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Bartonella Infections in Deer Keds (*Lipoptena cervi*) and Moose (*Alces alces*) in Norway

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Infections with *Bartonella* spp. have been recognized as emerging zoonotic diseases in humans. Large knowledge gaps exist, however, relating to reservoirs, vectors, and transmission of these bacteria. We describe identification by culture, PCR, and housekeeping gene sequencing of *Bartonella* spp. in fed, wingless deer keds (*Lipoptena cervi*), deer ked pupae, and blood samples collected from moose, *Alces alces*, sampled within the deer ked distribution range in Norway. Direct sequencing from moose blood sampled in a deer ked-free area also indicated *Bartonella* infection but at a much lower prevalence. The sequencing data suggested the presence of mixed infections involving two species of *Bartonella* within the deer ked range, while moose outside the range appeared to be infected with a single species. *Bartonella* were not detected or cultured from unfed winged deer keds. The results may indicate that long-term bacteremia in the moose represents a reservoir of infection and that *L. cervi* acts as a vector for the spread of infection of *Bartonella* spp. Further research is needed to evaluate the role of *L. cervi* in the transmission of *Bartonella* to animals and humans and the possible pathogenicity of these bacteria for humans and animals.

The deer ked (*Lipoptena cervi*) is a blood-sucking ectoparasitic fly prevalent in Europe and Asia which has been introduced to North America (1). In the Nordic countries, it was until recently restricted to Denmark and the southernmost part of Sweden, but during the last few decades *L. cervi* has shown a remarkable increase in abundance and is currently rapidly expanding its range northward in Finland, Sweden, and Norway (2). Its predominant hosts are cervids, but the insect may attack a wide range of animals (3). They are generally considered a nuisance due to their habit of swarming in large numbers and landing on humans, whom they not infrequently bite. While several authors have reported the presence of persistent pruritic papules on humans bitten by the insect (4–6), their role as vector of pathogens/disease has been poorly elucidated.

Over the years, several insect vectors and mammal hosts have been associated with *Bartonella* sp. infections (See Tsai and coauthors for a review [7]). Dehio et al. isolated *Bartonella schoenbuchensis* from the blood of roe deer (*Capreolus capreolus*) (8). Subsequently, the same workers also found that deer keds collected from individual roe deer or red deer (*Cervus elaphus*) were either negative or positive for this bacterium, indicating that the keds became infected when feeding on bacteremic individuals (9). The midgut of infected insects contains large numbers of *B. schoenbuchensis* bacteria (9). In a concurrent study (10), *B. schoenbuchensis* was detected in deer ked collected from roe deer in France, and the flies were again suggested as a vector. Since then, *Bartonella* DNA of closely related species has been reported from ticks (*Ixodes ricinus*) parasitizing roe deer in Poland (11), southern deer keds (*Lipoptena mazamae*) from white-tailed deer (*Odocoileus virginianus*) in Georgia and South Carolina (12), deer ked from white-tailed deer in Massachusetts (13), and forest flies (*Hippobosca equina*) and blood from rusa deer (*Cervus timorensis russa*) in New Caledonia (14). A single report has also described *B. schoenbuchensis* in blood from a French cow (15).

Several bartonellae are regarded as potential or established emerging zoonotic infections (see Chomel and Kasten for a recent review [16]). Although deer keds have not been directly associated with human bartonellosis, Dehio and coauthors (9) suggested that a risk of transmission of *B. schoenbuchensis* to humans exists through the bite of the insect and that *Bartonella* infection could be the cause of deer ked dermatitis.

The main objective of this study was to determine whether deer keds could be a candidate vector of *Bartonella* infection in moose (*Alces alces*) in Norway. Data from culture, PCR screening, and sequence analysis of *Bartonella* DNA from moose blood and deer keds at different developmental stages is presented.

MATERIALS AND METHODS

Collection of material. A total of 41 moose (*Alces alces*) were sampled within the distribution range of deer ked in southeastern Norway. The studied material comprised 11 carcasses submitted for necropsy in conjunction with an outbreak of deer ked-associated alopecia (thoroughly described by Madslien and coauthors [17]) and free-ranging, presumably healthy moose chemically immobilized in association with radio-collaring. Wingless (fed) deer ked imagines and pupae were collected from the carcasses. Samples were taken from the liver, spleen, and (if available) blood at necropsy, while blood samples were taken from all live animals (Table 1). In addition, blood samples taken from 28 free-ranging, presumably healthy moose immobilized in association with radio-collaring in the Stor-Elvdal area (approximately 38 km north of the recognized deer ked distribution front) were included in the analyses. The collection also included winged deer keds (i.e., imagines that have not yet fed), caught in two localities: (i) Østfold, within a well-established deer ked area, and (ii)

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TABLE 1 Screening for *Bartonella* from moose and deer ked samples by culturing and PCR

Sampling site	Sample type (no. of moose tested)	No. of samples cultivated (in pools of *n*)	No. of samples DNA extracted (in pools of *n*)	No. (%) of positive samples	
				Culture	PCR[b]
Inside deer ked zone	Moose carcasses (11)				
	Liver	11	11	0	0
	Spleen	10	10	0	0
	Blood	7	7	0	0
	Wingless fed deer ked imagines	35 (3–4)	50 (5)	1 (10)	10 (100)
	Deer ked pupae[a]	20 (2–4)	50 (5)	0	5 (50)
	Blood from live moose (30)	29	29	17 (57)	21 (70)
	Winged unfed deer ked imagines	44 (7–8)	50 (5)	0	0
Outside deer ked zone	Blood from live moose (28)	28	28	0	10 (37)

[a] Sampled from only eight carcasses.
[b] Both seminested and qPCR detection.

Akershus, at the deer ked expansion front (see Välimäki [18] for a more thorough description). The deer keds were captured as they settled on a study person walking slowly through a forested area. Blood samples from live animals were collected into EDTA plastic tubes (Becton, Dickinson, Franklin Lakes, NJ) and frozen at −80°C together with the other sampled materials on arrival in the laboratory.

Bacterial culture. Culture was attempted from samples obtained from necropsied moose carcasses, live moose, and winged unfed deer ked imagines as mentioned above by using Columbia agar medium with 5% horse blood (CA) and incubated for a total of 6 weeks with 5% CO_2 at 37°C. Deer ked imagines and pupae were previously surface sterilized in 70% ethanol. While culture was attempted on all tissue samples and imagines from all carcasses, pupae from only eight carcasses were investigated in this way due to scarcity of material. Presumptive *Bartonella* isolates were identified by colony morphology, subsequently confirmed by DNA sequencing. Pure cultures were obtained by successive streaking on agar plates under the same culturing conditions as those described above.

DNA extraction. Prior to DNA extraction, deer ked imagines and pupae were surface disinfected by immersion in 0.5% hypochlorite (5 min) and 70% ethanol (5 min), followed by three rinses in sterile water. These were transferred into a sterile 2-ml microcentrifuge tube containing one 3-mm tungsten carbide bead (Qiagen) and 0.5 g 0.1-mm glass beads (Biospec Products, Bartlesville, OK). To this was added 180 μl QIAamp DNA minikit tissue lysis buffer (Qiagen, Valencia, CA), and the flies were mechanically disrupted in a mini-bead beater (Biospec) for 2 min, with the speed set to "homogenize." The homogenates were held at −20°C for 5 min to reduce foaming and centrifuged at 10,000 × *g* for 1 min to pellet debris. Proteinase K (1 mg/ml) was added to the supernatant and incubated overnight at 55°C. Thereafter, total genomic DNA was purified using the Qiagen QIAamp DNA minikit (Qiagen) according to the manufacturer's protocol. DNA templates from tissues and blood samples were either obtained directly using the same kit as mentioned above or from cultured isolates by boiling cell suspensions in a phosphate buffer for 10 min at 95°C.

PCR detection of *Bartonella* DNA. DNA extracts from the individual samples were subjected to three different PCR strategies for the detection of *Bartonella*. Quantitative PCR (qPCR) and seminested PCR were initially used to screen the samples for *Bartonella* DNA, based on previously described protocols (19, 20). Both assays detect a region in the *gltA* gene. For further analysis of positive samples, conventional PCR was used to produce amplicons from five housekeeping genes (*gltA*, *rpoB*, *ftsZ*, *ribC*, and *groEL*) as described previously (21). Primers for *ribC* and *groE*L were, however, redesigned to amplify a wider range of *Bartonella* species. All primers were purchased from Invitrogen, and their respective sequences are listed in Table 2. Amplifications were performed in 25-μl reaction mixtures containing 1× *Taq* buffer, 0.4 μM forward and reverse primers, 0.2 mM deoxynucleoside triphosphate (dNTP) mix, 1.5 mM $MgCl_2$, 1 U *Taq* DNA polymerase, and 2 to 3 μl DNA template. Each PCR was carried

TABLE 2 Primers and probe used in this study

Gene	Forward sequence (5′→3′)	Reverse sequence (5′-→3′)	Probe sequence (5′→3′)[d]	Amplicon size (bp)	Reference
gltA	GGGGACCAGCTCATGGTGG	AATGCAAAAAGAACAGTAAACA	NA	~350	22
gltA[a]	GGGGACCAGCTCATGGTGG	CGTGGATCATAATTTTTATA	CCAAAACCCATAAG GCGGAAAGGATCATTT	~143	19
gltA[b]	GTTATCCTATTGACCAA AACTCTTGCCGCTATGG	CCAAAACCCATAAGGCG TATTCTTCACAAGGAAC	NA	~685 ~401/387[c]	20
rpoB	GCACGATTYGCATCAT CTTTTCC	CGCATTATGGTCGTATTTGTCC	NA	~333	21
groEL	ATGGACAAAGTTGGC AATGAA	TTCCACCACCAGCAACAATA	NA	~720	This study
ribC	TAACCGATATTGGTTGT GTTGAAG	TAAAGCTAGAAAGTCTGGCA ACATAACG	NA	~588	This study
ftsZ	CATATGGTTTTCATTAC TGCYGGTATGG	TTCTTCGCGAATACGATTAG CAGCTTC	NA	~515	21

[a] qPCR.
[b] Seminested PCR.
[c] Seminested PCR product.
[d] Dually labeled oligonucleotide probe with 5′-6-carboxyfluorescein and 3′-black hole quencher 1. NA, not applicable.

out in a PTC-100 programmable thermal controller (MJ Research Inc., Watertown, MA) with the following thermal cycling conditions: initial denaturation cycle at 95°C for 3 min; followed by 35 cycles of amplification at 95°C for 60s s, 55 or 60°C (depending on the gene) for 60 s, and 72°C for 60 s; and a final extension at 72°C for 10 min. With the exception of *groEL* (annealing at 60°C), all other genes were amplified at annealing temperature of 55°C. PCR products were identified by electrophoresis. In all analyses, positive and negative controls were included within each PCR assay.

Multilocus sequence analysis (MLSA) and phylogeny of *Bartonella* DNA. PCR products of the five examined housekeeping genes were purified with the Nucleospin purification kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's protocol and sequenced in both directions on an automatic DNA sequencer (ABI 3130 Genetic Analyzer; Applied Biosystems, Foster City, CA). Sequencing reactions were performed in a PTC-100 programmable thermal cycler using the amplicon target PCR primers at a concentration of 2.5 μM. Cycling conditions for the sequencing reactions were as described by Platt and coauthors (23). Raw chromatograms (both directions) were assembled, inspected visually for errors, and edited using Sequencher 4.5 (Gene Codes, Ann Arbor, MI). Consensus individual gene sequences aligned in ClustalX 2.1 (24) were compared to reference sequences obtained from the GenBank database.

Phylogenies for individual genes generated using a neighbor-joining algorithm with the Kimura 2 parameter model (1,000 bootstrap replicates) in PAUP 4.0b10 (25) provided congruous tree topologies, and so the sequences were concatenated and reanalyzed using a maximum likelihood algorithm in PhyML (26) with a GTR substitution model (estimated using jModelTest [27]) implemented through the University of Oslo bioportal (www.uio.no/bioportal/).

Nucleotide sequence accession numbers. Sequences obtained during the present study have been submitted to GenBank under the following accession numbers: *gltA*, JN990623 to JN990630; *rpoB*, JN990603 to JN990615; *groEL*, JN990631 to JN990639; *ribC*, JN990640 to JN990650; and *ftsZ*, JN990616 to JN990622.

RESULTS

Bacterial culture. *Bartonella* spp. (as identified by subsequent MLSA) were successfully cultured from a single pooled sample of 10 pools of wingless/fed deer ked imagines and from 17 of 29 blood samples from live moose within the deer ked distribution range. No *Bartonella* spp. were cultured from deer ked pupae, winged/unfed imagines, moose tissues or blood from carcasses, or blood samples from live moose outside the deer ked distribution range (Table 1). Culture from necropsy samples was generally severely compromised by nonspecific bacterial contamination. With the exception of a single carcass, deer ked imagines and pupae were identified on all animals sampled within the deer ked distribution range but not on animals immobilized in the ked-free area (Stor-Elvdal).

Prevalence of *Bartonella* infection. On qPCR screening, positive signals were obtained from 8 of 10 pools of wingless/fed deer keds and 1 of 10 pools of pupae. The cycle threshold (C_T) values, representing the number of genome copies in the sample, were in the range of 20 to 30, indicative of a heavy *Bartonella* presence. In addition, weak positive signals ($C_T > 30$) were identified from 2 of 10 pools of wingless/fed imagines and 4 of 10 pools of pupae examined, while the winged/unfed flies were negative for *Bartonella* DNA (Table 1). Although some inhibition was apparent, all tissues from the carcasses were also negative. An overall prevalence of 87% was detected directly from blood and blood-cultured samples from 30 individual moose within the deer ked range. Of these, 17 were positive by qPCR, and the remaining were detected on seminested PCR amplification and by culture. Generally, qPCR gave weak signals from blood ($C_T > 36.9$), and some culture-positive samples were negative by qPCR. Positive but generally weak PCR signals ($C_T > 36.4$) were also obtained from 10 of 28 blood samples from live moose in the presumed ked-free area, resulting in 36% prevalence (Table 1). The identity of these positive samples was confirmed by sequencing.

Multilocus sequence analysis. Good-quality sequences for all 5 genes were not obtained from all individual samples tested. However, a total of 16 (*gltA*), 17 (*rpoB*), 17(*groEL*), 18 (*ribC*), and 15 (*ftsZ*) sequences were retrieved from 7 wingless/fed imagine deer ked pools, 4 cultured bacterial isolates, and 10 moose blood extracts from within and outside the deer ked zone. Sequence chromatograms retrieved from some deer ked imagines and blood samples originating within the deer ked zone suggested the existence of a mixed infection of related *Bartonella*, as a small number of ambiguous bases were consistently identified in most genes, while all sequences from cultured isolates were identical with no ambiguous bases. Interestingly, most of the ambiguous bases observed in this study were at positions which are diagnostic for species identification in other ruminant-infecting *Bartonella* spp. (Table 3). Sequences obtained from moose blood sampled outside the deer ked zone displayed a very low level of ambiguity (Table 3). For phylogenetic placement, representative sequences obtained directly from blood and blood cultured isolates displaying no ambiguity in nucleotide sequence were used. The consensus maximum likelihood phylogeny based on concatenated sequences from all 5 studied genes resulted in two *Bartonella* lineages (Fig. 1; see also Fig. S1 in the supplemental material). Lineage I was confined within the deer ked zone and clustered closely with *Bartonella chomelii*, *B. schoenbuchensis*, and *Bartonella capreoli*, all infectious bacteria of ruminants within a single clade of *Bartonella*. Identity levels between examined samples and the type sequences of ruminant bartonellae deposited in GenBank were different for each of the 5 loci (see Table S1 in the supplemental material), and it proved difficult to subscribe the bacterial isolate concerned or other sequences generated to an individual *Bartonella* species. On the other hand, lineage II was identified both inside (in 10 of 26 infected moose) and outside (in 10 of 10 infected moose) the deer ked zone. Although the lineage II sequences were almost identical, sequences retrieved from samples originating outside the zone displayed very few ambiguous bases compared to those originating within the zone. As lineage II sequences displayed only limited identity to lineage I isolates/strains (approximately 95% at *ftsZ*, 97% at *rpoB*, 96% at *gltA*, 92% at *ribC*, and 97% at *groEL*) and to other sequences in GenBank (see Table S1), it should be considered a genetically distinct, previously undescribed clade of ruminant-infecting *Bartonella*.

DISCUSSION

The current report describes *Bartonella* infection in moose (*Alces alces*) and deer ked (*Lipoptena cervi*) feeding on this host. Our data indicated a higher prevalence of *Bartonella* DNA in moose within the deer ked zone than in animals outside the zone. Such variation may be due to levels of fly infestation, as there was no indication of deer ked, ticks, or other common cervid-parasitizing hippoboscids in the deer ked-free areas. The strong qPCR signals, equating to large numbers of *Bartonella* genomes in all but two pools of wingless/fed deer ked imagines, is consistent with the proliferation of *Bartonella* in the gut of this insect as described by Dehio and coworkers (9). The signal from moose blood was weak but posi-

TABLE 3 Comparison of the sites with ambiguous bases[a]

Gene	Position	Ambiguous base(s) (no. of samples)						
		Inside the ked zone	Outside the ked zone	*B. chomelii*	*B. capreoli*	*B. schoenbuchensis*	*B. melophagi*	*B. bovis*
ftsZ	411	A/G (2)	G	G	G	G	G	
	432	**A/G (2)**	**A**	**A**	**G**	**A**	**A**	
	453	A	A/G (3)	A	A	A	A	
	489	A/G (5)	A	A	A	A	A	
	492	A	A/G (3)	A	A	A	A	
	497	G/T (1)	T	T	T	T	T	
	624	**A/G (2)**	**A**	**G**	**G**	**G**	**A**	
gltA	**909**	**T/C (4)**	**ND**	**C**	**T**	**C**	**C**	**T**
	918	**T/C (5)**	**ND**	**C**	**C**	**C**	**C**	**T**
	936	**T/C (3)**	**ND**	**C**	**T**	**C**	**T**	**T**
	1077	**T/C (3)**	**ND**	**T**	**C**	**T**	**C**	**C**
groEL	**861**	**A/G (4)**	**ND**	**A**	**A**	**G**		
ribC	**147**	**T/C (3)**	**ND**	**C**	**C**	**C**	**C**	**G**
	306	T/C (2)	**ND**	T	T	T	T	T
rpoB	**1833**	**T/C (1)**	**T/C (2)**	**C**	**C**	**C**	**T**	**T**
	1854	**G/T (3)**	**G/T (1)**	**G**	**G**	**G**	**A**	**G**
	1894	**A/G (4)**	**G**	**A**	**A**	**A**	**A**	**G**
	1902	T/C (2)	T	T	T	T	T	T
	1915	A/G (9)	A/G (1)	G	G	G	G	G
	1923	**A/G (3)**	**A**	**G**	**G**	**G**	**A**	**A**
	1974	**A or G**	**A/G (2)**	**G**	**G**	**G**	**G**	**A**
	2013	A/G (7)	A	A	A	A	A	A
	2019	A/G (3)	A	A	A	A	A	A

[a] Ambiguous bases within sequenced gene fragments of the examined samples and relevant nucleotides in the type sequences of ruminant-infecting bartonellae. The position in the gene is indicated by nucleotide number and with the number of samples in which they were found. Bold indicates the polymorphic sites of ruminant-infecting *Bartonella* spp., where the ambiguities in samples from moose occurred. ND, no data.

tive, consistent with low numbers of circulating *Bartonella*-infected cells. Most interestingly, a strong signal was also obtained from a single pool of pupae and a weaker signal from a further four pools of pupae, suggesting the possibility of vertical transmission. Although this may have represented contamination of the pupae by bacteria from the genital tract of the female after pupal formation (hippoboscids produce fully developed pupae in the female reproductive tract), extensive hypochlorite and ethanol disinfec-

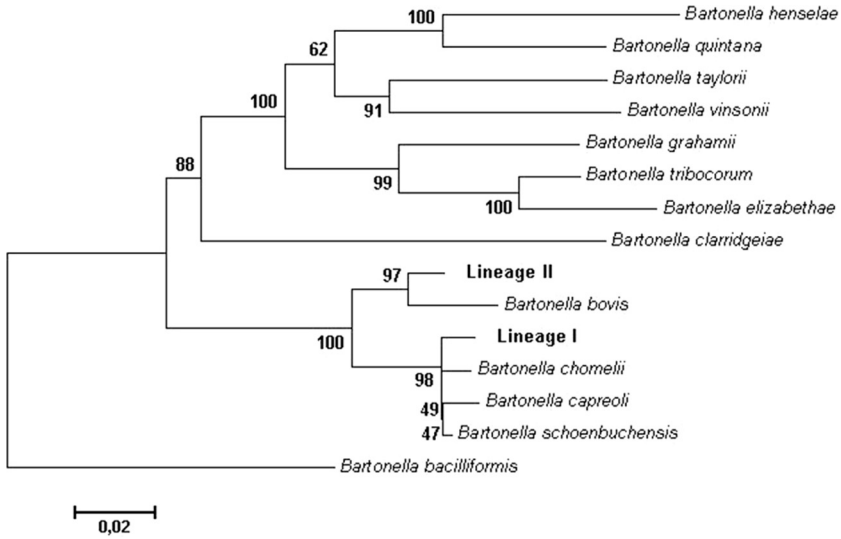

FIG 1 Concatenated phylogenetic tree of *Bartonella*-type isolates and a representative isolate of this study, based on fragments of 3 genes, *gltA*, *rpoB*, and *ribC* (832 bp in total), generated using the maximum likelihood algorithm in PhyML with a GTR substitution model (1,000 replicates; bootstrap values indicated at the nodes).

tion would have been expected to denature surviving bacterial DNA on the surface of the cuticle. The most likely explanation is, therefore, that bacteria are transferred from mother to larva *in utero*, as suggested by Zacharias (28).

Generally, species designation in *Bartonella* has been based on housekeeping gene sequence analysis. La Scola et al. (29) reported that *gltA* and *rpoB* were the most appropriate targets for species differentiation in the genus *Bartonella*. Cutoff values of ≤96.0% in *gltA* and ≤95.4% in *rpoB* gene sequences were proposed for the designation of novel *Bartonella* species. In the present study, we identified two *Bartonella* lineages, with lineage I showing high similarity to corresponding sequences from *B. chomelii*, *B. schoenbuchensis*, and *B. capreoli*, a clade of species primarily infecting ruminants (8, 30–32). The association between lineage I *Bartonella* infection in unwinged flies and moose exclusively within the deer ked range strongly suggests that deer keds may be potential vectors for the transmission of this *Bartonella* species in the sampled moose population. Lineage II sequences could be clearly differentiated as originating from a distinct *Bartonella* clade based on *gltA* and other housekeeping genes. Based on the concatenated sequences, sequences from lineage II form a distinct cluster separate from other known *Bartonella* species and therefore appear to constitute a novel, previously undescribed clade. As strains in lineage II were commonly found in the moose population both inside and outside the deer ked range, they may represent a long-standing endemic infection, for which the means of transmission is entirely unknown.

It is unknown whether these two bacterial species are pathogenic in moose. A single report has described *B. bovis*-associated endocarditis in cattle (33), but in another study, no effect of bacteremia was found on milk production or reproduction in cattle (34), suggesting that ruminant-infecting *Bartonella* species are of little clinical importance. In spite of a thorough examination, no pathological lesions associated with *Bartonella* were found in the necropsy cases in our study (17), and no signs of disease were reported in immobilized animals. The observed prevalence of *Bartonella* in moose blood, however, indicates that the detected strains are able to cause a persistent and systemic infection in this cervid host. As chronic, asymptomatic infection with long-term bacteremia is common for *Bartonella* spp. in their reservoir host (35–37), this may suggest that the moose is a primary host. This seems to be comparable with findings in roe deer, where ~80% of the population was found to be positive for *Bartonella* DNA (8).

Many *Bartonella* spp. are important pathogens causing morbidity or mortality in humans (38–41). While Dehio and coauthors (9) suggested that *B. schoenbuchensis* transmitted with the bites of deer ked may establish a local infection in the skin and thereby contribute to the etiology of deer ked dermatitis, there is limited evidence to support a role for the ruminant-infecting *Bartonella*, such as *B. schoenbuchensis*, *B. capreoli*, *B. melophagi*, or *B. chomelii*, as zoonotic agents. Recently, Maggi and coauthors (42) reported isolation of the closely related "*Candidatus* Bartonella melophagi" from the blood of two diseased women, but no causal relationship between the disease and the infection was proven.

Interestingly, it was suggested that in Sweden, *Bartonella*-induced subacute myocarditis was the cause of sudden unexpected cardiac death (SUCD) in orienteers (43). PCR amplification of a short fragment of the *gltA* gene revealed sequences that could be consistent with *B. quintana* or *B. henselae* in samples from five orienteers who had succumbed to SUCD (43). Four of these five cases, as well as two other orienteers with cardiomyopathy and 31.3% of elite orienteers (compared with 6.8% of healthy blood donors), had antibodies against *Bartonellaceae* (43, 44). The specific identity of the *Bartonella* sp. concerned is, however, uncertain, as species determination was based on PCR and sequencing of a short fragment of the *gltA* gene alone, a gene that is prone to recombination (21). How the Swedish orienteers were exposed to *Bartonella* was not determined, although vector-borne transmission via blood-sucking arthropods was suspected (43). In light of our findings, it is pertinent to mention that the accumulation of SUCD among orienteers coincided with a major increase in the abundance and distribution range of the deer ked in Fennoscandia (2). Given the uncertainty over the identity of the *Bartonella* spp. identified from orienteers, it could be speculated that transmission of *Bartonella* spp. by deer keds, which frequently bite orienteers, may be one of the factors behind the observed high seroprevalence and disease among these sportsmen.

In conclusion, the presented findings show the presence of a potential vector-borne pathogen within a prevalent reservoir host (the moose) and a prevalent and geographically invasive vector which frequently attacks humans and other animals. *Bartonella* infection in the Norwegian moose population involves at least two different clades of *Bartonella*, one potentially transmitted by *Lipoptena*, the other almost certainly not. The high prevalence of infections both inside and outside the deer ked distribution range may suggest transmission of *Bartonella* by different vectors. This warrants further research on *Lipoptena cervi* and *Bartonella* spp. (45).

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