EPIDEMIOLOGY OF BARTONELLA INFECTIONS IN DOGS AND MOOSE:
Animals as sentinels for human disease

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DOCTORAL DISSERTATION

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To Justin and Helena
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ABSTRACT

The incidence of arthropod-borne infections is increasing worldwide and Fennoscandia is no exception. In the last decades, infections transmitted by ticks are being diagnosed more frequently in people living in the Nordic countries. *Ixodes ricinus*, the sheep or castor bean tick, which is the most common tick in North-Western Europe, is widely distributed in Finland. *Ixodes* ticks are vectors of a broad spectrum of pathogens of medical and veterinary importance, such as *Babesia* spp., *Borrelia* spp., *Anaplasma phagocytophilum* (Ap), *Bartonella* spp., tick-borne encephalitis virus (TBEV), and *Francisella tularensis*. To date, there is limited information regarding the prevalence of many vector borne diseases in companion animals in Finland, and therefore the majority of available data come from human medicine studies. Infections caused by *Bartonella* species are considered an emerging zoonosis. One peculiarity of this genus of bacteria is its ability to cause long lasting bacteremia in reservoir hosts. Also, it appears that no other infectious agent is transmitted by more vectors. The deer ked, *Lipoptena cervi*, is an ectoparasite of moose (*Alces alces*), which carries *Bartonella* DNA. Deer keds, which are a nuisance for people, can occasionally bite humans and cause deer ked dermatitis. Whether or not the deer ked can successfully transmit bartonellae to ruminants or humans has not been determined. Because many of the arthropod-borne infections that affect dogs can cause serious disease in people, dogs are considered to be effective sentinel animals to assess the risk of human infection. Also, pets represent a large reservoir for human *Bartonella* infection because most of the species that infect them are zoonotic. The objective of the present research project was threefold: first, to establish the serological and molecular prevalence of selected tick borne diseases in a large group of dogs in Finland; second, to retrospectively compare different diagnostic approaches and clinicopathologic findings in dogs infected with *Bartonella* spp.; and third, to explore the role of the deer ked in the transmission of *Bartonella* spp. to Finnish moose. The serological results from dogs in this study indicate that Finnish dogs are exposed to at least one of four tested arthropod borne pathogens. Dogs were most frequently exposed to Ap (5.3%) followed by Bb (2.9%). Exposure rates were significantly higher in dogs living in Åland. No Finnish dog in this study was infected with *Bartonella* spp., based on PCR. *Bartonella*-infected dogs from the USA were most often infected with *B. henselae*, based on BAPGM enrichment PCR. Interestingly, for most of these dogs, no positive antibodies against *Bartonella* spp were detected. Clinicopathologic abnormalities in dogs with *Bartonella* infection were similar to those dogs suspected to have other vector-borne infection. The presence of *Bartonella* DNA (*B. schoenbuchensis* and *B. bovis*) was demonstrated in deer ked pupae samples and in one winged adult, which indicates transstadial transmission of this bacterium in the deer ked. The same *Bartonella* species were identified in blood samples from free ranging moose in Finland. Furthermore, a high prevalence of *Bartonella* infection was found in moose, which was significantly lowest in northern Lapland, a region considered deer-ked free. These findings further support the potential of *L. cervi* as vector of *Bartonella*. 
## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>Ap</td>
<td><em>Anaplasma phagocytophilum</em></td>
</tr>
<tr>
<td>Bb</td>
<td><em>Borrelia burgdorferi</em></td>
</tr>
<tr>
<td>Ec</td>
<td><em>Ehrlichia canis</em></td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>TBEV</td>
<td>Tick-borne encephalitis virus</td>
</tr>
<tr>
<td>IFA</td>
<td>Immunofluorescent antibody assay</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>Bvb</td>
<td><em>Bartonella vinsonii subsp. berkhoffii</em></td>
</tr>
<tr>
<td>Bh</td>
<td><em>Bartonella henselae</em></td>
</tr>
<tr>
<td>VBDDL</td>
<td>Vector Borne Diseases Diagnostic Laboratory</td>
</tr>
<tr>
<td>NCSU-CVM</td>
<td>North Carolina State University, College of Veterinary Medicine</td>
</tr>
<tr>
<td>BAPGM</td>
<td>Bartonella alpha-Proteobacteria growth medium</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diaminetetraacetic acid</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>SPG</td>
<td>Sucrose-phosphate glutamate</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco’s phosphate buffered saline</td>
</tr>
<tr>
<td>rpoB</td>
<td>Beta subunit of the RNA polymerase gene</td>
</tr>
<tr>
<td>OD</td>
<td>Odds ratio</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence Intervals</td>
</tr>
<tr>
<td>Bk</td>
<td><em>Bartonella koehlerae</em></td>
</tr>
<tr>
<td>Bvl</td>
<td><em>Bartonella volans-like</em></td>
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LIST OF ORIGINAL PUBLICATIONS


*both authors contributed equally

1. REVIEW OF THE LITERATURE

1.1. Arthropod borne diseases in dogs

Diseases transmitted by arthropods are a worldwide problem, causing many clinical illnesses in humans and domestic animals. For multiple reasons, their epidemiology in Europe is gradually changing.\(^1,2\) Cases of tick borne infections are more often being reported in non-endemic areas, both in human and veterinary medicine. Ticks and the diseases they carry have a zoogeographical distribution determined by host movement as well as climatic factors. For instance, changes in temperature and humidity, especially global warming, affect arthropod abundance, its distribution and vector capacity. *Ixodes ricinus*, the sheep or castor bean tick, which is the most common tick in North-Western Europe, is widely distributed in Finland.\(^3\) *Ixodes* ticks are vectors of a broad spectrum of pathogens of medical and veterinary importance,\(^1\) including *Babesia* spp., *Borrelia* spp., *Anaplasma phagocytophilum* (Ap), *Bartonella* spp., tick-borne encephalitis virus (TBEV) and *Francisella tularensis*.

To date, there is limited information regarding the prevalence of many vector borne diseases in companion animals in Finland, and therefore the majority of available data come from human medicine studies. Many arthropod borne infections described in dogs can cause serious disease in people; thus, dogs are considered excellent sentinels to assess the risk of human exposure and infection.\(^1\)

Infections with Ap, the etiologic agent of human granulocytic ehrlichiosis in the US, have been increasingly diagnosed in people and dogs living in Lyme-endemic areas.\(^1,4\) *Anaplasma* infections, also called Tick-borne fever, have been reported in cattle and sheep in Finland.\(^5-7\) In the last years, Ap infection has also been described in one cat,\(^8\) two dogs,\(^9\) and one horse living in Finland.\(^10\) This organism was recently found in *Ixodes ricinus* ticks from the southeastern part of the country.\(^11\)
*Borrelia burgdorferi* (Bb) sensu lato complex causes Lyme disease, which is the most frequently reported arthropod-borne disease in Europe.\(^1,^2\) The prevalence of Bb infection varies geographically and is determined by the distribution of *Ixodes ricinus* and *Ixodes persulcatus*, the primary vectors of Bb. Lyme disease is known to be endemic in Finland.\(^3\) *I. persulcatus* is found along the western coast whereas *I. ricinus* is distributed in the southern and central parts of the country.\(^3\) To date, infection with Bb has been associated with neuroborreliosis, erythema migrans, arthritis and other musculoskeletal symptoms in humans.\(^1\) In contrast, the majority of exposed dogs remain asymptomatic.\(^4\) To the authors’ knowledge, the Bb exposure rate has not been studied in dogs in Finland and much remains unknown regarding the epidemiology of canine Bb infection in the country. In addition to Bb, *I. ricinus* also harbors a great diversity of organisms potentially pathogenic for humans and dogs, including *Bartonella* spp, Ap or *Babesia* spp.\(^5\)

*Ehrlichia canis* (Ec), which is transmitted by the brown tick *Rhipicephalus sanguineus*, causes monocytic ehrlichiosis in dogs. Three clinicopathologic stages of ehrlichiosis have been recognized in dogs:\(^1\) an acute stage, where dogs may show variable signs such as fever, lethargy, lymphadenomegaly, epistaxis; a sub-acute phase, characterized by hyperglobulinemia and cytopenia; and a third or chronic stage, where dogs may have variable clinicopathologic findings and remain seropositive. To date, no studies have reported the rates of Ec exposure in dogs living in a non-endemic country like Finland.

*Bartonella* spp. are Gram-negative hemotropic bacteria that are transmitted by several arthropod vectors, including *Ixodes* ticks, as well as blood transfusion, scratches, and bites.\(^6\) There appears to be a wide spectrum of arthropods that might be potential vectors for *Bartonella* species,\(^5\) for instance, the deer ked, *Lipoptena cervi*, an ectoparasite of Finnish moose (*Alces alces*), which has been found to harbor *Bartonella* DNA.\(^7\) Keds may incidentally bite humans in Finland and they are a nuisance for people who spend
time in forested areas during late summer and early autumn. It is unclear whether dogs which get tick and deer ked bites become infected with *Bartonella* spp. Although case reports of people infected with *Bartonella* spp. have been previously described in Finland,\textsuperscript{18,19} to date no dog infected with *Bartonella* spp. has been reported in Finland.

1.2. *Bartonella* infection in dogs: diagnosis and clinicopathologic abnormalities

The genus *Bartonella* contains a great number of species that are considered to be emerging animal and human pathogens. During the last decade, infection with several *Bartonella* species has been reported in dogs.\textsuperscript{16,20} Members of this genus of bacteria are able to cause chronic intravascular infection and a relapsing pattern of bacteremia in humans, cats and possibly other mammals.\textsuperscript{21-23} Due to its fastidious nature, the definitive diagnosis of *Bartonella* infection has proven to be notably challenging. Conventional diagnostic tests, such as bacterial isolation on agar plates, Enzyme-like immunofluorescence antibodies (ELISA), immunofluorescence antibody assays (IFA), and polymerase chain reaction (PCR) amplification of *Bartonella* DNA after direct extraction from samples have considerable limitations, making these tests relatively insensitive.\textsuperscript{24} For example, in two small case series, approximately 50% of *Bartonella vinsonii* subsp. *berkhoffii* (Bvb) and *Bartonella* (Bh) infected dogs did not have detectable IFA antibodies to the infecting *Bartonella* sp.\textsuperscript{25,26} In 2004, the vector borne diseases diagnostic lab (VBDDL) of the North Carolina State University (NCSU) described a unique diagnostic platform that includes *Bartonella* PCR after direct extraction of DNA from the patient sample, PCR following enrichment culture in an optimized insect cell culture-based growth medium (*Bartonella alpha-Proteobacteria* Growth Medium or BAPGM) and PCR if visible growth occurs following subculture of the BAPGM enriched sample onto a blood agar plate, which is incubated for 4 weeks.\textsuperscript{24}
To date, there is limited information regarding the clinicopathologic abnormalities in dogs diagnosed with *Bartonella* infection. Previous reports were based primarily on serology results and included individual cases or small case series. In addition, only a few studies have described the clinicopathologic findings in dogs experimentally infected with *Bartonella* spp.\(^{27,28}\) Importantly, naturally-infected dogs and human patients infected with *Bartonella* spp. share many similar disease manifestations.\(^{20}\) In a study in which dogs were tested for *Bvb* antibodies, thrombocytopenia, anemia, neutrophilia, and eosinophilia were the most frequently reported hematological abnormalities in *Bvb* seroreactive dogs.\(^{29}\) Another study reported thrombocytopenia in 44% of *B. henselae* (*Bh*) seroreactive dogs.\(^{30}\) A seroepidemiological study found highly variable clinicopathologic abnormalities among individual dogs and only eosinophilia was significantly associated with *Bartonella* seroreactivity.\(^{31}\)

1.3. Deer keds as possible vectors of *Bartonella* spp

The deer ked (*Lipoptena cervi*) is a hematophagous ectoparasite of cervids, especially moose (*Alces alces*).\(^{32}\) Since the deer ked invaded Finland 50 years ago, its distribution has progressively spread northward to the southern parts of Finnish Lapland.\(^{32,33}\) A prerequisite for the deer ked to be a successful *Bartonella* vector is vertical transmission from the mother to its progeny and transstadial transmission from the pupa to the adult. When an adult deer ked attaches to a mammalian host, it drops its wings and remains on the same host for the rest of its life.\(^{34}\) Following a blood meal, the female ked gives birth to larvae, which pupate during the autumn and winter. In Finland, pupae drop to the ground or snow and hatch the following summer- or early autumn. Then, emerged winged unfed adults have a short flying time to search for a suitable host (Fig. 1).\(^{32,34,35}\)
Deer keds in different developmental stages, except the winged adult stage, collected in Germany, France, the United States, and Norway have been shown via PCR or culture to harbor Bartonella spp. However, the question of whether the deer ked is a competent vector for the transmission of Bartonella spp. remains open. Although Bartonella spp. have been isolated or amplified from both pupae and adult wingless deer keds, transstadial transmission has not been demonstrated.

Given that deer keds incidentally bite humans there is potential risk for transmission of B. schoenbuchensis. In fact, B. schoenbuchensis has been considered a possible etiological agent of deer ked dermatitis, which resembles cat scratch disease, a usually self-limiting febrile illness caused by Bartonella henselae. Since the 1970s, there have been an increasing number of people in Finland suffering from recurrent and
occasionally long-lasting dermatitis associated with deer ked bites.\textsuperscript{41}

1.4. \textit{Bartonella} spp infection in moose

\textit{Bartonella} spp. are small Gram-negative bacilli that belong to the alpha-proteobacteria group.\textsuperscript{42} Phylogenetically, this genus of bacteria has been classified in 4 different lineages.\textsuperscript{43} Lineage II is associated with those strains that infect ruminants, which include \textit{B. bovis}, \textit{B. schoenbuchensis} \textit{B. capreoli}, \textit{B. chomelii} as well as \textit{B. melophagi}. Based on epidemiological studies, the prevalence and distribution of \textit{Bartonella} infection in cattle is highly variable around the world, from 0 to 90\%,\textsuperscript{44} however, to date, no data regarding \textit{Bartonella} infection in cattle from Finland are available.

Several hematophagous ectoparasites, such as fleas, ticks and mites have been implicated as bartonellae- transmitting vectors.\textsuperscript{45} For instance, the deer ked (\textit{L. cervi}), a habitual ectoparasite of ruminants, has been proposed as a vector of \textit{Bartonella} spp.\textsuperscript{17,36} Keds in different developmental stages collected in several countries have been found to harbor \textit{Bartonella} spp.\textsuperscript{36-40,46} Recently, we demonstrated transstadial transmission of \textit{Bartonella}, by PCR and DNA sequencing, in deer keds from Finland, which supports the potential of the deer ked for vector competence of \textit{Bartonella} spp.\textsuperscript{46}

Moose (\textit{Alces alces}) are currently the most important host species for \textit{L. cervi} in northern Europe.\textsuperscript{47} \textit{Bartonella} infection, with species closely related to \textit{B. bovis}, \textit{B. capreoli}, \textit{B. chomelii} and \textit{B. schoenbuchensis}, have been described in moose.\textsuperscript{40} In a previous study we identified \textit{B. schoenbuchensis} and \textit{B. bovis} in deer keds and moose from Finland. In that study, however, only a small number of moose from the deer ked infested region were tested.

Whether chronic \textit{Bartonella} bacteremia has any impact on the health of moose deserves further research. Although most of the data suggest that ruminant-associated species are not pathogenic, endocarditis due to \textit{B. bovis} has been diagnosed in cows.\textsuperscript{48,49}
Additionally, *B. bovis* has also been isolated in cats\textsuperscript{50} and in dogs.\textsuperscript{16} The zoonotic potential of *B. bovis* remains unclear.
AIMS OF THE STUDY

The aim of this research was to establish the prevalence of selected arthropod-borne diseases in dogs in Finland, with special focus on Bartonella infections. The diagnosis and clinicopathologic findings of dogs confirmed to be infected with Bartonella spp is also investigated. Finally, the role of the deer ked and moose in the transmission cycle of Bartonella spp is explored.

The specific aims of the studies were the following:

1. To establish the serological and molecular prevalence of selected tick borne diseases, including Bartonella spp, in dogs in Finland, and investigate the risk factors associated with exposure and/or infection. (Study I)
2. To describe the variety of Bartonella species identified in diagnostic samples from sick dogs and compare the results of the IFA seroreactivity to the BAPGM enrichment platform results. (Study II)
3. To compare clinicopathologic findings in dogs diagnosed with Bartonella infection to Bartonella spp negative dogs suspected of a vector-borne disease (Study III)
4. To investigate whether Finnish deer keds carry bartonellae, and if they do, to determine the molecular diversity, prevalence and geographic distribution of the identified Bartonella species. A second objective of this part of the study was to seek evidence of possible vertical and/or transstadial transmission of Bartonella spp. in deer keds; and lastly, to investigate whether Finnish moose and deer keds carry the same Bartonella species, which would further support the role of the deer ked in the transmission of Bartonella spp. (Study IV)
5. To screen the prevalence of Bartonella infection in moose from Finland and compare it with the geographic distribution of the deer ked. (Study V)
3. MATERIALS AND METHODS

3.1. Survey of selected vector borne diseases in Finland (Study I)

3.1.1. Dog population

Anticoagulated blood and serum samples from 340 dogs living in Finland were included in the study. Of these samples, 219 were prospectively collected from client-owned dogs examined in private practices around the country and at the veterinary teaching hospital of the University of Helsinki, in the fall (September to November) of 2011 and 2012. Previously, veterinary clinics from all around Finland had been randomly contacted per email to inform them about this research study. The contact information had been obtained via the internet (www.fonecta.fi, key words: eläinlääkari suomi). In less populated areas of Finland, (Lapland, Åland), the veterinarians were contacted by phone. After the veterinarians had agreed to participate in the study, a pre-paid envelope with detailed instructions for blood collection/storing/shipping was given. Ethylene diaminetetraacetic acid (EDTA) and serum tubes were also sent. In order to avoid any bias selection, veterinarians were specifically asked to collect blood from any dog presented at their clinic/hospital during one week between September and October, regardless of the clinical signs of the dog. In addition, 50 healthy hunting dogs were included in the study, from which blood samples were collected during a hunting dog show in September of 2011. Furthermore, one hundred and twenty-one samples from client-owned Finnish dogs, which had been collected in the fall of 2010 and 2011 and had been stored in a blood bank at -30°C, were included in the study.

3.1.2. Data collection

The date of sample collection, age, breed, size (defined as small $\leq$ 10 kg, medium 11-25 kg, and large $>$ 25 kg), sex, and neuter status, as well as municipality and zip code, were
recorded for the pet dog group. Any travel history outside Finland was also noted. Finally, the health status of the animal at the time of blood collection was also reported.

For the healthy hunting dog group, date of collection, age, sex, municipality, and zip code were recorded. For statistical purposes, municipalities and zip codes were categorized into 6 regions (historical provinces) in Finland: Lapland, Oulu, Eastern Finland, Western Finland, Southern Finland, and from island of Åland.

3.1.3. Serological testing

Serum samples were tested for the presence of antibodies against *Ec*, *Ap*, and *Bb* using ELISA 4DX SNAP® (IDEXX Laboratories). This test detects IgG and IgM antibodies against p44/msp2 of *Ap*, antibodies for *Ec* proteins p30 and p30-1, and C6 peptide of *Bb* sensu lato as well as the antigen for *Dirofilaria immitis*, the agent of heartworm disease in dogs.

3.1.4. DNA extraction and PCR amplification

DNA was extracted from 300 μl of each dog’s frozen EDTA-blood pellet using a commercially available GFX Genomic Blood DNA Purification Kit (Qiagen, Germany). The final eluted volume was 200 μl per sample. PCR screening for *Bartonella* DNA was performed targeting the intergenic spacer (between 16S sRNA and 23S rRNA region) using primers (BsppITS325s: 5’ CCTCMGATGATGATCCCAAGCCTTYTGGCG 3’ and BsppITS1100as: 5’-GAACCGACGACCCTGCCCTGTTGCAAAGCA-3’) as described previously. Amplification was performed in a 25 μL final volume reaction containing 12.5 μl of the Phusion Flash master mix (Fisher Scientific, USA), 200 nM of each primer, and 5 μL of DNA template. For the detection of *Anaplasma* DNA, a quantitative PCR based upon amplification of the multicopy *msp2* gene was performed, modified from a
previously described protocol. Briefly, the reaction was performed at 20 µl final volume containing 10 µl of PerfeCta qPCR ToughMix 2x (Quanta BioSciences, USA), 750 nM of the forward primer 5’-GAAGATGAWGCTGATACAGTA-3’, 750 nM of the reverse primer 5’-CAACHGCCTTAGCAAAACT-3’, 200 nM of the probe Fam-TTATCAGTCTGTCCAGTAACA -Tamra, and 5 µl of template DNA. The Stratagene MX3005P thermocycler was used to run the program with an initial denaturation step of 1 min at 95 °C, followed by 50 cycles of 10 s at 95 °C, 10 s annealing at 53 °C and 8 s extension and measurement at 72 °C.

3.2. Diagnosis and clinicopathologic findings in dogs with *Bartonella* infection (Study II and III)

3.2.1. Study population and review of medical records

This study was performed in North Carolina, USA. The laboratory data from 924 diagnostic samples from 663 sick dogs, which had been submitted to the NCSU-CVM-VBDDL for testing using the BAPGM platform, were retrospectively reviewed. Serology for *Bh* and *Bvb* was performed, in those cases where serum samples were available. From the VBDDL database, all dogs in which infection with a *Bartonella* spp. was confirmed by PCR amplification and DNA sequencing prior to or after enrichment culture in BAPGM were included in this study. Medical records from each dog were reviewed. Data collected included duration of illness as well as concurrent administration of antibiotics or corticosteroids at the time of diagnosis of bartonellosis. For those samples submitted from other institutions, complete medical records were requested by email, fax, or telephone, and reviewed retrospectively.

3.2.2. Control group

The control group, used for the study regarding clinicopathologic abnormalities in
dogs with \textit{Bartonella} infection, included ill dogs suspected of a tick-borne disease that were negative for \textit{Bartonella} using the BAPGM enrichment PCR. Dogs were not matched based on age, sex, or concurrent illness.

3.2.3. Medical record review

Medical records were reviewed in detail by the first author (Cristina Pérez Vera). Veterinarians were contacted by telephone, fax or electronic mail to retrieve the medical records. Data in the medical record were considered adequate when complete signalment and demographic information was recorded, results of physical examinations, complete blood-cell count and serum chemistry panel were available for review. Information regarding travel history as well as environment (presence of other animals in the household, history of ectoparasite exposure) was also obtained in some cases. Geographic coordinates corresponding to zip code were determined. When available, the results of any additional test (aspirates, fluid analyses, histopathology) were reviewed. Also, all available radiographic, ultrasonographic, echocardiographic, and magnetic resonance imaging (MRI) findings were reported. Lastly, BAPGM enrichment culture platform results (including the sample from which \textit{Bartonella} sp. was isolated) were reviewed, and concurrent treatments were summarized.

3.2.4. BAPGM diagnostic platform

A portion of each sample (200\(\mu\)l–2ml of aseptically-obtained EDTA-anticoagulated blood or body fluid) was initially inoculated into liquid medium (BAPGM) and incubated as previously described. After a 7-10 day incubation period, a 1 ml aliquot from the liquid culture was sub-inoculated onto 10\% blood-agar plates (TSA with 10\% sheep blood) and incubated for 4 weeks. Multiple bacterial colonies were collectively swabbed from the surface of the blood-agar plate, re-suspended in sucrose-phosphate glutamate (SPG) buffer,
and stored at -80 °C until processed for DNA extraction. After that, total nucleic acids were extracted from the original diagnostic samples, BAPGM liquid culture samples, and blood-agar plate colonies using the QIAamp DNA minikit (QIAGEN Inc., Valencia, CA). All samples were eluted in nuclease free water and stored at -20 °C until use. An uninoculated BAPGM culture flask was processed simultaneously and in an identical manner with each batch of patient samples tested. PCR screening for *Bartonella* DNA was performed targeting the intergenic spacer (between 16SsRNA-23SsRNA region) using primers sets 325s-1100as and 438s-1100as (BsppITS438s: 5’ GGTTTTCCGGTTTATCCGGAGGGC 3’ and BsppITS1100as: 5’ GAACCGACGCCCTGCTTGCAAAAGCA 3’) as described previously. Primer set 438s-1100as, tested and validated at the Intracellular Pathogens Research Laboratory, NCSU, showed a detection sensitivity of 2.5 genome copies on 98% of *Bh* positive controls.

### 3.2.5. Serological analyses

Serology was performed using modifications of a previously described IFA test. *Bartonella vinsonii* subsp. *berkhoffii* and *Bh* antibodies were determined following traditional IFA practices with fluorescein conjugated goat anti-dog IgG. *Bartonella vinsonii* subsp. *berkhoffii* genotype I (isolate 93-CO-1 from the NCSU-IPRL, ATCC #51672) and *Bh* (strain Houston-1, ATCC #49882) were passed from agar grown cultures of each organism into DH82 (a continuous canine histiocytic cell line) cultures. Heavily infected cell cultures were spotted onto 30-well Teflon coated slides (Cel-Line/Thermo Scientific), air dried, acetone fixed, and stored frozen. Serum samples were diluted in phosphate buffered saline (PBS) solution containing normal goat serum, Tween-20 and powdered nonfat dry milk to block non-specific antigen binding sites. Patient sera were screened at dilutions of 1:16 to 1:64. The cut off titers were 1:64. All sera that remained
reactive at a titer of 1:64 were further tested with twofold dilutions out to a final dilution of 1:8192.

3.3. Detection of *Bartonella* spp in deer ked pupae and winged adult keds (Study IV)

3.3.1. Sample collection

Deer ked pupae were collected from the surface of the snow on moose bedding sites during the winter of 2007, and unfed winged adults were collected in autumn 2008 by walking in the forest and capturing deer keds that attached to the investigators. The pupae and the adults were stored at −70 ºC and −20 ºC, respectively, until processing. Moose blood samples (N=8) were collected by Finnish hunters during the hunting season in 2012. After shooting the moose, 7–10 ml of whole blood from jugular veins or thoracic cavity were collected from each moose into 10 ml EDTA tubes.

3.3.2. Homogenization and DNA extraction

Deer ked pupae and adults were homogenized in porcelain mortars with sterile sand in Dulbecco’s phosphate buffered saline (DPBS) supplemented with 0.2% (w/v) of bovine serum albumin. For the pooled pupae, DPBS was supplemented with antibiotics (10 U/ml penicillin and 0.1 mg/ml streptomycin). The individual pupae and the adults were surface-sterilized before homogenization with 75% EtOH for 5 min with no antibiotics. The DNA of the pooled pupae was extracted with Tripure isolation reagent (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s instructions, except that DNA was eluted in Tris EDTA buffer (pH 8.0). DNA extraction from individual pupae and adults as well as moose blood was performed using DNeasy Blood & Tissue Kits (Qiagen, Hilden, Germany).
3.3.3. Bartonella PCRs

Detection of Bartonella spp. was performed using two PCR techniques. First, all samples were screened with a nested PCR targeting the 16S rRNA gene of Bartonella spp. as described previously. To improve the sensitivity of the PCR method, nested primers were designed using the Primer3 software (Whitehead Institute for Biomedical Research, MIT, Cambridge, MA, USA) based on obtained sequences and those from the GenBank nucleotide sequence database. Amplification was performed in a 25 μL final volume reaction containing 2 mM MgCl₂, 1x Taq Buffer with KCl, 0.2 mM dNTP, 0.4 μM of each primer (16Si-F 5’-CAG CTC GTG TCG TGA GAT GT-3’ and 16Si-R 5’-CAG AGT GCA ATC CGA ACT GA-3’), 2.5 U of Recombinant Taq polymerase (Fermentas, Pittsburgh, PA, USA), and 2.5 μL of DNA template. The primers amplified a 250 bp amplicon. The first round of the nested PCR was performed as previously described; with the exception that the number of the cycles was decreased from 45 to 40 and 2.5 U of TrueStart Taq polymerase (Fermentas) was used. The second round was performed with 1 μl of the first round product in a total volume of 50 μl, and the annealing temperature was set to 54 °C with a total of 30 cycles. To avoid nested PCR contamination, sample preparation, DNA extraction, PCR preparation, and nested PCR amplification and analysis were performed in separate rooms. For species identification of the positive samples, PCR was used to produce amplicons from rpoB gene. The following rpoB oligonucleotides prAPT0244 (5’-GATGTGCATCCTACGCATTATGG-3’) and prAPT0245 (5’-AATGGTGCCTCAGCATATAAG-3’) were used in a previously described protocol. For the second round of the nested PCR, 1 μL of the PCR product of the first round was used as template. DNA from a Finnish human patient with B. quintana-associated endocarditis was used as a positive PCR control in both methods. Distilled water was used as negative control. PCR products were analyzed by 2% agarose gel electrophoresis under UV
exposure. The sizes of the amplicons were determined by comparison with the molecular weight of a standard marker (SM0323, Thermo Fisher Scientific, Schwerte, Germany).

3.3.4. Sequences and phylogenetic tree

All PCR products were directly sequenced and unique sequences deposited into GenBank. The accession numbers for the 16S rRNA sequences of the pupae are JN542708–JN542712 and JN542713 for the winged adults. The accession numbers for the *rpoB* sequences are KJ739719–KJ739723 (two from pupae and three from moose). *Bartonella* species annotation was performed by comparing similarities with other sequences deposited in the GenBank database using the Basic Local Alignment Search Tool (Blast v2.0, McAfee, Santa Clara, CA, USA). Obtained and selected sequences listed in the GenBank nucleotide sequence database were aligned with BioEdit (Ibis Biosciences, Carlsbad, CA, USA) and the phylogenetic tree was constructed based on the *rpoB* gene with the DNAML program from the PHYLIP package.

3.4. *Bartonella* infection in moose from Finland (Study V)

3.4.1. Sample collection

In August 2012 a letter was published at the Finnish hunters’ magazine called “Metsästäjä” requesting the collaboration of hunters from all around the country. All hunters who pay their game management fee receive this magazine 6 times per year. Approximately 300000 hunters are subscribed to this magazine. After the publication of the letter, those hunters willing to collaborate contacted us for further details and pre-paid shipping boxes were sent to each of them, with detailed information about the study, identification stickers and 10 ml EDTA tubes (the number of tubes depended on the number of moose that the hunting group anticipated to hunt). The hunting season in Finland starts at the end of September and ends at the end of December. Each group of
hunters was instructed to collect 7–10 ml of whole blood from the jugular vein or thoracic cavity from each moose, as soon as the animal was shot, and to keep it refrigerated until shipping. The following information was reported from each blood sample: name of the hunter and contact information, the geographic location where the moose was captured (coordinates, zip code or municipality), gender and approximate age of the moose. The latitude and longitude of Finland is approximately 64° 00’ N and 26° 00’ E. Moose were divided into categories as follows: male, female, calf (< year old) and adult (>1 year old). Using the zip code and/or municipality where the sample was obtained, a map was created to depict the origin of the moose samples.

### 3.4.2. DNA extraction and PCR detection of *Bartonella* DNA.

DNA was extracted from 70 μl of each moose’s EDTA-blood using DNeasy Blood & Tissue Kits (Qiagen, Hilden, Germany). The final eluted volume was 200 μl per sample. Detection and characterizing of the *Bartonella* spp. was performed using a multilocus sequence analysis strategy. The samples were first screened for the presence of *Bartonella* DNA by PCR targeting the ITS 16SrRNA-23SrRNA spacer region using the primers BsppITS325s: 5’ CCTCMGATGATGATCCCAAGCCTYTGGCG 3’ and BsppITS1100as: 5’ GAACCGACGACCCCCTGCTTGCAAAGCA as described previously. Amplification, which yields a 400-750 base pair amplicon (depending on species/strain), was performed in a 25 μL final volume reaction containing 12.5 μl of the Phusion Flash Mastermix (10043967, Thermo Fisher Scientific, USA), 0.2 μl of each 10 uM primer, 7.3 μl of molecular-grade water and 5 μl of extracted DNA template. The PCR was performed under the following conditions: a pre-denaturation step at 98°C for one minute, 40 cycles of denaturation at 98°C for one second, annealing at 69°C for five seconds and extension at 72 °C for 15 seconds. The PCR reaction was completed by a final cycle at 72 °C for one minute.
For preliminary species identification, a nested-PCR targeting the beta subunit of the RNA polymerase gene (rpoB) was done for the 107 ITS-PCR positive samples. The outer rpoB oligonucleotides were 1400F 5’-CGCATTGGCTTACTTCGTATG-3’ and 1400R 5’-GTAGACTGATTAGAACGCTG-3’, and the inner oligonucleotides were prAPT0244 (5’-GATGTGCATCCTACGCATTAGTGG-3’) and prAPT0245 (5’-AATGGTGCCCTCAGCATATAAG-3’) as described. The PCR mix used for the first round was 12.5 μL of the Phusion Flash master mix, 5.5 μL of molecular-grade water and 1 μL of each 10 μM primer dilution with 5 μL of template DNA. For the second round of the nested PCR, two μL of the PCR product of the first round was used as template and the amount of water was adjusted accordingly. The PCR reactions were performed with 10 second initial denaturation at 98°C followed by 30 cycles of one second at 98°C, five second annealing at 50°C for the 1st round and 55°C for the 2nd round and 15 second extension at 72°C and ended by a final extension with one minute at 72°C. DNA isolated from a dog with B. henselae infection was used as a positive PCR control. The PCR products (of all PCR reactions) were visualized by agarose gel electrophoresis with GelRed stain (41003, Biotium, CA, USA). Further characterization of selected eight samples was done by PCR and sequence analysis of the citrate synthase gene (gltA) and transfer-messenger RNA gene (SsrA). The primers used for gltA were BhCS.781p 5’ GGGGACCAGCTCATGGTGG 3’ and BhCS.1137n 5’ AATGCAAAAAAGAACAGTAACA 3’ and the primers for SsrA were ssrA F 5’ GCTATGGTAATAATGGACAATGAATAA 3’ and ssrA R 5’ GCTTCTGTGCGCAGGTG 3’. The basic reaction set up used for both PCRs was the same as for The ITS PCR. The program used was 98 °C for one min followed by 40 cycles of 98 °C for three seconds, 58 °C (GltA) and 55 °C (SsrA) annealing for five seconds and 72 °C for 15 seconds, with a final extension of 72 °C for one minute.
Approximately half of the ITS PCR products, representing the different regions, were selected for sequencing, and all the positive samples from the \( rpoB \) PCR. The products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) prior to sequencing. The products were then sequenced using specific PCR primers with Sanger sequencing and the unique sequences were deposited into the GenBank. The accession numbers for the ITS sequences were XXX-YYY. The \textit{Bartonella} species annotation was performed by comparing similarities with other sequences deposited in the GenBank database using the Basic Local Alignment Search Tool (Blast v2.0, McAfee, Santa Clara, CA, USA). The resulting sequences were aligned with ClustalX and analyzed using BioEdit (Ibis Biosciences, Carlsbad, CA, USA), and the phylogenetic tree was constructed based on the \( rpoB \) gene using Bayesian approach implemented in BEAST.

3.5. Statistical analysis (I-V)

The following statistical analyses were performed:

I: Logistic regression analysis was carried out in order to assess associations between each factor (sex age-group, size-group, geographic region, travel history, and health status) and the prevalence to each of the arthropod borne diseases studied. Each factor was first analyzed separately with univariate logistic regression. Each model included only the factor at hand as a fixed effect. A liberal alpha value was selected \((P \leq 0.1)\) as an entry criterion for exact logistic regression analysis. Variables that were significant at the univariate analysis were subsequently individually entered into a multivariable logistic regression analysis, for which significance was set at \(P \leq 0.05\). With the multivariable model, the possible correlations of the factors could be taken into account. In the modeling, the differences between groups were quantified with odds ratios (OR) and their 95\% confidence intervals (CI). All the models were constructed to model the risk of having a
tick borne disease. Statistical analyses were performed using 4Pharma Ltd using SAS® System for Windows, version 9.3 (SAS Institute Inc., Cary, NC, USA).

II: Data from medical records were tabulated and analyzed using statistical software (JMP, SAS Institute Inc., Cary, NC). Results from PCR amplifications of *Bartonella* DNA from BAPGM liquid culture and plate isolates were combined as a single group (BAPGM group) to be compared with PCR results from original samples. PCR results from body fluids such as pleural effusion, pericardial effusion, synovial liquid, cerebrospinal fluid, and urine were combined in a single group to be compared against blood and tissue samples. Serology results were defined as positive and negative based upon the cut-off titers. Illness duration was classified into two groups: less or equal to one month (acute) and more than one month of duration (chronic). The following variables were considered for association analysis: *Bartonella* spp. infection status, *Bartonella* spp. exposure status, specimen type (blood, tissue, body fluids), illness duration, antibiotic therapy, and corticoid therapy. Despite the fact that multiple samples were tested from some subjects, each dog was considered to be a unique event for analysis. Univariate associations were initially evaluated using Chi-Square or Fisher’s Exact Test at a significance level of 0.2. When potential associations were detected, a multivariable logistic regression was performed, with significance level of 0.05. Tests of agreement between matched data (PCR from original samples compared to PCR after enrichment culture, PCR results compared to serology results) were performed using the non-parametric test of McNemar with the continuity correction, with a null hypothesis of agreement between both tests, and a significance level of 0.05. Level of agreement between these diagnostic techniques was determined by Cohen's kappa coefficient and 95% confidence intervals.

III. Descriptive statistics were obtained for all demographic variables, historical clinical signs and physical exam findings, clinicopathologic abnormalities, and previous/current treatments. Univariate analysis was carried out through Chi-squared test
to assess associations between each variable and *Bartonella* infection status. Fisher’s exact test was used when cell size was <5. Variables with more than 5% missing values were not included in the analysis. At this early stage of the analysis, a liberal alpha value was selected (alpha ≤ 0.25). The effect of each significant variable on the outcome variable was adjusted in separate logistic regression models controlling for age and current treatment with doxycycline at the time of blood collection. Variables with a cell size of <5 during initial univariate analysis were not included in the multivariate analysis, with the exception of ‘joint effusion’ and ‘neutropenia’, which had a minimum cell size of 4, and were of particular clinical interest. Significance was set at a *P* value <0.10. Statistical analyses were performed using SAS/STAT 9.2 for Windows (SAS Institute Inc., Cary, NC, 2008).

V: Descriptive statistics were obtained for demographic variables (sex, age, location). For statistical purposes, age was categorized into two categories: calf (< 1 year of age) and adult (>1 year). Municipalities and zip codes were categorized into two regions in Finland based on the last published distribution area of the deer ked (deer ked area and deer-ked-free area).

Univariate associations between infection with *Bartonella* and sex, age, and region were evaluated by Fisher’s exact test. The level of significance was set at *P* < 0.05. Statistical analyses were performed using MedCalc® Statistical Software Version 12.3.0.0 (MedCalc Software, Mariakerke, Belgium).
4. RESULTS

4.1 Survey of selected vector borne diseases in Finland (Study I)

Our study included 340 client-owned pet dogs and 50 healthy hunting dogs. Dog samples were sent from veterinary clinics and hospitals from the South (163 dogs, 10 clinics), Western (92 dogs, 6 clinics) and Eastern Finland (24, 6 clinics), Oulu (28, 2 clinics), Åland (20, 2 clinics), and Lapland (12, 3 clinics). In total, 193 zip codes were recorded (Figure 2). Travel history was available for 193 dogs, of which 27 had a history of staying abroad (Germany, Poland, Sweden, Denmark, Norway, Estonia, France, Spain, USA, Italy and Latvia). Additional demographic information recorded from client owned dogs is available in Table 1.
Table 1. Association between signalment, size, origin, health status, and travel history between dogs that were seropositive and seronegative to any of the tick borne diseases tested (Study I).a

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Categories</th>
<th>Seropositive (N=29)</th>
<th>Seronegative (N=311)</th>
<th>Univariate P valueb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>N (%)</td>
<td>N (%)</td>
<td></td>
</tr>
<tr>
<td>Origin</td>
<td>South</td>
<td>0</td>
<td>156 (96.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>East</td>
<td>1 (4)</td>
<td>24 (96)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>West</td>
<td>10 (10.9)</td>
<td>82 (89.1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Oulu</td>
<td>0</td>
<td>28 (100)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lapland</td>
<td>0</td>
<td>12 (100)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Åland</td>
<td>12 (60)</td>
<td>8 (40)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Missing data</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>≤2 years</td>
<td>6 (5.6)</td>
<td>101 (94.4)</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td>2-8 years</td>
<td>18 (9.6)</td>
<td>170 (90.4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt;8 years</td>
<td>4 (9.1)</td>
<td>40 (90.9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Missing data</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>Male Intact</td>
<td>7 (5.1)</td>
<td>130 (94.9)</td>
<td>0.3896</td>
</tr>
<tr>
<td></td>
<td>Male castrated</td>
<td>2 (12.5)</td>
<td>14 (87.5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Female intact</td>
<td>12 (7.9)</td>
<td>139 (92.1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Female spayed</td>
<td>3 (14.3)</td>
<td>18 (85.7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Missing data</td>
<td>5</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Size</td>
<td>&lt;10 kg</td>
<td>10 (15.2)</td>
<td>56 (84.8)</td>
<td>0.0747</td>
</tr>
<tr>
<td></td>
<td>10-25 kg</td>
<td>8 (9.6)</td>
<td>75 (90.4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt;25 kg</td>
<td>10 (5.7)</td>
<td>165 (94.3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Missing data</td>
<td>1</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Travel history abroad</td>
<td>Yes</td>
<td>4 (14.8)</td>
<td>23 (85.2)</td>
<td>0.6179</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>19 (11.4)</td>
<td>147 (88.6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Missing data</td>
<td>1</td>
<td>141</td>
<td></td>
</tr>
<tr>
<td>Health status</td>
<td>Healthy</td>
<td>28 (10.0)</td>
<td>251 (90)</td>
<td>0.0652</td>
</tr>
<tr>
<td></td>
<td>Sick</td>
<td>1 (1.6)</td>
<td>60 (98.4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Missing data</td>
<td>19 (40.4)</td>
<td>51 (54.8)</td>
<td></td>
</tr>
</tbody>
</table>

aAll data presented as number of dogs (%). bUnivariate analysis performed with Fisher’s exact test. Results statistically significant are highlighted in bold.

In this study Ap antibodies were detected in 5.3% (18/340) client-owned Finnish dogs. Ten (2.9%) dogs, which had not been abroad, were seroreactive to Bb antigens and one was seroreactive to Ec antigens. The seropositivity rate to Ap and Bb was significantly higher in Åland Island, where the seroprevalences were 45% and 20%, respectively. The different seroprevalences per region are available in Figure 2.
Figure 2. Distribution of the 193 zip codes recorded from 340 pet dogs included in our study. The dots denote the dog samples that were seronegative to all vector borne diseases tested, whereas the triangles show the samples that were seropositive to at least one infectious disease. The seroprevalence for every vector borne disease in each region in reported in the table (Study I).

<table>
<thead>
<tr>
<th>Region</th>
<th>Number of Seropositive Dogs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lapland</td>
<td>E.canis 0, B. burgdorferi 0, A.phagocytophilum 0</td>
</tr>
<tr>
<td>Oulu</td>
<td>E.canis 0, B. burgdorferi 0, A.phagocytophilum 0</td>
</tr>
<tr>
<td>Eastern</td>
<td>E.canis 0, B. burgdorferi 0, A.phagocytophilum 0</td>
</tr>
<tr>
<td>Western</td>
<td>E.canis 0, B. burgdorferi 3 (3.3), A.phagocytophilum 6 (6.5)</td>
</tr>
<tr>
<td>South</td>
<td>E.canis 1 (0.6), B. burgdorferi 3 (1.8), A.phagocytophilum 2 (1.2)</td>
</tr>
<tr>
<td>Åland</td>
<td>E.canis 0, B. burgdorferi 4 (20), A.phagocytophilum 9 (45)</td>
</tr>
</tbody>
</table>
One client-owned dog, which was healthy, was infected with Ap, however seronegative to Ap antigens, which is compatible with an acute infection. Also, one dog from Åland was seropositive to both Ap and Bb. Based on logistic regression analysis, there was no significant difference in age, breed, sex, health status, and travel history between seropositive and seronegative dogs (Table 1); however, living in Åland [OR = 26.65; 95% CI: 9.58-74.12] was strongly associated with an increased likelihood of being seropositive for a vector borne disease.

Table 2: Results of the multivariate logistic regression analysis (Study I).

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Odds Ratio (OR)</th>
<th>Standard error</th>
<th>95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy vs Sick</td>
<td>3.176</td>
<td>1.065</td>
<td>0.391</td>
<td>25.792</td>
</tr>
<tr>
<td>Small vs medium</td>
<td>1.392</td>
<td>0.614</td>
<td>0.416</td>
<td>4.660</td>
</tr>
<tr>
<td>Small vs large</td>
<td>2.710</td>
<td>0.575</td>
<td>0.874</td>
<td>8.409</td>
</tr>
<tr>
<td>Medium vs large</td>
<td>1.948</td>
<td>0.569</td>
<td>0.636</td>
<td>5.963</td>
</tr>
<tr>
<td>East vs South</td>
<td>1.012</td>
<td>1.113</td>
<td>0.113</td>
<td>9.041</td>
</tr>
<tr>
<td>West vs South</td>
<td>3.106</td>
<td>0.552</td>
<td>1.048</td>
<td>9.205</td>
</tr>
<tr>
<td>West vs East</td>
<td>3.070</td>
<td>1.084</td>
<td>0.364</td>
<td>25.901</td>
</tr>
<tr>
<td>Åland vs South</td>
<td>55.212</td>
<td>0.743</td>
<td>12.802</td>
<td>238.122</td>
</tr>
<tr>
<td>Åland vs East</td>
<td>54.565</td>
<td>1.187</td>
<td>5.284</td>
<td>563.502</td>
</tr>
<tr>
<td>Åland vs West</td>
<td>17.755</td>
<td>0.693</td>
<td>4.544</td>
<td>69.525</td>
</tr>
<tr>
<td>Åland vs rest of Finland</td>
<td>26.647</td>
<td>0.52</td>
<td>9.580</td>
<td>74.118</td>
</tr>
</tbody>
</table>

Results statistically significant are highlighted in bold.

Fifty healthy hunting dogs living in Southern Finland were also included in our study. Because not all the demographic information was available for all of them and only a few of them (3) were seropositive, they were not included in the statistical analysis. The majority of the dogs were Finnish hounds (34). Other reported breeds were: Swedish Elkhound (4), Labrador Retriever (3), German Shorthaired Pointer (2), English Springer Spaniel (2), German Hunting Terrier (2), West Siberian Laika (1), Finnish Spitz (1), and
working Jack Russell Terrier (1). Eighteen were female and 32 were males, but the neuter status was unknown. Within this group, 4% (2/50) and 2% (1/50) had detectable antibodies against Ap and Bb, respectively. Like in the pet dog population, one dog was infected with Ap but seronegative to Ap antigens. For *Bartonella* spp., none of the dogs tested (pet and hunting dogs) were positive by PCR.

**4.2 Bartonella infection in dogs**

**4.2.1. Diagnosis of Bartonella infection in dogs in the USA (Study II)**

Of the 663 dogs that had been tested using the BAPGM platform, 61 dogs (9.2%) were *Bartonella* positive by PCR. Twenty-eight dogs were examined at NCSU-CVM-VTH and 33 at other veterinary teaching hospitals or private veterinary clinics. A total of 924 diagnostic samples were submitted from North Carolina, South Carolina, New York, Virginia, Minnesota, Tennessee, Texas, Connecticut, Mississippi, Pennsylvania, and New Hampshire. For 18 (41.8%) dogs, the duration of illness was less or equal to 1 month, for 4 (6.6%) dogs between 3 and 6 months, and for 13 (21.3%) dogs longer than 6 months. Twenty-seven (44.3%) dogs were receiving at least one antibiotic at the time of Bartonella testing, 19 of which were being treated with multiple antibiotics. Antimicrobials included tetracyclines (14.8%/27 dogs), macrolides (74%), rifamycins (22.2%), fluoroquinolones (29.6%), and cephalosporins (14.8%). Four of 61 dogs (6.6%) dogs were treated with corticosteroids. *Bartonella sp.* DNA was amplified and sequenced directly from either the samples, from BAPGM enrichment cultures, or from agar plate isolates for 28 (45.9%), 29 (47.5%) and 16 (26.2%) specimens, respectively. *Bartonella* spp. DNA was not amplified from any negative control sample at any time during the study. Forty-five specimens (73.8%) were PCR+ following enrichment culture, of which only 8 samples (13.1%) were also PCR+ following extraction of sample DNA. Thirty-two dogs (52.5%) had a positive diagnostic sample for *Bartonella* DNA only after the BAPGM enrichment steps. The
Cohen’s Kappa coefficient of PCR from original samples compared to PCR after BAPGM enrichment culture was 0.19 (95% CI of 0.05 - 0.32), and statistically these two approaches were not associated ($P = 0.17$, McNemar test).

Of the sixty-one infected dogs, $Bh$ was amplified and sequenced from 30 (49.2%), $Bvb$ from 17 (27.9%), $B.koehlerae$ ($Bk$) from 2 (3.2%), $B.volans-like$ ($Bvl$) from 2 (3.2%), and $B.bovis$ from 1 (1.6%). The remaining 9 (14.8%) dogs were co-infected with more than one $Bartonella$ species (Table 3).
Table 3. Serology and PCR results for 61 (9.2%) Bartonella-positive dogs, of 663 sick dogs from which blood and other fluid samples were tested by BAPGM enrichment culture. Serology results are reported for 30 of the 61 Bartonella-positive dogs (49%) (Study II).

<table>
<thead>
<tr>
<th>Bartonella species detected in 61 infected dogs N (%)</th>
<th>Bartonella IFA results (number positive results/number tested)</th>
<th>Bartonella PCR results (number positive results/number tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bh 30 (49%)</td>
<td>7/19 (37%) 4/17 (24%)</td>
<td>15/30 (50%) 14/30 (47%)</td>
</tr>
<tr>
<td>Bvb17 (28%)</td>
<td>4/10 (40%) 4/10 (40%)</td>
<td>8/17 (47%) 6/17 (35%) 6/17 (35%) 10/17 (59%) 9/17 (53%)</td>
</tr>
<tr>
<td>Bh + Bvb 7 (11%)</td>
<td>0/2 (0%) 0/2 (0%) 2/7 (29%)</td>
<td>6/7 (86%) 3/7 (43%) 6/7 (86%) 5/7 (71%)</td>
</tr>
<tr>
<td>Bk 2 (3%)</td>
<td>NP NP 1/2 (50%)</td>
<td>1/2 (50%) 1/2 (50%) 1/2 (50%)</td>
</tr>
<tr>
<td>Bvl 2 (3%)</td>
<td>0/1 (0%) NP 0/2 (0%)</td>
<td>0/2 (0%) 0/2 (0%) 2/2 (100%) 2/2 (100%) 2/2 (100%)</td>
</tr>
<tr>
<td>B. bovis 1 (1.6%)</td>
<td>NP NP 1/1 (100%)</td>
<td>1/1 (100%) 0/1 (0%) 0/1 (0%) 0/1 (0%)</td>
</tr>
<tr>
<td>Bvb + Bk 1 (1.6%)</td>
<td>NP NP 1/1 (100%)</td>
<td>1/1 (100%) 1/1 (100%) 1/1 (100%) 0/1 (0%)</td>
</tr>
<tr>
<td>Bvb + Bvl (1.6%)</td>
<td>0/1 (0%) 0/1 (0%) 1/1 (100%)</td>
<td>1/1 (100%) 1/1 (100%) 1/1 (100%) 0/1 (0%)</td>
</tr>
<tr>
<td>Total 61 (100%)</td>
<td>11/33 (33%) 8/30 (27%) 29/61 (48%)</td>
<td>29/61 (48%) 16/61 (26%) 40/61 (66%) 32/61 (53%)</td>
</tr>
</tbody>
</table>

Bh Bartonella henselae; Bvl Bartonella volans-like; Bvb Bartonella vinsonii berkhoufii, Bk Bartonella koehlerae

Infection with one or more Bartonella spp. was confirmed by PCR amplification and DNA sequencing of 61 blood and 6 serum samples. Bartonella sp. infection was detected
at the same time from 18 tissue samples, and 9 body fluid or effusion samples (thoracic, pleural, peritoneal, seroma, joint fluid, cerebrospinal fluid, and urine). Of 18 PCR positive tissue samples, (liver, pancreas, lymph node, bone marrow, lung, and heart), 12 (66.6%) contained Bh, 2 (11.1%) contained Bvb, 2 (11.1%) were co-infected with Bh and Bvb and 2 (11.1%) dogs were infected with Bk. Of the 9 PCR positive effusion or fluid samples, 4 (44.4%) contained Bh, 3 (33.3%) contained Bvb, and 2 (22.2%) contained Bh and Bvb. When compared with other Bartonella species or co-infections with multiple species, Bvb DNA was more frequently amplified directly from blood ($P = 0.0136$). Bartonella infection was not statistically associated with illness duration or concurrent antibiotic and corticosteroid therapy.

Overall, Bh and Bvb serology results were available for 30/61 (49%) Bartonella infected dogs. Only 5/20 (25%) Bh and 5/10 (50%) Bvb infected dogs were seroreactive to Bh and Bvb antigens (IFA reciprocal titers of 64 or greater), respectively. None of the co-infected dogs were seropositive. There was no statistical association between serology and Bh or Bvb infection, as determined by PCR.

4.2.2. Clinicopathologic abnormalities in dogs with Bartonella infection: case-control study (Study III)

For this study, 47 Bartonella positive dogs and 93 controls met the inclusion criteria. Twenty-two (46.8%) infected and 75 (80.6%) control dogs had been evaluated at the NCSU-VTH. The rest of the dogs were examined at other university hospitals or private clinics. Samples from the South region represented 87.9% (123 of 140) of the samples, whereas other regions represented 12.1% (17 of 140). Thirty-five (74.5%) Bartonella-infected dogs were from the South, 7 (14.9%) from the Northeast, 3 (6.4%) from the Midwest, and 2 (4.2%) from the West. The majority of the controls (87, 93.5%) were from the South, 4 (4.3%) from the Northeast, and 2 (2.2%) from the West. None of the control
dogs were from the Midwest of the United States.

Blood samples from Bartonella-PCR positive dogs were more likely to be sent from places other than NCSU ($P=0.0001$; Table 4). Given the low number enrichment culture-positive dogs from each state, the association between Bartonella infection and the state of origin was not investigated. There was no significant difference in age, breed, size, sex, or neuter status between Bartonella-infected dogs and controls.

**Table 4.** Association between signalment and history of sick dogs with Bartonella infection detected by enrichment PCR, compared with control dogs (Study III).a
<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No</td>
<td>22 (46.8)</td>
</tr>
<tr>
<td></td>
<td>Missing data</td>
<td>19 (40.4)</td>
</tr>
<tr>
<td>Outdoor access</td>
<td>Indoor Only</td>
<td>10 (21.3)</td>
</tr>
<tr>
<td></td>
<td>Indoor and Outdoor</td>
<td>11 (23.4)</td>
</tr>
<tr>
<td></td>
<td>Outdoor Mostly</td>
<td>9 (19.2)</td>
</tr>
<tr>
<td></td>
<td>Missing data</td>
<td>17 (36.2)</td>
</tr>
<tr>
<td>Cats in household</td>
<td>Yes</td>
<td>4 (8.5)</td>
</tr>
<tr>
<td></td>
<td>No</td>
<td>30 (63.8)</td>
</tr>
<tr>
<td></td>
<td>Missing data</td>
<td>13 (27.6)</td>
</tr>
</tbody>
</table>

*aAll data presented as number of dogs (%). bUnivariate analysis performed with Fisher’s exact test. cP value not calculated due to the large proportion of missing values. Results statistically significant are highlighted in bold.

4.2.2.1 Results of the enrichment PCR

*Bartonella* DNA was detected by PCR and DNA sequencing of blood samples from all 47 dogs. In addition, infection was confirmed in 8 tissue samples and 4 body fluid specimens (thoracic, pleural, peritoneal, seroma, joint fluid, and cerebrospinal fluid). DNA of five different *Bartonella* sp. was amplified and sequenced from these dogs. The frequency of each species, as well as the number of samples positive by PCR prior to and after BAPGM enrichment culture is summarized in Table 5.
Table 5. Agreement between results of *Bartonella* PCR from original samples and after enrichment PCR platform from 47 clinically-ill dogs from the United States (Study III).a

<table>
<thead>
<tr>
<th>Species</th>
<th>Original sample</th>
<th>Enrichment PCR platform</th>
<th>Combined results from original sample and enrichment PCRb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive PCR</td>
<td>BAPGM culture</td>
<td>BAPGM and/or plate culture positive, but negative PCR from original sample</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Positive PCR</td>
<td>Positive PCR</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BAPGM Positive PCR</td>
<td>Plate culture positive PCR</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>BAPGM and/or plate culture positive, but negative PCR from original sample</td>
</tr>
<tr>
<td>B. henselae</td>
<td>10</td>
<td>23</td>
<td>5</td>
</tr>
<tr>
<td>B. vb</td>
<td>9</td>
<td>15</td>
<td>8</td>
</tr>
<tr>
<td>B. volans-like</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>B. koehlerae</td>
<td>1</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>B. bovis</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bartonella spp.²</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Co-infection with more than one species</td>
<td>0</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a All data presented as number of dogs. Because some dogs were infected with more than one *Bartonella* species, the sum of each row or column exceeds the total. b Percentages calculated based on the total number of dogs in this study. ² The species of *Bartonella* could not be identified in this sample.

4.2.2.2 Clinical Signs and Medical History

Compared to controls, presence of joint effusion, skin lesions, and history of weight loss were significantly associated with *Bartonella* infection by univariate analysis \( P < 0.05 \). Based on logistic regression analysis, history of weight loss [OR = 2.82; 95% CI: 1.08-7.56] was the only parameter associated with an increased likelihood of testing positive via enrichment PCR. The most common historical findings in both groups were nonspecific, and the diagnoses for both groups were highly variable. Among the 9 *Bartonella*-infected lame dogs, 4 dogs had joint effusion, all of which were diagnosed with polyarthritis based on cytopathology of an arthrocentesis sample. No arthrocentesis was performed in the remaining 5 dogs. In the vector-borne disease control group, 26 dogs had lameness and 23 of those had joint effusion on physical examination. Four out of 7 dogs, which had arthrocentesis performed, had joint fluid analysis consistent with polyarthritis.
Neutrophilic inflammation was concurrently found in joint and cerebrospinal fluid from one control dog.

**Table 6.** Association between clinical signs or physical exam findings and *Bartonella* infection in dogs detected by enrichment PCR, compared with control dogs (Study III).a

<table>
<thead>
<tr>
<th>Physical examination finding</th>
<th><em>Bartonella</em>-infected</th>
<th>Controls</th>
<th>Uncontrolled Univariate Analysisb</th>
<th>Exact Logistic Regression Analysis controlled for Age and Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N (%)</td>
<td>N (%)</td>
<td>Odds Ratio (OR) 95% CI P value</td>
<td>Adjusted OR 95% CI P value</td>
</tr>
<tr>
<td>Joint Effusion</td>
<td>4 (8.5)</td>
<td>23 (24.7)</td>
<td><strong>0.28</strong> 0.07-0.91 <strong>0.024</strong></td>
<td>0.26 0.06-0.94 0.058</td>
</tr>
<tr>
<td>Weight Loss</td>
<td>16 (34)</td>
<td>18 (19.4)</td>
<td><strong>2.14</strong> 0.89-5.10 0.063</td>
<td><strong>2.82</strong> 1.08-7.56 <strong>0.033</strong></td>
</tr>
</tbody>
</table>

a Only variables with P value <0.05 at the univariate or at the exact logistic regression analysis are shown.

b Univariate analysis performed with Fisher’s exact test. Variables with marginal frequencies < 10 during univariate analysis were excluded from exact logistic regression analysis. Results statistically significant are highlighted in bold.

### 4.2.2.3 Hematologic and Serum Biochemistry Findings

The majority of dogs from which one or more *Bartonella* sp. were detected had normal white blood cell counts, hematocrits, and platelet counts. Variables that were significant at the univariate analysis were individually entered into exact logistic regression analysis to control for age (≤ 96 months vs. > 96 months) and origin (dogs seen at NCSU-VTH vs. other sites). As two of the authors worked as internal medicine clinicians at the NCSU-VTH, there were a higher number of samples submitted to the VBDDL from NCSU. Thus, origin was determined to be a confounder. Given the potential influence of age on laboratory values (for example, azotemia) and the fact that older dogs were more likely to be exposed to vector-borne infections throughout their life, age was also considered to be a confounding variable. Based on logistic regression analysis, hypoglobulinemia [OR = 4.26; 95% CI: 1.31-14.41] was associated with an increased
likelihood of testing positive via enrichment PCR, whereas neutrophilia [OR = 0.36; 95% CI: 0.14-0.87] was associated with a decreased likelihood of being infected with a *Bartonella* sp.

**Table 7.** Association between serum biochemical and hematological abnormalities and *Bartonella* infection in dogs detected by enrichment PCR, compared with control dogs (Study III).a

<table>
<thead>
<tr>
<th>Clinicopathologic abnormalities</th>
<th><em>Bartonella</em>-infected N (%)</th>
<th>Controls N (%)</th>
<th>Uncontrolled Univariate Analysisb</th>
<th>Exact Logistic Regression Analysis controlled for Age and Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N (%)</td>
<td>N (%)</td>
<td>Odds Ratio (OR)</td>
<td>95% CI</td>
</tr>
<tr>
<td>High creatinine</td>
<td>10 (21.7)</td>
<td>37 (41.6)</td>
<td>0.39</td>
<td>0.15-0.94</td>
</tr>
<tr>
<td>Hypoglobulinemia</td>
<td>11 (24.4)</td>
<td>9 (10.6)</td>
<td>2.71</td>
<td>1.92-7.20</td>
</tr>
<tr>
<td>Neutrophilia</td>
<td>11 (23.4)</td>
<td>47 (50.5)</td>
<td>0.30</td>
<td>0.12-0.69</td>
</tr>
<tr>
<td>Neutropenia</td>
<td>7 (14.9)</td>
<td>4 (4.3)</td>
<td>3.85</td>
<td>1.07-15.06</td>
</tr>
</tbody>
</table>

*a Only variables with *P* value <0.05 at the univariate or at the exact logistic regression analysis are shown. Univariate analysis performed with Fisher’s exact test. Variables with marginal frequencies < 10 during univariate analysis were excluded from exact logistic regression analysis. Results statistically significant are highlighted in bold.

4.2.2.4 Treatment

Information about antibiotic and corticosteroid treatments given at the time of the sample collection, as well as later therapy, was available for all dogs. There were no differences in treatments at the time of testing between groups.
4.2.2.5 Results of serologic testing for other vector-borne organisms

Overall, 60% of all dogs were tested for at least one additional vector-borne pathogen, and 7.9% of the *Bartonella*-infected, and 17.1% of the controls, were seropositive for at least another tick-borne disease. Due to the insufficient number of dogs not tested for other organisms, a statistical comparison among groups was not possible.

4.3 Deer keds as possible vectors of *Bartonella* spp (Study IV)

We collected a total of 1154 pupae from 13 different locations within the deer ked distribution area and 118 adult winged keds from a single location (Pulkkila). All pupae samples were analyzed either in pools (54 pools of 2–40) or individually (102 pupae). Fifty-nine pools (2 keds per pool) of adult, winged deer keds were screened. Using 16S rRNA *Bartonella* genus-specific primers, *Bartonella* DNA was successfully amplified from 12/156 (7.7%) deer ked pupae and 1/59 (1.7%) deer ked pools from eight different locations (Fig. 3).
Figure 3. Map of Finland depicting the geographical locations of *Bartonella*-positive samples obtained from deer keds (1–8) and moose (*, #). 1, Siikainen; 2, Yläne; 3, Mynämäki; 4, Kuhmoinen; 5, Kitee; 6, Kuopio; 7, Lemi; 8, Pulkkila; *, Liperi and #, Hyvinkää (Study IV).

All locations where more than three pools of pupae were collected contained at least one sample that was positive for *Bartonella* (Table 8). All samples from locations with three or less pools remained negative (Pörtom 0/1, Juva 0/2, Nilsiä 0/1, Kontiolahti 0/2, Laukaa 0/1, Leppävirta–Heinävesi 0/3 samples). Pulkkila, the location with the positive winged adult, is close to the northernmost limit of the deer ked distribution area.59

In addition, 8 EDTA-blood samples were collected from free-ranging moose for *Bartonella* testing. Five samples were collected in Liperi and 3 in Hyvinkää (Fig. 3). All 8 moose samples were *Bartonella* PCR positive. The overall prevalence of *Bartonella* DNA-
positive pupae in the pools was approximately 0.7% (7/1052), assuming there was at least one *Bartonella* DNA-positive pupa per one positive pool. In addition, 5 out of the 102 (4.9%) individually processed pupae collected from 3 locations were positive and 1/59 (1.7%) of the adult winged deer keds harbored *Bartonella* DNA; likewise, if assumed that only one ked in a positive pool of two carried *Bartonella*, 1/118 (0.8%) of the adults were positive (Table 8).

**Table 8.** Geographical origin of the *Lipoptena cervi* samples and the prevalence of *Bartonella* infection based on PCR and DNA sequencing (Study IV).

<table>
<thead>
<tr>
<th>Location (number in Fig. 2)</th>
<th>Number of positive samples/total number of pooled samples (%)</th>
<th>Total amount of pupae/keds*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Siikainen (1)</td>
<td>2/40 (5)</td>
<td>230</td>
</tr>
<tr>
<td>Yläne (2)</td>
<td>3/73 (4.1)</td>
<td>228</td>
</tr>
<tr>
<td>Mynämäki (3)</td>
<td>1/3 (33.3)</td>
<td>96</td>
</tr>
<tr>
<td>Kuhmoinen (4)</td>
<td>3/9 (33.3)</td>
<td>144</td>
</tr>
<tr>
<td>Kitee (5)</td>
<td>1/2 (50)</td>
<td>62</td>
</tr>
<tr>
<td>Kuopio (6)</td>
<td>1/2 (50)</td>
<td>29</td>
</tr>
<tr>
<td>Lemi (7)</td>
<td>1/17 (5.9)</td>
<td>134</td>
</tr>
<tr>
<td>Pulkkila (8)*</td>
<td>1/59 (1.7)</td>
<td>118</td>
</tr>
</tbody>
</table>

* all winged adult keds were collected at one location, Pulkkila

The amplified product ranged from 200–250 bp in length. Sequence analysis confirmed the presence of *Bartonella* DNA in the screened deer ked and moose samples but identification at the species level was not possible due to a high level of sequence conservation. However, two distinct PCR sequence groups, identical either to *B. bovis* or *B. schoenbuchensis*, were identified, based on one signature nucleotide substitution. Five deer keds (from which DNA samples were available) and all moose samples that were
Bartonella-positive using the 16S rRNA PCR were subsequently tested for the rpoB gene, of which 4/5 and 8/8 were positive. A maximum likelihood phylogenetic tree was generated from the alignment of the rpoB fragments from all deer ked and moose sequences described as well as representatives of Bartonella isolates and Bartonella spp. deposited in Genbank (Fig. 4). The phylogeny demonstrated that there are two Bartonella lineages in deer keds in Finland: the majority of the sequences from deer keds (10/13, 76.9%) and moose (5/8, 62.5%) clustered with B. schoenbuchensis, whereas 23% (3/13) of the sequences from deer keds and 37.5% (3/8) of the moose clustered within another clade more closely related to B. bovis. Sequence analysis demonstrated no geographical clustering of either of the clades.
Figure 4. Maximum likelihood phylogenetic tree based on partial nucleotide sequences of the \( rpoB \) gene, estimated using DNAML program from PHYLIP. Bootstrap support values are given for the major nodes including sequences derived in this study. Clustering pattern of additional samples, from which 16S sequences were derived, is indicated in the boxes. The 16S signature is \( A \) at the position 16 of the 182 nt fragment for the lineage I, and \( C \) for the lineage II. The scale bar indicates evolutionary distance of 0.03 nucleotides per position in the sequence (Study IV).

4.4 \textit{Bartonella} spp. infection in moose

A total of 352 moose blood samples were collected by Finnish hunters. Altogether 148 specimens were selected for \textit{Bartonella} prevalence analyses to represent the deer ked area (32 from the South, 27 from the West, 30 from the East and 25 from Oulu) and the
area outside the deer ked range (34 from northern Lapland). Sixty-eight moose were bulls (45.9%) and 53 (35.8%) were cows. The gender was not recorded for 27 moose (18.2%). Thirty (34.4%) were calves and 57 (65.5%) were adults. The ages for 61 moose were not available. The origin of the samples in relation to the overall deer ked distribution is shown in Figure 5.

**Figure 5.** Geographical origin of the 148 moose samples included in our study. Dots denote the municipalities where moose were hunted. A horizontal line shows the most current distribution of the deer ked in Finland based on observations from 2006-2009 (Study V).33
The overall prevalence of *Bartonella* infection in moose was 72.9% (108/148) using the ITS PCR. Geographically, the prevalence was highest in the South (90.6%) and lowest in Lapland (55.9%). Based on the logistic regression analysis, no significant difference was observed in the age and gender between *Bartonella* positive and *Bartonella* negative moose (Table 9); however, moose from outside the deer ked zone were significantly less likely to be *Bartonella* infected (p<0.015) compared to moose within the deer ked zone.

**Table 9.** Association between geographical location, age and sex between *Bartonella*-positive and *Bartonella*-negative moose (Study V).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Categories</th>
<th>Bartonella positive (N=108) N (%)</th>
<th>Bartonella negative (N=47) N (%)</th>
<th>Univariate P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Origin</td>
<td>Deer ked areas</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>South</td>
<td>29 (90.6)</td>
<td>3 (9.3)</td>
<td>0.0152</td>
</tr>
<tr>
<td></td>
<td>East</td>
<td>19 (63.3)</td>
<td>11 (36.7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>West</td>
<td>24 (88.9)</td>
<td>3 (11.1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Oulu</td>
<td>17 (68)</td>
<td>8 (32)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>89 (78.1)</td>
<td>25 (21.9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Deer ked free areas</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>Calf</td>
<td>21 (70)</td>
<td>9 (30)</td>
<td>0.6371</td>
</tr>
<tr>
<td></td>
<td>Adult</td>
<td>36 (63.1)</td>
<td>21 (36.8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Missing data</td>
<td>51 (83.6)</td>
<td>10 (16.4)</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>48 (70)</td>
<td>20 (30)</td>
<td>0.6835</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>38 (71.7)</td>
<td>15 (28.3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Missing data</td>
<td>22 (81.5)</td>
<td>5 (18.5)</td>
<td></td>
</tr>
</tbody>
</table>

Of the 108 samples that tested positive using the ITS PCR, sequence analysis confirmed the presence of *Bartonella* DNA in 44 selected samples (16 from the South, 14 from the West, three from the East and 11 from Lapland). In 9 additional samples submitted for sequence analysis, no adequate sequences were retrieved. As the partial ITS sequence cannot be used to identify the *Bartonella* species, 107 positive moose samples (one sample was lost) were also tested for the *rpoB* gene, 44 of which (16 from the South, 10 from the West, 6 from the East, 10 from Oulu and two from Lapland) were confirmed to be *Bartonella* positive based on DNA sequencing. All unique sequences were submitted
We detected two different *Bartonella* lineages in Finnish moose. Thirty-nine *rpoB* sequences were closest to *B. bovis* whereas 5/44 were clustered closely with *B. schoenbuchensis*, Candidatus *B. melophagi*, *B. chomelii* and *B. capreoli*, all ruminant bartonellae, as shown in Figure 6. These samples were from the South (3/5), the West (1/5) and Oulu (1/5). In two moose samples, DNA from both lineages, *B. bovis* and *B. schoenbuchensis*-like species, was found. We were further able to obtain good quality sequence from five samples from the gltA gene and six samples from the ssrA gene. The sequencing of these four partial genes, however, yielded insufficient data to fully determine the exact species.

**Figure 6.** A phylogenetic tree based on the partial *rpoB* gene sequences showing the two lineages of *Bartonella* detected in the Finnish moose. Eight unique *rpoB* sequences cluster with *B. schoenbuchensis*, Candidatus *B. melophagi*, *B. chomelii* and *B. capreoli*, while eighteen sequences cluster with *B. bovis*. Bayesian Posterior Probability values are given at the critical nodes.
5. DISCUSSION

5.1. Survey of selected arthropod borne diseases in Finland

This is the first study to investigate exposure to multiple tick borne pathogens in domestic and hunting dogs in Finland. Previous studies are limited to case reports.\textsuperscript{8-10} Our results show that dogs living in Finland are exposed to at least one of four vector-borne pathogens. Our pet dog population was most frequently exposed to Ap, followed by Bb. Examination of a few epidemiologic variables permitted the assessment of possible associations with seropositivity to a vector borne disease. No link was detected between sex, age, travel history, and health status, however small dogs were more likely to be Ap seropositive ($P =0.07$) compared larger dogs. Uni- and multivariate analyses found a strong association between Åland and being seropositive to Ap or Bb. The Åland Islands in Finland, with a population of 28,000, are known to be endemic for tick borne diseases.\textsuperscript{60,61} Åland is an archipelago that includes Main Åland and more than 6,000 smaller islands. The incidence of Bb infection in people in this region is 50 times higher than in mainland Finland.\textsuperscript{62} An epidemiologic study performed in the islands revealed that 85% of the people in Åland had suffered from tick bites.\textsuperscript{60} However there are no published data regarding human granulocytic anaplasmosis in the Åland Islands. Our results suggest that Ap infections, in addition to Lyme disease, may be endemic in this region. Future studies are required to determine whether the prevalence in people correlates with the results observed in dogs.

Dogs in our study were most frequently exposed to Ap compared to the other infectious diseases tested. \textit{Anaplasma} spp. is maintained in the environment by a wide range of hosts such as cattle as well as wild rodents and cervids. A study from 2013 found that 100% of the Swedish moose (\textit{Alces alces}) tested (n=234) were Ap seroreactive.\textsuperscript{62} Therefore, these animals may also be a reservoir for \textit{Anaplasma} spp. Migratory birds may also contribute to the expansion of Anaplasma-infected ticks to new regions.\textsuperscript{63} Ap has been
detected in ticks from Finland and neighboring countries.\textsuperscript{64,65} Even though the prevalence of Ap infection has not yet been studied in Finnish people, studies in Denmark showed a high incidence of human granulocytic ehrlichiosis in people exposed to ticks.\textsuperscript{66}

An older study from Denmark found that up to 16.1\% of healthy dogs were Bb seropositive\textsuperscript{67} however they were not tested for Ap antibodies. Comparably, a higher prevalence of Ap antibodies (20.7\%), in contrast to Bb seroprevalence (4.7\%), was found in Swedish dogs tested between 1991-1994.\textsuperscript{68}

The Snap test used in this study has been described as highly sensitive and specific.\textsuperscript{69} However, serological cross-reactivity between Ap and other related species such as \textit{A. platys}, \textit{E. ewingii}, and \textit{E. chaffeensis} has been reported.\textsuperscript{70} Because no infection associated with any of these \textit{Ehrlichia} spp. has yet been reported in Finland, it is unlikely that the prevalence observed here is based on cross-reactivity. Dogs were only tested once, between September and October, right after the tick season. For this reason, exposure to Ap may have been underestimated. Some of the dogs might have been recently exposed or infected with Ap and perhaps they did not have time to develop detectable antibodies.\textsuperscript{4,70} If dogs had been retested a few weeks later, some of them may have seroconverted. The lack of DNA amplification of Ap from dogs that were seropositive could be due to immunological elimination after infection or a low concentration of DNA in the blood sample. Information regarding any previous clinical signs compatible with a tick borne infection was not obtained from the owners, thus it remains unclear whether the seropositive dogs had been previously infected and subsequently eliminated the infection, therefore remaining seropositive.

Two healthy dogs (one pet dog and one hunting dog, none of which lived in Åland) were infected with Ap in the present study. Even though Ap infections usually cause an acute illness in dogs, subclinical infections have been diagnosed\textsuperscript{70}. Since no follow-up
PCR or serology was carried out in these two dogs, it is unknown whether they were able to eliminate the infection without treatment and become PCR negative.

Altogether, 10 client-owned dogs and one hunting dog were seroreactive to Bb antigens. Because the SNAP 4Dx test only detects antibodies as a result of active infection, it is possible that the rate of exposure is higher than reported here. Previously, Wilhelmsson et al. detected up to 6 different Bb species in ticks that had bitten humans in Åland, which included B. afzelii, B. garinii, B. valaisiana, B. burgdorferi sensu stricto, B. miyamotoi, and B. spielmanii. Neither Bb DNA nor the Bb genospecies were investigated in our dogs, thus future studies are necessary to determine the diversity of Bb species in dogs living in Finland.

Based on the high exposure of hunting dogs to ticks and deer keds, which have been found to harbor Bartonellae, the authors hypothesized that hunting dogs would be subclinically infected with Bartonella spp. However, no dog tested positive for Bartonella spp DNA using PCR in the present study. Even though hunting dogs have frequent outdoor access and may be at higher risk of acquiring a vector borne infection, no higher prevalence could be determined for any tested organism in hunting dogs, compared to the pet dog population. Our data should be cautiously interpreted, because our hunting dog population included hunting dogs that attended dog shows, which may have created a possible bias (the owners of dogs that attend shows may be routinely applying acaricides to their dogs). It is also possible that the population of hunting dogs in our study may not representative of the whole hunting dog population in Finland.

The definitive molecular diagnosis of Bartonella infection has proven to be extremely challenging due to the fastidious nature and intracellular tropism of these bacteria for erythrocytes and endothelial cells. Previously, it was demonstrated that enrichment culture and subculture, followed by PCR amplification, enhances molecular diagnostic sensitivity in dogs. Of the 61 Bartonella infected dogs in that study, BAPGM
(Bartonella alpha-Proteobacteria Growth Medium) enrichment culture was required for molecular diagnosis of 36 (59%) dogs.\textsuperscript{16} Thus, it is possible that our PCR could have missed some positive cases, but the results indicate a very low prevalence – if any - at the population level. Intravascular infection with Bartonella spp. has been associated with a relapsing pattern of bacteremia at 5-day intervals.\textsuperscript{72} Consequently, a diagnosis using blood samples collected from a single point of time remains challenging. In fact, obtaining three sequential blood samples during a one-week period may be recommended to increase the sensitivity of the PCR.\textsuperscript{73} Optimally, antibody screening against Bartonella antigens would have been included in the study. However, serology is diagnostically insensitive. In a previous study, only 25\% of B. henselae infected dogs and only 50\% of the Bvb infected dogs were seroreactive by IFA.\textsuperscript{16}

Finland is situated in the northernmost distribution range of Ixodes ticks and therefore climate change, in particular, may have a substantial impact on the epidemiology of vector borne infections in this country. Currently, the Åland Islands have adequate temperature conditions for the establishment of Ixodes ticks, whereas the temperature in the rest of Finland is not yet optimal for its life cycle. This may explain the geographic differences in seroprevalence observed in our study. If mean annual temperatures continue to increase, as predicted, it is likely that the population of ticks will continue to expand northward. As a result, the prevalence of seropositive dogs will probably increase gradually in the next decades, which may also correlate with a higher incidence of arthropod borne zoonosis in people.

Several additional factors, together with climate change, have probably led to the observed emergence of arthropod borne diseases, such as the improvement in the available diagnostic techniques, the development of commercial serological screening tests and an increased awareness among veterinarians and owners about diseases transmitted by arthropods.\textsuperscript{1} Outdoor recreation is inherent to the Finnish way of life: a large majority of
Finns participate in outdoor activities and visit nature during the course of a year. Popular outdoor activities include walking, swimming in natural waters, spending time at a summer cottage, picking berries and mushrooms, biking, hunting, picnicking, and collecting wood for household use. These activities increase the risk for people and pet dogs for being bitten by ticks, deer keds, and other arthropods.74

5.2 Diagnosis and clinicopathologic findings in dogs with *Bartonella* infection

This retrospective study shows that sick dogs can be infected with various *Bartonella* species and that enrichment culture and subculture, followed by PCR amplification, has a higher sensitivity than IFA testing. Overall, 9.2% (61 of 663) of the dogs tested were infected with one or more *Bartonella* spp., with co-infection documented in 14.7% of the infected dogs. *Bh* and *Bvb* were the most frequently amplified *Bartonella* sequences, accounting for 49.2% and 27.9%, respectively, while less frequently detected species included *Bk*, *Bvl*, and *B.bovis*.

In this study almost 50% of dogs were receiving antibiotics at the time of sample collection. Antibiotic therapy could have decreased the diagnostic sensitivity of the BAPGM platform, particularly the post-enrichment steps. However, *Bartonella* DNA was frequently detected despite antibiotic therapy, which may be consistent with cases of treatment failure. In fact, *Bartonella* resistance genes have been recently delineated, however, the extent to which resistance or failure of antibiotics to achieve adequate intracellular concentrations contribute to treatment failures remains unknown.75 If one suspects canine bartonellosis, these results suggest that a microbiological diagnosis may still be achieved despite concurrent antibiotic treatment. Four dogs (6.6%) were receiving corticosteroids (unknown doses). There was no statistical association with enhanced PCR detection, however only a few dogs were treated with steroids thus it remains unclear whether steroid therapy may have helped the molecular diagnosis of *Bartonella* infection.
Infections associated with a diversity of *Bartonella* spp. are being diagnosed more frequently. This may be due to a combination of increased awareness among veterinarians and physicians, as well as improvements in diagnostic techniques. In the last decade, at least eight *Bartonella* species have been implicated as canine pathogens.\textsuperscript{21-23} In 2004, *Bh*, which for many years was thought to infect cats, but not dogs, was isolated for the first time from a dog from Gabon.\textsuperscript{76} Before its successful isolation, *B. henselae* DNA was amplified and sequenced from lymph nodes (generalized granulomatous lymphadenitis),\textsuperscript{77-79} liver tissue, and from a dog with peliosis hepatis.\textsuperscript{77} In this study, *Bh* was the most frequently detected *Bartonella* sp., which suggests that diagnostic and research attention should be focused on *Bh* infection in dogs.

With regard to less frequently detected species (9.2\% of the cases in this study), *B. elizabethae* DNA was amplified and sequenced from the blood of a dog with chronic weight loss and sudden death and from the lymph node of a Golden retriever.\textsuperscript{53,78} Kosoy and colleagues identified various rodent-associated *Bartonella* species, including *B. elizabethae*, in 31.3\% (60/192) of stray dogs in Thailand.\textsuperscript{76} *Bartonella washoensis* infection was diagnosed in a dog with mitral valve endocarditis,\textsuperscript{80} and *B. clarridgeiae* was associated with endocarditis\textsuperscript{80,81} and lymphocytic hepatitis.\textsuperscript{77} *B. quintana* has also been identified in two dogs with endocarditis.\textsuperscript{82} Most recently, *B. rochalimae* was isolated from three domestic dogs and from 22 gray foxes, a presumed wildlife reservoir in California,\textsuperscript{83} and from a Californian dog with endocarditis.\textsuperscript{84}

Because DNA carryover during the postmortem or histopathological processing of animal tissues has been described\textsuperscript{85} the possibility of DNA carryover should be considered when reading reports in which a *Bartonella* sp. was amplified from processed paraffin-embedded tissues. Additionally, blood as well as other diagnostic fluid samples, should be collected aseptically to avoid contamination with rapidly growing bacteria, which could negate detection of *Bartonella* spp. through the enrichment process.
Based on serologic studies, exposure to *Bh* has been reported in 3.0% (3/100) of dogs in the United Kingdom, 7.7% (4/52) of dogs in Japan, and 27.2% (82/301) of sick dogs in North Carolina. In the present study, infection with *Bh* was found more frequently in sick pet dogs than *Bvb* (49.2% versus 27.9%). In comparison, *Bvb* antibodies were reported in 10% (4/40) of dogs from Israel and in 38% (19/49) of dogs from Thailand, where tick transmission of *Bvb* was suspected. Based upon the results of this study, it appears that serology underestimates the prevalence of *Bartonella* infection in epidemiological studies as well as in clinical cases involving dogs.

This study also provides initial documentation for infection with a Bvl, closely related to *Candidatus B. volans*, a novel *Bartonella* sp. in dogs from the southeastern United States. Bvl was isolated from flying squirrels in the southeastern US. We also report *B. koehlerae* bacteremia in two dogs. Aortic valve endocarditis was also diagnosed in one dog from Israel infected with *Bk*. Our results, in conjunction with previous case reports, suggest that a wide spectrum of *Bartonella* species are capable of infecting pet dogs. Also, this study provides additional evidence that *Bartonella* spp. DNA can be found in a spectrum of patient samples. Bartonella DNA was found in blood (EDTA/serum), tissue biopsies, body fluids (including joint and cerebrospinal fluid, seroma or cavitary effusions), lymph node, and bone marrow aspirates. Based on consecutive blood culture data, a relapsing pattern of bacteremia occurs in experimentally-infected cats, in rodents and most likely in human patients. This pattern of bacteremia may also take place in infected dogs. For this reason, sampling at a single time point could lead to a false negative result. Future prospective studies are necessary to determine which sample source would be most likely to yield positive test results in a given patient and the frequency in which blood cultures should be performed for optimal detection.
In our study, only 25% of \( Bh \) infected dogs and only 50% of the \( Bvb \) infected dogs were seroreactive. Unfortunately, concurrently obtained serology results were only available for 49% of the \( Bartonella \)-infected dogs in this study. Similar discrepancies between serology and PCR analyses are frequently observed in chronic intra-erythrocytic and occult vector borne infections, including babesiosis and leishmaniasis.\(^{97,98}\) Genetic methods, such as PCR amplification of organism-specific DNA sequences, provide a more sensitive and specific means of detecting acute infections. Seronegativity may be due to a short period of time after, as almost half of the dogs diagnosed with bartonellosis in this study had a short duration of clinical signs (less than 1 month). Following experimental infection, 6/6 dogs seroconverted by day 7 post-infection, but the degree and rapidity of antibody production was variable.\(^{28}\) Unfortunately, data regarding the canine humoral response after natural infection are lacking. As the production of antibodies in infected people against the infecting \( Bartonella \) sp. is highly variable, serology appears to be diagnostically insensitive in both dogs and human patients.\(^{99-104}\) In conclusion, as with the diagnosis of many other highly fastidious infectious diseases, concurrently obtaining serology, PCR, and enrichment culture results will improve its diagnostic sensitivity.

Based upon the results of the our study,\(^{105}\) dogs diagnosed with \( Bartonella \) infection had, for the most party, clinicopathologic findings that were similar to the non-specific findings reported in dogs suspected of other arthropod-borne infections. Dogs with \( Bartonella \) infection were often lethargic, febrile, and had lymphadenopathy, similar to those findings of dogs infected with other arthropod borne pathogens.\(^{106,107}\) Weight loss, which was identified in 30 (32.3%) and 18 (19.4%) controls, was significantly associated with \( Bartonella \) infection on multivariate analysis, as compared to sick dogs in which a vector-borne disease was suspected and the enrichment PCR was negative.

Blood samples from \( Bartonella\)-PCR positive dogs were more likely to be submitted from institutions different than NCSU. This association may be due to the fact
that more frequent testing resulted in a higher number of control-dog medical-records being available for review from NCSU-VTH and due to the fact that enrichment PCR testing for *Bartonella* was suggested during telephone or email consultations with non-NCSU-VTH veterinarians.

Many dogs with *Bartonella* infection in our study had normal total white blood cell and platelet counts. Previously, thrombocytopenia was reported as a frequent hematological finding,\(^{29-31}\) however thrombocytopenia was found in only 8 (18%) *Bartonella*-infected dogs in that study. Here, hypoglobulinemia was also associated with *Bartonella* infection, the mechanism of which is not known. Of the 11 dogs with *Bartonella* infection and hypoglobulinemia, 3 suffered from idiopathic chylothorax, 2 had polyarthritis, 1 had chronic lymphoplasmacytic colitis, 1 had endocarditis and secondary heart failure, and 1 was diagnosed with lymphoma on necropsy. None of these dogs received corticosteroids prior to blood sample collection, and none had concurrent hypoalbuminemia, which would indicate a protein losing nephropathy or enteropathy. Based on studies including *Bv*b-experimentally-infected dogs, *Bartonella* infection may cause immune suppression, characterized by defects in monocyctic phagocytosis, cyclic CD8+ T lymphopenia, as well as impaired antigen presentation within lymph nodes.\(^{27,28}\) Given the retrospective nature of the present study, it is unclear whether hypoglobulinemia was secondary to infection-induced immune suppression caused by *Bartonella*.

Limitations of this study are primarily related to its retrospective nature, variability related to the duration of illness, and the possibility of concurrent illnesses, potentially causing biases with regard to the clinicopathologic findings found in each dog. Additionally, the low number of cases with specific pathologic abnormalities of interest (such as endocarditis) limited their inclusion for statistical analysis. One of the limitations of serology is the low sensitivity during the acute-phase of an infection and the inability to confirm ongoing infection based solely on a detectable antibody response. In this study,
serological and PCR testing requested by attending veterinarians varied, therefore targeted infections were frequently limited to one or only a few vector borne organisms. Consequently, the statistical analysis of the influence of other vector borne pathogens in the occurrence of clinical signs and laboratory abnormalities for each group in this study could not be determined. However, a disproportionate number of dogs seroreactive to other vector-borne diseases was not subjectively identified in the Bartonella-PCR positive group, suggesting that co-infections with one or more vector-borne pathogens did not induce a major bias for the results reported in this study. Finally, weight loss and hypoglobulinemia were significantly associated with Bartonella infection, however they may also occur in association with other infectious and non-infectious diseases.

5.3 Detection of Bartonella spp in deer ked pupae and winged adult keds

We provide the initial molecular evidence of Bartonella spp. DNA in an unfed winged adult deer ked, demonstrating that this organism or at least its DNA could survive in the deer ked from one life stage to another (transstadial transmission).

Bartonella spp. are Gram-negative bacteria that infect erythrocytes as well as endothelial cells. Due to its ability to reside within erythrocytes of several hosts, there is a considerable chance that these bacteria are uptaken by several hematophagous arthropods. Bartonella spp. are transmitted by lice, fleas, sandflies (Phlebotominae), and ticks. Bartonella spp. have also been detected by PCR or culture from several other arthropods, such as deer keds. In previous studies, B. schoenbuchensis-like bacteria were detected in deer keds collected from cervids. However, all keds had dropped their wings and they might have started consuming blood. On the other hand, B. bovis was previously reported in the USA and in France. Recently, researchers in Norway investigated the presence of Bartonella spp. in pools of winged unfed deer ked imagines. However, Bartonella DNA was not successfully amplified nor could the bacteria be
cultured. Thus, our data demonstrate the first preliminary evidence for transstadial transmission of *Bartonella* spp. in the deer ked. These results need to be confirmed with larger studies and isolation of viable bacteria.

Based on DNA sequencing, the same two species were identified in both deer keds and moose with practically exact sequences. In a recent study in Norway, two different *Bartonella* clades were found in deer keds and moose after PCR and sequencing.\(^4^0\) One lineage was similar to *B. schoenbuchensis*, *B. chomelii*, and *B. capreoli*, and a distinct lineage of *Bartonella* was found both inside and outside the deer ked range. We identified one *Bartonella* lineage similar to the corresponding sequences from *B. schoenbuchensis* and a different lineage, closely related to *B. bovis*, both within the deer ked distribution range.

Although the number of blood samples was limited, our data suggest a high prevalence of *Bartonella* infection in Finnish moose. Similarly, a high prevalence of *Bartonella* spp. infection was reported in moose blood (70%) collected in Norway.\(^4^0\) In that study, the prevalence of *Bartonella* infection in moose from the deer ked zone was higher than in moose in deer ked-free areas (70% vs. 37%), which suggested that the deer keds may have transmitted the infection. In our study, no geographical cluster was observed in the sequence analysis. However, we were unable to test moose blood samples from outside the deer ked zone.

In a recent study the prevalence and distribution of *Bartonella* infection were investigated in cattle living in Kenya, Thailand, Japan, Georgia, and Guatemala, as well as in buffaloes (*Bubalus bubalis*) from Thailand.\(^4^4\) The authors reported an extremely variable prevalence of *Bartonella* infection across the regions studied. Future epidemiologic studies with a larger number of moose-blood samples are necessary to determine if geographic differences in *Bartonella* prevalence in moose also occur in Finland.
Since its first diagnosis in a patient with endocarditis in 1993, *Bartonella* infection has become an important cause of culture-negative endocarditis in humans. Endocarditis has also been reported in cats (*Felis catus*) associated with *B. henselae* as well as in dogs due to *B. henselae* and *B. schoenbuchensis*. To date, it is unknown whether chronic bacteremia with *B. bovis* and *B. schoenbuchensis* has any impact on the health of the moose. In Finland, up to 17,000 keds can be found on adult moose bulls whereas counts are lower on cows and calves. According to a Finnish study, moose in deer ked-free areas did not show better indices of health compared to infested animals. Although ruminant-infecting *Bartonella* are considered non pathogenic, *B. bovis* has been found in diseased heart valves of cows by PCR and also in a cow diagnosed with *B. bovis* endocarditis by PCR and DNA sequencing, serology, and culture. Given the close association between the deer ked and its ruminant host, as well as the incidental infestation of humans with this arthropod, the deer ked may serve as a competent vector for the transmission of *Bartonella* within ruminants and to people.

### 5.4. *Bartonella* infection in moose from Finland

To the best of the authors’ knowledge this is the first epidemiological study investigating the prevalence of *Bartonella* infection in a large population of moose in Finland. The overall prevalence of *Bartonella* bacteremia was 72.9%, though this rate varied from 60 to 90% depending on the region studied. Moose living in deer ked-infested areas were more likely to be *Bartonella* bacteremic than those moose sampled in the northern Lapland, an area considered deer-ked free. These findings support the likelihood that deer keds may be successful vectors of *Bartonella* spp.

*Lipoptena cervi* is a hematophagous ectoparasite of cervids and domesticated animals, and in Finland, especially moose. Since the deer ked occupied Finland roughly 50 years ago, its geographic distribution has gradually spread at a rate of 50 km per year,
extending now to the southern parts of Lapland. High moose densities are thought to be responsible of the rapid expansion in the deer ked population. In Finland, not only are deer keds a nuisance for those who spend time outdoors, but they may also occasionally bite humans and cause moderate to severe dermatitis. This dermatitis, also known as deer ked dermatitis, is suspected to be associated with *B. schoenbuchensis* infection.

In the last few years, the vector potential of *L. cervi* has been investigated. *Bartonella schoenbuchensis* was first isolated from the gut of wingless adult deer ked. Subsequent studies detected *B. schoenbuchensis* in wingless adult deer keds and in fully developed pupae. Recently, researchers from Hungary demonstrated the presence of *B. schoenbuchensis* DNA in wingless females, developing larvae as well as in fully developed pupae, indicating vertical transmission of bartonellae from female *L. cervi* to their offspring. In addition, vertical transmission has also been reported in *Melophagus ovinus*, a different hippoboscid species.

Overall, more than 70% of moose tested were infected with *Bartonella* spp. *Bartonella* infection in cattle has been extensively screened worldwide and a highly variable prevalence across countries have been found. In Europe, the prevalence of *B. bovis* infection in cattle varies greatly, from 6.8% in Poland, 24.2% in Italy and 59% in France. The diversity of arthropod vectors and its abundance may partially explain these differences across regions.

Our findings parallel those from an earlier study, which detected a higher prevalence of *Bartonella* infection in moose within deer-ked areas in Norway. *Bartonella* DNA was found in 70% (21/29) of moose samples collected in deer ked areas, and in 37% (10/28) of moose from deer ked free areas, respectively. Additionally, moose and keds inside the distribution range of the ked were infected with a lineage of *Bartonella* bacterium closely related to *B. chomelii, B. schoenbuchensis*, and *B. capreoli*, whereas moose outside the distribution range were infected with a different clade of ruminant-infecting *Bartonella*. 
Our results are comparable. Here, the majority of moose in Finland were infected with *B. bovis* based on PCR and DNA sequencing, which was found inside and outside of the deer ked range; however DNA from a different lineage, which could be clearly differentiated from *B. bovis*, was also detected in five moose samples, all of them collected inside the deer ked zone. Not finding this different lineage in the northern parts of Lapland further supports that moose are reservoirs for *B. bovis*, and that *L. cervi* may be transmitting a different Bartonella species, in the *B. schoenbuchensis* lineage, to moose. Controlled experiments of feeding of keds on experimentally infected moose would be necessary to confirm the findings from this as well as other observational studies.

Here, moose from inside the deer ked range were more likely to be infected with *Bartonella* spp than moose from the northern Lapland. However, some moose from outside the deer ked zone were also infected with *Bartonella* spp. How and when these moose got infected remains undetermined. One hypothesis would be that moose migrate around the country, which could explain that moose originally from inside the deer ked range could have been sampled outside the deer ked distribution range. Although moose change pastures and make seasonal migration from winter pastures to summer pastures and vice versa, long-distance movements are actually uncommon and difficult to observe. The distances they move when changing pastures are 10-20 km.\(^{119}\) It is also possible that other vectors, in addition to the deer ked, may be capable of transmitting bartonellae, and this would explain, at least partially, why moose outside the deer ked were also infected with *Bartonella* spp.

Similarly to the Norwegian study, the sequences from a small number of moose confined in the deer ked zone displayed a high level of ambiguity and clustered closely with *B. schoenbuchensis*, *B. chomelii*, *B. melophagi* and *B. capreoli*. Despite performing PCRs targeting four different housekeeping genes (ITS, *rpoB*, *ssrA*, *gltA*), distinguishing the species was not possible. This reflects the highly fastidious nature of this genus of
bacteria, which makes the identification and definitive characterization of *Bartonella* species notably difficult. As previously reported in cattle from Israel\textsuperscript{58} as well as in wild rodents,\textsuperscript{120} the taxonomic classification of *Bartonella* species using direct detection of housekeeping genes is challenging. Co-infection with more than one *Bartonella* species from different clades, which was observed in two specimens, could have contributed to the ambiguity in species identification.\textsuperscript{58} Since not all the moose samples were sequenced, the co-infection rate could not be determined. Bacterial cultures would have been necessary to clearly identify those samples with co-infection. However a different type of sampling protocol would have been required, as our samples were not obtained aseptically. Future studies should consider culturing *Bartonella* from aseptically obtained blood samples, for instance by trapping live moose.

Our study has a few limitations. In order to maximize the number of moose blood samples, hunters performed the collection of the blood specimens during the hunting season. Consequently, it is unknown whether the prevalence of *Bartonella* infection would be different if examined at a different time of the year. In addition, the health status from the moose in our study is unknown. Even though *Bartonella* infections usually result in persistent asymptomatic bacteremia in reservoir hosts, endocarditis due to *B. bovis* has been previously described in cows.\textsuperscript{48,49} The pathogenic potential of *Bartonella* in moose, however, needs further investigation.

We successfully documented the presence of *Bartonella* spp DNA in moose blood at one specific time point, and we found an overall prevalence of *Bartonella* infection of 72\%. Based on human data, a triple draw approach (obtaining blood on three different occasions within a one week period) will increase overall diagnostic sensitivity. This is most likely a result of the relapsing pattern of bacteremia that has been documented to occur in experimentally infected cats and rodents.\textsuperscript{72} Consequently, it is possible that the prevalence of *Bartonella* infection in moose could be greater than here reported.
In conclusion, our data confirm that Finnish moose are natural reservoirs of ruminant-infecting *Bartonella* species, with a highly variable prevalence of infection, ranging between 56% (in northern Lapland) and 90% (in the South). We successfully identified two distinct *Bartonella* lineages, represented by *B. bovis* and *B. schoenbuchensis*. Although the numbers are small, *B. schoenbuchensis* lineage, previously documented in Finnish deer keds, was only found in deer-ked infested areas and not in Lapland, which further supports the potential of *L.cervi* as vector of *Bartonella*. 
CONCLUSIONS

- To the authors’ knowledge, this is the first epidemiologic study to investigate exposure to multiple tick-borne pathogens in dogs in Finland. The results show that dogs are exposed to at least one of four vector-borne pathogens, including Ap, Bb, and Ec. Statistical analysis found a strong association between Åland and being seropositive to Ap and Bb. In addition to Bb and TBEV, Ap infection may be endemic in the Åland Islands. Because *I. ricinus* and *I. persulcatus* are capable of transmitting both Ap and Bb to people and small animals, dogs serve as effective sentinel animals to assess the risk of human infection.

- A wide range of *Bartonella* species can be isolated or PCR amplified from diagnostic specimens from sick dogs. In agreement with seroepidemiologic studies, dogs are more often exposed to and infected with *Bh*, as compared to *Bvb* or other *Bartonella* spp. Dogs can also, although less frequently, be infected with *Bk*, *B. bovis*, or *Bvl*.

- The use of the BAPGM diagnostic platform improves the microbiological documentation of *Bartonella* infection in dogs and it can aid in the detection of novel *Bartonella* sp.

- Dogs infected with *Bartonella* sp. have clinicopathologic abnormalities that are similar to those reported in association with other vector borne infections.

- The same two *Bartonella* lineages, *B. bovis* and *B. schoenbuchensis*, are found in deer keds and moose.

- Finnish moose are natural reservoirs of ruminant-infecting *Bartonella* species, with a highly variable prevalence of infection, ranging between 56% (in northern Lapland) and 90% (in the South). We successfully identified two *Bartonella* lineages, represented by *B. bovis* and *B. schoenbuchensis*. Although the numbers
are small, *B. schoenbuchensis* lineage, previously documented in Finnish deer keds, was only found in deer-ked infested areas and not in Lapland, which further supports the potential of *L.cervi* as vector of *Bartonella*. 
7. FUTURE STUDIES

It is anticipated that this research provides the basis for further epidemiologic studies in the Nordic countries.

- In this study, no healthy dog in Finland was found to be infected with *Bartonella* spp. Future studies should include clinically-ill dogs, especially those with a diagnosis (clinical and/or histopathological) of endocarditis.

- Future studies could focus on disease associations between infection with a selected vector borne disease and immune mediated illnesses (immune mediated anemia, polyarthritis...).

- One of our studies showed a high prevalence of Ap seropositivity in dogs in Åland. This may reflect a high seropositivity also in humans, however that remains to be determined.

- An experimental study, including moose and keds, would be necessary to demonstrate the vector potential of the deer ked

- The University of Helsinki has a blood bank containing moose serum from decades ago. If the deer ked (which invaded the country 50 years ago) is thought to be responsible for the high prevalence of *Bartonella* infection among moose, it would be interesting to develop an IFA assay and retrospectively investigate the prevalence of *Bartonella* antibodies in moose from decades ago, and compare it to moose serum obtained in the present time.
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REFERENCES


