*, *B. schoenbuchensis* and *B. capreoli*, described only in areas where the vector deer ked (*Lipoptena cervi*) is present (Duodu et al., 2013; Korhonen et al., 2015; Pérez Vera et al., 2016; Razanske et al., 2018), and an additional lineage closely related to *B. bovis*, identified both inside and outside this vector's distribution area (Duodu et al., 2013; Pérez Vera et al., 2016). To the authors' knowledge, there is no information regarding *Bartonella* spp. infections in reindeer (*Rangifer tarandus*) or its presence in northern Norway.

We hypothesized that the transmission of *Bartonella* outside the deer ked distribution area could be driven by different vector species. The goals of this study were (a) to survey the prevalence of *Bartonella* infections in moose, red deer and reindeer outside the deer ked distribution area in Norway, (b) to investigate whether *Bartonella* is present in sheep ticks (*Ixodes ricinus*) collected from wild cervids (moose and red deer) and in biting midges of the genus *Culicoides* (Diptera: Ceratopogonidae) and (c) to study the phylogenetic relationships of the identified *Bartonella* nucleotide sequences and the remaining *Bartonella* species.

2 | MATERIALS AND METHODS

2.1 | Samples

Blood samples from 141 cervids of three different species were evaluated: moose ($n = 65$), red deer ($n = 41$) and reindeer ($n = 35$) sampled from 2014 to 2018 in Norway. Information regarding the date and the location (municipality of origin), age

class (calf, juvenile, adult, unknown) and sex (male, female, unknown) of the sampled animals is provided in Table 1. All moose and reindeer were chemically restrained and had blood samples collected by venipuncture of the jugular vein. The anaesthesia was performed following established protocols (Kreeger & Arnemo, 1996). Regarding red deer, blood samples were collected from 26 chemically restrained and 15 hunted specimens. In the latter, the blood was withdrawn from the thoracic cavity by the hunters with a plastic Pasteur pipette and transferred to EDTA tubes. All blood samples were kept frozen at −80°C until testing. DNA extraction was performed using the QIAsymphony DSP Virus/Pathogen Midi Kit (Qiagen) in a QIAsymphony system (Qiagen), following the manufacturer's instructions, except in 14 moose blood samples of northern Norway. A volume of 200 µl of blood was lysed in 500 µl MagNA Pure 96 bacterial lysis buffer (Roche), followed by DNA extraction using the DNA Viral NA Large Volume Kit (Roche) in MagNA Pure 96 (Roche) automatic extraction instrument. All cervid captures and immobilizations were carried out according to national regulations on animal health and welfare, and use of animals for scientific purposes. All protocols and necessary licences over the different years included in this study were approved and obtained from the Norwegian Environmental Agency (NEA) and the Norwegian Food Safety Authority (NFSA), which enforces regulations and issues permits for biological sampling of wild animals in Norway. As a part of the National Health Surveillance of wildlife in Norway, NEA requires the submission of blood samples to the Norwegian Veterinary Institute (NVI) from all free-ranging cervids captured in the scientific context. Hence, all the blood samples in our study originate from such statutory blood samples submitted to NVI over the past years and the authors have therefore no access to the individual capture projects' field permit numbers.

Additionally, we analysed 44 pooled samples of 187 *Ixodes ricinus* ticks (Acari: Ixodida) attached to cervids (27 pools of 124 ticks collected from 74 red deer and 17 pools of 63 ticks collected from 45 moose ([up to six ticks per pool]), several of them partially or fully engorged, collected in 2013 in different municipalities of southern Norway (Birkenes, Halden, Lardal, Larvik, Steinkjer and Trysil [moose], and Etne, Flora, Luster, Lærdal, Masfjorden, Namsos, Rauma, Suldal and Vindafjord [red deer]). All these municipalities are located outside the deer ked distribution area, with the exception of Birkenes, Halden, Lardal, Larvik and Trysil. A total of 120 pooled samples of 6,710 *Culicoides* biting midges collected in 2012 in the municipalities of Lund, Farsund, Hå, Kragerø and Sirdal were also tested. Tick pools were created according to host species and municipality of origin, whereas *Culicoides* biting midge pools were made according to municipality of origin and number of collected individuals in each location.

TABLE 1 Age class (C = calf, J = juvenile, A = adult, U = unknown) and sex (M = male, F = female, U = unknown) of the tested moose (*Alces alces*), red deer (*Cervus elaphus*) and reindeer (*Rangifer tarandus*)

Species	District/municipality		Number of animals	Age class				Sex		
				C	J	A	U	M	F	U
Moose	Northern Norway	Porsanger	12	0	1	11	0	7	5	0
		Tana	1	0	0	1	0	0	1	0
		Nesseby	1	0	0	1	0	0	1	0
	Southern Norway	Selbu	24	2	0	22	0	3	7	14
		Vega	27	11	0	16	0	13	14	0
	Subtotal		65	13	1	51	0	23	28	14
Red deer	Aurland		4	0	0	4	0	2	2	0
	Hol		6	0	0	6	0	2	4	0
	Kvinnherad		11	0	3	8	0	8	3	0
	Lærdal		16	0	2	13	1	3	13	0
	Ørsted		4	0	3	1	0	3	1	0
	Subtotal		41	0	8	32	1	18	23	0
Wild reindeer	Hardangervidda		13	0	0	13	0	1	12	0
	Lesja		1	0	0	1	0	0	1	0
	Nordfjella		10	0	0	10	0	2	8	0
	Oppdal		4	0	0	4	0	0	4	0
	Rondane Sør		2	0	0	2	0	1	1	0
	Setesdal Ryfylke		4	0	0	4	0	2	2	0
	Sunndal		1	0	0	1	0	0	1	0
	Subtotal		35	0	0	35	0	6	29	0
Total			141	13	9	118	1	47	8	14

2.2 | Molecular study

The invertebrate samples were disrupted in 1.5-ml Eppendorf tubes containing 500 μl of ATL lysis buffer (Qiagen) and one tungsten carbide bead (Qiagen) in a mixer mill (Retsch MM301; GmbH & Co.), at 12 times/s during 30 min. After that, the DNA and RNA were extracted using NucliSENS easyMAG (bioMérieux), according to the manufacturer's protocol.

The initial screening for *Bartonella* sp. was conducted using a novel conventional PCR protocol to amplify an approximately 161-bp fragment of the RNA polymerase beta-subunit gene (*rpoB*) of *Bartonella bovis*, *B. capreoli*, *B. schoenbuchensis*, *B. chomelii* and *Bartonella* sp. strains previously described in moose, using the consensus primer set rpoB-F: 5′-TTGAAAGTCCATATCGCAAAATT-3′ and rpoB-R 5′-ACCTGCGTGACGGCAAAC-3′. The final PCR volume of 25 μl contained 10.5 μl of RNase-free water, 0.2 μM of each primer, 1.25 U HotStarTaq™ Master Mix (Qiagen) and 1–4 μl DNA template. The thermocycler programme was set at 95°C for 15 min, followed by 40 amplification cycles of 95°C, 30 s; 55°, 30 s; and 72°C, 30 s. The final extension step was performed at 72°C for 3 min. To confirm our results and identify the *Bartonella* species, two additional housekeeping genes were amplified by PCR: an approximately 588-bp fragment of the riboflavin synthase gene (*ribC*) and a 350-bp fragment citrate synthase gene (*gltA*), as previously described (Duodu et al., 2013; Norman, Reqnery, Jameson, Greene, & Krause, 1995, respectively). PCR products were analysed using 1.5% agarose gels stained with GelRed (Biotium). Selected amplicons were confirmed using direct Sanger sequencing.

Sequence reads were assembled using ClustalW alignment in MEGA7.0 (Kumar, Stecher, & Tamura, 2016) and compared with those available in GenBank/EMBL/DDBJ database using online BLASTn search (http://www.ncbi.nlm.nih.gov/blast). The genetic distance to the closest sequences was calculated based on p-distance, after excluding the primers from the obtained sequences.

Multiple sequence alignments for *rpoB* and *gltA* were made using the ClustalW algorithm in MEGA7.0 (Kumar et al., 2016), selecting representative sequences obtained in our study, other *Bartonella* sequences with a similar size amplified from cervids, ruminant-associated *Bartonella* species (lineage II) and other recognized *Bartonella* species, *Candidatus* B. dromedarii and *Candidatus* B. tamiae. After that, the phylograms were constructed using MEGA7.0. *Brucella melitensis* and *Rickettsia rickettsii* were selected as an outgroup for *rpoB* and *gltA*, respectively.

2.3 | Statistical analysis

The Kruskal–Wallis test (function 'kurskall.test') was used to assess the relation between species and *Bartonella* sp. The chi-square test (function 'chisq.test') was used to assess the relation between *Bartonella* sp. prevalence and sex, age and origin (north, south in moose) variables. All statistical analyses were performed in R software (R Development Core Team, 2013) with a significance level of $p < .05$.

3 | RESULTS

3.1 | PCR amplification

The *rpoB* gene was amplified in 75.4% (49/65) of the moose blood samples: 92.9% (13/14) of moose from northern and 70.6% (36/51) of moose from southern Norway. The same gene was detected in 4.9% (2/41) of the red deer blood samples. All the reindeer blood samples ($n = 35$) were negative (Table 2). Additionally, *rpoB* gene was amplified in two out of 17 tick pool samples from moose (a minimum percentage of ticks from moose harbouring *Bartonella* DNA, 3.2% [2/63]), while all the red deer tick pools samples ($n = 27$) were negative. The same gene was also amplified in three out of 120 pool samples of *Culicoides* biting midges (a minimum percentage of biting midges harbouring *Bartonella* DNA, 4.5×10^{-4} [3/6710]).

The *ribC* gene was amplified in all *rpoB*-positive samples, except in blood samples from two moose and one red deer, and in one tick pool from moose and three *Culicoides* biting midges pools. The *gltA* gene was amplified in all *rpoB*-positive samples (Table 2), with the exception of the above mentioned *Culicoides* biting midges.

3.2 | Sequencing

We selected 37 *rpoB* gene-PCR-positive blood samples for sequencing (from 30 moose, two red deer, two ticks from moose and three *Culicoides* biting midges), as well as the *ribC* and *gltA* amplicons available for these samples (Appendix S1).

Two different *rpoB* sequences were obtained from cervid blood samples and ticks: one in moose ($n = 25$), red deer ($n = 1$) and ticks ($n = 2$), and an additional sequence in moose ($n = 4$, identified in the moose v65, v76, v81 and v177nm). These sequences differed in only a single nucleotide position. The amino acid (aa) and nucleotide (nt) identities within these *rpoB* sequences and the closest relatives retrieved from GenBank are summarized in Table 3. The *rpoB* sequence obtained from one *Culicoides* biting midge sample (BM81) was most similar (81.7% sequence identity) to a *Bartonella* sp. identified in lesser mouse-eared bat (*Myotis blythii*) from Georgia (KX300129) and with *Bartonella taylorii* amplified in striped field mouse (*Apodemus agrarius*) from South Korea (JN810827). The closest amino acid identity (90%) was with the same *Bartonella* species previously described, and with other sequences, such as *B. bovis* obtained in cattle Poland (AFP47207), and *Bartonella* sp. from moose in Norway (AFI57917 and AFD02569) and Finland (AIT18334).

Two different *ribC* sequences were found when looking at data from moose and ticks. The sequences differed in a single position when contrasting data from 19 moose samples with that found in moose samples v65, v90nm and v92nm as well as a tick sample. All *gltA* nt sequences obtained from moose, red deer and ticks were identical. The nt and aa identities within the *ribC* and *gltA* sequences, and the similar sequences found in GenBank are summarized in Table 3. In summary, they are highly similar to *B. bovis*.

TABLE 2 Results for the *Bartonella* PCRs conducted in cervid samples in this study, listed by species and district of origin

Species	District/municipality		Number of samples	*Bartonella* PCR		
				rpoB +	*ribC* +	*gltA* +
Moose	Northern Norway	Porsanger	12	12	12	12
		Tana	1	1	0	1
		Nesseby	1	0	0	0
	Southern Norway	Selbu	24	12	12	12
		Vega	27	24	23	24
	Subtotal		65	49	47	49
Red deer	Aurland		4	0	0	0
	Hol		6	1	1	1
	Kvinnherad		11	0	0	0
	Lærdal		16	1	0	1
	Ørsted		4	0	0	0
	Subtotal		41	2	1	2
Wild reindeer	Hardangervidda		13	0	0	0
	Lesja		1	0	0	0
	Nordfjella		10	0	0	0
	Oppdal		4	0	0	0
	Rondane Sør		2	0	0	0
	Setesdal Ryfylke		4	0	0	0
	Sunndal		1	0	0	0
	Subtotal		35	0	0	0
Total			141	51	48	51

TABLE 3 Nucleotide (nt) and amino acid (aa) identities of the *rpoB*, *ribC* and *gltA* sequences obtained from cervids and ticks

Gene	Sequence	nt	aa
rpoB	LC497326 (moose)	100% *Bartonella* sp. of moose from Finland and Norway (e.g. KU254131, KU254132, KJ739722, JQ411079, JN990608) 98.3% *B. bovis* (e.g. KR733194, KF218220)	100% *Bartonella* sp. moose of Finland and Norway (e.g. KU254131, KU254133, JQ411079), *B. bovis* (e.g. KR733194, KF218220) and other *Bartonella* species as *B. schoenbuchensis* (MH598359.1), *B. taylorii* (MH547319.1) and *B. capreoli* (AB703143)
	LC497327 (moose), LC497328 (red deer) and LC497329 (tick)	100% *Bartonella* sp. of moose from Finland (KU254133) 97.5% *B. bovis* (e.g. KR733194, KF218220)	
ribC	MN094879 (moose) and MN094881 (tick)	99.8% *Bartonella* sp. of moose from Norway (JN990644)[a] 96.8% *B. bovis* (KF218216, KF218215)	100% *B. bovis* (WP_010702375) 100% *Bartonella* sp. of moose of Norway (JN990644)[a]
	MN094880 (moose)	99.6% uncultured *Bartonella* of moose from Norway (JN990644)[a] 96.6% *B. bovis* (KF218216, KF218215)	99.4% *B. bovis* (WP_010702375) 99.3% *Bartonella sp.* of moose of Norway (JN990644)[a]
gltA	MN094882 (moose), MN094883 (red deer) and MN094884 (tick)	97.6% *B. bovis* (e.g. KJ909848, KJ909819, KF199898, DQ358232)	99.1% *B. bovis* (e.g. KJ909848, KJ909819, KF199898, DQ358232) and to a sequence of *Candidatus* B. dromedarii (KM371034)

[a]The sequence JN990644 is shorter in length: 454 nt versus 533 nt of our *ribC* sequences after excluding primers.

Representative *rpoB* sequences obtained in this study were submitted to the DNA Data Bank of Japan (DDBJ) under accession numbers LC496324 (biting midges [BM81]), LC497326 (northern moose [v99nm]), LC497327 (moose [v65]), LC497328 (red deer [v108]) and LC497329 (pool of five sheep ticks [TC36]), while representative *ribC* and *gltA* sequences were submitted to GenBank under accession numbers MN094879 to MN094884 (that correspond with *ribC* sequences from northern moose [v99nm], moose [v65] and sheep ticks [TC36], and with *gltA* sequences from northern moose [v99nm], red deer [v108] and sheep ticks [TC36], respectively).

3.3 | Phylogeny

The *Bartonella* sp. sequences obtained from moose, red deer and ticks clustered with other ruminant-associated *Bartonella* comprising lineage II (Figure 1). In addition, the *gltA* sequences from moose, red deer and ticks clustered with *B. bovis*. It was not possible to solve the phylogenetic placement of the *rpoB* sequence obtained from one *Culicoides* biting midge.

3.4 | Statistics

Significantly higher prevalence ($K = 79.5$, $p = .0001$) of *Bartonella* sp. was observed in moose compared with red deer and reindeer (75.4%, 4.9% and 0.0%, respectively). No differences in respect to sex, age or location were found in any of the sampled species.

4 | DISCUSSION

In this study, we demonstrate that bacteria of the genus *Bartonella* are present in cohorts of moose across Norway, providing the first data from the northern part of the country. The same *rpoB* and *gltA* *Bartonella* sequences were also detected in red deer. All analysed blood samples from moose, red deer and wild reindeer were obtained from areas with no confirmed presence of deer ked, a significant vector of *Bartonella* (Korhonen et al., 2015; Razanske et al., 2018) present in southern Norway (Valimaki et al., 2010).

The *Bartonella* DNA prevalence in moose was 75.4% (49/65), with prevalence of 92.9% (13/14) and 70.6% (36/51) in northern and southern moose, respectively. The overall high prevalence of this bacterium in moose suggests that this species is a potential *Bartonella* reservoir. Contrary to other infectious agents, the prevalence of *Bartonella* in the reservoir species is usually high (Breitschwerdt, Maggi, Chomel, & Lapin, 2010). Previous studies in Fennoscandia found lower *Bartonella* prevalence in moose from outside the deer ked distribution area, that is 55.9% (19/34) in northern Finland (Pérez Vera et al., 2016) and 35.7% (10/28) and 17.2% (5/29) in deer ked-free areas of southern Norway (Duodu et al., 2013; Razanske et al., 2018), respectively. Conversely, moose cohorts from deer ked-positive regions presented high prevalence, ranging from 82.4% (89/108) to 100% (8/8; Korhonen et al., 2015; Pérez Vera et al., 2016) in Finnish moose (considering that, Korhonen et al., 2015 only tested 8 animals), and from 40.2% (51/127) to 70% (21/30) in Norwegian moose (Duodu et al., 2013; Razanske et al., 2018). Statistically significant differences were observed by Pérez Vera et al. (2016) between both deer ked-free and deer ked-infested areas, higher inside the deer ked distribution area. Nevertheless, Malmsten et al. (2018)

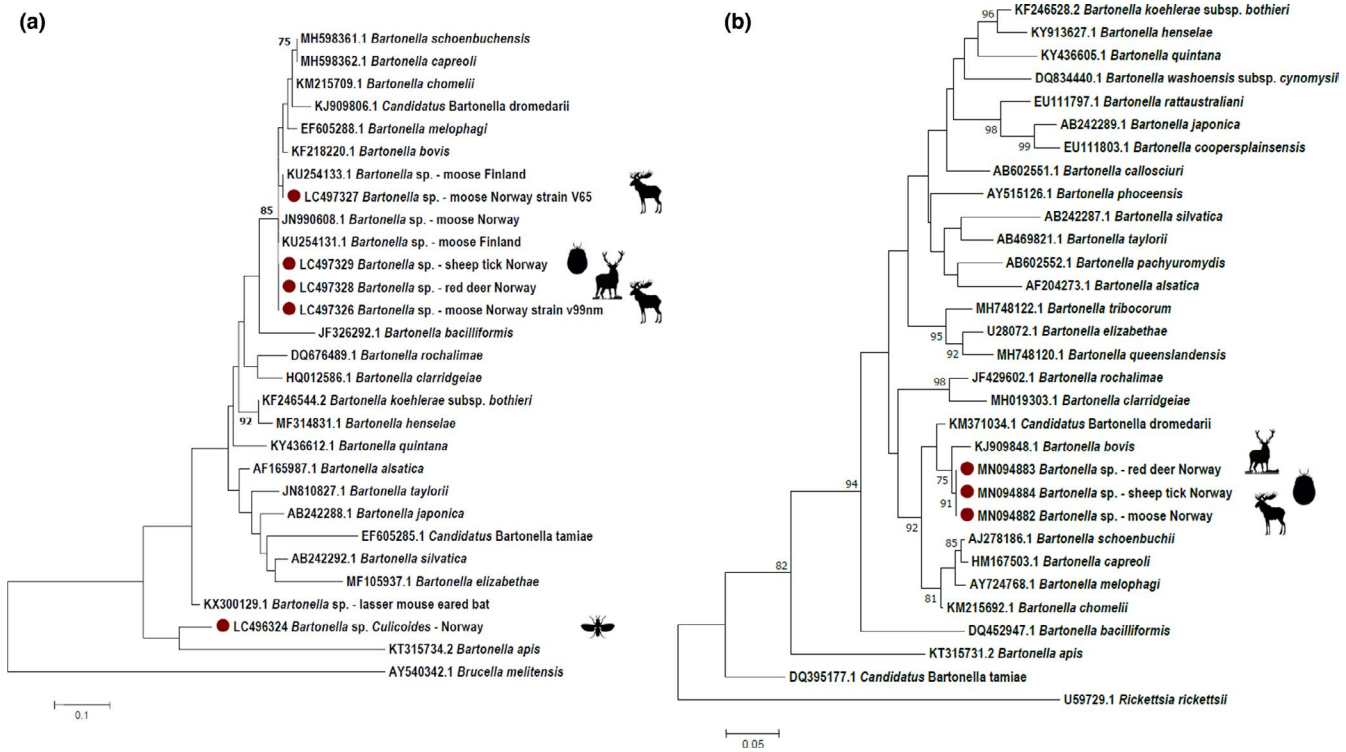

FIGURE 1 Phylogenetic relationships of *Bartonella* species inferred from maximum-likelihood phylograms of the alignment of (a) RNA polymerase beta-subunit gene (*rpoB*) nucleotide sequences of representative *Bartonella* obtained in this study (marked with red dots) and selected *Bartonella* sequences retrieved from GenBank, including known species classified in lineages I, II, III and IV. *Brucella melitensis* was selected as out-group. (b) Citrate synthase (*gltA*) of representative nucleotide sequences obtained in this study (marked with red dots) and selected *Bartonella* sequences retrieved from GenBank, including known species included in lineages I, II, III and IV. *Rickettsia rickettsii* was selected as out-group. The reliability of the trees was tested by bootstrap analysis with 1,000 replicates, and those bootstrap values lower than 70 were omitted

found sequences highly similar to *B. schoenbuchensis* in only 0.7% (4/615) of the moose spleen samples from southern Sweden, an area where deer keds are present. Yet, the employed methodology—a novel high-throughput real-time PCR to amplify the *ssrA* gene—could be less sensitive than those used in previous studies. We believe that the high *Bartonella* prevalence outside the deer ked distribution area could be caused by uncharacterized vector(s). High *Bartonella* prevalence was also reported in other cervid species, that is 90.5% (38/42) of the evaluated mule deer from the United States (Chang, 2000), 80.0% (4/5) in roe deer from Germany (Dehio et al., 2001) and 61.8% (34/55) in ectoparasite-infested (deer keds and ticks) Sika deer from Japan (Sato et al., 2012).

Regarding red deer, we observed a low prevalence for the same strain reported in moose (4.9%, 2/41), mimicking the low *Bartonella* prevalence (15%, 15/100) observed in free-ranging elk (*Cervus elaphus*) in the United States (Chang, 2000), and in other red deer from Norway (17.6%, 3/17) living outside the deer ked distribution region, although the red deer from inside showed a prevalence of 50% (10/20; Razanske et al., 2018). High prevalence of *Bartonella* DNA (35%, 7/20) was also observed in red deer from Poland, and prevalence appeared to be higher in spleen than blood (Adamska, 2008).

All the tested wild reindeer were negative. This is the first study on the prevalence of *Bartonella* in this species. It is significant that all tested specimens were free-ranging animals, living at high altitude in the Norwegian mountains, and likely not exposed to the same potential vectors than the cervids living in forested lowlands, such as moose and red deer. In the mountains, exposure to ectoparasites is potentially lower, due to windy environments adverse for small flying insects, which may be potential vectors for *Bartonella*. In addition, *Ixodes ricinus* ticks are absent in the mountains—the main habitat for wild reindeer. These data suggest that the absence of ectoparasites could be associated with the absence of *Bartonella*. However, the possible expansion on the geographic range of potential *Bartonella* vectors such as deer keds or other unidentified blood-sucking vectors to novel niches as mountain areas driven by the climate change should be considered (Mills, Gage, & Khan, 2010) and could represent a threat to the last free-ranging reindeer population in Europe (Vistnes, Nellemann, Jordhøy, & Strand, 2004). Alternatively, the reindeer could be infected by *Bartonella* species not amplified by the *rpoB* primers used for the initial screening, since that primer set was specifically designed for ruminant-associated *Bartonella* species. Furthermore, research about the prevalence of *Bartonella* in semi-domestic reindeer is warranted, as they live in sympatric areas with the *Bartonella*-positive northern moose.

Phylogenetic analyses of *rpoB* and *gltA*—considered the best markers for *Bartonella* species demarcation (La Scola et al., 2003), place all the sequences amplified from cervids and ticks into lineage II, which also includes ruminant-associated *Bartonella* species. Additionally, the sequences from moose, red deer and ticks clustered with *Bartonella bovis* in the *gltA* phylogram. Our sequences showed a high degree of similarity with the *Bartonella* clade detected in other studies in cervids of Fennoscandia from inside and outside the deer ked distribution area (Duodu et al., 2013; Korhonen et al., 2015; Pérez Vera et al., 2016; Razanske et al., 2018) and with *B. bovis*. Therefore, the *Bartonella* DNA found in moose, red deer and ticks could possibly stem from this species. In a moose from Sweden, the infection by a strain of *B. bovis* able to infect bovine endothelial cells was previously confirmed by whole-genome shotgun (Guy et al., 2013).

Two pools of *I. ricinus* from moose were positive, thus a minimum of two *I. ricinus* samples directly collected from moose hosted *Bartonella* DNA, but no sequences were obtained from *I. ricinus* of red deer. To the authors' knowledge, this is the first report of *Bartonella* DNA in ticks from Fennoscandia. Until now, no *Bartonella* DNA had been identified in ticks from Finland (Sormunen et al., 2016), Sweden (La Scola, Holmberg, & Raoult, 2004) or Norway (Quarsten, Skarpaas, Fajs, Noraas, & Kjelland, 2015). *I. ricinus* is commonly found in Europe (Sanogo et al., 2003) and has been previously shown to be positive for *Bartonella* species, for example *B. henselae*, *B. doshiae*, *B. grahamii*, *B. bovis* and *B. schoenbuschensiis* (Adamska, 2008; Matsumoto, Berrada, Klinger, Goethert, & Telford, 2008; Müller, Reiter, Schötta, Stockinger, & Stanek, 2016; Sanogo et al., 2003). *Bartonella* has also been identified in ticks collected from other cervid species aside from moose, including 4 (2.0%, 4/203) *I. dammini* ticks from white-tailed deer (*Odocoileus virginianus*) from the United States that were positive to *B. schoenbuchensis* (Matsumoto et al., 2008), 11 (3.0%, 11/363) ticks of different species (*Haemaphysalis flava*, *H. longicornis*, *I. persulcatus*) collected from Korean water deer (*Hydropotes inermis argyropus*) positive for *Bartonella* spp. (Kang et al., 2016), 73 (60%, 73/121) *I. ricinus* ticks from roe deer from the Netherlands positive to *Bartonella* sp. (Schouls, Van de Pol, Rijpkema, & Schot, 1999) and 2 (1.9%, 2/103) from Poland positive to *B. schoenbuchensis* and *B. bovis*, respectively (Adamska, 2008).

Ticks are considered vectors of several protozoan, bacterial and viral diseases that affect humans, and wild and domestic animals, including babesiosis, Lyme borreliosis, some rickettsial diseases and tick-borne encephalitis (Chauvin, Moreau, Bonnet, Plantard, & Malandrin, 2009; Parola, Davoust, & Raoult, 2005; Piesman & Gern, 2004; Süss, 2011). The role of ticks as *Bartonella* vectors, as well as their potential epidemiological relevance, is still under discussion. Several studies reported the presence of *Bartonella* DNA in ticks, with the prevalence ranging from 0.0% to 60.0% (Regier et al., 2018). Moreover, *Bartonella* spp. have been detected in questing and engorged adult ticks and nymphs (Chang, Chomel, Kasten, Romano, & Tietze, 2001; Cotté, Bonnet, Cote, & Vayssier-Taussat, 2010; Sanogo et al., 2003). The presence of *Bartonella* DNA in engorged ticks could be due to contamination with infected host blood, as observed by Matsumoto et al. (2008) in ticks collected from deer, which could also explain our findings. Nevertheless, the amplification of *Bartonella* DNA in questing ixodid tick collected from the environment (Chang et al., 2001) suggests a possible transstadial transmission, since this type of tick only feeds once per stage. The transstadial transmission of *Bartonella* in ticks feeding on artificial skin or mice has been previously reported (Cotté et al., 2008; Reis et al., 2011). Transovarial transmission of *Bartonella* in ticks requires further clarification.

There are some indirect evidences of *Bartonella* transmission to the host via ticks, such as infections by different species of *Bartonella* in Lyme borreliosis-seronegative human patients from rural areas, with undifferentiated chronic illness and preceding tick bite; however, in some cases the patients reported having been bitten several years prior the diagnosis (Vayssier-Taussat et al., 2016). Among the direct evidences of tick-borne *Bartonella* transmission, there are some of note: (a) the experimental transmission from *Bartonella bacilliformis*-infected non-human primates to healthy specimens through the bite of *Dermacentor andersoni* tick, maybe acting as a mechanical vector (Noguchi, 1926), (b) transmission of *B. henselae* to cats through salivary contents of infected *I. ricinus* using an artificial feeding system (Cotté et al., 2008) and (c) the transmission of *Bartonella birtlesii* by different stages of *I. ricinus* in a murine model, demonstrating vector competence (Reis et al., 2011). Ticks may play an important role in the transmission of *Bartonella* spp. in cervids, once, in comparison with ticks, other arthropod vectors (e.g. fleas) are uncommon in these species (Chang et al., 2001). *Ixodes ricinus* is currently expanding its latitudinal and altitudinal distribution in central Europe, and their latitudinal presence in Fennoscandia, a phenomenon driven by climatic, ecological (such as the increase in wild cervid populations acting as tick hosts), landscape and anthropogenic changes (Medlock et al., 2013).

The *rpoB* sequence obtained from one *Culicoides* biting midge was significantly different from the closest *Bartonella* species available at GenBank (with similarities of only 81.7% and 90% for nucleotide and amino acid sequences, respectively). The *rpoB* gene has been proposed as the most accurate for distinguishing *Bartonella* species, along with the *gltA* gene; nucleotide identities lower than 95.4% in 825-bp fragments of *rpoB* and 96.0% for 327-bp fragments of *gltA* genes when compared with the closest known validated species are consistent with novel *Bartonella* species (La Scola et al., 2003). Despite the small size of the nucleotide sequence obtained from one pool of *Culicoides* biting midges (120 bp, excluding primers), the observed differences support the hypothesis that it could correspond to a novel *Bartonella* species. Unfortunately, it was not possible to amplify the *gltA* gene or to clarify the phylogeny of the *rpoB* sequence obtained from the *Culicoides* biting midges. It is not possible to state if the *Bartonella* species identified in these *Culicoides* biting midges could be transmitted to mammals, given that no similar sequences have been retrieved from mammals. We hypothesize the hitherto unknown *Bartonella* species is an endosymbiont, possibly part of the gut microbiome of *Culicoides*, as observed in other arthropods (e.g. carrion beetles, butterflies, honey bees, ants and numerous ectoparasites, Frank et al., 2018).

The *Culicoides* biting midges are grouped with the order Diptera. Other species included in the same order—the sand fly *Lutzomyia verrucarum*, is recognized as a *B. bacilliformis* vector, and several other species of *Lutzomyia* could also be involved in *Bartonella* transmission (Caceres, 1993; Lydy, Lascano, Garcia-Perez, Williams-Newkirk, & Grijalva, 2018). Additionally, the horn fly (*Haematobia* sp.) and the stable fly (*Stomoxys* spp.) can harbour *B. bovis* and *B. henselae*, respectively (Chung et al., 2004). To the authors' knowledge, this is the first report of *Bartonella* in insect of the family Ceratopogonidae.

From a public health perspective, it is important to remark that is not possible to exclude the spillover of the *Bartonella* identified in cervids (moose and red deer) and ticks in this study to humans and domestic animals. In humans, the spillover of *Bartonella* from distantly related species has been described several times, including one from roe deer (HG977196; Frank et al., 2018), and another from the ruminant-associated species *B. schoenbuchensis*, previously described in deer, elk and cattle (Vayssier-Taussat et al., 2016). Frank et al. (2018) affirmed that the observed high *Bartonella* host specificity could be partially explained by ecological factors related to exposure than by immunological incompatibility and consequent lack of susceptibility. Humans in close contact with cervids, as hunters, butchers or veterinarians, could be more exposed to *Bartonella* infection in comparison with the rest of the human population. A One Health approach is necessary to clarify the epidemiology of the *Bartonella* species herein identified in cervids through interactions among humans, and domestic and wild mammals, and vectors.

5 | CONCLUSIONS

This study contributes to the understanding of epidemiology and potential vectors of *Bartonella* species in wild cervids from Norway living outside the deer ked distribution area. Our findings corroborate that a species very similar to *B. bovis* infects moose and red deer outside the deer ked distribution area, with moose as a possible reservoir for this strain. The absence of *Bartonella* species in wild reindeer suggests this species could be a useful sentinel to survey the expansion of this pathogen. We report the first detection of *Bartonella* DNA in ticks from Fennoscandia and in *Culicoides* biting midges worldwide. Future studies are warranted to establish whether *Bartonella* sp. infect semi-domestic reindeer from northern Norway and to identify other potential arthropod vectors.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support our findings are available in the manuscript and in the supplementary material.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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