ABSTRACT

PULTORAK, ELIZABETH LAUREN. The Epidemiology of Lyme Disease and Bartonellosis in Humans and Animals. (Under the direction of Edward B. Breitschwerdt).

The expansion of vector borne diseases in humans, a variety of mammalian hosts, and arthropod vectors draws attention to the need for enhanced diagnostic techniques for documenting infection in hosts, effective vector control, and treatment of individuals with associated diseases. Through improved diagnosis of vector-borne disease in both humans and animals, epidemiological studies to elucidate clinical associations or spatio-temporal relationships can be assessed.

Veterinarians, through the use of the C6 peptide in the SNAP DX test kit, may be able to evaluate the changing epidemiology of borreliosis through their canine population. We developed a survey to evaluate the practices and perceptions of veterinarians in North Carolina regarding borreliosis in dogs across different geographic regions of the state. We found that veterinarians’ perception of the risk of borreliosis in North Carolina was consistent with recent scientific reports pertaining to geographic expansion of borreliosis in the state. Veterinarians should promote routine screening of dogs for *Borrelia burgdorferi* exposure as a simple, inexpensive form of surveillance in this transitional geographic region.

We next conducted two separate studies to evaluate *Bartonella* spp. bacteremia or presence of antibodies against *B. henselae, B. koehlerae*, or *B. vinsonii* subsp. *berkhoffii* in 296 patients examined by a rheumatologist and 192 patients with animal exposure (100%) and recent animal bites and scratches (88.0%). Among 296 patients examined by a
rheumatologist, prevalence of antibodies (185 [62%]) and Bartonella spp. bacteremia (122 [41.1%]) was high. In the population exposed to animal bites and scratches, we documented Bartonella spp. seroreactivity or bacteremia in 49.5% (n=95) and 23.9% (n=46) of the patients, respectively. In both studies, serology, in conjunction with blood, serum, and BAPGM enrichment culture PCR, facilitated the diagnosis of Bartonella spp. bacteremia. Patients in both studies frequently reported symptoms including fatigue, sleeplessness, joint pain, and muscle pain. However, neither study can establish a causal link between Bartonella spp. infection and these symptoms in our populations. In our rheumatology population, B. henselae bacteremia was significantly associated with prior referral to a neurologist, most often for blurred vision, subcortical neurologic deficits, or numbness in the extremities, whereas B. koehlerae bacteremia was associated with examination by an infectious disease physician. The contribution of Bartonella spp. infection to these symptoms should be systematically investigated.

Due to low levels of bacteremia in nonreservoir-adapted hosts, which result in diagnostically low levels of circulating bacteria in the bloodstream at a given point in time, Bartonella detection at a single time point may result in false negatives. Therefore, we next sought to determine if the testing of specimens collected serially over a 1-week period significantly improved PCR documentation of Bartonella bacteremia in human patients compared to the testing of specimens from a single time point. Detection was improved when patients were tested three times within a one week period (OR = 3.4 [1.2-9.8]; p = 0.02). Obtaining three sequential blood samples during a one-week period should be considered as a diagnostic approach when bartonellosis is suspected.
Finally, based upon the established oncogenic properties of *Bartonella*, we hypothesized that *Bartonella* spp. can be molecularly detected in canine cutaneous histiocytoma (CCH) and can be localized within skin neoplasms using indirect immunofluorescence (IIF). There were no significant differences in the prevalence of *Bartonella* spp. between our CCH group and controls (p=0.63), and *Bartonella* was identified in only 2/4 (50.0%) CCH tissues using IIF. *Bartonella* spp. are unlikely to cause CCH. Though Bartonella can be visualized in CCH using IIF, cellular localization of *Bartonella* within the skin has reduced sensitivity due to low organism load, a limitation well-supported by previous attempts by our laboratory to localize the bacterium in various tissues and lesions.
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The Epidemiology of Lyme Disease and Bartonellosis in Humans and Animals

by
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DEDICATION

To my sweet boy, little lady, and dear husband.
BIOGRAPHY

Elizabeth received her Bachelor of Science in Molecular and Cell Biology from the University of Illinois, Urbana-Champaign and her Master of Science in Epidemiology and Biostatistics from the University of Illinois, Chicago.
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CHAPTER 1.

EPIDEMIOLOGY OF LYME DISEASE AND BARTONELLOSIS IN HUMANS AND ANIMALS

Excerpts as submitted for publication in Confronting Emerging Zoonoses: The One Health Paradigm.

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Lyme Disease

Introduction

Lyme Disease, the most common vector-borne disease in the Northern Hemisphere, is a multi-system inflammatory disorder caused by gram-negative spirochetes belonging to the genus *Borrelia* (Marques, 2010; Stanek, Wormser, Gray, & Strle, 2012; Tilly, Rosa, & Stewart, 2008). In Europe and Asia, three species of *Borrelia*, including *B. burgdorferi* sensu strictu (s.s.), *B. garinii*, and *B. afzelii*, are responsible for most human cases of disease. Collectively, these species are referred to as *B. burgdorferi* sensu lato. In the United States, Lyme disease is caused by the single species *B. burgdorferi* s.s. Lyme disease first gained attention from scientific and medical communities in the mid-1970s, during an outbreak of oligoarthritis, mainly in children, in rural communities clustered around the town of Lyme, Connecticut (Steere, 1997). Many of these children were initially misdiagnosed as having juvenile rheumatoid arthritis before researchers observed an expanding, annular skin rash before the onset of arthritis in one-quarter of patients (Benach et al., 1983). Now known as ‘bulls-eye rash’, or erythema migrans, this lesion is a defining characteristic of acute-Lyme disease. Early field investigations led to the discovery of pathogen transmission through the bite of infected hard ticks of the genus *Ixodes*, specifically *Ixodes scapularis* and *Ixodes pacificus* (Maggi, Reichelt, Toliver, & Engber, 2010; Marques). Later, DNA-DNA hybridization studies revealed the pathogen to be a new species of the *Borrelia* genus, and was soon after named *Borrelia burgdorferi* (Hyde & Johnson, 1984). *B. burgdorferi* is distributed widely throughout the Northern Hemisphere, particularly in temperate zones.
Climate and landscape modeling of Lyme disease has placed the Northeast and upper Midwest portions of the United States as high risk areas for Lyme disease transmission (Diuk-Wasser et al., 2012). Lower elevation, low vapor pressure deficit, and low seasonal extremes in minimum temperature is associated with the presence of spirochete infected nymphs. Areas with these characteristics overlap with ‘Lyme disease endemic’ counties, which are defined as counties with at least two confirmed, locally acquired cases or in which established populations of a known tick vector are infected with *B. burgdorferi* (Diuk-Wasser et al.). Lyme disease accounts for roughly 90% of all vector borne diseases in the United States; however, recently the Centers for Disease Control and Prevention (CDC) announced that the actual number of cases of Lyme disease is substantially underreported in the United States, and that the actual number is three times what was initially thought.

**Lifecycle**

**Vectors**

Four species of *Ixodes* tick are able to transmit *B. burgdorferi*. In the United States, *I. scapularis* and *I. pacificus* can transmit the pathogen, whereas *I. ricinus* and *I. persulactus* can transmit the pathogen in Europe and Asia, respectively (Kurtenbach et al., 2006; Piesman & Sinsky, 1988). Other hard ticks are unable to efficiently acquire Lyme disease spirochetes from a blood meal, or fail to maintain the spirochetes throughout their life cycle (Mather, Telford, Moore, & Spielman, 1990). The lifecycle of *Ixodes* ticks has a distinct seasonality, with *I. scapularis* and *I. pacificus* nymphs being most active from early summer to early autumn (Stanek et al.). Interestingly, a 3-month difference occurs between peak nymph and
larva activity. Researchers theorize this allows for the reservoir host that has been infected by nymphs to become infective for larvae (Eisen, Eisen, & Lane, 2002). *Ixodes* ticks have a four-stage lifecycle- egg, larva, nymph, and adult; the tick feeds only once during every stage. Feeding time has been shown to vary by stage, and is estimated to be 3 days for larvae, 5 days for nymphs, and 7 days for adult females. *B. burgdorferi* is acquired by tick larvae after feeding on an infected reservoir host. Following a molt into the nymphaial stage, infected ticks can feed on a range of animals and transmit the pathogen to a new reservoir and perpetuate its life cycle. A feeding period of 36 hours or greater is required for transmission (des Vignes et al., 2001). Nymphs then molt into the adult stage; during this stage, the ticks feed primarily on large mammals, which are dead end hosts for *B. burgdorferi* (Lane & Stubbs, 1990). Following a feed, the tick will drop off its host and reside on the soil surface until it develops into the next developmental stage. *Ixodes* ticks require a relative humidity of 80% humidity for survival (Stanek et al.). The overall life cycle of an *Ixodes* tick can last from 2-6 years and is dependent upon environmental factors, such as climate and host availability (Piesman, Zeidner, & Schneider). In order for *B. burgdorferi* to adapt to a variety of hosts, the pathogen varies its gene expression to produce a variety of proteins that allow it to adapt in different environments. Most notably, *B. burgdorferi* must adapt to its hosts temperature and be able to survive and grow within mammals, that regulate body temperature between 37-39°C, and within ticks, that regulate temperature to align with ambient temperature. Additionally, the pH of mammalian tissue and blood is neutral, compared to the more basic pH of the tick midgut (Carroll, Cordova, & Garon, 2000; Schwan & Piesman, 2000; Yang et al., 2000).
Hosts

The lifecycle of *Borrelia* involves infection in multiple reservoir hosts for continued propagation, such as mice, chipmunks, and other small rodents; however, infection in inadvertent hosts, such as dogs and humans, may occur (Radolf, Caimano, Stevenson, & Hu, 2012; Tilly et al., 2008). Vertebrate reservoirs for *B. burgdorferi* include small mammals, such as mice and voles, and species of birds (Figure 2). Larva feed on a variety of animals, including mice, squirrels, and birds, and continue to feed on these hosts through the nymphal stage. Adult ticks feed primarily on larger mammals, including deer. Deer are essential for the maintenance of tick populations, as they are able to feed sufficient numbers of ticks and tick mating occurs on deer, but they are not competent reservoirs for *B. burgdorferi*. Smaller populations of deer within a tick habitat are shown to be indicators of Lyme borreliosis risk due to the variety of species that include reservoir hosts that may be present (Stanek et al.). In larger populations of deer, Lyme borreliosis risk decreases, as ticks are more likely to feed on deer (dead end host) than reservoir hosts. Studies have also shown that cattle and sheep are accidental hosts (Ogden, Nuttall, & Randolph, 1997). Habitats consisting of deciduous or mixed woodland, with a great amount of understory and ground vegetation, support the presence of ticks and the range of hosts involved in the *B. burgdorferi* life-cycle (Stanek et al.). In the southern United States, *I. scapularis* ticks are adapted to feed off lizards and skinks, which may account for a lower incidence of Lyme borreliosis in that particular area (Diuk-Wasser et al.).
Molecular and Cellular Characteristics

*B. burgdorferi* is a Gram-negative organism that is 10-30 um long and 0.2-0.25 um wide and has a genome made up of a small linear chromosome (approximately 900 kb) and over 20 naturally occurring genetic elements (S. R. Casjens et al., 2012; Fraser et al., 1997). The small chromosome carries the majority of the housekeeping genes, while the plasmids exhibit a great variability in gene content and encode differentially expressed outer-surface lipoproteins that are essential to the maintenance of the enzootic cycle and help the organism to survive in very different environments (S. R. Casjens et al.). The genome of *B. burgdorferi* has no classically defined virulence factors, but instead uses the lipoproteins to trigger components of the mammalian innate immune system. Lipoproteins are very abundant within the genome and account for 7.8% of all open reading frames (Samuels). While many of these plasmids are essential for the enzootic cycle, they are not essential for propagation in vitro (Purser & Norris; Schwan, Burgdorfer, & Garon). The loss of plasmids during in vitro experiments creates a barrier for developing techniques to genetically manipulate the bacterium. Complete sequencing of *B. burgdorferi* reveals no obvious virulence factors, creating difficulties in studying the pathogenesis of Lyme disease (S. R. Casjens et al.; Fraser et al.). *B. burgdorferi* is an auxotroph, and is unable to synthesize its own amino acids, nucleotides, and fatty acids. Therefore, it parasitizes all nutrients from its host and has over 52 genes that encode transporters or binding proteins of carbohydrates, peptides, and amino acids to successfully do so (S. Casjens; Gherardini). *B. burgdorferi* also does not have genes to encode enzymes for the tricarboxylic acid cycle and oxidative phosphorylation, and instead derives energy by glycolysis and the fermentation of sugars to lactic acid (Fraser et
al.; Gherardini). An additional hallmark characteristic of *B. burgdorferi*, as with all spirochetes, is the use of flagella that are contained entirely within the periplasmic space and move the organism posteriorly by propagating planar waves (Goldstein, Charon, & Kreiling). Cryoelectron tomographs have shown a ribbon of nine flagellar filaments that wrap around the inner membrane of the spirochete in a right-handed helix and are attached at each cell pole to flagellar motors, which can create motive force for motility (Lu, Wan, Hou, Hao, & Geng). Adjacent to these motors are methyl-accepting chemotaxis proteins that are able to transmit environmental signals to the motors to modulate motility (Goldstein et al.).

**Bacterial proteins**

**Lipoproteins**

In contrast to other proteobacteria, the outer membrane of *B. burgdorferi* is a lipid membrane that contains phosphatidylcholine, phosphatidlyglycerol, and non-inflammatory glycolipids, but does not contain lipopolysaccharide (S. Casjens; LaRocca et al.). The number and diversity of lipoproteins that span the outer membrane is notably different from that of other proteobacteria, and may play an important role in the pathogenesis of *B. burgdorferi* (Bergstrom, Bundoc, & Barbour). The characterization of these lipoproteins on the outer membrane has been an important focus of recent research, including vaccine development. The bacterium uses a variable major protein-like system (*vls*) to create highly diverse epitopes of the outer membrane lipoprotein vlsE during mammalian infection (Fraser et al.). vlsE recombination has been observed in the mouse and rabbit models of infection. In mouse models, antigenic variation of the lipoprotein begins four das following initial
mouse infection, which results in the parental vlsE sequence being eliminated within 28 days post infection (Zhang & Norris). It has been shown that the variation rate of vlsE is faster in immunocompetent mice as compared to immunocompromised mice; this suggests that vlsE is a target of the host immune response (McDowell, Sung, Labandeira-Rey, Skare, & Marconi). Further studies have shown that antibodies against the parental vlsE sequence have reduced affinity for the recombinant vlsE.

**Outer surface proteins (Osps)**

Outer surface proteins, or Osps, are expressed during various times in the pathogen’s lifecycle. OspA is expressed on the surface of *B. burgdorferi* and is present when the pathogen is in the midgut of the tick. OspA may act as an adhesion and bind to tick midgut epithelial cells and maintain *B. burgdorferi*’s adherence to the midgut during feeding (Pal et al.). As *B. burgdorferi* moves into the tick salivary glands, OspA is downregulated and OspC is expressed. OspC is a major protein expressed during the transmission of spirochetes from ticks to mammals (Schwan; Schwan & Piesman). There are conflicting reports in terms of the role of OspC during spirochete dissemination through tick tissues, but OspC is required by *B. burgdorferi* to establish early infection in mouse models. Following transmission from the tick, it has been suggested that OspC may play a role in the colonization in host tissues (Schwan & Piesman).

**Animal Models**

Experimental models have been utilized to study infection of *B. burgdorferi* and subsequent disease manifestations. Small mammals, such as hamsters and rabbits are able to become infected with the pathogen, but not reproduce signs of disease similar to that of
humans. Hamsters do not show signs of disease, although arthritis can be induced in vaccinated animals (R. C. Johnson, Marek, & Kodner). While rabbits develop a rash similar to erythema migrans found in humans, they are able to clear the infection quite readily (Kornblatt, Steere, & Brownstein). In contrast, infected dogs develop arthritis and can become chronically infected (Appel et al.). Rhesus monkeys also develop manifestations similar to humans, including skin rash, arthritis, and neuroborreliosis (Philipp et al.; Roberts et al.). However, mice have been shown to function as an popular model for the natural infectious cycle of the pathogen. Mice can be infected with *B. burgdorferi* through inoculation or tick feeding and develop a serological response and remain chronically infected (Kang, Barthold, Persing, & Bockenstedt; Schwan, Kime, Schrumpf, Coe, & Simpson). Clinical disease varies by strain of mouse utilized; some strains are disease resistance and are studied extensively to determine the factors associated with disease resistance among mice (Crandall et al.; Kang et al.). Other strains, however, are considered to be disease susceptible and develop ankle swelling and inflammation that resembles arthritis in response to infection (Kang et al.). Following infection, mice are able to develop antibodies against various *B. burgdorferi* proteins (Magnarelli; Schwan et al.). While infected mice produce neutralizing antibodies and are able to limit the spirochete load, the host immune response is unable to completely eradicate infection. *B. burgdorferi’s* ability to persist despite an antibody response suggests a bacterial niche in which the bacterium resides to evade the host response, or the bacterium’s ability to vary antigens and mask reactive proteins to evade antibody reactivity (Barthold, Sidman, & Smith). However, studies in SCID mice have shown that antibodies are able to attenuate disease and pathogenesis; infected
SCID mice, which lack antibody components of the immune response, contain higher spirochete loads and exhibit more severely arthritic joints compared to control mice (Barthold et al.). The host immune response to lower spirochete load creates a diagnostic complication, in that low levels limit direct direction of the bacterium in clinical samples.

**Human Borreliosis**

In humans, disease typically manifests in stages: early localized disease, early disseminated disease, and persisting late disease. Early localized disease is typically defined by a ‘bulls-eye rash’ that develops at the site of a tick bite three to 30 days after the bite, in addition to chills, fever, muscle pain, and headache, and begins days after the initial tick bite (Marques; Stanek et al.; Tilly et al.). Erythema migrans typically begins as a red papule and expands over the course of days to weeks as the spirochetes spread within the skin. Approximately 85% of patients experience this skin rash, and it is frequently located around the knees, axilla, and groin (Gerber & Shapiro; Nadelman & Wormser). Stage 2, or early disseminated disease, may begin weeks after the initial tick bite, and includes paralysis of the muscles of the face, heart problems, numbness, and abnormal muscle movement (Stanek et al.; Tilly et al.). Additional systemic manifestations associated with *B. burgdorferi* infection include fatigue, myalgia, arthralgia, headache, fever and/or chills, and stiffness of the neck (Nadelman & Wormser). Stage 3, or disseminated Lyme disease, is characterized by carditis, neuroborreliosis, and arthritis (Stanek et al.; Tilly et al.). Carditis may develop weeks to months after infection, though case series have shown carditis to have a low incidence of less than 1% (Gerber & Shapiro). Neuroborreliosis can occur after long periods of latent infection in up to 10% of untreated patients. Symptoms of neuroborreliosis
typically include peripheral neuropathy, paresthesias, encephalopathy, and encephalomyelitis, and typically develop within weeks after the onset of early-localized disease. Lyme arthritis, characterized by intermittent episodes of joint pain and swelling in the large joints occurs weeks to months after infection and occurs in approximately 60% of untreated patients (Hengge et al.). *B. burgdorferi* does not produce toxins, and it is thought that most damage occurs as a result of inflammatory reactions (Strle & Stanek). Autoimmune mechanisms have been proposed for individuals with Lyme arthritis due to an increase in frequency of HLA-DRA4 alleles (Steere, Gross, Meyer, & Huber). In the synovia from these patients, often vascular proliferation, infiltration of mononuclear cells, and synovial hypertrophy can be seen. Of the patients that develop Lyme arthritis, 10% may develop persisting inflammatory joint disease that may ultimately lead to destruction of the joints. Autoimmunity may occur within affected joints due to molecular mimicry between an immunodominant T-cell epitope of a *B. burgdorferi* outer protein (OspA) and a human adhesion molecule expressed on T-cells in the synovium. In these patients, a high concentration of T-cells that react to OspA concentrate in the joints (Meyer et al.). Interestingly, studies have shown that *B. burgdorferi* DNA can be detected in the synovial fluid of up to 85% of patients with Lyme arthritis through the use of PCR; however, the organism is difficult to culture from the joint fluid using traditional microbiological methods (Hengge et al.; Nocton et al.).
Host immune response

Innate response

The mechanism by which \textit{B. burgdorferi} interacts with the host immune system is incompletely understood. Some individuals, following exposure to \textit{B. burgdorferi}, fail to develop clinical disease, most likely due to the innate immune response. Studies have shown that mice that are genetically deficient in granulocytes and NK cells develop less severe arthritis when infected with \textit{B. burgdorferi} compared to wild type mice, which suggests a role for NK cells in the induction of Lyme arthritis (Barthold). However, this study has also suggested that NK cells may contribute to, but is not required in, the development of Lyme arthritis, as removal of NK cells in infected mice failed to affect the severity of arthritis. Additional support for the contribution of NK cells in the immune response to \textit{B. burgdorferi} lies in the ability of OspA to augment the activation of NK cells and induce inflammatory mediators such as TNF-\(\alpha\), IL-8, and macrophage inflammatory protein 1 (Brown & Reiner). Additionally, activated dendritic cells can produce IL-2 to stimulate NK cells to stimulate an overaggressive, pathogenic response to infection, which can lead to Lyme arthritis. In addition to the NK cell, neutrophils are abundant within the synovial fluid of individuals with Lyme arthritis (Brown & Reiner; Steere et al.). Neutrophils are able to kill \textit{B. burgdorferi} and release granules that can cause local tissue damage (Garcia et al.; Lusitani, Malawista, & Montgomery) (Lusitani D 2002; Garcia R 1998). Interestingly, tick saliva contains factors that can inhibit the action of neutrophils at the site of infection; studies have shown that EM lesions typically do not contain neutrophils (Montgomery, Lusitani, de Boisfleury Chevance, & Malawista). Additionally, neutrophils are able to inhibit \textit{B. burgdorferi}’s spread to the
joint (Xu, Seemanapalli, Reif, Brown, & Liang), suggesting that neutrophils are an essential component of the immune response against infection. However, studies in mice have shown that Lyme arthritis is able to develop independently of neutrophils (Barthold & de Souza). Additionally, the ability of neutrophils to produce inflammatory cytokines, such as IL-1, IL-8, TNF-α, and IL-15, of which a depletion of can prevent arthritis in untreated controls, suggest that neutrophils contribute to arthritis (Amlong et al.; Faldt, Dahlgren, & Ridell).

While the site of infection is typically devoid of neutrophils, macrophages and dendritic cells are often found in EM lesions (Salazar et al.). These cells are able to bind *B. burgdorferi*’s lipoprotein in a TLR-2 dependent manner, which induces the expression of inflammatory cytokines, chemokines, and mediators. More specifically, interaction of *B. burgdorferi* with macrophages results in the production of inflammatory mediators including nitric oxide, IL-1, TNF-α, IL-6, IL-12, and matrix metalloproteinase 9 (MMP-9), a mediator of tissue destruction (Zhao, Fleming, McCloud, & Klempner; Zhao, McCloud, Fleming, & Klempner). A study in hamsters has demonstrated a role for macrophages in the induction of arthritis. Macrophages previously exposed to *B. burgdorferi* were transferred into infected hamsters, which resulted in the development of severe arthritis that correlated with the number of transferred macrophages (DuChateau et al.). In this study, the transfer of unprimed macrophages failed to induce arthritis, implicating macrophages in the development of Lyme arthritis.

**Adaptive response**

Studies have suggested that Th1 cells dominate the immune response to *B. burgdorferi* within the joints of patients with Lyme arthritis. The presence of T-cells without
B-cells has been shown to worsen arthritis and carditis in mouse models, indicating that T-cells are not necessary for the clearance of disease (McKisic & Barthold). The humoral response has been shown to be necessary for clearing *B. burgdorferi* infection (Connolly & Benach, 2005). The T-cell independent production of antibodies, particular IgM, functions to reduce the initial burden of spirochete infection; T-cell dependent production of IgG typically appears during the second week of infection (Tunev et al., 2011). The production of antibodies can occur independently of TLR signaling, as demonstrated in TLR-2 and MYD88-deficient mice (Bolz & Weis, 2004; Wooten et al., 2002). Additionally T-cells have been implicated in the pathogenesis of severe arthritis using the hamster model of infection. Lyme node T-cells from hamsters vaccinated with *B. burgdorferi* are able to confer susceptibility to severe destructive arthritis when transferred into naïve hamsters challenged with *B. burgdorferi*. However, this was not seen when the hamsters were infused with normal T-cells and later challenged with *B. burgdorferi* (McKisic & Barthold, 2000).

**Molecular mimicry**

In the early 1990s, researchers discovered that patients suffering with chronic Lyme arthritis had increased frequencies of the HLA-DR4 and HLA-DR2 alleles (Steere, Berardi, Weeks, Logigian, & Ackermann, 1990). Soon after, it was observed that patients with chronic Lyme arthritis that failed antibiotic therapy were more likely to have immune responses to the outer-surface protein (OspA) than patients that responded to treatment (Kalish, Leong, & Steere, 1993). In one study, 93% of patients with antibiotic-refractory Lyme arthritis had immune responses to OspA, compared to 35% of arthritis patients that responded to treatment (Chen et al., 1999). It has been shown that the OspA epitope has a
partial sequence homology with a peptide containing human lymphocyte function-associated antigen (hLFA) (Gross et al., 1998). Therefore, scientists have postulated that antibiotic resistant Lyme arthritis may be due to molecular mimicry between OspA and LFA-1 epitopes, resulting autoimmunity within affected synovial tissue, leading to arthritis. However, it has been shown that T-cell epitopes are determined by the receptor structure and not its amino acid sequence. Benoist and Mathis (2001) have suggested that the reactivity of T-helper 1 cells to LFA-1 is due to the common cross-reactive phenotype of the T-cell receptor, and that a particular peptide binding to a T-cell receptor is not prediction of molecular-mimicry induced autoimmune disease, since one T-cell receptor can bind $10^5$-$10^6$ different peptides.

**Diagnosis**

The culture of *B. burgdorferi* from patients with erythema migrans and early-localized disease allows for definition diagnosis. However, in areas in which Lyme disease is endemic, diagnosis is often based solely upon clinical signs and symptoms (Hengge et al., 2003). Diagnosis of Lyme disease is based upon clinical signs and symptoms and a history of possible exposure to infected *Ixodes* ticks. The CDC currently recommends a two-step laboratory testing process for testing the blood of patients for antibodies against *Borrelia burgdorferi*. Enzyme immunoassays (EIA) typically comprise the first tier of testing; following a positive or indeterminate immunoassay result, a second immunoblot test, a western blot, that detects IgM and IgG antibodies to individuals *B. burgdorferi* antigens is performed (Hengge et al., 2003). The combined results of the enzyme immunoassay and the Western blot immunoassay are required to make a diagnosis. False positive EIA’s can be
caused autoimmune disorders, syphilis, Tick-borne relapsing fever, and various other bacterial and viral infections; therefore, the western blot is an important component of diagnostic testing (Norman, Antig, Bigaignon, & Hogrefe, 1996). Studies have shown that the protein flagellin, or FlaB, is an important immunodominant antigen, and strong IgM and IgG responses are developed within a few days after infection (Aguero-Rosenfeld, Nowakowski, McKenna, Carbonaro, & Wormser, 1993). Similarly, plasmid encoded OspC protein, which is expressed during tick feeding while B. burgdorferi is still in the tick hindgut, is important during early infection. IgM and IgG antibodies may persist in a patient’s serum for years following successful therapeutic treatment, though may decrease slightly. Studies have shown that following successful antibiotic therapy, B. burgdorferi antigens have a half life of 112 +/- 92 days SD, compared with 271 +/- 115 SD days after unsuccessful antibiotic therapy (Panelius, Seppala, Granlund, Nyman, & Wahlberg, 1999).

**Treatment**

The recommended treatment for Lyme disease varies by the type of infection. In early and localized disease, treatment with an antibiotic, such as doxycycline and amoxicillin, for 14-21 days is recommended (Luft, Volkman, Halperin, & Dattwyler, 1988). Transiently intensified symptoms, including rash, fever, and arthralgia, is typically seen within 24 hours. Phase III clinical trials have shown no difference in the benefit of a 20-day treatment regimen versus a 10-day regimen (Wormser et al., 2003). Multicenter studies on individuals with early-localized disease have shown 90% treatment efficacy. Patients with early-disseminated disease are typically treated with a 2-4 week intravenous course of cefotaxime; signs and symptoms of disease may resolve within weeks, though chronic
residual deficits may still occur (Logigian, Kaplan, & Steere, 1990, 1999). Late disseminated
disease, such as Lyme arthritis, is also treated with intravenous or oral regimens including
ceftriaxone and doxycycline, respectively (Dattwyler, Halperin, Volkman, & Luft, 1988). In
a small percentage of patients, subjective symptoms, including musculoskeletal pain,
neurocognitive difficulties, or fatigue, may persist for years; this collection of symptoms is
referred to as ‘chronic Lyme borreliosis’, and typically occurs more frequently in patients
when treatment is delayed (Seltzer, Gerber, Cartter, Freudigman, & Shapiro, 2000; Shadick
et al., 1999).

**Canine Borreliosis**

The clinical manifestations of Lyme disease in dogs is somewhat different than that
observed in humans, in that 95% of dogs remain asymptomatic after infection (S. A. Levy &
Magnarelli, 1992). Dogs with clinical disease often present with transient fever, anorexia,
and arthritis. Experimental studies in which infected *Ixodes* ticks were placed on Beagles
demonstrated that adult dogs were able to seroconvert but did not exhibit any clinical signs.
In contrast, puppies that were exposed to infected *Ixodes* ticks presented with
oligoarthropathy in the limb closest to the tick bite approximately 2-5 months following
exposure (Appel et al., 1993; Straubinger, 2000; Straubinger, Straubinger, et al., 1997;
Straubinger et al., 1998; Straubinger, Summers, Chang, & Appel, 1997). While disease in
these puppies was self-limited and resolved shortly after treatment, some puppies developed
similar episodes several weeks apart. Case series have reported renal, cardiac, neurological,
or dermatologic manifestations due to *B. burgdorferi* infection in dogs. A seroprevalence
study reported that fewer than 5% of seropositive dogs demonstrated lameness during a 20-
mont observation period, similar to that seen in seronegative dogs (S. A. Levy & Magnarelli, 1992). A separate study reported 57% of polyarthropathy dogs to be Lyme-positive, compared to 37% of the general population (Rondeau, Walton, Bissett, Drobatz, & Washabau, 2005). In endemic areas, approximately 70-90% of healthy and clinically ill dogs may be seropositive to *B. burgdorferi* (Magnarelli & Anderson, 1987; Magnarelli, Anderson, & Schreier, 1990). Similar antibody tests used for humans, such as ELISAs and Western blot immunoassays have historically been used in dogs. However, false positives can result in Lyme vaccinated dogs. The OspA antigen is typically used in the vaccination of dogs against *B. burgdorferi*; it has recently been shown that the OspA antigen can be expressed in dogs during carrier, subclinical, or chronic phases of *B. burgdorferi* infection, which may lead to false positives on a Western blot (Guerra, Walker, & Kitron, 2000; Littman, 2003). A synthetic peptide, known as C6, has recently been derived from the VlsE antigen, is expressed when transmitted into the dog, is not expressed in the tick, nor is it present in Lyme vaccines. Therefore, antibodies against the C6 peptide indicate natural exposure (Bacon et al., 2003; S. Levy, O'Connor, Hanscom, & Shields, 2002; Liang, Jacobson, Straubinger, Grooters, & Philipp, 2000). Studies have shown that antibodies to this peptide correlate with spirochete load. Additionally, antibodies against this peptide can be detected 3-5 weeks post-infection and have been shown to decrease with antibiotic treatment (Liang et al., 2000). Therefore, antibodies against this peptide may indicate current infection. Given proclivity of dogs for tick exposure, canine seroprevalence has shown to be a marker for human Lyme disease risk (Mead, Goel, & Kugeler, 2011). Canine seroprevalence of >5% has been determined to be a sensitive, but non-specific marker of human risk, while
seroprevalence <1% is associated with low risk of human infection. Therefore, the dog is an excellent sentinel to characterize the risk of *B. burgdorferi* transmission to humans in a defined geographical area (Duncan, Correa, Levine, & Breitschwerdt, 2004).

**Summary**

Lyme disease accounts for roughly 90% of all human vector borne diseases in the United States, and is able to infect a variety of reservoir hosts, including mice, chipmunks, and other small rodents, in addition to inadvertent hosts, including dogs and humans. *B. burgdorferi* is able to induce both acute and chronic disease in human hosts, and produce massive inflammatory reactions that are able to produce a variety of systemic manifestations, including chronic arthritis, neuroborreliosis, and carditis. Diagnosis of Lyme disease is technically difficult, and relies upon multi-step laboratory testing process based upon the presence of antibodies to *B. burgdorferi* antigens. Additionally, in a small percentage of patients with late disseminate disease, treatment may fail and result in subjective symptoms, including musculoskeletal pain, neurocognitive difficulties or fatigue, which persists for years. As such, surveillance of the presence of *B. burgdorferi* in a particular area remains a high priority of great public health significance. Dogs have been shown to make excellent sentinels for Lyme disease surveillance. Epidemiological studies using the surveillance of *B. burgdorferi* in dogs can be used to further understand the transmission, spread, and ecology dynamics of *B. burgdorferi* in a given area.
Bartonellosis

Introduction

The genus *Bartonella* is comprised of over 30 species of zoonotic, vector-transmitted intracellular bacteria that are able to infect a large number of vertebrate hosts (Breitschwerdt & Kordick, 2000; Breitschwerdt et al., 2013; Chomel, Boulouis, Maruyama, & Breitschwerdt, 2006; Maggi, Duncan, & Breitschwerdt, 2005). Because *Bartonella* spp. can infect a range of mammalian hosts and are found in a spectrum of arthropod vectors, understanding the ecology and transmission dynamics of these pathogenic bacteria requires an integrated approach, more rigorous than typically used for other vector-borne diseases. The existence of Bartonellosis in humans and animals dates back hundreds of years. However, due to advances in biotechnology, molecular diagnostic techniques, and subsequent newly discovered syndromes associated with infection, this genus of bacteria has only recently been acknowledged as an emerging pathogen.

Within the past 25 years, the number of *Bartonella* species that have been identified in a variety of mammals has dramatically increased. On an evolutionary basis, *Bartonella* species have become highly adapted to one or more mammalian reservoir hosts, and are able to induce a long-lasting intra-erythrocytic or endotheliotropic infections (Breitschwerdt & Kordick, 2000; Breitschwerdt et al., 2013). Species within the *Bartonella* genus are transmitted mainly by arthropod vectors, such as fleas, sand flies, lice, biting flies, potentially ticks and other vectors (Billeter, Levy, Chomel, & Breitschwerdt, 2008).
Hosts

Humans

Trench Fever

Frequent reports of *Bartonella* infection began in early 1915 during World War I with *Bartonella quintana*, the causative agent of trench fever (D. J. Brenner, O'Connor, Winkler, & Steigerwalt, 1993; Kostrzewski, 1950; Swift, 1920) which affected over 1 million soldiers in the trenches of the Western front and across Europe and the Middle East during World War I, and a smaller proportion of soldiers during World War II (Relman, 1995). The manifestations of trench fever ranged from a mild influenza-like illness, including fever, malaise, bone pain, and transient macular rash, to debilitating protracted and recurrent disease (Relman, 1995; Swift, 1920; Vinson, Varela, & Molina-Pasquel, 1969). Patterns of fever had been noted in several soldiers with recurrent disease, including a single episode of fever, continuous fever for 5-7 days, and recurrent episodes of fever occurring every 4-5 days (McNee JW, 1916; Vinson et al., 1969). The cyclical nature of febrile illness in soldiers with trench fever caused it to be nicknamed ‘5-day fever’. Once a soldier became infected, *B. quintana* could circulate in the blood for weeks to months to longer than a year (Vinson et al., 1969).

Soon after the first reports of trench fever, scientists began to emphasize that transmission of *B. quintana* occurred between persons in frequent contact with each other (Byam & Lloyd, 1920). Though initially described as a virus, the bacterium was observed in the blood of infected patients and the feces of human body lice (Byam & Lloyd, 1920). Early
experimental transmission studies proved that lice were the vector of trench fever. Researchers could successfully transmit the disease by allowing lice to first feed on several patients with trench fever before infecting healthy volunteers (Byam & Lloyd, 1920). In the 1960’s, Vinson successfully fulfilled Koch’s postulates by inoculating volunteers with *B. quintana* and observed them develop clinical disease consistent with trench fever (Vinson et al., 1969). The incidence of trench fever increased during cold, winter months, due to people staying indoors in close contact, and the wearing of underclothing for long periods of time (Kostrzewski, 1950).

**Immunocompromised populations**

Trench fever had long been regarded as a disease occurring only in wartime, or associated with louse infestation and crowded, unsanitary conditions. Until recently, this Gram-negative bacterium was rarely recognized within industrialized countries, including the United States. In the 1980’s, two members of the *Bartonella* genus, identified as *B. quintana* and *B. henselae*, were implicated as agents of severe or fatal disease in individuals infected with Human Immunodeficiency Virus (HIV) (Regnery, Childs, & Koehler, 1995). In 1990, an organism with DNA sequences closely related to *B. quintana* was identified in an HIV patient with bacillary angiomatosis, a vascular proliferative disorder that occurs in HIV patients (Koehler, Quinn, Berger, LeBoit, & Tappero, 1992). In 1986, special blood culture techniques identified a novel *Bartonella* species in patients with HIV, in addition to immunocompetent individuals with relapsing illness (Slater, Welch, Hensel, & Coody, 1990). This novel *Bartonella* species was named *B. henselae* (Regnery et al., 1992; Welch, Pickett, Slater, Steigerwalt, & Brenner, 1992). The spectrum of clinical manifestations in
immunocompromised HIV patients, concomitantly infected with *Bartonella*, has since increased to include blood culture-negative endocarditis, vasoproliferative lesions of the skin, liver, lung, bone, and brain and osteolytic or osteoproliferative lesions. Prior to the diagnostic recognition of this genus, undiagnosed *Bartonella* infection in HIV-positive patients was shown to produce fatal consequences (Cockerell, Whitlow, Webster, & Friedman-Kien, 1987; Koehler & Tappero, 1993; Spach et al., 1993).

In addition to HIV-positive patient populations, documentation of infection with *Bartonella* spp. began to emerge in other immunocompromised populations, where infection has caused persistent fever in transplant patients and acute graft rejection complications in 2 renal transplant patients (Lienhardt, Irani, Gaspert, Weishaupt, & Boehler, 2009; Thudi et al., 2007). In liver transplant patients, *B. henselae* can cause hepatic granulomas, hepatic masses, or disseminated bacteremic disease (Bonatti et al., 2006; Humar & Salit, 1999). It has been shown that *Bartonella* species can survive throughout the storage period of red blood cell units, and therefore transmission of *Bartonella* from asymptomatic blood donors to blood transfusion recipients may cause additional morbidity and mortality in immunocompromised populations (Magalhaes et al., 2008).

In the early 1990’s, reports from the United States and France described cases of *B. quintana* in various homeless populations (Drancourt et al., 1995; Spach, Kanter, Daniels, et al., 1995; Spach, Kanter, Dougherty, et al., 1995). In 1995, researchers in Seattle reported 10 patients, not infected with HIV, with bacteremia caused by *B. quintana*. These patients reported symptomatic febrile illness that was clinically similar to classical descriptions of trench fever (Spach, Kanter, Dougherty, et al., 1995). The infections were mostly in
homeless individuals suffering from chronic alcoholism - a condition facilitated by close contact with other individuals with poor hygiene, - previously described risk factors for trench fever. Later studies would show that alcoholism is significantly associated with positive antibody titers (>64) to *B. quintana* (Jackson et al., 1996).

A simultaneously published report from France described *B. quintana* in HIV-negative homeless individuals who later developed endocarditis (Drancourt et al., 1995). *B. quintana* was also found to be a significant cause of *Bartonella* blood culture-negative endocarditis, which had a poor prognosis with a 20% fatality rate, became a frequent diagnosis in HIV-negative homeless individuals, and may have contributed to increases in endocarditis-related deaths among the homeless (Fournier, Lelievre, et al., 2001). It was later reported that approximately 5-10% of homeless people in Marseilles, France had persistent *B. quintana* bacteremia (Brouqui, Lascola, Roux, & Raoult, 1999). Other epidemiological studies, including one conducted in emergency rooms in Marseilles, have shown that 30% of 71 tested homeless persons had antibody titers against *B. quintana* and that 14% were bacteremic (Brouqui et al., 1999). A second study from Marseilles demonstrated that 50 (5.4%) of 930 non-hospitalized, homeless persons tested during 4 years (2000–2003) had *B. quintana* bacteremia (Brouqui et al., 2005).

The presence of *B. quintana* in body lice has recently been reported in Moscow, Russia (12.3%), rural Andean villages in Peru (1.4%), and various countries in Africa (2.3%-93.9%) (Fournier, Ndihokubwayo, Guidran, Kelly, & Raoult, 2002; Raoult & Roux, 1999). In Mexico, the presence of *B. quintana* in body lice was first reported in 1954 from a homeless population in Mexico City (Varela, Fournier, & Mooser, 1954). Current reports
estimate a *B. quintana* infection rate of 28% in body lice from prisoners and homeless people in Mexico City (Alcantara, Rolain, Eduardo, Raul, & Raoult, 2009). In Tokyo, Japan, 57% of homeless patients had immunoglobulin (Ig) G titers >128 for *B. quintana* (Sasaki et al., 2006). A later study in Japan examined clothing from 12 homeless persons for body lice. These authors found that lice from 2 (16.7%) of 12 homeless persons were positive for *B. quintana* by PCR (Sasaki, Kobayashi, & Agui, 2002). The recent emergence of *B. quintana* among homeless populations throughout the world may be due to increases in poverty and recrudescent human body louse infestations. A higher seroprevalence of *B. quintana* has been reported in injection drug users (IDUs), and high frequency of injection has been associated with a higher prevalence of *Bartonella* antibodies (Comer, Flynn, Regnery, Vlahov, & Childs, 1996). However, it is currently unclear if transmission can occur through needling sharing of IDUs. Additionally, while not previously documented, transmission of *B. quintana* in homeless/IDU populations through direct contact should not be excluded. Several factors in the homeless population, such as crowded and unsanitary living conditions, inability to change clothes regularly, and close exposure to people potentially carrying ectoparasites, predispose homeless individuals to louse infestations.

**Carrion’s disease**

In addition to *B. quintana*, humans are also the natural host reservoir for *B. bacilliformis*, the causative agent of Carrion’s disease, which characteristically causes massive hemolytic anemia and vasoproliferative skin lesions, and is found primarily in the Andes region of Peru, Columbia, and Ecuador (Ihler, 1996). Until 1993, *B. bacilliformis* was the only member of the *Bartonella* genus. At that time, the *Rochalimaea* and *Bartonella*
genera were merged to accommodate an expanding list of *Bartonella* species, including *B. henselae*, *B. quintana*, *B. vinsonii*, and *B. elizabethae* (D. J. Brenner et al., 1993). *B. bacilliformis* was first identified in erythrocytes in 1905 by Alberto Barton, though archeological evidence suggests its presence in human society for several millennia (Minnick & Battisti, 2009).

Oroya fever, or verruga peruana, a sequelae of chronic infection by *B. bacilliformis*, has been documented in anthropomorphic pottery jugs and carvings made by pre-Columbian Indians. In the 1540’s, tales of epidemic acute febrile illness followed by ‘warts full of blood’ were passed down from Spanish conquistadores in South America (Minnick & Battisti, 2009). Infection with *B. bacilliformis* can be relatively short lived and potentially life threatening, due to the severe hemolytic anemia (Minnick & Battisti, 2009).

**Cat Scratch Disease**

In stark contrast to the often acute and fulminant nature of *B. bacilliformis* infections, other major pathogenic *Bartonella*, including *B. henselae*, *B. quintana*, *B. vinsonii* subsp. *berkhoffii*, and *B. koehlerae*, can establish a chronic intracellular parasitism of erythrocytes, endothelial cells and macrophages in humans (Minnick & Battisti, 2009). As previously discussed, *Bartonella* was first documented as a cause of disease in immunocompromised individuals. Subsequently, *B. henselae* was identified as the predominant cause of cat scratch disease (CSD). CSD is typically characterized by self-limiting lymphadenopathy and generalized flu-like symptoms in immunocompetent individuals. However, in 10% to more recently 20% of human cases, atypical symptoms of CSD can manifest, including encephalopathy, endocarditis, granulomatous hepatitis and nephritis, bacillary angiomatosis
and peliosis hepatis, osteomyelitis, arthropathy, myalgia, and neurological issues. (Armengol & Hendley, 1999; Breitschwerdt, Maggi, Nicholson, Cherry, & Woods, 2008; Giladi et al., 2005; Houpikian & Raoult, 2005; Kahr et al., 2000; Koehler et al., 1997; Maman et al., 2007). While immune suppression is a risk factor for more severe clinical manifestations of CSD, it has been recently shown that a subset on immunocompetent individuals can remain chronically infected with Bartonella and produce more atypical symptoms, as listed above. Evidence suggests that at least 14 Bartonella spp. can cause pathology in humans, with a wide range of symptoms being reported by persistently bacteremic individuals. Pathogenic Bartonella spp. species can be found in Table 1.

**Rodents and small mammals**

Several Bartonellae found in small mammals, such as wild mice, squirrels and rats, have been linked to human disease, including B. grahamii with ocular syndromes, B. elizabethae with endocarditis, B. vinsonii subsp. arupensis with fever and bacteremia, and B. washoensis with fever and myocarditis (Gil et al., 2010). Recent evidence from southeast Asia suggests that rodent Bartonella spp. may play an important and previously unrecognized role as a source of infection in stray dogs and febrile human patients (Bai, Kosoy, et al., 2012; M. Kosoy, Bai, Sheff, et al., 2010). Small mammals and rodents are responsible for maintaining the highest number of Bartonella species, including currently unnamed Bartonella (Gundi, Taylor, Raoult, & La Scola, 2009; Inoue et al., 2010). Bartonella species are continuing to be discovered in novel potential reservoir species of small mammal and rodent populations around the world, including Europe, Africa, Asia, North and South America (Castle et al., 2004; Gundi et al., 2004; Jardine et al., 2005; M.
Based upon several lines of evidence, studies support the hypothesis that *Bartonellae* have very effectively co-evolved with rodent species as reservoir hosts (Telfer, Clough, et al., 2007).

First, closely related phylogenetic groups of *Bartonella* are present in populations of related species of rodents separated by considerable geographical distances and land barriers (Bai, Kosoy, Ray, Brinkerhoff, & Collinge, 2008; Castle et al.; M. Y. Kosoy et al., 1997). Additionally, experimental infection of some rodents, including cotton rats (*Sigmodon hispidus*) and white-footed mice (*Peromyscus leucopus*), resulted in bacteremia only when variants were originally isolated from the same species or a close taxonomic relative (M. Y. Kosoy et al., 2000). However, the diversity of *Bartonella* species that can exist within a single rodent species or individual is highly variable. Multiple distinct *Bartonella* species have been documented in a single rodent species (Birtles et al., 2001) or within a rodent population within a single site (M. Y. Kosoy et al., 1997).

The relative abundance of each *Bartonella* spp. within these rodent populations is thought to be dependent on host density of the rodent species, density of flea species (with peak activity during peak flea season, or seasonal changes in host behavior that result in significant amounts of transmission by means other than fleas), and spill-over infection between each species of rodent (Telfer, Begon, et al., 2007). Importantly, transplacental transmission has been documented for some rodent species (M. Y. Kosoy et al., 1998). It has recently been shown that species of bats in Kenya, southwest England, Taiwan, Central

**Domestic and feral cats**

*B. henselae* was first isolated from cats in the early 1990s (Koehler, Glaser, & Tappero, 1994). Domestic cats are the primary, but seemingly not the sole reservoir for *B. henselae*, the etiologic agent of cat scratch disease (CSD), *B. clarridgeiae*, and *B. koehlerae*, and have been found to be accidental hosts for other species of *Bartonella*, such as *B. quintana* and *B. bovis*, which are less adapted to the cat host and therefore able to produce a variety of severe clinical manifestations (Breitschwerdt & Kordick, 2000; Breitschwerdt, Maggi, Sigmon, & Nicholson, 2007; Chomel, Boulouis, & Breitschwerdt, 2004; Chomel, Kasten, Henn, & Molia, 2006). Cats are carriers of the *B. henselae*, showing few clinical manifestations of infection. However, experimental studies of cats with *B. henselae* have shown that some cats may experience transient fever, anorexia, and lymphadenopathy following infection (Boulouis, Chang, Henn, Kasten, & Chomel, 2005; Guptill et al., 1997). Bacteremia in cats may last for months to years, which may allow for *Bartonella* to be more efficiently transmitted among cat populations by fleas, and potentially other vectors (Chomel, Kasten, et al., 2006; Guptill et al., 2004). Mechanisms of transmission cat-to-cat and cat-to-human are provided below.
Seroprevalence studies have indicated that a high proportion of cats worldwide have been exposed to *Bartonella* spp., and can therefore act as a substantial reservoir for infection in humans and other animals. However, recent molecular microbiological evidence indicates that the predominant *B. henselae* strains isolated from cats are not the predominant strains isolated from human patients with CSD (Bouchouicha et al., 2009). Seroprevalence to *B. henselae* antigens in cats tends to vary by geographic region and is highest (40 to 70%) in areas with warm or humid climates that are well suited to support flea populations.

In the United States, regional seroprevalence in domestic and feral cats can vary from 13% to 90%, and a higher seroprevalence correlates to outdoor exposure (Guptill et al., 2004; Jameson et al., 1995). Elsewhere around the world, seroprevalence has ranged from 7% to 48%.\(^1\) There is often a great discrepancy between antibody and bacteremia prevalence in cats, with no correlation between seropositivity and active infection (McElroy, Blagburn, Breitschwerdt, Mead, & McQuiston, 2010). Studies have shown that cats younger than 1 year of age are more likely to be bacteremic than older cats (Guptill et al., 2004), and that feral cats are more likely to be bacteremic for *Bartonella* spp. than pet cats from within the same

\(^1\) Studies have documented seroprevalences in cats of 47% in Hawaii, 15% in Japan, 40.6% and 41.8% in pet and feral cats in the United Kingdom, respectively, 54% in Indonesia, 53% in France, 40% in Israel, 11% in Egypt, 7% in Portugal, 48% in Singapore, 21% in South Africa, and 24% in Zimbabwe (Barnes, Bell, Isherwood, Bennett, & Carter, 2000; Childs et al., 1995; Demers et al., 1995; Kelly et al., 1996; Maruyama et al., 1996; Nasirudeen & Thong, 1999)
geographic region, presumably due to the higher prevalence of parasite infestation within feral cat populations (Breitschwerdt & Kordick, 2000).

Historically, it has been thought that *B. henselae* and *B. clarridgeiae* can exist as part of a healthy intravascular microbial flora in cats for months to years (Breitschwerdt & Kordick, 2000). While most naturally infected cats are able to tolerate the chronic bacteremia of *Bartonella* spp. infection without obvious clinical abnormalities, epidemiological evidence suggests a link between *Bartonella* infection and various clinical manifestations in cats. Several case reports and studies have suggested a link between *B. henselae* and ocular diseases in cats, such as uveitis and conjunctivitis (Ketring, Zuckerman, & Hardy, 2004; Lappin & Black, 1999; Lappin, Kordick, & Breitschwerdt, 2000). *B. henselae* DNA has also been amplified from cats with fatal pyogranulomatous myocarditis and diaphragmatic myositis in the United States (Varanat, Broadhurst, Linder, Maggi, & Breitschwerdt, 2012).

In a non-controlled retrospective study, *B. henselae* antibodies were associated with seizures and other neurological manifestations in cats (Leibovitz, Pearce, Brewer, & Lappin, 2008; Pearce, Radecki, Brewer, & Lappin, 2006). In a very recent study, *B. henselae* antibody titer was associated with hyperglobulinemia, which suggests that persistent feline infection with *B. henselae* may be of pathogenic importance, potentially contributing to feline immune complex diseases (Whittemore, Hawley, Radecki, Steinberg, & Lappin, 2012). However, further studies are needed to fully elucidate any potential role of *Bartonella* spp. in these and other feline diseases.
B. henselae and B. clarridgeiae have been shown to be transmitted through inoculation of infected cat blood (Kordick, Brown, Shin, & Breitschwerdt, 1999). Therefore, cats should be screened by blood culture and serology, and cats that are seropositive or blood culture positive should not be used as feline blood donors. Prevention of Bartonella infection in cats can be accomplished by avoiding exposure to fleas by routine use of arthropod control measures and by limiting exposure to other infected animals. B. henselae can be transmitted from cats to humans via a bite or scratch, resulting in clinical illness in the non-reservoir human host. Kim et al. documented B. henselae DNA in over 40% of the saliva, nails, and blood samples of feral cats in Korea (Y. S. Kim et al., 2009). Bartonella DNA has also been detected in the saliva of cats in Iran (Oskouizadeh, Zahraei-Salehi, & Aledavood, 2010), in addition to the United States, where either B. henselae or B. clarridgeiae was amplified from 31% of skin biopsies, 18% of both gingival and claw bed swabs, and 57% of blood samples of feral cats (Lappin & Hawley, 2009). It is thought that transmission may occur via bite or scratch if cat blood or flea excrement contaminates the bite site, cat saliva, or nail bed. Despite the risk of transmission via cat scratch, there is no evidence or studies indicating that declawing cats decreases the probability of transmission of B. henselae among cats and to non-reservoir species (Guptill, 2010). Currently, there is no evidence to support direct cat-to-cat transmission through bites or scratches that may occur during tomcat fights.

Canids

Bartonellae have been reported in wild canids, such as coyotes (Canis latrans), gray foxes (Urocyon cinereargenteus), red foxes (V. vulpes), and domestic dogs across North
America and Europe, and wild canids have been implicated as reservoir hosts for *B. vinsonii* subsp. *berkhoffii* (Gabriel et al., 2009; Henn et al., 2009; Marquez, Millan, Rodriguez-Liebana, Garcia-Egea, & Muniaín, 2009). Based upon sequence differences in the 16s-23s intergenic spacer region (ITS) and bacteriophage associated heme binding protein Pap31 gene, four genotypes of *B. vinsonii* subsp. *berkhoffii* have been described (Breitschwerdt, Maggi, Duncan, et al., 2007; C. C. Chang, Kasten, et al., 2000; Maggi, Chomel, Hegarty, Henn, & Breitschwerdt, 2006). Studies have reported a *B. vinsonii* subsp. *berkhoffii* seroprevalence of 35%-76% in coyotes in California and a PCR detection rate of 28% (C. C. Chang, Kasten, et al., 2000; C. Chang et al., 1999). The seroprevalence of *Bartonella* in gray foxes in the United States is extraordinarily high, ranging from 89% in Northern California (Sykes, Henn, Kasten, Allen, & Chomel, 2007), 63% in Santa Rosa Island, Florida (Schaefer, Kasten, Coonan, Clifford, & Chomel, 2011), to 50% in Texas (Schaefer, Moore, Namekata, Kasten, & Chomel, 2012).

*B. vinsonii* subsp. *berkhoffii* is able to cause persistent, prolonged bacteremia in domestic dogs that may result in severe pathophysiological and clinical manifestations (Breitschwerdt & Kordick, 2000; Kordick & Breitschwerdt, 1998). There is evidence for substantial strain variation. The variability among strains may reflect host adaptation, strain variation due to geographic origin, or random events (Cadenas et al., 2008). *B. vinsonii* subsp. *berkhoffii* was first isolated from a domestic dog with endocarditis in 1995 (Breitschwerdt, Atkins, Brown, Kordick, & Snyder, 1999). A later study using molecular methods found a prevalence of *Bartonella* infection in 28% of dogs with infective endocarditis at the UC-Davis School of Veterinary Medicine in California, United States.
Since that time, *Bartonella* infection in dogs has been serologically associated with or detected in dogs with myocarditis, cardiac arrhythmias, anemia/thrombocytopenia, arthropathy, epistaxis, neurological and neurocognitive abnormalities, granulomatous inflammatory diseases, vasoproliferative disorders, such as *peliosis hepatis*, bacillary angiomatosis, and splenic disease, such as hemangiosarcoma (Breitschwerdt et al., 1999; Breitschwerdt et al., 2004; Breitschwerdt, Hegarty, Maggi, Hawkins, & Dyer, 2005; Cadenas et al., 2008; Diniz, Billeter, et al., 2009; Kitchell et al., 2000; MacDonald et al., 2004; Pappalardo, Brown, Gookin, Morrill, & Breitschwerdt, 2000; Pesavento, Chomel, Kasten, McDonald, & Mohr, 2005; Smarick, 2004; Tuttle, Birkenheuer, Juopperi, Levy, & Breitschwerdt, 2003; Varanat, Maggi, Linder, & Breitschwerdt, 2011; Yager et al., 2010).

In the context of One Health, dogs may serve as an important naturally-occurring model for human disease manifestations and vice versa (Breitschwerdt et al., 2013). Domestic dogs may also serve as environmental sentinels, as dogs can be infected with several *Bartonella* spp. or subspecies\(^2\). (Breitschwerdt, Maggi, Chomel, & Lappin, 2010), and the clinical manifestations of *Bartonella* infection in dogs are similar to those seen in human patients. Early studies documented a seroprevalence of 4% to *B. vinsonii* subsp. *berkhoffii* antibodies among sick dogs in North Carolina and Virginia (Pappalardo, Correa, etc.)

\(^2\) Including *B. vinsonii* subsp. *berkhoffii*, *B. henselae*, *B. vinsonii* subsp. *arupensis*, *B. claridgeiae*, *B. washoensis*, *B. elizabethae*, *B. quintana*, *B. koehlerae*, *B. bovis*, *B. rochalimae*
York, Peat, & Breitschwerdt, 1997). A more recent study that measured the molecular prevalence of Bartonella spp. documented 9% of dogs with Bartonella spp. DNA in their blood (Perez, Maggi, Diniz, & Breitschwerdt, 2011). Seroprevalence studies in other parts of the world have documented higher exposure rates of Bartonella spp. in dogs, including 38% in Bangkok, Thailand, 7% in Sri Lanka, 0.8%-6% in San Paulo, Brazil, 0% in rural dogs in Vietnam, and 47% of dogs in Iraq (E. C. Brenner et al., 2013; Chomel et al., 2012; Diniz et al., 2007; Suksawat et al., 2001). Dogs have been implicated in recent reports of Bartonella transmission to humans. In a prevalence study in 52 dogs, Bartonella DNA was found in five of nine oral swabs and five of nine nail clippings (Tsukahara, Tsuneoka, Iino, Ohno, & Murano, 1998). Studies have also identified Bartonella spp. in the saliva of dogs (Duncan, Maggi, & Breitschwerdt, 2007). A recent seroprevalence study in China identified an association between having being bitten by a dog and human Bartonella seropositivity, suggesting that a large stray dog population may pose a risk for Bartonella spp. transmission, as is the case for rabies transmission (Sun et al., 2010).

**Additional domestic animals: cattle and horses**

*B. bovis* (formerly *B. weissii*), *B. chomelii*, *B. schoenbuchensis*, and *B. henselae* have been identified in cattle (Cherry, Maggi, Cannedy, & Breitschwerdt, 2009; Raoult, La Scola, Kelly, Davoust, & Gomez, 2005; Rolain, Rousset, La Scola, Duquesnel, & Raoult, 2003), and research over the past decade implicates cattle as the natural reservoir for *B. bovis*. *B. bovis* has been documented in cattle around the world, including North America (C. C. Chang, Chomel, et al., 2000), Europe (Bermond et al., 2002; Rolain et al., 2003), and Africa (Raoult et al., 2005). In the United States, studies have shown a high prevalence of *B. bovis*
in cattle; in one study, nearly half of all beef and dairy cattle sampled in Oklahoma and California were positive for *Bartonella* spp. (C. C. Chang, Chomel, et al., 2000), while nearly 90% of North Carolina beef cattle tested PCR positive in a second study (Cherry et al., 2009). The high prevalence of *B. bovis* in cattle indicates that *B. bovis* may have successfully co-evolved with cattle as its primary host, similar to the evolutionary adaptation of *B. henselae* and cats (Cherry et al., 2009). However, recent reports have described *B. bovis* in a cow that died of endocarditis, indicating *B. bovis* may be able to induce clinical manifestations in some cows (Erol et al., 2013). In addition to cattle, preliminary reports suggest that other ruminants, such as deer and elk may also be possible reservoirs of *Bartonella* spp. infection (C. C. Chang, Chomel, et al., 2000; Chitwood, Maggi, Kennedy-Stoskopf, Toliver, & Deperno, 2013; C. Dehio et al., 2001; C. Dehio, Sauder, & Hiestand, 2004; Lobanov, Gajadhar, Al-Adhami, & Schwantje, 2012).

*Bartonella* spp. was first reported in horses in 2008 when *B. henselae* was isolated from a 7 year old mare with presumptive vasculitis and from an 11 year old gelding with chronic arthropathy (Jones, Maggi, Shuler, Alward, & Breitschwerdt, 2008). In contrast to cattle, horses are not thought to be a reservoir host for any *Bartonella* species. Various case reports have suggested that infection with *Bartonella* spp. in horses may potentially lead to severe clinical illness, including high grade hemolytic anemia (Cherry, Maggi, Rossmeisl, Hegarty, & Breitschwerdt, 2011), spontaneous abortion (R. Johnson, Ramos-Vara, & Vemulpalli, 2009), and musculoskeletal disease (Cherry, Jones, Maggi, Davis, & Breitschwerdt, 2012), though additional studies are necessary to elucidate the association between *Bartonella* infection and these clinical manifestations in horses.
Wildlife

*Bartonella* also have been identified in a variety of other wildlife species, including marine mammals. Recent reports have identified infections in stranded harbor porpoises (*Phocoena phocoena*), harbor seals (*Phoca vitulina*), clinically normal loggerhead sea turtles (*Caretta caretta*), free-ranging wild bottlenose dolphins (*Tursiops truncatus*), and captive and hunter harvested beluga whales (*Delphinapterus leucas*) (C. A. Harms et al., 2008; Maggi, Harms, et al., 2005; Maggi et al., 2008; Turnbull & Cowan, 1999). *Bartonella* spp. has been identified in the seal louse (*Echynophthyrus horridus*), which can infest pinneped such as harbor seals (*Phoca vitulina*), though the mechanism of transmission from terrestrial mammals to sea mammals is not readily apparent (C. A. Harms et al., 2008). *Bartonella* spp. have been implicated in the development of neurologic disorders in animals and people, and therefore the relationship between *Bartonella* spp. infection and the stranding of marine mammals warrants investigation (C. A. Harms et al., 2008).

*Bartonella* spp. have also been amplified from blood samples taken from feral pigs in the southeastern United States. Feral pigs may represent a transmission risk to domesticated pigs and other livestock, in addition to hunters and butchers that are exposed to large quantities of pig blood (Beard et al., 2011). *Bartonella* spp. DNA and antibodies have been identified in an expanding list of wildlife species, including raccoons (*Procyon lotor*), Florida panthers (*Puma concolor coryi*), mountain lions (*P. concolor stanleyana*) (Henn et al., 2009; Rotstein, Taylor, Bradley, & rieitscherdt, 2000), and African cheetahs (Kelly, Rooney, Marston, Jones, & Regnery, 1998).
Vectors

As scientists, clinicians, and epidemiologists discovered more about the medical importance of *Bartonella* spp. infection, increasing efforts were placed on the study of known and suspected arthropod vectors. Reports have shown that *Bartonella* spp. have been identified in a variety of arthropod vectors (Billeter et al., 2008). Many cases of *Bartonella* spp. detection in arthropods have been determined by culture or by PCR, which documents the presence of the pathogen within the arthropod or the arthropod’s feces, but does not provide definitive proof of vector competence. Proof of vector competence of an arthropod requires demonstration of both the susceptibility of the particular arthropod to acquire a pathogen and also the arthropod’s ability to transmit the pathogen (Eldridge, 2003). Vector competence must be proven through experimental studies that demonstrate reliable transmission between the vector and the host (Billeter et al., 2008). However, it is not simply enough to document whether an arthropod is a competent vector of a given pathogen; the epidemiological importance of the vector-pathogen relationship must also be investigated at the population level (Eldridge, 2003). A competent vector may have no current epidemiologic relationship to the pathogen, may produce rare or occasional transmission involvement (as a secondary vector), or may provide the primary means of pathogen transmission among a population (primary vector). Medical entomologists have detailed a description of criteria (Barnett, 1962) that can be used to incriminate pathogen vectors in humans and animals as follows:
1. Demonstration that members of the suspected arthropod population frequently feed upon vertebrate hosts of the pathogen or make effective contact with the hosts under natural conditions.

2. Demonstration of a biological association between the suspected vectors and clinical or subclinical infections in vertebrate hosts in both time and space.

3. Repeated demonstration that suspected vectors harbor the infective stage of the pathogen under natural conditions.

4. Demonstration of efficient transmission of the identifiable pathogen by the suspected vectors under controlled experimental conditions.

An extraordinarily varied amount of information exists pertaining to the vector competence and vector potential of arthropods for *Bartonella* spp. A majority of the publications are epidemiologically based upon case reports or case-controlled studies. However, several experimental transmission studies have been conducted to examine the vector competence of several arthropod species for *Bartonella* transmission. With the exception of louse and sandfly transmission of *B. quintana* and *B. bacilliformis* respectively, the role of other vectors, such as the common, worldwide-distributed cat flea, is not known.

**Lice**

As previously mentioned, the human body louse (*Pediculus humanus humanus*) has been identified as a vector of *B. quintana* for several decades. Scientists, including Töpfer, Jungmann and Kuczynski, and da Rocha-Lima, described Rickettsia-like organisms within the intestinal mucosa and feces of infected lice (Swift, 1920), and experimental studies demonstrated the lack of transovarial transmission of *B. quintana* to offspring of infected lice.
A more recent study conducted by Fournier et al. (2001) demonstrated the ability of body lice to excrete viable \textit{B. quintana} (Fournier, Minnick, Lepidi, Salvo, & Raoult, 2001). The observations from these studies indicate that transmission of \textit{B. quintana} by body lice occurs when an adult louse becomes infected, by way of a blood meal, and maintains viable organisms in its intestinal tract. In contrast to other louse-borne pathogens that cause obstruction of the louse intestinal tract, as seen with Rickettsial bacteria and \textit{Yersinia pestis}, \textit{Bartonella} are able to multiply in the gut lumen without interfering with louse viability and are excreted in the feces (Fournier, Minnick, et al., 2001). Transmission to humans occurs during contamination of the louse bite site or when a wound becomes contaminated with louse feces.

\textit{Bartonella} spp. have been detected in a number of other louse species, including human head lice. The presence of \textit{B. quintana} DNA has been detected in Nepalese children that were heavily infested with the head louse (\textit{P. humanus capitis}) (Sasaki et al., 2006), and in head lice from a homeless population in San Francisco, California (Bonilla, Kabeya, Henn, Kramer, & Kosoy, 2009). In addition to human lice, \textit{Bartonella} spp. has been detected in various lice infesting rodents and mammals. \textit{Bartonella} spp. DNA, including \textit{B. henselae}, \textit{B. phoceensis}, \textit{B. tribocorum}, and \textit{B. rattimassiliensis}, has been detected in rodent and small mammal infesting lice within the \textit{Neohaemotopinus Hoplopleur}, \textit{Polyplax}, and \textit{Echinophtirius} genera throughout the United States, Egypt, and Taiwan (Durden, Ellis, Banks, Crowe, & Oliver, 2004; Nelder, Reeves, Adler, Wozniak, & Wills, 2009).
Sandflies

While *B. quintana* infection with louse transmission is the most widely known *Bartonella* in many parts of the world, the first *Bartonella* species to be described was *B. bacilliformis*. Arthropod transmission of *B. bacilliformis* was proposed in the early 1900s by Strong et al, and the sandfly (*Lutzomyia verrucarum*) was hypothesized to be the potential vector for *B. bacilliformis* in 1913 (Strong RP, 1915). As previously mentioned, *B. bacilliformis* is the causative agent of Oroya fever and verruga peruana, a clinical manifestation of the bacterium that is restricted to the Andean cordillera in Peru, Ecuador, and Colombia, with sporadic cases in Bolivia, Chile, and possibly Guatemala (Gray et al., 1990; Schultz, 1968). In 1913, a British scientist named C.H.T. Townsend observed that the distribution and feeding habits of *Lu. verrucarum* overlapped with cases of Oroya fever in the Peruvian Andes. Townsend’s speculation heightened when a British sailor on his ship willingly became exposed to wild sandflies and later developed intermittent fever and papules, presumably Oroya fever, though a diagnosis could never confirm *B. bacilliformis* as the cause of illness (Townsend, 1914).

Additional culture experiments detected Gram-negative rod organisms in the proboscis of female sandflies (Hertig, 1942). Interestingly, similar organisms, presumably *B. bacilliformis*, have been detected in male sandflies, which do not take a blood meal, and in unfed females. Though this would suggest transovarian transmission, limited studies have been conducted on the replication/survival/transmission of *B. bacilliformis* in the sandflies, and scientists have speculated that transmission of *Bartonella* spp. between sandflies may
occur through the commingling of breeding areas, contaminated water supplies and various other locations (Billeter et al., 2008).

**Fleas**

The cat flea (*Ctenocephalides felis*) has been experimentally and epidemiologically shown to be an important vector of *B. henselae*, the agent of cat scratch disease. Studies have shown that the seroprevalence of *Bartonella* spp. in cats is dependent on average daily temperature and annual precipitation. The seroprevalence is higher in warmer climate areas, where fleas are more common (Jameson et al., 1995). The first epidemiological studies conducted on patients with cat scratch disease identified owning a cat or kitten with fleas as a major risk factor for *B. henselae* infection (Zangwill et al., 1993). Various experimental studies have been conducted to demonstrate the successful acquisition of *B. henselae* by cats or kittens through cat flea transmission (Chomel et al., 1996; Foil et al., 1998; Higgins, Radulovic, Jaworski, & Azad, 1996). Other studies have demonstrated transmission of *Bartonella* spp. through flea feces. In 1998, *B. henselae* was transmitted via intradermal injection of saline containing infected flea feces to five cats (Foil et al., 1998). It has been hypothesized that *Bartonella* spp. can remain reproductively viable in flea feces within the environment, and that transmission of *Bartonella* to humans or other animals can occur via inoculation of contaminated flea feces onto the skin through an animal bite, scratch, or other open wound (Finkelstein JL, 2002). It is also possible that infection might occur by inhalation of infected flea feces, potentially resulting in a pulmonary presentation for bartonellosis (Breitschwerdt, Maggi, Robert Mozayeni, et al., 2010).
It is thought that cat fleas can maintain infection with other Bartonella species and transmit the organism among cats, which can subsequently transmit these species to people via bite or scratch (Breitschwerdt, Maggi, Duncan, et al., 2007). Cat scratches are able to transmit Bartonella through both cat blood and flea feces, in addition to blood that has been ingested by fleas that may contaminate cat claws. Bartonella spp. have also been detected in fleas from various other mammals, including human fleas (Pulex spp.) infesting people in Peru. The species identified in these fleas was closely related to B. vinsonii subsp. berkhoffii, which has been associated with endocarditis and myocarditis in humans and dogs (Parola et al., 2002). Human fleas (P. irritans) have also been shown to harbor B. quintana DNA collected from a pet monkey (Cercopithecus cephus) in Gabon, Africa (Rolain, Bourry, Davoust, & Raoult, 2005), B. rochalimae collected from red foxes (Vulpes vulpes) in Hungary (Sreter-Lancz, Tornyai, Szell, Sreter, & Marialigeti, 2006) and dogs in Chile (Perez-Martinez et al., 2009), while a similar flea, P. simulans, has been shown to harbor B. vinsonii subsp. berkhoffii and B. rochalimae in gray foxes (Urocyon cineroagentus) in northern California (Gabriel et al., 2009).

Additional studies have suggested that other species of fleas, including rodent and bat fleas, may be important vectors for transmission of Bartonella spp. Experimental studies have indicated that wild-caught Ctenophthalmus nobilis are competent vectors for transmission of B. grahamii and B. taylorii to bank voles (Myodes glareolus) (Bown, Bennet, & Begon, 2004). Many other rodent fleas have been tested for the presence of Bartonella DNA. Species of Bartonella, including B. elizabethae, B. vinsonii-like, B. quintana, B. henselae, B. clarridgeiae, B. schoenbuchensis-like, B. birtlesii, B. koehlerae, B. taylorii,
B. tribocorum, B. queenslandensis, and B. rochalimae, have been detected in various flea genera in China, Indonesia, Egypt, Israel, Thailand, Myanmar, Taiwan, including Xenopsylla, Nosopsyllus, Sternopsylla, and Leptopsylla (Winoto, Goethert et al. 2005, Loftis, Reeves et al. 2006, Li, Liu et al. 2007, Reeves, Rogers et al. 2007, Tsai, Chuang et al. 2010, Reeves, Rogers et al. 2007, Morick, Krasnov et al. 2010). Recently, a proposed new species of Bartonella (Candidatus B. antechini) was detected in fleas collected on yellow-footed antechinus (Antechinus flavipes) in Western Australia (Kaewmongkol et al., 2011). While it is unclear how many of these flea species are competent vectors for Bartonella transmission, the increasingly identified number of flea and host species and diversifying geographic range suggest that current literature may only be reporting a fraction of species and ranges that Bartonella may be present in.

Ticks

It is currently unclear whether ticks are involved in the transmission of Bartonella spp. to humans and animals under natural conditions. However, the high prevalence of Bartonella spp. in various tick species highlights the need for increased discussion relative to transmission of Bartonella spp. following a tick bite. The first documentation of Bartonella spp. detection or isolation from ticks was in the mid-1990’s, when a Bartonella strain was cultured from a questing Ixodes ricinus tick collected from a park in Poland (Kruszewska & Tylewska-Wierzbanowska, 1996). Since then, there have been numerous reports of Bartonella spp., mostly based on PCR amplification of organism specific DNA sequences, though rarely by culture, from various tick species. In the early 2000’s, two studies out of California, United States, provided evidence that two separate tick species might serve as
vectors for *Bartonella* transmission. The first study identified several pathogenic *Bartonella*, including *B. henselae*, *B. quintana*, *B. washoenisis*, *B. vinsonii* subsp. *berkhoffii*, and *B. bovis* in 19.2% of questing adult *I. pacificus* ticks that were collected by flagging local vegetation (C. C. Chang, Chomel, Kasten, Romano, & Tietze, 2001). The second study identified *Bartonella* spp. in adult and nymph *I. pacificus* ticks, in addition to *Dermacentor variabilis* and *D. occidentalis* ticks (C. C. Chang et al., 2002). Near that same time, researchers identified *Bartonella* spp. from *I. scapularis* deer ticks that were collected from the household of two patients co-infected with *B. henselae* and *Borrelia burgdoferi* (the etiologic agent of Lyme disease) detected from their spinal fluid (Eskow, Rao, & Mordechai, 2001), and from 34.5% of field collected *I. scapularis* in New Jersey (Adelson et al., 2004).

*Bartonella* spp. DNA has since been detected in ticks throughout the world, including *Carios kelleyi*, *I. ricinus*, *I. persulcatus*, *Dermacentor reticulates*, *Hyalomma longicornis*, *Rhipicephalus microplus*, *I. tasmani*, *I. sinensis*, *H. rufipes*, and *H. longicornis*. (Loftis et al., 2005)

Additional epidemiological and experimental evidence exists that suggests ticks may be relevant vectors for disease transmission. Ticks have been identified as a risk factor for *B. vinsonii* subsp. *berkhoffii* infection in dogs; a case control study determined that *Bartonella* seropositive dogs are 14 times more likely to have been exposed to previous heavy tick infestations (Pappalardo et al., 1997). In a clinical survey performed in Connecticut, United States, patients were more likely to have been bitten by a tick than controls (Zangwill et al., 1993). Several case reports describe human *Bartonella* infection shortly following a tick bite (Lucey et al., 1992) (Angelakis et al., 2010) (Eskow et al., 2001), and co-infections with
*Bartonella* and *Borrelia burgdorferi*, the etiologic agent of Lyme disease, have been frequently reported (Halperin & Wormser, 2001). A recent report detected a high prevalence of bacteremia with and antibodies against *Bartonella* spp. in patients diagnosed with Lyme disease or chronic arthralgia and myalgia, and statistically associated *Bartonella* spp. bacteremia with neurologic symptoms (Maggi et al., 2011). While some researchers may argue that cases of Bartonellosis may be potentially misdiagnosed as Lyme disease, rigorous scientific studies will clarify the role of *Bartonella* infection with rheumatic and neurologic symptoms.

Experimental transmission of *Bartonella* by ticks has also been demonstrated. In one study, *I. ricinus* ticks were infected with *B. henselae* in artificially infected ovine blood using an artificial feeding system. The ticks maintained infection throughout the molt, and were able to transmit *B. henselae* during a subsequent blood meal through an artificial feeding system (Cotte et al., 2008). A more recent study demonstrated vector competence of *I. ricinus* through transmission of *Bartonella* by ticks from an infected mouse and subsequent transmission to a naïve mouse, in addition to localization of *Bartonella* within the adult ticks (Reis et al., 2011). Also, Billeter (2009) demonstrated invasion and replication of seven *Bartonella* species in an *Amblyomma americanum* tick cell line in 2009, visualized *Bartonella* within the cells and quantified bacterial growth through qPCR amplification (Billeter et al., 2009). Recently, Billeter was able to detect *B. vinsonii* subsp. *berkhoffii* DNA in *R. sanguineus* ticks post-capillary tube feeding, as well as in tick feces (Billeter et al., 2012). Similar to other vectors for *Bartonella* that may transmit the bacterium through contamination of arthropod feces, it has been hypothesized that *R. sanguineus* may transmit
Bartonella through inoculation of tick feces through broken skin. Despite the evidence that Bartonella spp. might be transmitted through a tick vector, many scientists argue that transmission of Bartonella spp. to animals or humans through ticks is unlikely. While no well-documented case of transmission by ticks to humans or animals has been reported, additional studies will clarify the role of ticks in the transmission of this pathogen.

**Biting flies and mites**

A potential role in the transmission of Bartonella spp. has been suggested for a variety of other insects and arthropods. A study conducted in France revealed that 86% of 83 sample Hippoboscidae flies, including 94% of deer keds (Lipoptena cervi), 71% of louse flies (H. equina), and 100% of sheep keds (Melophagus ovinus) harbored Bartonella DNA, notably B. schoenbuchensis or B. chomelii (Halos et al., 2004). Dehio, et al. (2004) isolated B. schoenbuchensis from deer keds, which are flies that lose their wings after finding a host animal, collected from roe deer and red deer in Germany and demonstrated the presence of large bacterial aggregates in the midgut of the flies, suggesting that the arthropod may be a viable vector for Bartonella transmission (C. Dehio et al., 2004). B. schoenbuchensis has been detected in deer keds in Georgia and South Carolina, United States (Reeves, Nelder, Cobb, & Dasch, 2006), and it has been hypothesized that B. schoenbuchensis and deer keds may be widely distributed by elk or caribou across continents (Matsumoto, Berrada, Klinger, Goethert, & Telford, 2008).

Bartonella has also been detected in pools of Mesostigmatid mites collected from wild rodents and insectivores in Korea, in addition to house dust mites (Dermatophagoides farina and D. pteronyssinus) in the United States (C. M. Kim et al., 2005; Valerio, Murray,
Arlian, & Slater, 2005). B. tamiae has been detected in chigger mite pools (Leptotrombidium, Schoengastia, and Blankarrtia) that had been collected on rodents in Thailand (Kabeya et al., 2010). The bat fly (Trichobius major Coquillett) and the Eastern bat bed bug (Cimex adjunctus Barber) are ectoparasites of bats. A bat fly collected from Florida caverns and an Eastern bat bed bug collected from the Santee Caves in South Carolina, USA, were shown to harbor a unique Bartonella species (Reeves, Loftis, Gore, & Dasch, 2005). Bartonella has also been detected in honeybees (Apis mellifera capensis Eschscholtz). It has been suggested that the organism was ingested through contact with the environment and therefore it does not appear as though Bartonella can commonly be found in honey bees (Jeyaprakash, Hoy, & Allsopp, 2003). Recently, Bartonella spp. has also been detected in woodlouse hunter spiders and their associated prey, the woodlouse, though vector competence has yet to be investigated in these arthropods (Mascarelli et al., 2013).

Pathogenesis of Bartonella spp.

Lifecycle and infection strategy

Interaction with endothelial cells

The common infection strategy of Bartonella spp. is initiated through inoculation of a mammalian host through a blood-sucking arthropod. Initially, Bartonella are unable to immediately colonize erythrocytes and must inhabit a ‘primary niche’ before entry into the blood stream, as intravenous injection of Bartonella does not result in immediate infection of red blood cells (Schulein et al., 2001). Upon inoculation through a blood-sucking arthropod, Bartonella enter into a migratory cell in the dermis/epidermis and are transported to the
‘primary niche’, presumed to be the vascular endothelium (Figure 3). From here, *Bartonella* persist intracellularly until they are seeded into circulation where they can infect erythrocytes or re-enter into the vascular endothelium. Following limited replication in erythrocytes, *Bartonellae* can then be taken up by a blood-sucking arthropod (A. Harms & Dehio, 2012). While there has been no conclusive evidence that directly reveals the vascular endothelium as the location of the primary niche, in vitro data, and the proximity of the vascular endothelium to the blood stream, implicate the vascular endothelium as a primary candidate (C. Dehio, 2005).

Frequent relapse of bacteremia following antibiotic treatment or clearance of infection through the immune system supports the existence of the primary niche, as bacteria within this location would be protected from complete clearance of infection by persisting intracellularly, and later re-establishing infection through the reseeding into circulation (Byam & Lloyd, 1920; Kordick et al., 1999). A cyclical seeding of bacteria from the primary niche into circulation at regular 8-day intervals has been observed in the *B. tribocrum* rat model of infection (Seubert, Schulein, & Dehio). Additionally, a cyclic model of infection may explain the relapse of symptoms, or ‘5 Day Fever’ as observed in *B. quintana* infection in humans, as described earlier (Byam & Lloyd, 1920; C. Dehio, 2005). In addition to endothelial cells and erythrocytes, in vitro data has shown that *Bartonella* is able to invade a myriad of cell types, including endothelial progenitor cells (Salvatore et al., 2008), hematopoietic progenitor cells (Mandle et al., 2005), monocytes/macrophages (Kyme et al.), microglial cells (Munana, Vitek, Hegarty, Kordick, & Breitschwerdt, 2001), and tick cells (Billeter et al., 2009).
Interaction with erythrocytes

Following residence in the primary niche, *Bartonella* are able to invade erythrocytes, where they continue to remain protected from treatment therapies and the immune system (A. Harms & Dehio, 2012). *B. bacilliformis* and *B. quintana* has been found to infect human red blood cells and *B. henselae* typically infects feline erythrocytes, though has been shown to infect human erythrocytes in vitro (Cuadra & Takano, 1969; Kordick & Breitschwerdt, 1995; Pitassi et al.; Rolain et al., 2002). *Bartonella* species are adapted to survive within mammalian erythrocytes without causing hemolysis, with the exception of *B. bacilliformis*, which is able to induce fatal hemolytic anemia in humans (Ihler, 1996). However, *B. bacilliformis* is able to infect 100% of red blood cells, whereas other *Bartonella* species infect a much lower proportion of erythrocytes (Maguina, Garcia, Gotuzzo, Cordero, & Spach, 2001). The percentage of infected red blood cells for *B. quinana* has been shown to be 0.001-0.005% (Rolain et al., 2002), and less than 1% in rats (Schulein et al., 2001). Studies in cats, the reservoir for *B. henselae*, have shown up to 6.2% of erythrocytes containing intracellular bacteria (Kordick & Breitschwerdt, 1995).

Immune evasion and immunomodulation

*Bartonella* species are stealth species of bacteria that are able to avoid elicitation of a host immune response. One strategy, as previously mentioned, is the bacterium’s ability to take residence in the primary niche and intraerythrocytic niche, where *Bartonella* is protected from both innate and adaptive immune systems. An additional strategy is through the stimulation of IL-10 secretion, which is an important part of *Bartonella's* immune
modulation. Elevated IL-10 secretion has been shown to favor a persistent, asymptomatic course of infection (Capo, Amirayan-Chevillard, Brouqui, Raoult, & Mege, 2003). In the mouse model of *B. birtlesii*, bacteremia was unable to be established in IL-10 knockout mice (Marignac et al., 2010). Humans infected with *B. quintana* have been shown to exhibit an attenuated inflammatory profile and elevated levels of IL-10 (Capo et al., 2003). Similarly, studies have shown that *B. quintana* LPS does not stimulate TLR-4, and that this species may act as a potent anti-TLR4 agent to reduce pro-inflammatory signals (Popa et al., 2007).

Studies on dogs infected with *B. vinsonii* subsp. *berkhoffii* have identified substantial impairment of the host immune response, including deficiency in bacterial phagocytosis, increase in MHC-II negative B-cells, an increase in naïve CH4+ T-cells, and a decrease in CD8+ cells (Pappalardo, Brown, Tompkins, & Breitschwerdt, 2001). Additionally studies have helped to clarify the mechanism of immune evasion of *Bartonella*. Bacteremia in CD4 knockout mice was longer and had higher bacterial titers than wild type mice. In contrast, bacteremia in CD8 knockout mice was not different than wild type mice (Marignac et al., 2010). The authors of this study concluded that phagocytes and the humoral response participate in the immune response against *Bartonella*, as both require T-helper cells. Similarly, in a separate study in mice, infected with *B. grahamii* in B-cell knockout mice exhibited prolonged bacteremia (Koesling, Aebischer, Falch, Schulein, & Dehio, 2001). Studies investigating the Th1 response in mice found that *B. henselae* failed to cause bacteremia with the secretion of IFN-γ that was able to eliminate the pathogen (Arvand, Ignatius, Regnath, Hahn, & Mielke, 2001; Karem, Dubois, McGill, & Regnery, 1999).
Animal models of infection

Many studies using animal models of infection have been performed using rats and *B. tribocorum*. Using the rat model and GFP-expressing *B. tribocorum*, intravenous inoculation of culture grown *B. tribocorum* into 6 week old rats has been used to study the kinetics of infection. In these rats, the bacterium was cleared from the blood within hours of inoculation, and the blood remained culture-negative for 4-5 days. Bacteremia reappeared in the bloodstream 4-5 days post infection. Similarly, periodic increases in infection occurred at 5-day intervals (Schulein et al., 2001). In this model, infection was self-limiting, and rats were able to completely clear the infection after 10 weeks. This study supported the existence of the primary niche, as mentioned above. Experiments using the rat model have shown that erythrocytes are typically infected initially with one to two bacteria, which then divide two to three times to produce 8 bacteria per erythrocyte that persist for the life of the infection (Schulein et al., 2001).

Other rodent models of infection, such as the mouse model, have yielded similar results. Female BALB/c mice infected with *B. birtlesii* became bacteremic on day 8 post infection. Bacteremia peaked on day 14, and bacteria were completely cleared by 10 weeks (Boulouis et al., 2001). Rodents have also been used to study the host immune response to *Bartonella* infection. Convalescent cotton rats experimentally infected with three different *Bartonella* did not result in reinfection when challenged with the same *Bartonella* species. However, when these rats were challenged with a different *Bartonella* species, the animals became bacteremic (M. Y. Kosoy, Regnery, Kosaya, & Childs, 1999). In cats infected with four different species or strains of *Bartonella*, researchers found that *B. henselae* type I is
protective against *B. henselae* type II, but not vice versa (Yamamoto et al., 2003). Additionally, these researchers found that *B. clarridgeiae* did not protect cats against infection with either strain of *B. henselae*. Dogs are excellent natural models for human bartonellosis; however, experimentally infected dogs have yielded limited results (Balakrishnan, Cherry, et al., 2013). Macaques have been found to be useful models for studying virulence factors associated with human *Bartonella* infection, though this model is both expensive and labor intensive (MacKichan, Gerns, Chen, Zhang, & Koehler, 2008).

**Virulence determinants**

Important virulence factors have been identified in *Bartonella* that contribute to the bacterium’s ability to enter, replicate, and persist in a host. Timeric autotransporter adhesins (TAAs) bind to host proteins on cell surfaces or in the extracellular matrix (ECM). Most research on *Bartonella* TAAs has been conducted on *Bartonella* adhesion A (BadA) of *Bartonella henselae*, which plays a key role in bacterial autoagglutination, adhesion to host cells and ECM, inhibition of phagocytosis, and induction of pro-angiogenic transcriptional program target cells (A. Harms & Dehio, 2012). Specifically, studies have shown that BadA is able to induce gene expression to activate NF-kB and HIF-1, which play key roles in angiogenesis (Rahman & McFadden, 2011). *Bartonella* also has three different type IV secretion systems, including VirB/VirD4, Vbh, and Trw, which act as host adaptability factors (Saenz et al., 2007). A functional VirB/D4 T4SS is an essential component of pathogenicity for *Bartonella* species, and plays a major role in the manipulation of host cells. VirB/D4 T4SS translocates *Bartonella* effector proteins (Beps) into host cells, where they subvert cellular functions, including inhibition of apoptosis, inhibition of endocytosis, and
contribute to replication and persistence of *Bartonella* in the intracellular niche (A. Harms & Dehio, 2012). Beps are able to interact with the host by mediating AMPylation, the covalent transfer of an AMP moiety onto hydroxyl side chains of target proteins, and subvert cell-signaling cascades through the phosphorylation of tyrosine kinase motifs (Backert & Selbach, 2005; Palanivelu et al., 2011; Roy & Mukherjee, 2009). The Trw T4SS is necessary for intraerythrocytic infection in the rat model of *B. tribocororum* infection (Seubert, Hiestand, de la Cruz, & Dehio, 2003). A deletion in the Trw T2SS results in the pathogen being unable to infect erythrocytes; however, Trw mutants are still able to infect the primary niche, as shown through the cyclical appearance within the blood of infected animals (Schmid et al., 2004; Schroder & Dehio, 2005). Trw has also been shown to mediate host-specific erythrocyte infection, as shown through cat and human specific *Bartonella* species expressing the Trw of the rat-specific *B. tribocororum* being able to infect rat erythrocytes (Vayssier-Taussat et al., 2010). The third T4SS is functionally redundant to the VirB/VirD4 T4SS, and is only functional in species in which VirB/D4 is absent (Saenz et al., 2007).

Species of *Bartonella* lineage 1 and 3, in contrast to species of lineage 4, possess a set of flagella and are therefore motile (A. Harms & Dehio, 2012). Studies have shown that flagella are important during the infection of erythrocytes; nonmotile *B. bacilliformis* are unable to invade red blood cells, and show decreased erythrocyte attachment and deformation (Mernaugh & Ihler, 1992). Additionally, treatment of bacteria with antiflagellin antibodies reduced the invasion of erythrocytes by 99.7% (Scherer, DeBuron-Connors, & Minnick, 1993). Species of *Bartonella* that are non-flagellated include *B. henselae*, *B. quintana*, and *B. tribocororum*. However, these species are able to invade erythrocytes through
the use of the Trw T4SS. An additional virulence factor essential for erythrocyte invasion is
the invasion-associated locus (ial), encoding IalA and IalB (Vayssier-Taussat et al., 2010).
IalA is aNudix family nucleoside polyphosphate hydrolase thought to be involved in reduce
levels of stress-induced dinucleotides during invasion and aiding bacterial survival
(Cartwright, Britton, Minnick, & McLennan, 1999). IalB is an outer membrane protein of B.
henselae (though an inner membrane protein of B. bacilliformis), and may be directly related
to erythrocyte invasion through interacting with one or more of erythrocyte surface proteins
that are bound by Bartonella. IalB expression of B. bacilliformis is shown to be strongly
upregulated under conditions that resemble the sand fly gut, which suggests that this protein
may be involved in the priming of bacteria during its time in an arthropod vector (Coleman &
Minnick, 2003).

**Role of Bartonella in chronic disease**

**Bartonella and rheumatic disease**

There has been increased interest in infective causes of autoimmune diseases,
such as rheumatoid arthritis. Autoimmune disease occurs as a result of stimulation of
the immune system by a self-antigen that cannot be cleared from the body. When T-
lymphocyte and B-lymphocytes are stimulated by self-antigen, they can induce a
powerful cell growth and survival pathway of immune cells that attack cells within the
body and cause organ/tissue damage (Goodnow, 2007). Innate and adaptive immune
responses can be induce autoimmune diseases through a variety of mechanisms
including molecular mimicry, epitope spreading, bystander activation, and polyclonal
activation. Molecular mimicry is characterized by the cross-reactivity between epitopes shared by both pathogen and host. The pathogen must be associated with the onset of the auto-immune disease, and it must induce a host response that can cross react with host antigens. A classic example of molecular mimicry includes acute rheumatic fever following Streptococcus pyogenes infection. Close molecular resemblance between the M-protein and human glycoproteins causes a breakdown of self-tolerance in genetically susceptible individuals (Kivity, Agmon-Levin, Blank, & Shoenfeld, 2009). Epitope spreading involves the diversification of epitope specificity that begins with molecular mimicry. The chronic autoimmune state induced through molecular mimicry to a host epitope can result in an additional immune response against a different host epitope. The immune response becomes no longer specific to the original mimicking antigen (Lehmann, Forsthuber, Miller, & Sercarz, 1992; Oldstone, 1998). In streptococcal induced myocarditis, the chronic autoimmune state against the cardiac myosin can result in an additional immune response against collagen or laminin. Bystander activation is a mechanism of autoimmune disease in which tissue damage can cause the release of a sequestered antigen, which can subsequently activate autoreactive lymphocytes that were not directly involved in the initial reactivity to the damaging agent. Cytokines, including TNF-α, lymphotoxin, and nitric acid, can lead to the bystander killing of uninfected cells and tissue and induce an autoimmune response (Duke, 1989). An additional mechanism of autoimmune disease can occur through persistent infection and polyclonal activation. Constant activation of the immune
response induces constant polyclonal activation and proliferation of B cells. This may cause a monospecific proliferation of immune cells to emerge that can induce damage to host tissues.

Early epidemiologic studies have shown that the risk of developing rheumatoid arthritis may be increased with animal exposure within five years prior to developing disease. Additionally, exposure to cats, specifically, has been shown to increase the likelihood of developing rheumatoid arthritis (OR = 4.9) (Gottlieb, Ditchek, Poiley, & Kiem, 1974). This association followed a dose-response effect, with more intimate exposure to cats being associated with a greater odds of developing disease. Further investigation of this hypothesis revealed that the risk of developing RA following intimate exposure to cats is increased in individuals with certain HLA-DRB1 alleles, specifically *0401, *0404, and *1501 (Penglis, Bond, Humphreys, McCluskey, & Cleland, 2000).

Due to the high prevalence of *B. henselae* circulating in the blood of cats throughout the world, it has been hypothesized that *Bartonella* may play a role in the development of RA or other rheumatic diseases. Rheumatic manifestations in individuals infected with *Bartonella* were originally described in children in the early 1990s (Al-Matar et al., 2002), and was seen in cases of myolitis and juvenile rheumatoid arthritis (Giladi et al., 2005; Hayem, Chacar, & Hayem, 1996). Case reports have described reactive arthritis as a manifestation of *Bartonella* infection following a cat bite (Jendro, Weber, Brabant, Zeidler, & Wollenhaupt, 1998). Additional rheumatic
manifestations associated with *Bartonella* infection have included erythema nodosum (inflammatory condition that may involve arthritis), leukocytoclastic vasculitis, myalgia, and arthralgia. Serologic evidence has implicated *Bartonella* as a potential causative agent of Henoch-Schonlein purpura, a systemic vasculitis characterized by purpura, arthritis, and abdominal pain (Carithers, 1985; Hashkes, Trabulsi, & Passo; Jacobs & Schutze). In this study, 67% (n=12) of patients with Henoch-Schonlein purpura were seropositive against *B. henselae*, compared to 14% (n=8) of unaffected individuals (Ayoub, McBride, Schmiederer, & Anderson, 2002). On a comparative medical basis, *B. henselae* and *B. vinsonii* subsp. *berkhoffii* has been isolated from blood, joint, and subcutaneous seroma fluids from dogs with chronic and progressive polyarthritis (Diniz, Wood, et al., 2009). *B. henselae* has also been detected in the blood of horses with chronic arthropathy and presumptive vasculitis. However, one study in humans failed to identify *Bartonella* species from the synovial fluid of 20 patients with chronic arthritis (Dillon, Cagney, Manolios, & Iredell, 2000). Infection with *Bartonella* has been identified in a number of reports involving immune cell proliferations, including polyclonal plasmacytosis (Balakrishnan, Jawanda, Miller, & Breitschwerdt, 2013), hypergammaglobulinaemia, and has also been documented in inflammatory induced osteomyelitis in a cat (Varanat et al., 2009), neutrophilic polyarthritis in dogs (Breitschwerdt et al., 2004). Classically, *Bartonella* are able to induce a B-cell associated granulomatous reaction that is characterized by infiltrating histiocytes, plasmacytoid monocytes, small lymphocytes, and plasma cells with intermingled B cells. Polyclonal
and monoclonal gammopathies have been reported in association with *Bartonella* infection (Krause et al., 2003); therefore, it is possible that *Bartonella* participates in producing an autoimmune response, and subsequent rheumatoid manifestations, through persistent infection and polyclonal activation. Additionally, it has been hypothesized that *Bartonella* may induce rheumatologic disorders by triggering the hosts altered immune response through molecular mimicry, though this mechanism has not been investigated (Breitschwerdt et al.; Tsukahara, Tsuneoka, Tateishi, Fujita, & Uchida, 2001).

**Bartonella and cancer**

Within the past 25 years, considerable research has been conducted on the oncogenic properties of infectious agents such as bacteria, viruses, mycoplasma, and protozoa. Currently, infectious agents are accepted as causes or co-factors in nearly 20% of human cancers worldwide (Pagano et al., 2004). A majority of these infectious agents are viruses, such as Epstein Barr virus, human papillomaviruses, and Kaposi’s sarcoma-associated herpesvirus, which have direct oncogenic properties by integration of viral genomes into host cells or by secretion of gene products into healthy cells to create tumor cells (Pagano et al., 2004). However, other infectious agents, such as bacteria, may lack the inherent oncogenic properties of their viral counterparts and indirectly promote cancer development through persistent replication, inflammation and chronic tissue damage (Coussens & Werb, 2002). *Helicobacter pylori*, for example, colonizes and replicates within the gastric mucosa and induces a chronic pro-inflammatory response, which promotes cancer risk and oncogenesis
of gastric cancer through functional changes associated with epithelial cell proliferation and apoptosis (Lax & Thomas, 2002; Unger et al., 2001).

*Bartonella* spp. comprise a genus of fastidious, Gram-negative bacteria that are highly adapted to the mammalian reservoir host and can cause long-lasting intra-erythrocytic bacteremia in both animals and humans (Breitschwerdt & Kordick, 2000; Breitschwerdt et al., 2013; M. Kosoy, Hayman, & Chan, 2012). Historically, disease manifestations associated with *Bartonella* infection in dogs have included endocarditis, peliosis hepatis, meningoencephalitis, arthritis, unexplained weight loss, and granulomatous lesions (Boulouis et al., 2005; Breitschwerdt & Kordick, 2000; Chomel, Boulouis, et al., 2006). In human patients with a history of animal contact or scratches, *Bartonella* often manifests itself as lymphadenopathy with an erythematous papule near the site of inoculation (Breitschwerdt, Maggi, Robert Mozayeni, et al., 2010; Carithers, 1985). Cutaneous lesions develop within 3-10 post inoculation and consist of diffuse inflammatory cell infiltrates containing neutrophils, histiocytes, and plasma cells (Carithers, 1985; Spach & Koehler, 1998). Often, epidermal hyperplasia may be present in the skin. *Bartonella* infections have been shown to be responsible for different vascular growths in the skin in humans, as in the case of *B. bacilliformis* and verruga peruana and *B. henselae* and bacillary angiomatosis (Koehler, 1995; Spach & Koehler, 1998; Yager et al., 2010).

Following vector-borne or direct transmission, *Bartonella* spp. do not immediately infect red blood cells, indicating that the bacteria persist in a primary niche prior to blood-stage infection (C. Dehio, 2005; Schulein et al., 2001). *In vitro* data supports extensive interactions with endothelial cells and indicates that the vascular endothelium represents a
target tissue for intra- and extracellular colonization of these bacteria (C. Dehio, 2005; A. Harms & Dehio, 2012; Schulein et al., 2001). *Bartonella* induced angiogenesis results in proliferation and migration of endothelial cells with subsequent reorganization into new capillaries (Kempf et al., 2001; Minnick, Smitherman, & Samuels, 2003). Angiogenesis is activated by a number of growth factors, such as vascular endothelial growth factor (VEGF), interleukin-8 (IL-8), and tumor necrosis factor-alpha (TNF-\(\alpha\)), and supplies nutrients and oxygen and removes metabolic waste from neoplastic growths (C. Dehio, 2005; A. Harms & Dehio, 2012; Kempf et al., 2001; Schulein et al., 2001); therefore angiogenesis is also required for tumor progression and tissue invasion. *Bartonella* is often seen as large bacterial aggregates surrounding the angiogenic endothelium, and eradication of the bacterium with antibiotic treatment will resolve these proliferative lesions (Kempf et al., 2001).

Current knowledge indicates that *Bartonella* can induce proliferation of endothelial cells through three pathways: mitogenic stimulation of endothelial cells, inhibition of apoptosis, and activation of paracrine vasoproliferative host factors (C. Dehio, 2005; M. Dehio, Quebatte, Foser, & Certa, 2005; A. Harms & Dehio, 2012; Kempf, Schairer, et al., 2005; Kempf et al., 2001; McCord, Cuevas, & Anderson, 2007). To date, these three pathways have been studied more intensively in endothelial cells than in other cell types. Cell-free extracts and live bacteria, are able to stimulate the proliferation of endothelial cells *in vitro*, indicating that soluble and secreted factors of *Bartonella* have a direct mitogenic effects on endothelial cell proliferation (McCord et al., 2007; Minnick et al., 2003).

*Bartonella* has also been shown to inhibit apoptotic events, such as caspase activation and DNA fragmentation in vitro, which may contribute in part to cellular proliferation (Kempf,
Schairer, et al., 2005; Schmid et al., 2006; Schmid et al., 2004). Apoptosis has been shown to be regulated by the ratio of apoptotic Bax and anti-apoptotic Bcl-2 proteins; following DNA damage, increases in Bax result in the release of apoptotic factors from the mitochondria to induce cell death (A. Harms & Dehio, 2012; McCord, Resto-Ruiz, & Anderson, 2006). *Bartonella* can induce the production of IL-8, an activator of angiogenesis, which also in turn increases the Bcl-2/Bax ratio, resulting in the cell being unable release the contents of the mitochondria following DNA damage to proceed through apoptosis (McCord et al., 2007; McCord et al., 2006). Therefore, *Bartonella* spp. may also be able to promote tumorigenesis through enhanced cell survival and enhanced vascular proliferation.

Following colonization of the vascular endothelium, inflammatory cells, including macrophages, neutrophils, and lymphocytes, are recruited to the site of *Bartonella* infection. *Bartonella* sp. may function in a pathologically similar manner to *H. pylori*, and may contribute to oncogenesis through a chronic inflammatory response that promotes angiogenesis and tumor growth. *Bartonella* has been associated with the activation of NF-κB, a primary regulator in the pro-inflammatory cascade, which induces the transcriptional activation of various inhibitors of apoptosis (Fuhrmann et al., 2001; Schmid et al., 2004). The NF-κB proinflammatory cascade can initiate chronic inflammation pathways that lead to elevated expression of angiogenic factors including ICAM-1, angiopoietin-2, IL-8, or prostaglandins E1 and E2 (Fuhrmann et al., 2001; A. Harms & Dehio, 2012). *Bartonella* spp. are also able to directly activate HIF-1α, a principle regulator of angiogenesis, which can stimulate expression of VEGF to increase oxygen delivery, via endothelial cells, to areas with hypoxic damage and generate new blood vessels to supply tumors with additional
oxygen (C. Dehio, 2005; Kempf, Lebiedziejewski, et al., 2005). The *Bartonella* induced recruitment of macrophages and monocytes to the site of infection facilitates production of VEGF, which can act on endothelial cells to stimulate their proliferation. VEGF has been shown to be specifically induced by *B. henselae* in vitro, and *in vitro* studies have shown that neutralizing anti-VEGF antibody is able to block the angiogenic activity of *Bartonella* in conditioned culture medium (C. Dehio, 2005; M. Dehio et al., 2005; Kempf et al., 2001).

**Summary**

*Bartonella* genus is comprised of over 30 species that are able to be transmitted by a myriad of arthropod vectors and induce a spectrum of clinical manifestations within a variety of animal hosts. With the ability of *Bartonella* to jump to accidental hosts, it is imperative that we determine the consequences of *Bartonella* spp. infection on new host populations and environments. The use of polymerase chain reaction (PCR) has given researchers and microbiologists the ability to rapidly detect and identify the DNA of Bartonella spp. in a variety of blood, effusion, and tissue specimens. Studies have indicated that a combinatorial approach that independently tests blood, serum, and enrichment culture by PCR can improve the diagnostic documentation of *Bartonella* spp. bacteremia (Maggi et al., 2011). Improvements in the diagnostic documentation of *Bartonella* spp. can be used to create controlled, insightful epidemiologic investigations to elucidate pathologies associated with infection. Co-factors that exacerbate pathologic manifestations, such as co-infections and immunosuppression, need to be investigated.
Figure 1. Statistically significant high and low risk areas for *B. burgdorferi* transmission. (Source: Diuk-Wasser MA, 2012)
Figure 2. The enzootic cycle of *B. burgdorferi*. (Source: Radolf JD, 2012)
Table 1. Pathogenic species of *Bartonella* spp.

<table>
<thead>
<tr>
<th>Species</th>
<th>Reservoir</th>
<th>Accidental Host</th>
<th>Vector</th>
<th>Distribution</th>
<th>Human Diseases</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. bacilliformis</em></td>
<td>Human</td>
<td>No</td>
<td>Sandfly (Lutzomyia verrucarum)</td>
<td>Andes (Peru, Ecuador, Colombia, Chile, Guatemala)</td>
<td>Carrions disease: Oroya fever and verruga peruana</td>
</tr>
<tr>
<td><em>B. quintana</em></td>
<td>Human</td>
<td>No</td>
<td>Body Louse (Pediculus humanis corporis)</td>
<td>Worldwide</td>
<td>Trench fever, persistent bacteremia, endocarditis, bacillary angiomatosis</td>
</tr>
<tr>
<td><em>B. clarridgeiae</em></td>
<td>Cat</td>
<td>Humans, dogs</td>
<td>Cat flea</td>
<td>Europe and the United States</td>
<td>Cat-scratch disease</td>
</tr>
<tr>
<td><em>B. elizabethae</em></td>
<td>Rats (Rattus norvegicus)</td>
<td>Humans, dogs</td>
<td>Fleas</td>
<td>Worldwide</td>
<td>Endocarditis, neuroretinitis</td>
</tr>
<tr>
<td><em>B. grahamii</em></td>
<td>Bank voles (Clethrionomys glareolus)</td>
<td>Humans</td>
<td>Fleas, Ticks?</td>
<td>Worldwide</td>
<td>Neuroretinitis, uveitis</td>
</tr>
<tr>
<td><em>B. henselae</em></td>
<td>Cat</td>
<td>Humans, dogs</td>
<td>Cat flea (Ctenocephalides felis), ticks?</td>
<td>Worldwide</td>
<td>Endocarditis, Cat Scratch Disease, Bacillary angiomatosis &amp; peliosis, neuroretinitis, bacteremia, peliosis hepatitis, Granulomatous lymphadenitis</td>
</tr>
<tr>
<td><em>B. koehlerae</em></td>
<td>Cat</td>
<td>Dogs, humans</td>
<td>Fleas (C. felis)</td>
<td>Worldwide</td>
<td>Endocarditis, Neurological disorders</td>
</tr>
<tr>
<td><em>B. vinsonii</em> subsp. arupensis</td>
<td>White-footed mice (Peromyscus leucopus)</td>
<td>Humans, dogs</td>
<td>Fleas? Ticks?</td>
<td>North America, Russia, Thailand, Nepal, France</td>
<td>Endocarditis, febrile illness</td>
</tr>
<tr>
<td><em>B. vinsonii</em> subsp. berkoffii</td>
<td>Coyotes (Canis latrans)</td>
<td>Humans, dogs, cats</td>
<td>Ticks?</td>
<td>Worldwide</td>
<td>Endocarditis, persistent bacteremia</td>
</tr>
<tr>
<td><em>B. washoensis</em></td>
<td>Ground squirrel (Spermophilus beecheyi)</td>
<td>Humans, dogs</td>
<td>Fleas? Ticks?</td>
<td>North America</td>
<td>Endocarditis, fever, myocarditis</td>
</tr>
<tr>
<td><em>B. alsatica</em></td>
<td>Rabbits (Oryctolagus cuniculus)</td>
<td>Humans</td>
<td>Unknown</td>
<td>Europe</td>
<td>Endocarditis, Granulomatous lymphadenitis</td>
</tr>
<tr>
<td>Species</td>
<td>Hosts</td>
<td>Vectors/Sources</td>
<td>Geographic Range</td>
<td>Disease(s)</td>
<td></td>
</tr>
<tr>
<td>--------------------</td>
<td>-----------------------------------------------</td>
<td>-----------------------</td>
<td>------------------</td>
<td>------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>B. alsatica</td>
<td>Rabbits (Oryctolagus cuniculus)</td>
<td>Humans</td>
<td>Europe</td>
<td>Endocarditis, Granulomatous lymphadenitis</td>
<td></td>
</tr>
<tr>
<td>B. birtlesii</td>
<td>Wood mice (Apodemus spp)</td>
<td>Unknown</td>
<td>Europe</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>B. bovis</td>
<td>Cattle, cat</td>
<td>Humans</td>
<td>Worldwide</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>B. capreoli</td>
<td>Roe Deer</td>
<td>Unknown</td>
<td>Europe, United States</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>B. chomelii</td>
<td>Cattle (B. taurus)</td>
<td>Unknown</td>
<td>Europe</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>B. weissii</td>
<td>Cattle, Cats</td>
<td>Unknown</td>
<td>Europe and the United States</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>B. doshiae</td>
<td>Meadow voles (M agrestis)</td>
<td>Unknown</td>
<td>Europe, Asia</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>B. peromysci</td>
<td>Deer, Mouse (Peromyscus spp)</td>
<td>Unknown</td>
<td>Europe, United States</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>B. phoceensis</td>
<td>Rat (Rattus spp.)</td>
<td>Unknown</td>
<td>Europe, Asia</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>B. rattimassiliensis</td>
<td>Rat (Rattus spp.)</td>
<td>Unknown</td>
<td>Europe, Asia</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>B. schoenbuchensis</td>
<td>Roe deer (C. capreolus)</td>
<td>Humans?</td>
<td>United States, Europe</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>B. talpae</td>
<td>Mole (Talpa europaea)</td>
<td>No</td>
<td>Europe, Asia</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>B. taylorii</td>
<td>Wood mice (Apodemus spp)</td>
<td>Unknown</td>
<td>Europe, Asia</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>B. tribocorum</td>
<td>Rats (R norvegicus)</td>
<td>Unknown</td>
<td>Europe, Asia</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>B. vinsonii subsp. vinsonii</td>
<td>Meadow voles(Microtus pennsylvanicus)</td>
<td>Dog</td>
<td>North America</td>
<td>Unknown</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3. Common infection strategy of Bartonellae. (Source: Harms, 2012)
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CHAPTER 2.

SURVEY OF VETERINARIANS’ PERCEPTIONS OF BORRELIOSIS IN NORTH CAROLINA

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Survey of veterinarians’ perceptions of borreliosis in North Carolina

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Abstract

Objective—To evaluate the practices and perceptions of veterinarians in North Carolina regarding borreliosis in dogs across different geographic regions of the state.

Design—Cross-sectional survey.

Sample—Data from 208 completed surveys.

Procedures—Surveys were distributed to veterinary clinics throughout North Carolina. Descriptive statistics were used to summarize perceptions pertaining to borreliosis among dogs in North Carolina.

Results—A significantly higher proportion of responding veterinarians believed that borreliosis was endemic in the coastal (67.2%) and Piedmont (60.9%) areas of North Carolina, compared with more western regions (37.5%). The 3 variables found to be significantly different between the north and south region of the state were the estimated number of borreliosis cases diagnosed by each responding veterinary clinic during the past year, the perception of borreliosis endemicity, and the perceptions related to the likelihood of a dog acquiring borreliosis in the state.

Conclusions and Clinical Relevance—Veterinarians’ perception of the risk of borreliosis in North Carolina was consistent with recent scientific reports pertaining to geographic expansion of borreliosis in the state. As knowledge of the epidemiologic features of borreliosis in North Carolina continues to evolve, veterinarians should promote routine screening of dogs for Borrelia burgdorferi exposure as a simple, inexpensive form of surveillance that can be used to better educate their clients on the threat of transmission of borreliosis in this transitional geographic region.
Introduction

Borreliosis, a multisystem inflammatory disorder caused by gram-negative spirochetes belonging to the genus *Borrelia*, is the most common vector-borne disease in the northern hemisphere.\(^1\text{--}^3\) *Borrelia burgdorferi*, the etiologic agent of borreliosis in the United States, is transmitted through the bite of infected hard ticks of the genus *Ixodes*, specifically *Ixodes scapularis* and *Ixodes pacificus*.\(^3\) Borreliosis–endemic regions of the United States have historically included the northeastern, upper Midwestern, and western United States, where most human cases are reported annually.\(^1,^4\) Because *Borrelia* infection is maintained through tick transmission to various wildlife vertebrate reservoirs, the density of *Ixodid* tick species, in combination with host species abundance and other environmental factors, may account for the variations in endemicity among geographic locations.\(^4\text{--}^9\) The diagnosis of borreliosis presently relies on serologic evidence of exposure to *B burgdorferi* in areas that have been identified by the CDC as endemic areas for transmission of borreliosis and documentation of clinical disease.\(^1\) Historically, North Carolina has not been considered a borreliosis–endemic state because it had a lower proportion of infected ticks and animal hosts than can be found in endemic areas with a higher prevalence of the disease.\(^1,^10\) Therefore, lack of travel history to a borreliosis-endemic state historically precluded veterinarians and physicians in North Carolina from making that diagnosis; often, humans or pets with borreliosis in North Carolina are assumed to have been exposed to *B burgdorferi* while traveling to borreliosis–endemic states.

A recent study\(^11\) that measured the prevalence of *Borrelia* spp in ticks collected from 7 coastal plains counties in North Carolina identified *B burgdorferi* or *Borrelia bissettii* DNA
in 63.2% of the collected *Ixodes affinis* ticks and none of the collected *I. scapularis* ticks.\(^\text{11}\) This high prevalence indicates that *Borrelia* spp may be widely distributed among *I affinis* ticks, which may or may not be able to effectively transmit *B burgdorferi* to dogs or humans.\(^\text{11,12}\) Despite the evolving understanding of the ecologic and epidemiologic features of *Borrelia* spp in North Carolina, researchers, physicians, and veterinarians have an incomplete picture of borreliosis risk in humans and pets throughout the state. Recently, it was found that a *B burgdorferi* seroprevalence in dogs > 5% may be a sensitive but nonspecific marker for predicting the risk of transmission of borreliosis to humans, because seroprevalence rates in dogs are correlated with increasing rates of human infections at state or county level.\(^\text{13}\) Therefore, dogs may be used as sentinels for the risk of *B burgdorferi* transmission to humans in North Carolina.\(^\text{13,14}\) A previous North Carolina study failed to identify any dogs that were *B burgdorferi* seropositive that did not have a travel history to a northeastern US borreliosis-endemic state.\(^\text{14}\) The epidemiologic features of borreliosis in North Carolina are changing and it is presently unclear how practicing veterinarians perceive the threat of *B burgdorferi* to dogs in the state, how frequently veterinarians use diagnostic testing to screen for exposure to tick-borne diseases, and whether the routine use of acaracides or borreliosis vaccination is recommended as a component of routine health care.

Enhanced understanding of the geographic differences in perceptions of the epidemiologic features of borreliosis may be used to promote ongoing surveillance of *B burgdorferi* and can facilitate continued education of veterinarians and other public health officials regarding the threat of borreliosis among pets and humans in North Carolina. Therefore, the purpose of the study reported here was to evaluate the practices and
perceptions of veterinarians in North Carolina regarding borreliosis in dogs across different geographic regions of the state.

Materials and Methods

Data collection

In 2001, a company\(^a\) that develops veterinary diagnostic products implemented a voluntary, passive system for the reporting of test results for selected canine blood-borne pathogens obtained with the company’s diagnostic testing kits. Participating veterinary clinics received a rebate toward the cost of the commercial in-house ELISA\(^b\) after submission of a log of test results. In January 2012, a survey was distributed from the North Carolina State University College of Veterinary Medicine Intracellular Pathogens Research Laboratory to all veterinary clinics that submitted commercial in-house ELISA\(^b\) results to the company from 2007 through 2011. Surveys were sent to the most senior veterinarian in each clinic to measure factors associated with borreliosis in dogs in North Carolina. Each clinic was given 5 months to respond to the survey; clinics that did not respond to the survey were excluded from analysis. The study protocol was reviewed and approved by the North Carolina State University Institutional Review Board.

Statistical analysis

Descriptive statistics were used to summarize survey results. The 3 natural landforms of North Carolina were used to categorize 3 east-west regions of the state: the coastal plains, the Piedmont, and the mountain region. Additionally, the state was categorized into north and south regions on the basis of the approximate geographic center of the state (79°27.3 W...
35°36.2'N) according to the US Geological Survey. Univariate analyses, including the 2 test, Fisher exact test, and Fisher-Freeman-Halton test, were used with statistical software to assess potential associations between the survey responses and clinic locations in North Carolina (3 east-west regions, north region, and south region). For all tests, $P \leq 0.05$ was considered significant.

Results

Descriptive statistics

Surveys were sent to 512 North Carolina veterinary clinics, and 208 responses were received within 5 months. Among participating clinics, 27 (12.9%) were in the mountain region, 119 (56.9%) were in the Piedmont region, and 63 (30.1%) were in the coastal plains region; 146 (69.8%) were located in the northern part of the state and 63 (30.1%) were located in the southern part of the state. Approximately 70 (33.5%) clinic respondents reported having clients who resided in urban locations, whereas 162 (77.5%) and 164 (78.5%) reported having clients who resided in rural and suburban locations, respectively. The median number of years spent practicing veterinary medicine by the responding veterinarian was 21.9 (interquartile range, 13.0 to 30.0 years). Responses were obtained for clinics in 64 counties throughout the state, whereas 36 counties had no responses from any clinic. The number of counties with veterinary clinics that did not respond to the survey did not vary by north-south region ($P = 0.45$), but did vary among the 3 east-west regions ($P = 0.03$). Of the counties in the mountain region, approximately half (53.8%) did not have a
veterinary clinic that responded to the survey, compared with 36.6% in the coastal plains and 21.2% in the Piedmont.

The estimated number of cases of canine borreliosis diagnosed during the past year varied greatly by clinic, with 103 (49.5%) clinic respondents reporting approximately 1 to 5 cases, 47 (22.6%) reporting approximately 6 to 26 cases, 11 (5.3%) reporting approximately 26 to 50 cases, and 4 (1.9%) reporting approximately > 50 cases. However, 43 clinic respondents (20.7%) reported no diagnoses of borreliosis during the past year. Estimated numbers of cases did not vary by east-west region of the state ($P = 0.06$) but did vary by north-south region ($P = 0.01$). Of the clinics in the northern region, 35.8% ($n = 52$) respondents reported ≥ 6 cases diagnosed during the past year, compared with 15.8% (10) in the southern region. However, 19.3% ($n = 28$) and 23.8% (15) of clinic respondents in the northern region and the southern region, respectively, reported that no cases were diagnosed during the past year.

For a majority ($n = 120; 57.4$%) of clinics, the senior veterinarian respondent believed that borreliosis is endemic in North Carolina. Perceptions of endemicity varied greatly by east-west and north-south geographic regions ($P = 0.04$ and $P = 0.005$, respectively). Of the clinics in the coastal and Piedmont regions of the state, 67.2% ($n = 41$) and 60.9% (66) of responding veterinarians, respectively, believed that borreliosis is endemic in North Carolina, compared with only 37.5% (9) in the mountain region. Additionally, of the clinics in the northern region of the state, 77.5% ($n = 93$) of responding veterinarians believed that borreliosis is endemic in North Carolina, compared with only 22.5% (27) in the southern region. For more than three-quarters ($n = 185; 88.5$%) of veterinary clinics located
throughout the state, the respondent veterinarian believed that at least 1 dog had acquired borreliosis in North Carolina during the past year. This perception did not vary by east-west region \((P = 0.40)\), but did vary by north-south region \((P < 0.001)\), with 97.1% \((n = 136)\) of clinic respondents in the northern region reporting that at least 1 dog had acquired borreliosis in North Carolina, compared with 77.7% \((n = 49)\) of clinic respondents in the southern region. Similarly, approximately two-thirds \((n = 136; 65.3\%)\) of the clinic respondents reported that a proportion of dogs with borreliosis in North Carolina were exposed to \(B\) burgdorferi in the state. Interestingly, 46 (22.0%) clinic respondents reported that they had not identified a dog that acquired \(B\) burgdorferi outside of the state (on the basis of results of a commercial in-house ELISA\(^b\)). This perception did not vary by east-west or north-south region \((P = 0.10\) and \(P = 0.37\), respectively). 

History of tick attachment in the clinic’s dog patient population varied, with 88 (42.3%) clinic respondents reporting a history of tick attachment in 0% to 25% of the population, 47 (22.5%) reporting a history of tick attachment in 25% to 50% of the population, and 71 (34.1%) reporting a history of tick attachment in > 50% of the population. Two-thirds of all clinic respondents \((n = 138; 66.8\%)\) reported that > 50% of their clients used tick- and flea-preventative products. Of the 188 (90.3%) clinic respondents who recommended vaccination against borreliosis, 34 (16.3%) routinely recommended vaccination for all dogs in the practice, 117 (55.9%) recommend vaccination for dogs with unusual risk factors (eg, travel to a highly endemic region to go deer hunting), and 37 (17.7%) recommended vaccination at the client’s request.
Discussion

Results of the survey indicated that veterinarians’ perceptions related to transmission of borreliosis in North Carolina varied widely across geographic regions of the state. A higher proportion of responding veterinarians believed that borreliosis is endemic in the coastal plains (67.2%) and Piedmont (60.9%) areas of North Carolina, compared with the mountain region (37.5%). These results correspond to current distribution maps of established populations of *I. scapularis* in North Carolina, which categorize the western regions of the state as unable to support *I. scapularis* populations. Additionally, the recent expansion of populations of *I. affinis* infected with *B. burgdorferi* in the coastal plains area may have contributed to a belief of heightened risk in that region. Although it is suggested that *I. affinis* rarely bite humans and therefore are seldom implicated in transmission to humans, reports indicate that *I. affinis* may play a more important role than *I. scapularis* in the maintenance of the enzootic cycle of *B. burgdorferi*; therefore, overlapping populations of these arthropod vectors may have important implications for the amplification, transmission, and spread of *B. burgdorferi*, particularly in southern states where such overlap is more common.

Current spatial predictions of risk indicate the expansion of borreliosis into an area in the northern coastal plains of North Carolina and define the coastal plains as transitional areas for borreliosis expansion, which are areas that cannot be defined as high or low risk based on the probability of infected nymphs within the area. Furthermore, Wake County, a central county in the Piedmont region, was declared endemic for human borreliosis for the first time in 2010, according to CDC case standards. The media attention surrounding the
human cases of borreliosis in the coastal plains and Piedmont regions may have contributed to the high proportion of clinic respondents in those regions who believed that borreliosis is endemic, or is becoming endemic, in North Carolina.\textsuperscript{19}

Three variables were also found to vary significantly between the north and south regions of the state, including the estimated number of canine borreliosis cases diagnosed in the past year at each participating clinic, the perception of borreliosis endemicity, and the perception related to the possibility of a dog acquiring borreliosis in the state. Historically, Virginia, which shares its southern border with North Carolina, has been considered to be a state of intermediate to low risk for borreliosis. However, according to the CDC, the incidence of borreliosis in Virginia has increased in recent years from 3.6 cases/100,000 persons in 2005 to 11.4 cases/100,000 persons in 2010.\textsuperscript{20} Additionally, in 2011, Virginia had declared roughly two-thirds of its counties endemic for borreliosis, including 5 along the Virginia-North Carolina border.\textsuperscript{18,21} Recent studies\textsuperscript{12,15} confirm the expansion of \textit{I. affinis} beyond the North Carolina border and into southeastern Virginia. Because \textit{I. affinis} play a more important role than \textit{I. scapularis} in the maintenance of the enzootic cycle of \textit{B. burgdorferi} the expanding distribution of this tick vector may contribute to further amplification and spread of \textit{B. burgdorferi} throughout Virginia. The expanding range of borreliosis within Virginia may subsequently influence the perceptions of veterinarians in the northern regions toward a heightened perception of risk of borreliosis.

Despite increasing reports of borreliosis in North Carolina, the presence of borreliosis throughout the southeast region of the United States has been a highly controversial issue for more than a decade.\textsuperscript{22,23} A borreliosis-like syndrome in humans known as STARI is prevalent
throughout the southeastern United States and is known to be transmitted by the lone star tick \textit{(Amblyomma americanum)}. The clinical signs and symptoms of STARI are similar to those of borreliosis, including an erythema migrans–like rash, fever, and muscle and joint stiffness.\textsuperscript{23–25} The recognition of STARI, in conjunction with northward movement of \textit{A americanum}, has lead to difficulties in differentiating the 2 diseases, making accurate reporting difficult and has further complicated accurate diagnosis and treatment of borreliosis versus STARI in the southeastern United States. Studies have failed to identify any specific microorganism as the cause of STARI. Although the pathogen \textit{Borrelia lonestari} was initially associated with this illness, more recent evidence fails to support this association.\textsuperscript{26}

Use of the C6 peptide in the commercial in-house ELISA\textsuperscript{b} has resulted in a highly sensitive and specific commercial enzyme immunoassay used for in-house detection of antibodies against \textit{B burgdorferi} in blood, serum, or plasma. Antibodies against the C6 peptide correlate with the number of infecting Borrelia organisms in dogs. Additionally, the C6 peptide is not found in sera from dogs that have received commercially available \textit{B burgdorferi} vaccines.\textsuperscript{28} In addition to detection of antibodies against \textit{B burgdorferi}, the commercial in-house ELISA\textsuperscript{b} detects \textit{Dirofilaria immitus} antigen, anti-\textit{Ehrlichia canis} antibodies, and antibodies against \textit{Anaplasma phagocytophilum}. Therefore, veterinarians can use an in-house test kit to test for exposure to 4 vector-borne infections in dogs. In the context of using dogs as sentinels for human borreliosis, serologic cross-reactivity occurs among \textit{Borrelia} spp, as well as \textit{Leptospira} spp, with a conventional indirect fluorescent antibody test; however, the commercial in-house ELISA\textsuperscript{b} kit provides 96% sensitivity and 100% specificity for detection of \textit{B burgdorferi} C6 peptide antibody.\textsuperscript{30} Therefore, the results
of the present study would be minimally influenced by cross-reactive antibodies, prior vaccination against *B. burgdorferi*, or exposure to *A. americanum* that could potentially transmit STARI. It is unlikely that veterinary clinicians would misinterpret commercial in-house ELISA for *B. burgdorferi* results and subsequently overestimate the potential risk of borreliosis in their canine patient population.

Although 512 invitations to participate in the survey were sent out, only 208 were returned. Current contact information for some veterinary clinics was not available. Additionally, missing responses varied significantly among the 3 east-west regions. Because of the limited sample size and high nonresponse rate, results of the survey may have been subject to selection bias. Veterinarians who responded to the survey may have had different impressions regarding the presence of borreliosis in North Carolina, compared with veterinarians who did not respond. Survey instructions indicated that the most senior veterinarian in each clinic should be the respondent, but this was not confirmed. Different veterinarians in each clinic may also have had different impressions pertaining to borreliosis in North Carolina. Subsequently, results of the survey may not reflect all veterinarians’ perceptions of borreliosis in North Carolina.

In addition, this survey did not obtain specific test results or demographic information such as travel history for individual dogs in each clinic; therefore, no assumption could be made about the specific demographics or travel history for individual cases within our aggregate data. All canine health-related information obtained from the surveys was based on information obtained from the owner and the veterinarian’s perception of the dog’s health status. Therefore, it is possible that responses may have been subject to recall bias.
Additionally, C6 peptide–positive dogs may have been exposed to *B burgdorferi* outside of North Carolina. We attempted to assess the proportion of the clinic’s clients that acquired borreliosis within North Carolina, rather than by traveling to a borreliosis endemic area. Although it is unlikely that the responding veterinarian was aware of every client’s detailed travel history, the influence of travel history likely had only a marginal effect on the results.

Results of previous studies\textsuperscript{13,14,31} suggest that dogs may serve as an appropriate sentinel for the risk of borreliosis in the eastern United States, and it has been reported that dogs are approximately 6 times more likely to be infected with *B burgdorferi* as humans because of more frequent environmental exposure to ticks. Because approximately 95% of infected dogs remain without clinical signs following infection with *B burgdorferi*, routine screening of healthy dogs for anti-*B burgdorferi* antibodies is an essential component of sentinel surveillance of borreliosis. Because erythema chronicum migrans has not been reported in dogs, there is no accepted case definition for borreliosis in dogs. A majority of veterinarians (76.1%) surveyed responded that they test healthy dogs for routine surveillance of *B burgdorferi* exposure or perform testing in conjunction with routine heartworm testing (15.7%) when clinical signs of borreliosis are not present. Continued education of veterinarians regarding the potential threat of borreliosis in North Carolina could potentially result in increased use of the ELISA for routine surveillance and also increase the number of veterinarians participating in the company’s voluntary, passive reporting system for the surveillance of blood-borne pathogens. Because veterinarians have an easy-access and cost-effective method for routine surveillance of *B burgdorferi* exposure in dogs through screening for the C6 peptide, sequentially obtained canine surveillance data should provide a
more complete picture of borreliosis risk for dogs and humans in North Carolina. The present study revealed that veterinarians’ perceptions of risk of borreliosis in North Carolina was consistent with current scientific reports pertaining to borreliosis expansion and transmission in the state. Understanding veterinarians’ perceptions of epidemiologic features of borreliosis will give insight into why they may or may not choose to use the ELISA or continue to participate in the passive reporting system. Identification of these factors can be used to create more targeted educational efforts to promote the routine testing of dogs for surveillance efforts across the state. As the epidemiologic features of borreliosis in North Carolina continue to be determined, veterinarians should promote routine testing of dogs for borreliosis as a simple, inexpensive form of surveillance to better educate their clients on the threat of borreliosis. Although this study was focused on North Carolina veterinarians and their perceptions, the results should have relevance for veterinarians and public health officials in other regions of *B burgdorferi* expansion. As with many vector-borne infections, a One Health approach to surveillance and disease prevention should benefit animal and human health.32

**Footnotes**

a. IDEXX Laboratories Inc, Westbrook, Me.

b. SNAP 3DX/SNAP 4DX tests, IDEXX Laboratories Inc, Westbrook, Me.

c. Proc Freq, SAS Institute Inc, Cary, NC.
References


7. Estrada-Peña A. Diluting the dilution effect: a spatial Lyme model provides evidence for the importance of habitat fragmentation with regard to the risk of infection. *Geospat Health* 2009;3:143–155.


CHAPTER 3.

*BARTONELLA* SPP. BACTEREMIA AND RHEUMATIC SYMPTOMS IN PATIENTS FROM A LYME DISEASE-ENDEMIC REGION

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Bartonella spp. bacteremia in rheumatic patients from a Lyme disease-endemic region

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Keywords: Bartonella, bacteremia, blood, arthritis, myalgia, PCR, DNA sequencing, bacteria, Lyme disease, rheumatic, bacteria

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Abstract

*Bartonella* spp. infection has been reported in association with an expanding spectrum of symptoms and lesions. Among 296 patients examined by a rheumatologist, prevalence of antibodies against *Bartonella henselae*, *B. koehlerae*, or *B. vinsonii* subsp. *berkhoffii* (185 [62%]) and *Bartonella* spp. bacteremia (122 [41.1%]) was high. Conditions diagnosed before referral included Lyme disease (46.6%), arthralgia/arthritis (20.6%), chronic fatigue (19.6%), and fibromyalgia (6.1%). *B. henselae* bacteremia was significantly associated with prior referral to a neurologist, most often for blurred vision, subcortical neurologic deficits, or numbness in the extremities, whereas *B. koehlerae* bacteremia was associated with examination by an infectious disease physician. This cross-sectional study cannot establish a causal link between *Bartonella* spp. infection and the high frequency of neurologic symptoms, myalgia, joint pain, or progressive arthropathy in this population; however, the contribution of *Bartonella* spp. infection, if any, to these symptoms should be systematically investigated.
Introduction

The genus *Bartonella* comprises at least 26 species or subspecies of vector-transmitted bacteria, each of which has evolved to cause chronic bacteremia in >1 mammalian reservoir hosts (1–4). Among these, bartonellae of 14 species or subspecies have been implicated in zoonotic diseases (5,6), including cat-scratch disease, which is caused by *B. henselae* transmission during a cat bite or scratch and characterized by acute onset of self-limiting fever and regional lymphadenopathy (7–9). Recent observations, however, are causing a paradigm shift from the assumption that infection with a *Bartonella* sp. consistently induces an acute, self-limiting illness to the realization that subsets of infected, immunocompetent patients can become chronically bacteremic (10–15). After *B. henselae* was confirmed as the primary cause of cat-scratch disease in the early 1990s, several reports described an association between the newly identified bacterium and rheumatic disease manifestations, variously described as rheumatoid, reactive, or chronic progressive polyarthritis (16–20). One study, however, failed to isolate *B. henselae* from synovial fluid of 20 patients with chronic arthritis (21). Because epidemiologic evidence supports an association between rheumatic symptoms and cat-scratch disease and because arthritis is a primary disease manifestation of *Borellia burgdorferi* infection (Lyme disease), we explored whether antibodies against and bacteremia with *Bartonella* spp. can be detected in patients examined for arthropathy or chronic myalgia. Our primary objective was to determine the serologic and molecular prevalence of *Bartonella* spp. bacteremia in patients referred to a clinical rheumatologist. We also compared self-reported symptoms, health history, and demographic factors with *Bartonella* spp. bacteremia as determined by an enrichment blood culture.
platform combined with PCR amplification and DNA sequencing, when possible, to
determine the *Bartonella* species and strain. This study was conducted in conjunction with
North Carolina State University Institutional Review Board approval (IRB# 164–08–05).

**Materials and Methods**

**Study Population**

For this cross-sectional study, we enrolled only patients examined by a rheumatologist in the
Because *Bartonella* spp. are known to primarily infect cells within the vascular system,
including erythrocytes, endothelial cells, and potentially circulating and tissue macrophages
(1,5,6), selection was biased by patients who had historical, physical examination, or
laboratory evidence of small vessel disease, including a subset of patients with a prior
diagnosis of Lyme disease or chronic post–Lyme syndrome. We also included patients with
chronic joint pain, prior documentation of synovial vascular inflammation, or a diagnosis of
rheumatoid arthritis.

A standardized 5-page questionnaire was mailed to each participant for self-report. The
questionnaire collected information about demographics, animal/arthropod exposure, history
of visiting a medical specialist, outdoor activity, self-reported clinical symptoms, and
concurrent conditions. Questionnaires were returned to the Intracellular Pathogens Research
Laboratory at North Carolina State University, College of Veterinary Medicine, Raleigh,
North Carolina, USA, where results were entered into an electronic database.
Sample Collection

From each patient, the attending rheumatologist aseptically obtained anticoagulated blood samples (in EDTA tubes) and serum samples and shipped them overnight to the laboratory. Patient variations included timing of sample collection relative to onset of illness, duration of illness, current illness severity, and prior or recent use of antimicrobial drugs. The samples were then processed in a limited-access laboratory.

Sample Processing

Immunofluorescence Antibody Assay

To determine the antibody titer to each \textit{Bartonella} species or subspecies, we used \textit{B. henselae}, \textit{B. koehlerae}, and \textit{B. vinsonii} subsp. \textit{berkhoffii} (genotypes I, II, and III) antigens in a traditional immunofluorescence antibody (IFA) assay with fluorescein conjugated goat anti-human IgG (Pierce Antibody; Thermo Fisher Scientific, Rockford, IL, USA) (10,12,22). To obtain intracellular whole bacterial antigens for IFA testing, we passed isolates of \textit{B. henselae} (strain Houston-1, ATCC #49882); \textit{B. koehlerae} (NCSU FO-1–09); and \textit{B. vinsonii} subsp. \textit{berkhoffii} genotypes I (NCSU isolate 93-CO-1, ATCC #51672), II (NCSU isolate 95-CO-2), and III (NCSU isolate 06-CO1) from agar-grown cultures into \textit{Bartonella}-permissive tissue culture cell lines: AAE12 (an embryonic \textit{Amblyomma americanum} tick cell line) for \textit{B. henselae}, DH82 (a canine monocytoid cell line) for \textit{B. koehlerae}, and Vero (a mammalian fibroblast cell line) for the \textit{B. vinsonii} genotypes. Heavily infected cell cultures were spotted onto 30-well Teflon coated slides (Cel-Line; Thermo Fisher Scientific), air dried, acetone fixed, frozen, and stored. Serum samples were diluted in a phosphate-buffered saline solution.
containing normal goat serum, Tween-20, and powdered nonfat dry milk to block nonspecific antigen binding sites and then incubated on antigen slides. All available patient serum was screened at dilutions from 1:16 to 1:64. Samples reactive at a 1:64 dilution were further tested with 2-fold dilutions to 1:8192. As in previous studies, we defined a seroreactive antibody response against a specific *Bartonella* sp. antigen as a threshold titer of 64 (10–15,23,24).

**Bartonella α Proteobacteria Growth Medium Enrichment Culture**

Each sample was tested by PCR amplification of *Bartonella* spp. DNA before and after enrichment of blood and serum in *Bartonella α* Proteobacteria growth medium (BAPGM) (10–14,23–26). The BAPGM platform incorporates 4 PCR steps, representing independent components of the testing process for each sample, as follows: step 1) PCR amplifications of *Bartonella* spp. after DNA extraction from whole blood and serum; steps 2 and 3) PCR after whole blood culture in BAPGM for 7 and 14 days; and step 4) PCR of DNA extracted from subculture isolates (if obtained after subinoculation from the BAPGM flask at 7 and 14 days onto plates containing trypticase soy agar with 10% sheep whole blood, which are incubated for 4 weeks). To avoid DNA carryover, we performed PCR sample preparation, DNA extraction, and PCR amplification and analysis in 3 separate rooms with a unidirectional work flow. All samples were processed in a biosafety cabinet with HEPA (high-efficiency particulate air) filtration in a limited-access laboratory.

Methods used to amplify *Bartonella* DNA from blood, serum, and BAPGM liquid culture and subculture samples included conventional PCR with *Bartonella* genus primers targeting
the 16S-23S intergenic spacer region (ITS) and a second PCR with *B. koehlerae* ITS species-specific primers, as described (13,25–29). Amplification of the *B. koehlerae* ITS region was performed by using oligonucleotides *Bkoehl*-1s: 5′-CTT CTA AAA TAT CGC TTC TAA AAA TTG GCA TGC-3′ and *Bkoehl*1125as: 5′-GCC TTT TTT GGT GAC AAG CAC TTT TCT TAA G-3′ as forward and reverse primers, respectively. Amplification was performed in a 25-μL final volume reaction containing 12.5 μL of Tak-Ex Premix (Fisher Scientific), 0.1 μL of 100 μM of each forward and reverse primer (IDT; DNA Technology, Coralville, IA, USA), 7.3 μL of molecular grade water, and 5 μL of DNA from each sample tested. Conventional PCR was performed in an Eppendorf Mastercycler EPgradient (Hauppauge, NY, USA) under the following conditions: 1 cycle at 95°C for 2 s, followed by 55 cycles with DNA denaturing at 94°C for 15 s, annealing at 64°C for 15 s, and extension at 72°C for 18 s. The PCR was completed by a final cycle at 72°C for 30 s. As previously described for the *Bartonella* ITS genus and *B. koehlerae*–specific PCRs, all products were analyzed by using 2% agarose gel electrophoresis and ethidium bromide under UV light, after which amplicon products were submitted to a commercial laboratory (Eton Bioscience Inc., Research Triangle Park, NC, USA) for DNA sequencing to identify the species and ITS strain type (13,15,28,30).

To check for potential contamination during processing, we simultaneously processed a noninoculated BAPGM culture flask in the biosafety hood in an identical manner for each batch of patient blood and serum samples tested. For PCR, negative controls were prepared by using 5 μL of DNA from the blood of a healthy dog. All controls remained negative throughout the course of the study.
Statistical Analysis

Descriptive statistics were obtained for all demographic variables, self-reported clinical symptoms and concurrent conditions, previous specialist consultation, and self-reported exposures. The $\chi^2$ test was used to assess associations between self-reported clinical symptoms and previous specialist consultation separately with PCR results for *B. henselae*; *B. koehlerae*; and *B. vinsonii* subsp. *berkhoffii* genotypes I, II, and III. The Fisher exact test was used when expected cell value was <5. For the initial analysis, a liberal $\alpha$ value ($\alpha<0.10$) was selected. The effect of each significant variable on the outcome variables was adjusted in separate multivariate logistic regression models controlling for age, sex, and health status. The models were repeated for different possible outcomes: PCR results for *B. henselae* or PCR results for *B. koehlerae*. Variables maintaining $p<0.05$ were considered significant. For some comparisons of potential interest, we were unable to estimate associations with the outcome(s) of interest because of low numbers (e.g., *B. vinsonii* subsp. *berkhoffii* genotypes I, II and III). Statistical analyses were performed by using SAS/STAT for Windows version 9.2 (SAS Institute Inc., Cary, NC, USA).

Results

Patient Characteristics

The age range of the 296 patients was 3–90 years; median ages were 46 years for women and 36 years for men (Table 1). Women made up $\approx 70\%$ of the study population. Most (68.2%) patients reported that they felt ill, whether chronically or infrequently, and 27.7% considered themselves to be generally healthy. The most common animal exposure reported was dog (n
= 252; 85.1%), followed by cat (n = 202; 68.2%) and horse (n = 86; 29.0%). Most patients reported having been bitten or scratched by an animal (n = 202; 68.2%) or exposed to ticks (n = 229; 77.4%) and biting flies (n = 160; 54.0%). Hiking was the predominant outdoor activity reported (52.0%). Most (273 [92.2%]) patients reported having had a condition diagnosed before visiting the rheumatologist. Previously diagnosed conditions included Lyme disease (46.6%), arthralgia/arthritis or osteoarthritis/rheumatoid arthritis (20.6%), chronic fatigue (19.6%), and fibromyalgia (6.1%) (Figure 1).

Serologic and BAPGM Findings

Of the 296 patients, 185 (62.5%) were seroreactive to >1 Bartonella sp. antigens and 122 (41.1%) were infected with B. henselae, B. koehlerae, B. vinsonii subsp. berkhoftii, or Bartonella spp. Of the 122 patients with Bartonella spp. infection, PCR results were positive but DNA sequencing was unsuccessful or did not enable species identification for 29 (23.7%). After subculture, 6 isolates were obtained from 5 samples: 3 B. henselae isolates, 2 B. koehlerae isolates, and 1 Bartonella sp. isolate that was not fully characterized. Of the Bartonella-infected patients, 120 (98.4%) had a positive PCR result after DNA extraction from blood, serum, or enrichment culture (Figure 2), and 2 (1.6%) had a positive PCR result only after subculture isolation.

For B. henselae, 67 (22.6%) patients were seroreactive and 40 (13.5%) had positive PCR results. Of these 40 patients, only 7 (17.5%) were concurrently B. henselae seroreactive, whereas 33 (82.5%) patients who had a positive PCR result were not seroreactive to B. henselae antigens. There was no association between B. henselae antibodies and bacteremia
For *B. koehlerae*, 89 (30.1%) patients were seroreactive and 54 (18.2%) had positive PCR results. Of these 54 patients, 24 (44.4%) were seroreactive to *B. koehlerae* by IFA assay, whereas 29 (53.6%) were not seroreactive to *B. koehlerae* antigens. One patient with a positive *B. koehlerae* PCR result did not have a concurrent IFA test result (serum not submitted). There was an association between *B. koehlerae* seroreactivity and bacteremia (*p* = 0.008); seroreactive patients were more likely to be infected (odds ratio [OR] 2.25 [1.22–4.15]).

For *B. vinsonii* subsp. *berkhoffii*, 148 (50.0%) patients were seroreactive by IFA testing to at least 1 of 3 genotypes, and 10 (3.4%) had a positive PCR. Of these 10 patients, 3 were infected with genotype I, 6 were infected with genotype II, and for 1 patient the genotype could not be defined on the basis of readable DNA sequence. Seroreactivity to genotypes I, II, and III was found for 77 (26.0%), 102 (34.5%), and 82 (27.7%) patients, respectively. There was no association between *B. vinsonii* subsp. *berkhoffii* seroreactivity and bacteremia.

Combined PCR and IFA assay results are summarized in Table 2. Of the patients with a positive PCR, 65% reported a prior diagnosis of Lyme disease (*n* = 138), bartonellosis (*n* = 29), or babesiosis (*n* = 14). Among the 138 patients with a prior diagnosis of Lyme disease, the prevalence of *Bartonella* spp. antibodies and bacteremia were 93 (67.4%) and 57 (41.3%), respectively.

**Factors Associated with *Bartonella* spp.**

PCRs indicated the following: *B. henselae* positivity was associated (*p*<0.05) with blurred
vision and numbness (Table 3), patients who had visited a neurologist were more likely than those who had not to be *B. henselae* positive, older median age was significantly associated with *B. koehlerae* positivity, and patients who reported paralysis were more likely to be positive for *B. vinsonii* subsp. *berkhoffii*. No associations were found for self-reported exposures (e.g., insect or animal exposure) and positive PCR for *B. henselae*, *B. koehlerae*, or *B. vinsonii* subsp. *berkhoffii*.

**Logistic Regression Analysis**

To identify factors associated with PCR positivity for *B. henselae* or *B. koehlerae*, we adjusted the models for 3 biological confounders: age, sex, and health status (Table 4). We identified the following factors as associated with *B. henselae*–positive PCR result: blurred vision (adjusted OR [aOR] 2.37, 95% CI 1.13–4.98), numbness (aOR 2.74, 95% CI 1.26–5.96), and previous consultation with a neurologist (aOR 2.76, 95% CI 1.33–5.73). No self-reported symptoms were significantly associated with PCR positivity for *B. koehlerae*. However, patients who had visited an infectious disease physician were more likely to have a *B. koehlerae*–positive PCR result (aOR 1.98, 95% CI 1.05–3.75).

**Discussion**

We identified unexpectedly high serologic and molecular prevalence for *B. henselae*, *B. koehlerae*, and *B. vinsonii* subsp. *berkhoffii* in patients who had been examined by a rheumatologist, of whom more than half reported a prior diagnosis of Lyme disease, bartonellosis, or babesiosis. However, the diagnostic criterion upon which these infections were based was not available for review because all prior diagnoses were self-reported.
Overall, 185 (62.5%) of 296 patients had antibodies to *B. henselae*, *B. koehlerae*, or *B. vinsonii* subsp. *berkhoffii*, and 122 (41.1%) were positive for *Bartonella* spp. according to PCR. In most instances, DNA sequencing of the amplified product facilitated identification of the infecting species. The prevalence of antibodies against *Bartonella* spp. (93 [67.4%]) and bacteremia [57 [1.3%]) among 138 patients with a prior diagnosis of Lyme disease did not differ from that of the overall study population. Because our analysis was restricted to patients selected by a rheumatologist practicing in a Lyme disease–endemic region, extrapolations to other regions or other rheumatology practices might not be applicable. Also, because the survey was self-administered, objective confirmation of symptoms, conditions, and diagnoses was not always possible; therefore, responses might have been subject to respondent bias. Similarly, because responses associated with symptoms, conditions, and exposures might have occurred over a protracted time, survey responses might also be subject to recall bias.

Despite these study limitations, *B. henselae* infections seemed to be more common in patients who reported blurred vision, numbness in the extremities, and previous consultation with a neurologist before referral to the rheumatologist. In a case series of 14 patients, the following were reported by 50% of patients infected with a *Bartonella* species, specifically *B. henselae*, *B. vinsonii* subsp. *berkhoffii*, or both: memory loss, numbness or a loss of sensation, balance problems, and headaches (10). Another 6 *B. henselae*–bacteremic patients reported seizures, ataxia, memory loss, and/or tremors; 1 of these patients was co-infected with *B. vinsonii* subsp. *berkhoffii*, and another was positive for *B. henselae* by PCR after enrichment of cerebrospinal fluid in BAPGM (23). An enrichment culture approach also
identified an association between intravascular infection with *B. vinsonii* subsp. *berkhoffii* genotype II and *B. henselae* and neurologic symptoms in a veterinarian and his daughter (12). Symptoms in the father included progressive weight loss, muscle weakness, and lack of coordination; symptoms in the daughter were headaches, muscle pain, and insomnia. For each patient, after repeated courses of antimicrobial drugs, blood cultures became negative, antibody titers decreased to nondetectable levels, and all neurologic symptoms resolved. Although no symptoms were statistically associated with *B. koehlerae* infection, patients infected with *B. koehlerae* were more likely to have previously consulted an infectious disease physician. Of the 54 *B. koehlerae* patients with a positive PCR result, 54% reported a prior diagnosis of Lyme disease (n = 25), bartonellosis (n = 3), or babesiosis (n = 1). Fatigue, insomnia, memory loss, and joint and muscle pain were frequent complaints among those with a positive PCR result for *B. koehlerae*, but these symptoms did not differ in frequency from those in patients with negative PCR. Similar symptoms were previously reported in a small case series involving *B. koehlerae*–bacteremic patients (13). Peripheral visual deficits, sensory loss, and hallucinations resolved in a young woman after antimicrobial drug treatment for *B. koehlerae* infection (30). Because of the small number of patients with positive PCR results for *B. vinsonii* subsp. *berkhoffii*, we restricted the multivariate analysis to those with positive results for *B. henselae* and *B. koehlerae*. Because limited sample size affected our ability to conduct multivariate analysis to control for potential confounders for *B. vinsonii* subsp. *berkhoffii* positivity, the $\chi^2$ associations with *B. vinsonii* subsp. *berkhoffii* positivity should be interpreted with caution.

Although the pathogenic relevance of the high *Bartonella* spp. seroprevalence and
bacteremia in this patient population are unclear, these results justify additional prospective studies involving more narrowly defined patient and control populations. Of the 92 patients infected with \textit{B. koehlerae}, \textit{B. henselae}, or \textit{B. vinsonii} subsp. \textit{berkhoffii}, 69 (75\%) had at least 1 discordant IFA assay result for \textit{Bartonella} spp. antigen seroreactivity and only 34 (30.6\%) had a concordant species-specific PCR and IFA result. Also, consistent with previous study findings (15), the PCRs depicted in Figure 2 illustrate an increased likelihood of positivity if blood, serum, and enrichment blood cultures are independently tested. According to these and previous results (7,18,31,32), a subset of \textit{Bartonella} spp.–bacteremic patients could be anergic and might not produce a detectable IFA response, or alternatively, the substantial antigenic variation among various \textit{Bartonella} strains might result in false-negative IFA assay results for some patients. In a study on \textit{Bartonella} serology conducted by the Centers for Disease Control and Prevention, IFA cross-reactivity among \textit{Bartonella} species occurred in 94\% of patients with suspected cat-scratch disease (33). Despite the lack of concordance between serologic results and BAPGM enrichment PCR results, most (185 [62.5\%]) patients in our study were seroreactive to \textit{Bartonella} spp., suggesting prior exposure to >1 \textit{Bartonella} spp. Because serologic cross-reactivity to \textit{Chlamydia} spp. and \textit{Coxiella burnettii} antigens has been reported, exposure to these or other organisms might have contributed to the high seroprevalence. In a previous study involving 32 healthy volunteers and patients at high risk for \textit{Bartonella} spp. bacteremia, seroprevalence rates for \textit{B. henselae}, \textit{B. koehlerae} and \textit{B. vinsonii} subsp. \textit{berkhoffii} genotypes I and II were 3.1\%, 0\%, 0\%, and 50\%, respectively, for the healthy population compared with 15.6\%, 9.2\%, 19.8\%, and 28.1\%, respectively, for the high-risk population (15). Although in that study and the study reported here, the same test
antigens and identical IFA assays were used and the same research technologist interpreted the results, the overall seroprevalence in the study reported here was higher than that among high-risk patients with extensive arthropod or animal contact (49.5%) and differed substantially from serologic results from healthy volunteers (15). However, in the study reported here, a large portion of the population (34.5%) was also seroreactive to \textit{B. vinsonii} \textit{berkhoffii} genotype II. Immunophenotypic properties giving rise to seroreactivity to this particular antigen among healthy control and patient populations have not been clarified but could be related to polyclonal B-cell activation, commonly found in patients with rheumatologic or chronic inflammatory diseases.

It is becoming increasingly clear that no single diagnostic strategy will confirm infection with a \textit{Bartonella} sp. in immunocompetent patients. Before the current study, we primarily used BAPGM enrichment blood cultures and PCR to test symptomatic veterinarians, veterinary technicians, and wildlife biologists, who seem to be at occupational risk for \textit{Bartonella} sp. bacteremia because of animal contact and frequent arthropod exposure (10–15,23). Cats are the primary reservoir hosts for \textit{B. henselae} and \textit{B. koehlerae}, whereas canids, including dogs, coyotes and foxes, are the primary reservoir hosts for \textit{B. vinsonii} subsp. \textit{berkhoffii} (4,6,29,34). Although infrequent when compared with cat transmission of \textit{B. henselae} resulting in classical cat-scratch disease, dogs have been implicated in the transmission of \textit{B. vinsonii} subsp. \textit{berkhoffii} and \textit{B. henselae} to humans (35,36). The predominant symptoms reported among occupationally at-risk patient populations have included severe fatigue, neurologic and neurocognitive abnormalities, arthralgia, and myalgia (10–13,23). In the study reported here, dog (85%) and cat (68%) contact were reported by
most respondents; however, no associations were found between infection with a *Bartonella* sp. and contact with a specific animal. Similarly, exposure to mosquitoes, ticks, fleas, and biting flies were all reported by >50% of the study population. The results of this study support documentation of *Bartonella* spp. bacteremia in patients seen by a rheumatologist in a Lyme disease–endemic area and provides the basis for future studies to ascertain the prevalence of *Bartonella* spp. in patients with rheumatic and neurologic symptoms.

This study was supported in part by the state of North Carolina, a grant from the American College of Veterinary Internal Medicine Foundation, and a monetary donation from Bayer Animal Health.

Dr Maggi is a research assistant professor in the Department of Clinical Sciences at North Carolina State University College of Veterinary Medicine. His research has focused on the development of novel or improved molecular diagnostic and culture methods for detection of *Bartonella* spp. infections in animals and humans.

E.B.B., in conjunction with Sushama Sontakke and North Carolina State University, holds US Patent No. 7,115,385, Media and Methods for Cultivation of Microorganisms, which was issued October 3, 2006. E.B.B. is chief scientific officer for Galaxy Diagnostics, a newly formed company that provides diagnostic testing for the detection of *Bartonella* species infection in animals and human patients. R.G. Maggi has lead research efforts to optimize the BAPGM platform and is the scientific technical advisor for Galaxy Diagnostics. R. Mozayeni was the attending physician for the patients described in this study and has recently joined Galaxy Diagnostics as the chief medical officer. All other authors have no potential conflicts.
Table 1. Characteristics and Bartonella spp. PCR results for 296 patients examined by a rheumatologist,
Maryland–Washington, DC, USA, August 25, 2008–April 1, 2009**
populatio
Characteristic
n, no.
Total
296 (100)
205 (69.3)
F
91 (30.7)
M
State of residence
Maryland
148 (50.0)
Virginia
76 (25.7)
Pennsylvania
26 (8.8)
District of Columbia
16 (5.4)
Other
30 (10.1)
Immunofluorescence antibody results
All Bartonella spp.
185 (62.5)
67 (22.6)
B. henselae
89 (30.1)
B. koehlerae
B. vinsonii subsp. berkhoffii
148 (50.0)
Self-report health assessment
82 (27.7)
Healthy
Infrequently Ill
53 (17.9)
Chronically Ill
149 (50.3)
No response
12 (4.0)
Animal contact
283 (95.6)
Yes
No
13 (4.4)
Type
252 (85.1)
Dog
Cat
202 (68.2)
Horse
86 (29.0)
Bird
59 (19.3)
Cattle
32 (10.8)
Poultry
30 (10.1)
Swine
25 (8.5)
Sheep
25 (8.5)
Other
12 (4.0)
Animal bites/scratches
Cat
154 (52.0)
Dog
118 (39.8)
Bird
12 (4.0)
Horse
14 (4.7)
Insect
exposure
256 (86.5)
Ticks
229 (77.4)
Fleas
148 (50.0)
Biting Flies
160 (54.0)
Lice
38 (12.8)
Spiders
5 (1.7)
Sarcoptes mite
3 (1.0)
Outdoor exposure
154 (52.0)
Hiking
Wildlife rescue/rehabilitation
22 (7.4)
Hunting
21 (7.1)
Other
36 (12.2)

Overall
positive
122 (41.4)
86 (29.0)
36 (12.2)

B. henselae
40 (13.5)
24 (11.7)
16 (17.6)

B. koehlerae
54 (18.2)
38 (18.5)
16 (17.5)

B. vinsonii
subsp. berkhoffii
10 (3.4)
7 (3.4)
3 (3.3)

58 (39.2)
37 (48.7)
9 (34.6)
5 (31.3)
13 (43.3)

20 (13.5)
13 (17.1)
2 (7.7)
1 (6.3)
4 (13.3)

27 (18.2)
19 (25.0)
3 (11.5)
1 (6.25)
4 (13.3)

5 (3.4)
0
2 (7.7)
1 (6.3)
2 (6.7)

13 (8.8)
7 (9.2)
3 (11.5)
2 (12.5)
4 (13.3)

77 (41.6)
24 (35.8)
38 (42.7)
59 (39.8)

25 (13.5)
7 (10.3)
10 (11.2)
21 (14.1)

33 (17.8)
8 (11.7)
24 (26.9)
21 (14.1)

4 (2.1)
2 (2.9)
3 (3.4)
3 (2.0)

20 (10.8)
8 (11.7)
5 (5.6)
18 (12.1)

32 (39.0)
26 (49.1)
54 (36.2)
10 (83.3)

12 (14.6)
7 (13.2)
17 (11.4)
4 (33.3)

13 (15.8)
14 (26.4)
31 (20.8)
6 (50.0)

3 (3.6)
3 (5.6)
4 (2.7)
0

7 (8.5)
5 (9.4)
15 (10.1)
2 (16.7)

116 (40.9)
6 (46.2)

38 (13.4)
2 (15.4)

51 (18.0)
3 (23.1)

9 (3.2)
1 (7.7)

27 (9.5)
2 (15.4)

104 (41.3)
77 (38.1)
41 (47.7)
26 (44.0)
11 (34.4)
13 (43.3)
10 (25.0)
12 (48.0)
12 (58.3)

33 (13.1)
24 (11.8)
12 (13.9)
8 (13.5)
3 (9.3)
6 (20.0)
5 (20.0)
6 (24.0)
4 (33.3)

45 (17.9)
34 (16.8)
14 (16.3)
8 (13.5)
4 (12.5)
3 (10.0)
2 (8.0)
2 (8.0)
1 (8.3)

7 (2.8)
7 (3.5)
2 (2.3)
2 (3.4)
0
0
0
0
0

27 (10.7)
19 (9.4)
13 (15.1)
9 (15.2)
4 (12.5)
4 (30.7)
3 (12.0)
4 (16.0)
2 (16.7)

64 (41.6)
52 (44.1)
10 (83.3)
9 (64.2)

21 (13.6)
18 (15.3)
3 (25.0)
2 (14.3)

27 (17.5)
22 (18.6)
4 (33.3)
3 (21.4)

6 (3.9)
2 (1.7)
2 (16.7)
1 (8.3)

14 (9.1)
13 (11.0)
3 (25.0)
3 (21.4)

106 (41.4)
96 (41.9)
66 (44.5)
68 (42.5)
17 (44.7)
4 (80.0)
1 (33.3)

37 (14.4)
29 (12.6)
23 (15.5)
25 (15.6)
7 (18.4)
1 (20.0)
0

46 (17.9)
43 (18.7)
26 (17.5)
27 (16.9)
3 (7.8)
2 (40.0)
0

8 (3.1)
10 (4.3)
7 (4.7)
5 (3.1)
0
0
0

24 (9.4)
23 (10.0)
16 (10.8)
16 (10.0)
7 (18.4)
1 (20.0)
1 (33.3)

66 (42.9)
7 (31.8)
9 (42.9)
16 (44.4)

21 (13.6)
2 (9.1)
1 (4.7)
6 (16.7)

28 (18.2)
2 (9.1)
4 (19.0)
8 (22.2)

5 (3.3)
0
0
2 (5.6)

16 (10.4)
3 (14.3)
4 (19.1)
1 (2.8)

Bartonella
spp.†
29 (9.8)
21 (10.3)
8 (8.8)

Positive sample and exposure categories are not mutually exclusive (i.e., some persons had positive test
results by both IFA and PCR, or could have been exposed to both cats and dogs). Median patient ages, for
women and men, respectively, were as follows: overall study population, 46.0 and 36.0 y; those with positive
results for overall Bartonella, 47.0 and 38.0 y, B. henselae, 44.0 and 41.0 y, B. koehlerae, 49.0 and 40.5 y, B.
vinsonii subsp. berkhoffii, 43.0 and 64.0 y, and Bartonella spp., 48.0 and 24.0 y.

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Figure 1. *Bartonella* spp. PCR results for the 15 most frequently reported previous diagnoses. OA, osteoarthritis; RA, rheumatoid arthritis.
Figure 2. *Bartonella* PCR amplification results from blood, serum, and enrichment blood culture with the *Bartonella α* Proteobacteria growth medium. Of 296 patients, 120 had positive PCR results in 1 component. Two patients, who had positive PCR results only after enrichment culture incubation and subculture onto agar, are not included. Each circle represents *Bartonella* PCR amplification results from blood, serum, or after enrichment blood culture. Each number represents the total (%) positive for each of the 4 possibilities within each of the 3 circles. For example, only 3 (1%) patients had positive results from blood, serum, and enrichment blood culture.
Table 2. Test results for *Bartonella* spp. in 296 patients examined by a rheumatologist, Maryland–Washington, DC, USA, August 25, 2008–April 1, 2009*

<table>
<thead>
<tr>
<th></th>
<th>IFA- / PCR-</th>
<th>IFA+ / PCR-</th>
<th>IFA+ / PCR+</th>
<th>IFA- / PCR+</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. henselae</em></td>
<td>196</td>
<td>60</td>
<td>7</td>
<td>33</td>
</tr>
<tr>
<td><em>B. koehlerae</em></td>
<td>177</td>
<td>65</td>
<td>24</td>
<td>29</td>
</tr>
<tr>
<td><em>B. vinsonii</em> subsp. <em>berkhoffii</em></td>
<td>141</td>
<td>145</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>Genotype I</td>
<td>217</td>
<td>75</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Genotype II</td>
<td>189</td>
<td>101</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Genotype III</td>
<td>213</td>
<td>82</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*IFA, indirect immunofluorescent antibody results obtained by using *B. henselae*, *B. koehlerae*, and *B. vinsonii* subsp. *berkhoffii* antigens; PCR, results obtained after PCR amplification by using Bartonella intergenic spacer primers, followed by attempted DNA sequencing of each amplicon.*
<table>
<thead>
<tr>
<th>Variable</th>
<th>( B. ) henselae</th>
<th>( B. ) koehlerae</th>
<th>( B. ) vinsonii subsp. berkhoffii</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCR+ (%)</td>
<td>PCR- (%)</td>
<td>P*</td>
</tr>
<tr>
<td>Median Age **</td>
<td>42.5</td>
<td>44.0</td>
<td>0.43</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Women</td>
<td>24 (60.0)</td>
<td>181 (70.7)</td>
<td>0.17</td>
</tr>
<tr>
<td>Men</td>
<td>16 (40.0)</td>
<td>75 (29.3)</td>
<td></td>
</tr>
<tr>
<td>Self-Reported Health Status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy</td>
<td>12 (33.3)</td>
<td>70 (29.2)</td>
<td>0.77</td>
</tr>
<tr>
<td>Infrequently Ill</td>
<td>7 (19.4)</td>
<td>46 (18.5)</td>
<td></td>
</tr>
<tr>
<td>Chronically Ill</td>
<td>17 (47.2)</td>
<td>132 (53.2)</td>
<td>21 (43.7)</td>
</tr>
<tr>
<td>Fatigue</td>
<td>38 (95.0)</td>
<td>226 (88.3)</td>
<td>0.27</td>
</tr>
<tr>
<td>Headache</td>
<td>25 (62.5)</td>
<td>155 (60.5)</td>
<td>0.81</td>
</tr>
<tr>
<td>Difficulty</td>
<td>32 (80.0)</td>
<td>174 (84.5)</td>
<td>0.12</td>
</tr>
<tr>
<td>Confusion</td>
<td>25 (62.5)</td>
<td>132 (51.5)</td>
<td>0.20</td>
</tr>
<tr>
<td>Disoriented</td>
<td>18 (45.0)</td>
<td>82 (32.0)</td>
<td>0.10</td>
</tr>
<tr>
<td>Irritability</td>
<td>30 (75.0)</td>
<td>153 (59.7)</td>
<td>0.06</td>
</tr>
<tr>
<td>Blurred Vision</td>
<td>23 (57.5)</td>
<td>100 (39.1)</td>
<td>0.03</td>
</tr>
<tr>
<td>Eye Pain</td>
<td>16 (40.0)</td>
<td>78 (30.5)</td>
<td>0.23</td>
</tr>
<tr>
<td>Sleepingness</td>
<td>32 (80.0)</td>
<td>188 (73.4)</td>
<td>0.37</td>
</tr>
<tr>
<td>Insomnia</td>
<td>22 (55.0)</td>
<td>153 (59.7)</td>
<td>0.56</td>
</tr>
<tr>
<td>Balance Problems</td>
<td>24 (60.0)</td>
<td>123 (48.0)</td>
<td>0.16</td>
</tr>
<tr>
<td>Tremors/Shaking</td>
<td>17 (42.5)</td>
<td>92 (35.9)</td>
<td>0.42</td>
</tr>
<tr>
<td>Muscle Weakness</td>
<td>28 (70.0)</td>
<td>161 (62.9)</td>
<td>0.38</td>
</tr>
<tr>
<td>Paralysis</td>
<td>3 (7.5)</td>
<td>13 (5.1)</td>
<td>0.52</td>
</tr>
<tr>
<td>Muscle Pain</td>
<td>31 (77.5)</td>
<td>176 (68.7)</td>
<td>0.26</td>
</tr>
<tr>
<td>Numbness</td>
<td>28 (70.0)</td>
<td>128 (50.0)</td>
<td>0.01</td>
</tr>
<tr>
<td>Joint Pain</td>
<td>31 (77.5)</td>
<td>199 (77.3)</td>
<td>0.97</td>
</tr>
<tr>
<td>Chronic Fatigue</td>
<td>27 (67.5)</td>
<td>180 (70.3)</td>
<td>0.71</td>
</tr>
<tr>
<td>Bowel/Bladder</td>
<td>17 (42.5)</td>
<td>95 (37.1)</td>
<td>0.51</td>
</tr>
<tr>
<td>Shortness of Breath</td>
<td>19 (47.5)</td>
<td>98 (38.3)</td>
<td>0.26</td>
</tr>
</tbody>
</table>
Table 3. (Continued)

| Poor Appetite | 8 (20.0) | 75 (29.3) | 0.22 | 12 (22.2) | 71 (29.3) | 0.29 | 1 (10.0) | 82 (28.6) | 0.29 |
| Weight Loss   | 7 (17.5) | 52 (20.3) | 0.67 | 6 (11.1) | 53 (21.9) | 0.07 | 1 (10.0) | 58 (20.3) | 0.69 |
| Depression    | 20 (50.0) | 126 (49.2) | 0.92 | 28 (51.9) | 118 (48.7) | 0.68 | 4 (40.0) | 142 (49.6) | 0.75 |
| Syncope       | 4 (10.0) | 41 (16.0) | 0.32 | 8 (14.8) | 37 (5.3) | 0.93 | 2 (20.0) | 43 (15.0) | 0.66 |
| Consultation w/ Neurologist | 23 (57.5) | 87 (33.9) | <0.01 | 22 (40.7) | 88 (36.4) | 0.56 | 3 (30.0) | 107 (37.4) | 0.63 |
| Consultation w/ Infectious Disease Doctor | 16 (40.0) | 104 (40.6) | 0.94 | 29 (53.7) | 91 (37.6) | 0.03 | 4 (40.0) | 116 (40.7) | 0.97 |

*Median ages, compared by using Wilcoxon rank-sum test, for positive and negative results, respectively, were *B. henselae*, 42.5, 44.0, *p* = 0.43; *B. koehlarae*, 48.0, 43.0, *p* = 0.03; and *B. vinsonii* subsp. *berkhoffii*, 46.5, 44.0, *p* = 0.64.
†Results of $\chi^2$ analysis (Fisher exact test used when expected cell value <5).
Table 4. Factors associated with positive PCR result for *Bartonella henselae* and *B. koehlerae* among 296 patients examined by a rheumatologist, Maryland-Washington, DC, USA, August 25, 2008–April 1, 2009*

<table>
<thead>
<tr>
<th>Variable</th>
<th>Adjusted OR [95% Confidence Interval], p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCR + vs. PCR -</td>
</tr>
<tr>
<td></td>
<td><em>B. henselae</em></td>
</tr>
<tr>
<td>Blurred Vision</td>
<td>2.37 [1.13-4.98], p = 0.03</td>
</tr>
<tr>
<td>Numbness</td>
<td>2.74 [1.26-5.96], p = 0.01</td>
</tr>
<tr>
<td>Infectious Disease Doctor</td>
<td>NS</td>
</tr>
<tr>
<td>Neurologist</td>
<td>2.76 [1.33-5.73], p &lt;0.01</td>
</tr>
</tbody>
</table>

*Results of logistic regression analysis. Variables adjusted for age, sex, and duration of illness. NS, not significant.
References


CHAPTER 4.

*BA RTONELLA* SPP. BACTEREMIA IN HIGH-RISK IMMUNOCOMPETENT PATIENTS

Manuscript as published in Diagnostic Microbiology and Infectious Disease.

Bartonella spp. bacteremia in high-risk immunocompetent patients

Running title: Intravascular Bartonella infection

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Abstract

Serum and blood samples from 192 patients, who reported animal exposure (100.0%) and recent animal bites or scratches (88.0%), were screened for antibodies by indirect immunofluorescence assays and for bacteremia using the BAPGM (*Bartonella* alpha Proteobacteria growth medium) platform. Predominant symptoms included fatigue (79.2%), sleeplessness (64.1%), joint pain (64.1%), and muscle pain (63.0%). *Bartonella* spp. seroreactivity or bacteremia was documented in 49.5% (n=95) and 23.9% (n=46) of the patients, respectively, however, IFA antibodies were not detected in 30.4% (n=14) of bacteremic patients. Regarding components of the BAPGM platform, *Bartonella* DNA was amplified from 7.5% of blood (n=21), 8.7% of serum (n=25) and 10.3% of enrichment culture samples (n=29). PCR on only extracted blood would not have detected *Bartonella* infection in 34.7% (16/46) of bacteremic patients. Serology, in conjunction with blood, serum, and BAPGM enrichment culture PCR, facilitates the diagnosis of *Bartonella* spp. bacteremia in immunocompetent patients.

Clinical Relevance: This manuscript describes clinical, serological and molecular findings in immunocompetent patients with extensive animal contact and arthropod exposure.

Keywords: *Bartonella*; BAPGM, whole blood culture; human; isolation; PCR detection, IFA
Introduction

Bacteria of the genus *Bartonella* (alpha subdivision of the class Proteobacteria) are fastidious, gram-negative, aerobic bacilli with more than 26 described species or subspecies (Boulouis, HJ et al., 2005; Boulouis HJ and Chomel BB, 2004, Breitschwerdt EB et al., 2007a, Breitschwerdt EB et al., 2010b; Breitschwerdt EB et al., 2007b; Chomel BB et al., 2004; Chomel BB et al., 2006; Houpikian P et al., 2005; Joblet C et al., 1995; Maurin M et al., 1997). Seventeen *Bartonella* spp. have been associated with an expanding spectrum of human diseases, ranging from acute fever, to more severe disease manifestations including encephalopathy, endocarditis, sensory and motor neuropathies, pleural and pericardial effusion, pneumonia, and hemolytic anemia. *Bartonella* are highly adapted to various distinct mammalian reservoir hosts, including domestic animals, human beings, marine mammals, rodents and ungulates, wherein these bacteria cause long-lasting intra-erythrocytic and intravascular infection, which can be associated with a relapsing pattern of bacteremia, as demonstrated in cats and rodents (Chomel BB et al., 2003; Dehio C, 2001; Houpikian P et al., 2005; Joblet C et al., 1995; Jones SL et al., 2008; Kordick DL and Breitschwerdt EB, 1995; Kordick DL and Breitschwerdt EB, 1998; Maggi RG et al., 2009). Conventional microbiological approaches for documentation of *Bartonella* infections, including ELISA, immunofluorescence antibody assays, and because of the fastidious nature of these bacteria isolation on agar plates, all remain relatively insensitive and of limited diagnostic utility, particularly when only one test is used. When infection cannot be confirmed by PCR, diagnostic confirmation has often relied on a combination of clinical (fever), epidemiological (history of cat scratch), and serological criteria, (Breitschwerdt EB et al., 2009; Maggi RG et
In conjunction with efforts to improve the diagnostic documentation of *Bartonella* bacteremia, our laboratory described a novel, chemically modified, insect cell culture-based liquid growth medium, *Bartonella* alpha Proteobacteria growth medium (BAPGM), that experimentally facilitated growth of at least seven *Bartonella* species (Maggi RG et al., 2006). Subsequently, we used BAPGM enrichment culture to increase the levels of bacteria in patient samples, so as to enhance detection by PCR amplification or by subculture isolation of a *Bartonella* sp. onto blood agar plates (Breitschwerdt EB et al., 2007a, Breitschwerdt EB et al., 2008; Breitschwerdt EB et al., 2007b, Maggi RG and Breitschwerdt EB, 2005; Maggi RG et al., 2005). BAPGM pre-enrichment medium is formulated using the basal biochemical composition of the insect growth media IPL-41 (Sigma-Aldrich Co. LLC, St. Louis, MO) which is supplemented with several amino-acids, proteins, precursors of biosynthesis (vitamins) and other nutrients. Experimentally, BAPGM also supported the growth of co-cultures composed of two *Bartonella* sp., which subsequently lead to the microbiological documentation of co-infections in the blood of dogs and human patients (Breitschwerdt EB et al., 2007a, Breitschwerdt EB et al., 2008; Breitschwerdt EB et al., 2007b, Maggi RG and Breitschwerdt EB, 2005; Maggi RG et al., 2005). More recently and for the first time, investigators at the Centers for Disease Control and prevention (CDC) used BAPGM to facilitate the isolation of a new *Bartonella* sp. (Candidatus Bartonella tamiae) from febrile patients in Thailand (Kosoy M et al., 2010). The same investigators subsequently used either BAPGM or cell culture enrichment to increase the quantity of *Bartonella* organisms prior to PCR amplification of *Bartonella* spp. DNA from the blood of dogs or febrile human patients in Thailand (Bai Y et al., 2010; Kosoy M et al., 2010; Kosoy
M et al., 2008). Despite the ability of BAPGM to support the growth of Bartonella spp. (Maggi RG et al., 2005), cumulative microbiological results using this liquid enrichment blood culture approach in a large at-risk human population has not been previously reported. Previous reports have estimated the Bartonella seroprevalence in healthy veterinarians at 7.1% (Noah DL et al., 1997). However, the prevalence of Bartonella spp. bacteremia in healthy or sick veterinary professionals who experience frequent animal bites and scratches or extensive arthropod exposure is currently unclear. In this study, we describe serological and microbiological results using the BAPGM platform from a low-risk control group and from 192 patients who had experienced frequent animal contact and arthropod exposures, of whom 46 were bacteremic with one or more Bartonella sp. Indirect immunofluorescence antibody (IFA) assays targeting B. henselae, B. koehlerae, and B. vinsonii subsp. berkhoffii antigens, were descriptively compared to PCR and DNA sequencing results obtained from whole blood, serum, BAPGM enrichment whole blood culture and subculture isolates. In addition, we describe self-reported symptoms, historical exposures to animals and vectors, and previous clinical diagnoses.

Patients Controls and Methods

Study Population

Between September 2007 and June 2010, 192 patients with extensive arthropod exposure and/or frequent animal contact voluntarily entered into the study. Participants became aware of the study through lay publications and by attending lectures on canine and feline bartonellosis at regional and national veterinary conferences. In most instances, patients
requested testing because of a history of chronic poorly-defined illness, fatigue, joint pain, arthritis and neurological or neurocognitive abnormalities. As samples were not prospectively or systematically solicited, the timing of sample collection relative to the onset of illness, the duration of illness, and the prior, concurrent or recent use of antibiotics were all highly variable among the study population. Demographic information, animal/arthropod exposure, history of visiting specialists, outdoor activity, self-reported clinical symptoms and co-morbid conditions for each patient were collected using a standardized 5-page survey instrument. Questionnaires were mailed to each study participant for self-report and entered into an electronic database upon return to the Intracellular Pathogens Research Laboratory at North Carolina State University. Whole blood and serum samples (n=32) from healthy employees at a local medical school were tested by the same methods as the patient group as low-risk controls. Collection and analyses of these data were approved by the North Carolina State University Institutional Review Board (IRB#s 4925-03,164-08 and 1960-11).

Serology

*B. vinsonii* subsp. *berkhoffii* (genotypes I, II, and III), *B. koehlerae* and *B. henselae* antibodies were determine following traditional immunofluorescence antibody assay (IFA) practices with fluorescein conjugated goat anti-human IgG, as described in previous studies from our laboratory (Breitschwerdt EB et al., 2007a; Breitschwerdt EB et al., 2010b; Breitschwerdt EB et al., 2003). Isolates of *B. vinsonii* subsp. *berkhoffii* genotypes I, II and III, *B. koehlerae* and *B. henselae* (Houston I strain) were passed from agar grown cultures into DH82 or AAE12 cell cultures to obtain intracellular whole bacterial antigens for IFA testing. Heavily infected cell cultures were spotted onto 30-well Teflon coated slides (Cel-
Line/Thermo Fisher Scientific: Rockford, IL 61101, USA), 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 and incubated on antigen slides. All available patient sera were screened at dilutions of 1:16 to 1:64. All sera that were reactive at a 1:64 dilution were further tested with twofold dilutions out to 1:8192. A threshold titer of 1:64 was used to define a seroreactive antibody response against a specific *Bartonella* sp. antigen. IFA testing was performed on 289 serum samples from 192 patients.

**Sample Processing for the BAPGM Platform**

Following aseptic preparation of the venipuncture site, 289 whole blood samples from 192 patients were collected into ethylenediaminetetraacetic acid (EDTA)-anti-coagulated blood and clot or serum separator tubes. Whole blood and serum were transported directly or by overnight express carrier to the Intracellular Pathogens Research Laboratory (IPRL) for processing. PCR sample preparation, DNA extraction, and PCR amplification and analysis were performed in three separate rooms to avoid DNA contamination. In addition, BAPGM cultures and agar plate sub-inoculation cultures were processed in a Biosafety Level III laboratory, which was used exclusively for testing human patient samples.

A previously described approach that combines PCR detection of *Bartonella* spp. DNA and enrichment culture of blood and serum in *Bartonella* alpha Proteobacteria growth medium (BAPGM) was used to test EDTA-anti-coagulated whole blood (n=278) and centrifuged serum (n=286) samples. (Breitschwerdt EB et al., 2007a; Breitschwerdt EB et al., 2007b; Diniz PP et al., 2007; Duncan AW et al., 2007; Maggi RG et al., 2005). The BAPGM
platform incorporates four separate PCR testing time points, each representing a different component of the testing process for each patient sample: 1&2) PCR amplification of *Bartonella* spp. following DNA extraction from whole blood and from serum; 3) PCR following BAPGM enrichment of whole blood culture incubated for 7 and 14 days; 4) and PCR from subculture isolates if obtained after sub-inoculation from the BAPGM flask onto plates containing trypticase soy agar with 10% sheep whole blood that are incubated for 4 weeks. To avoid DNA carryover, PCR sample preparation, DNA extraction, and PCR amplification and analysis were performed in three separate rooms with a unidirectional workflow. All patient samples were processed in a biosafety cabinet with Hepa filtration located in a limited access laboratory. Forty-four patients (22.9%) had multiple samples processed, either from the same collection period (48-72 hours) or from other pre or post-treatment time points.

*Bartonella* DNA was amplified from blood, serum, BAPGM enrichment culture and subculture isolates using conventional *Bartonella* genus PCR primers targeting the 16S-23S intergenic spacer region (ITS), and PCR using *B. koehlerae* ITS species-specific primers, as previously described (Breitschwerdt EB et al., 2010c; Cadenas MB et al., 2008; Duncan AW et al., 2007, Maggi RG and Breitschwerdt EB, 2005; Maggi RG et al., 2006; Maggi RG et al., 2005). Amplification of the *B. koehlerae* ITS region was performed using oligonucleotides Bkoehl-1s: 5’ CTT CTA AAA TAT CGC TTC TAA AAA TTG GCA TGC 3’ and Bkoehl1125as: 5’ GCC TTT TTT GGT GAC AAG CAC TTT TCT TAA G 3’ as forward and reverse primers, respectively. Amplification was performed in a 25-µl final volume reaction containing 12.5 µl of Tak-Ex® Premix (Fisher Scientific: Rockford IL, 61101,
USA), 0.1 µl of 100 µM of each forward and reverse primer (IDT® DNA Technology: Coralville, Iowa 52241, USA), 7.3 µl of molecular grade water, and 5 µl of DNA from each sample tested.

Conventional PCR was performed in an Eppendorf Mastercycler EPgradient® (Eppendorf: Hauppauge, NY 11788, USA) under the following conditions: a single cycle at 95ºC for 2s, followed by 55 cycles with DNA denaturing at 94ºC for 15s, annealing at 64ºC for 15s and extension at 72ºC for 18s. The PCR reaction was completed by a final cycle at 72ºC for 30s. As previously described for the ITS genus and B. koehlerae PCR assays, all products are analyzed by 2% agarose gel electrophoresis with detection using ethidium bromide under ultraviolet light after which amplicon products were sequenced to identify the species and ITS strain type. PCR negative controls were prepared using 5 µl of DNA from the blood of a healthy dog and B. henselae (Houston 1 strain) was used as a PCR positive control during the entire course of this study. In no instance was B. henselae or any other Bartonella sp. amplified in the negative control lane on any PCR gel. To assess for potential contamination during blood sample processing in BAPGM, an un-inoculated BAPGM culture flask was processed simultaneously and in an identical manner with each batch of patient blood and serum samples tested. For all components of the BAPGM platform (PCR from blood, serum, enrichment cultures and subcultures) PCR negative controls remained negative throughout the course of the study. In addition, subcultures of un-inoculated BAPGM medium (culture control) at 7 and 14 days did not yield bacterial growth.
Results

Controls

Serology

IFA serology results for *B. vinsonii* subsp. *berkhoffii* (genotypes I and III) and *B. koehlerae* were negative in all 32 control samples. One control was *B. henselae* seroreactive at a reciprocal titer of 64. Unexpectedly, 16 controls were *B. vinsonii* subsp. *berkhoffii* genotype II seroreactive at reciprocal titers of 64 to 512. Seroreactivity to genotype II antigens in the patient population was 28.1% (54/192).

BAPGM Platform

All components (blood, serum, 7 and 14 day enrichment cultures, 4 PCRs per platform) of the BAPGM platform were negative for all 32 control patients and there was no bacterial growth on subcultures.

Patient Population

Patient characteristics and self-reported exposures are presented in Table 1. Samples from 137 females, 55 males, ranging in age from 8 to 86 years, were tested. The majority of patients resided in the Southern United States (77.1%), while 10.4% resided in the Northeast US, 6.3% in the Western US, and 4.1% in the Midwest. Four patients resided outside of the United States (Denmark, England, Norway, and Canada). The most common animal exposures reported by patients were domestic dog (96.8%), domestic cat (89.0%), bird (54.2%), and horse (46.4%). Three-quarters of participants reported being bitten or scratched
by a cat within the past year (77.6%), while 73.4% reported being bitten or scratched by a
dog. Similarly, 86.9% reported flea exposure and 90.6% reported tick exposure within the
past year. The most common clinical signs and symptoms reported by the patient population
included fatigue (79.2%), sleeplessness (64.1%), muscle pain (63.0%), joint pain (64.1%),
chronic fatigue (63.0%), irritability (59.9%), and headaches (61.5%). A majority reported a
previous consultation with a neurologist (52.1%), while 28.1% had a previous consultation
with an infectious disease physician, and 28.1% with a rheumatologist. Patients reported a
wide-spectrum of previous diagnoses, notably multiple sclerosis (n = 19, 9.9%) and Lyme
disease (n = 18, 9.3%).

**Serology**

Among the patient population, 49.5% (95/192) of patients were IFA seroreactive to at least
one antigen- 18 (9.4%) were seroreactive to *B. koehlerae* antigens, 30 (15.6%) to *B. henselae*
antigens, and 83 (43.3%) to one or more *B. vinsonii* subsp. *berkhoffii* genotypes I, II or III
antigens. Of the 46 *Bartonella* sp. bacteremic patients, 14 (30.4%) were not seroreactive to
any *Bartonella* spp. antigens by IFA testing. Seroreactivity to specific IFA antigens
compared to PCR results are summarized in Table 2. Overall, of the 289 serum samples
available for IFA testing, 130 (44.9%) were seroreactive to *B. henselae*, *B. koehlerae*, or *B.
vinsonii* subsp. *berkhoffii* genotypes I, II or III antigens. Forty six (15.9%) samples were
seroreactive to *B. henselae* antigens and 30 (10.4%) samples were seroreactive to *B.
koehlerae* antigens. Surprisingly, 109 (37.7%) samples were seroreactive to *B. vinsonii*
subsp. *berkhoffii* genotypes I, II or III antigens. Reciprocal *B. henselae* antibody titers
ranged from 64 to 512, whereas reciprocal \( B. \) koehlerae and \( B. \) vinsonii subsp. berkhoftii antibody titers ranged from 64 to 1024.

**BAPGM Platform**

Forty-six out of 192 patients (23.9%) were PCR positive for a Bartonella spp. prior to or following BAPGM enrichment blood culture. Sixteen (8.3%) patients were PCR negative following DNA extraction from whole blood and serum, but were PCR positive after BAPGM enrichment culture (Figure 1). The patient from Denmark was infected with \( B. \) koehlerae, whereas Bartonella DNA was not amplified from the patients from England, Norway, and Canada. Species-specific test results can be found in Table 3. For fifteen PCR+ patients, sequencing of the Bartonella species in either the pre or post-enrichment samples was not successful, most likely as a consequence of a co-infection or due to a low DNA concentration in an amplified product. For five patients, concordant Bartonella sequencing results were obtained from both pre- and post-enrichment samples. Four patients were infected with \( B. \) henselae and \( B. \) koehlerae, whereas three \( B. \) henselae-infected and three \( B. \) koehlerae-infected patients were PCR+ in a component of the BAPGM platform, but as sequencing was not successful, it was not possible to determine if these patients were co-infected with another Bartonella sp. Following subculture, no Bartonella sp. isolates were obtained.

Overall, 63 out of 289 samples (21.8%) tested using the BAPGM platform were Bartonella PCR-positive prior to or following BAPGM enrichment culture. Bartonella DNA was amplified from 21 out of 278 (7.5%) whole blood samples and 24 out of 286 (8.7%) serum samples. However, only 14.3% \((n=5/35)\) of simultaneously collected serum and whole blood
samples produced concordant results (i.e. both samples PCR+). Overall, *Bartonella* DNA was amplified from 29 (10.3%) samples after BAPGM enrichment whole blood culture, of which 23 samples (7.9%) were only PCR positive after BAPGM enrichment blood culture (i.e. whole blood and serum both PCR-).

**Discussion**

It is well recognized that *Bartonella* spp. are transmitted by various arthropod vectors and by animal bites and scratches (Boulouis HJ et al., 2005; Boulouis HJ and Chomel BB, 2004; Breitschwerdt EB et al., 2009; Breitschwerdt EB et al., 2007a; Chomel BB et al., 2004; Chomel BB et al., 2006). Based upon serological testing using a panel of five antigens, exposure to *Bartonella* species was common (49.5%) among the study population and nearly a fourth of the patients were bacteremic, a subset of whom (30.4%) did not have detectable *Bartonella* antibodies by IFA testing. In addition, independently performing PCR from blood, serum, and enrichment blood culture using an optimized insect-based cell culture growth medium increased diagnostic sensitivity in this high-risk patient population. If only whole blood and serum PCR results were obtained, *Bartonella* spp. infection would not have been confirmed in 34.7% of bacteremic patients, potentially resulting in misdiagnosis and inappropriate treatment. Fatigue (79.2%), joint pain (64.1%) and muscle pain (63.0%) were the predominant symptoms among the study population. While nearly 70% (130/192) of patients reported a previous consultation with a medical specialist prior to entry into the study, only 4 (2.1%) patients reported a previous differential diagnosis of *Bartonella* sp. infection. This is not unexpected as chronic *Bartonella* sp. bacteremia was only recently identified in immunocompetent patients. When whole blood, serum and enrichment culture
PCR results were combined, 23.9% of the patients tested in this study were infected with one or more *Bartonella* spp. Although this high prevalence of bacteremia is biased by testing at risk patients, who have increased animal exposure and/or frequent arthropod contact, these results clearly demonstrate that intravascular infection with one or more *Bartonella* sp. may be much more common in immunocompetent patients than was previously suspected. Excluding the *B. vinsonii* subsp. *berkhoffii* genotype II serology data found in the control population, there was minimal serological evidence to support *Bartonella* spp. exposure and no PCR evidence to support intravascular infection among low-risk healthy individuals. Although *Bartonella* spp. bacteremia was not found in the low-risk control population using the BAPGM platform, healthy veterinary professionals were not tested as a component of this study, which limits the extrapolation from our results.

Cat scratch disease (CSD), caused predominantly or solely by *B. henselae*, is characterized by fever, regional lymphadenopathy, and a history of a bite or scratch. Medically, CSD is considered a self-limiting infection and antibiotic therapy is considered to be of minimal benefit in altering the prototypical clinical course of disease (Chomel BB et al., 2003; Rolain JM et al., 2004). Endocarditis is another well-recognized form of pathology, induced by several *Bartonella* sp. in human patients (Chomel BB et al., 2003). However, CSD and endocarditis may represent only two of the many clinical presentations that can be induced in patients with intravascular *Bartonella* spp. infections. We have previously described patients with chronic *Bartonella* sp. bacteremia, whose predominant symptoms included memory loss, numbness or a loss of sensation, balance problems and headaches, seizures, ataxia, and/or tremors (Breitschwerdt EB et al., 2007a; Breitschwerdt EB et al., 2010b;
Breitschwerdt EB et al., 2008). Concurrent or sequential intravascular infection with *B. vinsonii* subsp. *berkhoffii* genotype II and *B. henselae* was also documented in two families with non-specific symptoms including progressive weight loss, muscle weakness, lack of coordination, headaches, muscle pain and insomnia (Breitschwerdt EB et al., 2010a; Breitschwerdt EB et al., 2010b). Intravascular infection with *B. koehlerae* has also been described in patients with fatigue, joint pain, decreased peripheral vision, sensory neuropathy, and neurocognitive abnormalities (Breitschwerdt EB et al., 2009; Breitschwerdt EB et al., 2010b; Breitschwerdt EB et al., 2008; Breitschwerdt EB et al., 2011). In conjunction with published case reports, the results of this study involving a large population of at-risk sick individuals, provides the basis for future studies to investigate clinical, epidemiological and clinicopathological data in patients with confirmed *Bartonella* spp. bacteremia as compared to age and sex matched low-risk patient controls.

While performing PCR on whole blood and serum will increase the sensitivity of *Bartonella* spp. detection, only 14.3% (n=5/35) of combined whole blood and serum sample sets produced concordant PCR results at identical time points. Differences in PCR amplification results from whole blood as compared to serum may be related to the partial PCR inhibition induced by hemoglobin when co-purified during the DNA extraction process. This inhibition would be minimized during DNA extraction from non-hemolyzed serum. Alternatively, amplification of *Bartonella* DNA from both blood and serum may be difficult to achieve due to low intravascular bacterial numbers, resulting in low PCR template targets, and thereby reflecting the limit of PCR detection. Additionally, all intravascular *Bartonella* may not be confined to an intra-erythrocytic location within the patient or the blood sample. Also,
potentially after whole blood is infused into a sample collection tube and begins to clot, some bacteria might leave erythrocytes in response to a “danger signal” and enter the serum. Previous results with the genus ITS primers used in this study have shown that PCR detection of *Bartonella* may exhibit a species bias towards *B. henselae* amplification (Maggi RG et al., 2005). Including co-infected samples, we were able to amplify and sequence *B. henselae* and *B. koehlerae* in 27 (42.8%) and 25 (39.6%) of all PCR positive samples, respectively; however, it should be noted that two PCR assays (*Bartonella* genus and *B. koehlerae*-specific) were used to amplify and sequence *B. koehlerae* DNA. *B. vinsonii* subsp. *berkoffii* was only detected in 3 (4.7%) samples. It is unclear whether the difference between *B. henselae*, *B. koehlerae* and *B. vinsonii* subsp. *berkoffii* reflects the true prevalence of these species in the study population, or is due to a selection bias induced by the PCR assays or the BAPGM enrichment step. As cats are the primary reservoir for *B. henselae* and *B. koehlerae*, and dogs and wild canids are the primary reservoir for *B. vinsonii* subsp. *berkoffii*, exposure to cats or their fleas may pose a greater risk for *Bartonella* spp. infection among veterinary professionals; i.e. eighty-one (42.2%) of our patients were occupationally exposed to animals. However, this possibility should not be over interpreted, as *B. vinsonii* subsp. *berkoffii* genotype II has been amplified from a chronically infected cat with osteomyelitis, both *B. henselae* and *B. koehlerae* have been reported in dogs with endocarditis, and *B. henselae* is the most frequent *Bartonella* sp. amplified from biological samples from sick dogs referred to a tertiary veterinary teaching hospital, using the BAPGM platform (Varanat M et al., 2009; Perez et al., 2011). In addition to PCR bias, BAPGM, the insect-based growth medium used to culture *Bartonella* in this study may selectively grow
one species or strain of *Bartonella* better than another. Preferential growth of one *Bartonella* species from the blood of a co-infected patient would influence the subsequent sensitivity of PCR after culture enrichment for amplification of both species and is likely responsible for discordant results between pre- and post- enrichment PCR detection in some co-infected samples in this study. Despite facilitating the enhanced molecular diagnosis of bartonellosis, obtaining stable agar plate isolates after subculture from liquid BAPGM at 7 or 14 days post-incubation remains a technical limitation of this platform and other isolation approaches (Breitschwerdt EB et al., 2010c; Duncan AW et al., 2007; La Scola B et al., 2003). Failure to obtain stable *Bartonella* sp. isolates remains a major patient management limitation, as routine testing for antibiotic sensitivity and resistance among patient isolates is in most instances not possible.

Of the 46 patients who were bacteremic with one or more *Bartonella* sp., 14 (30.4%) were not seroreactive to *B. henselae*, *B. koehlerae*, or *B. vinsonii* subsp. *berkhoffii* antigens by IFA testing. Based upon this cumulative study and previous case reports from our laboratory, it is likely that some *Bartonella* bacteremic patients are anergic and do not produce a detectable IFA antibody response. Alternatively, as previously reported, antigenic variation among *Bartonella* strains could result in false negative IFA results reported in some patients. (Breitschwerdt EB et al., 2009; Breitschwerdt EB et al., 2010c) Previous studies have shown that IFA test sensitivity among CSD patients can vary substantially from 14-100% (Bergmans AM et al., 1997; Murakami K et al., 2002; Sander A et al., 1998; Tsuneoka H et al., 2000; Tsuneoka H et al., 1998; Tsuneoka H et al., 1999; Tsuneoka H et al., 2005; Tsuneoka H et al., 2001; Tsuneoka H et al., 2004). When testing individual patient sera,
varying antigenic expression between *B. henselae* strains appears to be a contributor to false negative *B. henselae* IFA results (Drancourt M et al., 1996). Therefore, despite using multiple *Bartonella* sp. antigens, serology can lack sensitivity, and as an additional limitation, can only be used to implicate prior exposure to a *Bartonella* sp. There was no seroreactivity to three of the *Bartonella* sp. antigens used in this study among the control population. However, one sample from a healthy control was *B. henselae* seroreactive, which is not unexpected, as the seroprevalence of *B. henselae* in healthy blood donors in the United States has been estimated at 2-6% (Jackson LA et al., 1996; Regnery RL et al., 1992; Zangwill KM et al., 1993). However, 50% of control sera were reactive to *B. vinsonii berkhoffii* genotype II antigens, which exceeded the 28.1% seroreactivity to this genotype among the high-risk patient population. The reason for the high prevalence of antibodies to this particular antigen among the healthy control and study populations requires additional investigation.

It is becoming increasingly clear that no single diagnostic strategy will confirm infection with a *Bartonella* sp. in every immunocompetent patient. The serological and cumulative PCR results from this study further emphasize the diagnostic challenges associated with documentation of intravascular infection with *Bartonella* spp. in immunocompetent patients. Laboratory contamination was considered an unlikely explanation for these results, as *Bartonella* spp. DNA was never amplified from a negative control. Additionally, numerous *Bartonella* species, subspecies, genotypes and strains (data not shown) were sequenced from different patients during the course of this investigation. As conventional whole blood culture approaches have lacked sensitivity, a majority of previous efforts to diagnose *Bartonella*
infection in immunocompetent individuals have relied solely on the amplification of
*Bartonella* DNA from blood or other tissues, particularly lymph nodes, or the use of
serological assays (Drancourt M et al., 2004; Drancourt M et al., 1995; Drancourt M et al.,
1996; La Scola B and Raoult D, 1999; Raoult D, 2006; Raoult D et al., 1996;). Although
serological testing is beneficial, independently testing blood, serum, and enrichment blood
culture by PCR is recommended to improve diagnostic documentation of *Bartonella* sp.
bacteremia.

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Dr. Breitschwerdt holds U.S. Patent No. 7,115,385; Media and Methods for cultivation of
microorganisms, which was issued October 3, 2006. He is the chief scientific officer for
Galaxy Diagnostics, a newly formed company that provides diagnostic testing for the
detection of *Bartonella* species infection in animals and in human patient samples. Dr.
Ricardo Maggi has lead efforts to optimize the BAPGM platform and is the Scientific
Technical Advisor and Laboratory Director for Galaxy Diagnostics.
Table 1. Demographic Characteristics and Clinical Signs and Symptoms\(^1\) of the Patients.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Study Population</th>
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<tbody>
<tr>
<td></td>
<td>PCR Positive N = 46</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Women</td>
<td>28 (60.9)</td>
</tr>
<tr>
<td>Men</td>
<td>18 (39.1)</td>
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<tr>
<td>Median Age (IQR)</td>
<td></td>
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<tr>
<td>Women</td>
<td>37.5 (28.5-49.5)</td>
</tr>
<tr>
<td>Men</td>
<td>52.5 (43.0-57.0)</td>
</tr>
<tr>
<td>Residence</td>
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<td>South US</td>
<td>33 (67.4)</td>
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<td>Northeast US</td>
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<td>West US</td>
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<td>Midwest US</td>
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<td>International</td>
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<td>Fatigue</td>
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<td>Chronic Fatigue</td>
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<tr>
<td>Insomnia</td>
<td>28 (60.8)</td>
</tr>
<tr>
<td>Difficulty Remembering</td>
<td>27 (58.7)</td>
</tr>
<tr>
<td>Muscle Weakness</td>
<td>19 (41.3)</td>
</tr>
<tr>
<td>Balance Problems</td>
<td>24 (52.1)</td>
</tr>
<tr>
<td>Loss of Sensation/Numbness</td>
<td>26 (56.5)</td>
</tr>
<tr>
<td>Blurred Vision</td>
<td>17 (36.9)</td>
</tr>
<tr>
<td>Confusion</td>
<td>19 (41.3)</td>
</tr>
<tr>
<td>Shortness of Breath</td>
<td>16 (34.7)</td>
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<tr>
<td>Depression</td>
<td>14 (30.4)</td>
</tr>
<tr>
<td>Eye Pain</td>
<td>10 (21.7)</td>
</tr>
<tr>
<td>Bowel/Bladder Dysfunction</td>
<td>11 (23.9)</td>
</tr>
<tr>
<td>Tremors/Shaking</td>
<td>11 (23.9)</td>
</tr>
<tr>
<td>Weight Loss</td>
<td>10 (21.7)</td>
</tr>
<tr>
<td>Disoriented</td>
<td>7 (15.2)</td>
</tr>
<tr>
<td>Poor Appetite</td>
<td>11 (23.9)</td>
</tr>
<tr>
<td>Syncope</td>
<td>11 (23.9)</td>
</tr>
<tr>
<td>Paralysis</td>
<td>2 (4.3)</td>
</tr>
<tr>
<td>Previous Consultation with:</td>
<td></td>
</tr>
<tr>
<td>Neurologist</td>
<td>27 (58.6)</td>
</tr>
<tr>
<td>Infectious Disease Doctor</td>
<td>15 (32.6)</td>
</tr>
<tr>
<td>Rheumatologist</td>
<td>12 (26.1)</td>
</tr>
<tr>
<td>Endocrinologist</td>
<td>9 (19.6)</td>
</tr>
<tr>
<td>Psychiatrist</td>
<td>1 (2.1)</td>
</tr>
</tbody>
</table>

IQR = Inter-quartile range  
\(^1\)Symptoms are arranged in the table in decreasing order of frequency
Table 2: Prevalence of Bartonella species antibodies, as determined by IFA testing (reciprocal titers greater than or equal to 1:64), in PCR positive patients as determined by the BAPGM Platform.

<table>
<thead>
<tr>
<th>Bartonella species</th>
<th>Bacteremic N = 46</th>
<th>Non-Bacteremic N = 146</th>
<th>Controls N = 32</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IFA + n (%)</td>
<td>IFA - n (%)</td>
<td>IFA + n (%)</td>
</tr>
<tr>
<td><em>B. henselae</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>13 (28.3)</td>
<td>33 (71.7)</td>
<td>17 (11.6)</td>
</tr>
<tr>
<td><em>B. vinsonii berkoffii</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype I</td>
<td>26 (56.5)</td>
<td>20 (43.4)</td>
<td>57 (39.1)</td>
</tr>
<tr>
<td>Genotype II</td>
<td>11 (23.9)</td>
<td>35 (76.1)</td>
<td>27 (18.4)</td>
</tr>
<tr>
<td>Genotype III</td>
<td>19 (41.3)</td>
<td>27 (58.7)</td>
<td>35 (23.9)</td>
</tr>
<tr>
<td><em>B. koehlerae</em></td>
<td>14 (30.4)</td>
<td>32 (69.5)</td>
<td>4 (2.7)</td>
</tr>
</tbody>
</table>

Values are shown as n (%). Totals do not sum to N because a patient may have been IFA positive for more than 1 Bartonella species.
Fig. 1. *Bartonella* PCR amplification results from blood, serum, and enrichment blood culture for 192 patients processed using the BAPGM platform (see article). Numbers represent the total number of positives for each of the platform's component steps (i.e., PCR from blood, PCR from serum, PCR from BAPGM enrichment culture).
Table 3. Detected species of *Bartonella* in 46 bacteremic patients following the BAPGM platform.

*Numbers do not sum to total because a patient could be positive for more than 1 *Bartonella* species or PCR positive in more than 1 testing step in the BAPGM platform.

<table>
<thead>
<tr>
<th>Species</th>
<th>Pre-BAPGM PCR</th>
<th>Post-BAPGM PCR</th>
<th>Overall Platform</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. henselae</em></td>
<td>15</td>
<td>11</td>
<td>22</td>
</tr>
<tr>
<td><em>B. koehlerae</em></td>
<td>11</td>
<td>7</td>
<td>17</td>
</tr>
<tr>
<td><em>B. vinsonii</em> subsp. <em>berkhoffii</em></td>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td><em>B. spp</em></td>
<td>8</td>
<td>8</td>
<td>15</td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
<td>30</td>
<td>46</td>
</tr>
</tbody>
</table>
References:


Breitschwerdt EB, Maggi RG, Mozayeni BR, Hegarty BC, Bradley JM, Mascarelli PE (2010c) PCR amplification of Bartonella koehlerae from human whole blood and enrichment whole blood cultures. Parasit Vectors 3:76.


CHAPTER 5.

SERIAL TESTING FROM A THREE-DAY COLLECTION PERIOD USING THE BAPGM PLATFORM MAY ENHANCE THE SENSITIVITY OF *BARTONELLA* SPP. DETECTION IN BACTEREMIC HUMAN PATIENTS

Manuscript as published in the Journal of Clinical Microbiology.

Serial testing from a three-day collection period using the *Bartonella Alpha* Proteobacteria Growth Medium platform may enhance the sensitivity of *Bartonella* spp. detection in bacteremic human patients

Running title: Serial testing and *Bartonella* spp. detection

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Abstract

*Bartonella* spp. bacteremic patients, tested using *Bartonella* alpha Proteobacteria growth medium (BAPGM), were retrospectively categorized into two groups that included blood collection once (Group 1; n=55) or three times (Group 2; n=36) within a one-week period. Overall, 19 (20.8%) patients were PCR positive for one or more *Bartonella* sp. using the BAPGM platform. Seven (12.7%) patients in Group 1 tested positive, compared to 12 (33.3%) patients in Group 2. Detection was improved when patients were tested three times within a one week period (OR = 3.4 [1.2-9.8]; p = 0.02). Obtaining three sequential blood samples during a one-week period should be considered as a diagnostic approach when bartonellosis is suspected.

Keywords: *Bartonella*, blood culture, PCR, relapsing bacteremia
Introduction

The genus Bartonella comprises over 30 species of fastidious, Gram-negative, aerobic intracellular bacteria that induce chronic intravascular infection in a variety of hosts, including humans (1–4). At least 17 Bartonella species have been associated with an expanding spectrum of human diseases, ranging from acute self-limiting illness to more severe disease manifestations, including encephalopathy, endocarditis, neurologic dysfunction, pleural and pericardial effusion, pneumonia, and hemolytic anemia (2, 5–8). To enhance the diagnostic documentation of infection with Bartonella spp., our laboratory has combined enrichment cultures that utilize an insect cell culture liquid growth medium, Bartonella Alphaproteobacteria growth medium (BAPGM), with highly sensitive PCR assays (7, 9). When used to test blood, cerebrospinal and joint fluids, or pathological effusions from dogs or horses, PCR following enrichment culture has enhanced the diagnostic sensitivity over blood PCR testing alone (10–13). We refer to this diagnostic approach as the BAPGM enrichment culture platform.

Despite ongoing advances in diagnostic sensitivity when using whole-blood enrichment culture approaches compared to traditional agar culture isolation, the documentation of intravascular infection with Bartonella spp. using specimens obtained from a single point in time remains challenging (10, 14–16). Intravascular infection with Bartonella spp. can be associated with a relapsing pattern of bacteremia at 5-day intervals, as demonstrated in rodents and as observed in humans with Bartonella quintana infection (trench fever, historically referred to as 5-day fever) (17–21). Similarly, bartonellae often induce very low levels of bacteremia in nonreservoir-adapted hosts, resulting in diagnostically low levels of
circulating bacteria in the bloodstream at a given point in time (2, 8, 10). Thus, screening enrichment blood cultures for documentation of infection with *Bartonella* spp. at a single time point might result in false-negative results. Serial testing is a widely accepted epidemiological approach designed to improve the diagnostic sensitivity of a laboratory test, thereby defining bacteremia positives as at least one positive out of multiple test results (22). For several reasons, including enhanced sensitivity, three blood cultures are typically recommended when bacterial sepsis is suspected (23). Using *Bartonella* testing and the BAPGM platform as an example, bacteremia would be confirmed if any of three serially obtained blood specimens taken over a 1-week span resulted in bacterial isolation or PCR amplification of *Bartonella* DNA from the patient’s blood, serum, or enrichment blood culture. Bacteremia would not be confirmed if all BAPGM platform PCR results from the three specimen sets were negative. Historically, testing of blood specimens using the BAPGM platform has consisted of testing one blood specimen drawn from a patient at a single point in time. When some high-risk patients (veterinary professionals) who initially tested negative were subsequently found to be *Bartonella* bacteremic upon retesting, we began to consider the value of obtaining three blood culture sample sets within a 1-week period. Therefore, the objective of this retrospective study was to determine if the testing of specimens collected serially over a 1-week period significantly improved PCR documentation of *Bartonella* bacteremia in human patients compared to the testing of specimens from a single time point.
Materials and Methods

Study Population

From February through December 2010, 91 voluntary patients with extensive arthropod exposure and/or frequent animal contact were entered into the study. For the most part, the participants became aware of the study through lay or scientific publications or by attending lectures on bartonellosis at regional and national veterinary conferences. In most instances, the patients requested testing because of a history of chronic poorly defined illness, fatigue, joint pain, arthritis, and neurologic or neurocognitive abnormalities. The individuals were not recruited into a specific study but were aware that their test results could be used in one or multiple studies. Prior to 2010, each patient submitted one sample set (blood and serum) for BAPGM enrichment blood culture-PCR. Beginning in 2010, we requested submission of three sample sets, but for logistical reasons most individuals only submitted one sample set. Also, during 2010 there was consistency in the medium, inoculation methods, PCR primers, and amplification conditions. Therefore, the 91 patients used in this analysis reflect the individuals who were tested for Bartonella spp. with the BAPGM enrichment blood culture-PCR platform during the same period of time and with identical laboratory techniques. Demographic information, animal/arthropod exposure, history of visiting specialists, self-reported clinical symptoms, and comorbid conditions for each patient were collected using a standardized 5-page survey instrument. Questionnaires were mailed to each study participant for self-re-port, and upon return to the Intracellular Pathogens Research Laboratory (IPRL) at North Carolina State University, they were entered into an electronic database. This study
was conducted in conjunction with North Carolina State University Institutional Review Board approval (IRB no. 164-08 and 1960-11).

**Sample Processing**

Patients had blood samples drawn from one or three collection time points within 1 week (7 days). Following aseptic preparation of the venipuncture site, 91 blood specimens from 91 patients were collected into EDTA-anticoagulated blood and in serum separator tubes. Unopened collection tubes were transported directly or by overnight express carrier to the Intracellular Pathogens Research Laboratory (IPRL) for processing. A previously described approach that combines PCR amplification of *Bartonella* spp. DNA and enrichment culture of blood and serum in BAPGM was used to test whole-blood (n = 91) and centrifuged serum (n = 91) specimens (10, 24). The BAPGM platform incorporates 4 separate PCR testing time points, each representing a different component of the testing process for each patient sample: PCR amplification of *Bartonella* spp. following DNA extraction from (i) whole blood and (ii) serum, (iii) PCR following BAPGM enrichment of the whole-blood culture incubated for 7 and 14 days, and (iv) PCR from the subculture isolates if obtained after subinoculation from the BAPGM flask onto plates containing Trypticase soy agar with 10% sheep whole blood that are incubated for 4 weeks. PCR specimen preparation, DNA extraction, and PCR amplification and analysis were performed in three separate rooms with unidirectional workflow to avoid DNA contamination. In addition, BAPGM cultures were processed in a biosafety cabinet with HEPA filtration in a limited-access biosafety level II laboratory. The methods used to amplify the *Bartonella* DNA from blood, serum, BAPGM liquid culture, and sub-culture isolates, if obtained, included conventional PCR with
Bartonella genus primers targeting the 16S to 23S intergenic spacer (ITS) region and amplification using Bartonella koehlerae species-specific ITS primers as described previously (10, 15, 24, 25). Amplification of the B. koehlerae ITS region was performed using oligonucleotides Bkoehl-1s (5′=-CTT CTA AAA TAT CGC TTC TAA AAA TTG GCA TGC-3′) and Bkoehl-1125as (5′=-GCC TTT TTT GGT GAC AAG CAC TTT TCT TAA G-3′) as forward and reverse primers, respectively. Amplification was performed in a 25-μl final volume reaction mixture containing 12.5 μl of Tak-Ex Premix (Fisher Scientific, Rockford, IL), 0.1 μl of 100 μmol/liter of each forward and reverse primer (IDT DNA Technology, Coralville, IA), 7.3 μl of molecular-grade water, and 5 μl of DNA from each sample tested. Conventional PCR was performed in an Eppendorf Mastercycler EP gradient (Eppendorf, Hauppauge, NY) under the following conditions: a single cycle at 95°C for 2 s followed by 55 cycles with DNA denaturing at 94°C for 15 s, annealing at 64°C for 15 s, and extension at 72°C for 18 s. The PCR was completed by a final cycle at 72°C for 30 s. As previously described for the ITS genus and B. koehlerae PCR assays, all products were analyzed by 2% agarose gel electrophoresis with detection using ethidium bromide under UV light, after which the amplicon products were sequenced to identify the species and ITS strain types. All positive test results were confirmed as Bartonella spp. through DNA sequencing. PCR-negative controls were prepared using 5 μl of DNA from the blood of a healthy dog. Bartonella henselae (Houston 1 strain) at a concentration of 1 genome copy/μl was used as a PCR-positive control during the entire course of this study. In no instance was B. henselae or DNA of any other Bartonella spp. amplified in the negative control lane on any PCR gel. To assess potential contamination during blood sample processing into
BAPGM, an uninoculated BAPGM culture flask was processed simultaneously and in an identical manner with each batch of patient blood and serum samples tested. For all components of the BAPGM platform (PCR from blood, serum, enrichment cultures at 7 and 14 days, and subcultures), PCR-negative controls remained negative throughout the course of the study. In addition, subcultures of uninoculated BAPGM (culture control) at 7 and 14 days did not yield bacterial growth.

Statistical Analysis

Patients were retrospectively divided into one of two groups: group 1 included individuals with blood specimens drawn from one collection day, and group 2 included individuals with blood specimens drawn from three collection days within a 1-week period. Statistical analysis was performed using SAS/STAT 9.2 for Windows (SAS Institute Inc., Cary, NC; 2008). The chi-squared test, Fisher’s exact test, and the Mann-Whitney U test were used to detect differences in demographic characteristics by collection group. The chi-squared test and univariate logistic regression were used to assess differences in the proportions of positive patients by group to determine if serial testing within 1 week increased the likelihood of a positive test result using the BAPGM platform.

Results

A total of 91 patients, for whom testing was performed in an identical manner, were included in the study population. Group 1 comprised 55 patients (60.5%), and group 2 comprised 36 patients (39.5%). Demographic characteristics of the study population, by collection group, are listed in Table 1. No significant differences were detected between collection groups for demographic information, risk factors, disease severity, prior antibiotic use, or self-reported
symptoms, with the exception of self-reported weight loss (P < 0.01). Overall, 19 (20.8%) patients were PCR positive for one or more Bartonella spp. using the BAPGM platform. All positive test results were confirmed as Bartonella spp. through DNA sequencing. The numbers of patients who tested positive with the BAPGM platform, by group, are listed in Table 2. Overall, 13 patients (14.3%) tested positive following direct extraction of blood or serum, and 10 patients (10.9%) tested positive following liquid enrichment culture. No patients tested positive through subculture isolation. B. henselae and B. koehlerae were detected in 6 (6.6%) and 15 (16.5%) patients, respectively (Table 3). Overall, seven patients (12.7%) in group 1 tested positive, and 12 patients (33.3%) in group 2 tested positive (P < 0.02). Based upon the observed proportion of individuals who tested positive in each group, group 2 patients were more likely to test positive than group 1 patients (odds ratio [OR], 3.4 [95% confidence interval, 1.2 to 9.8]; P < 0.02) for the overall BAPGM platform and during the liquid enrichment culture stage of the BAPGM platform (P < 0.04) (Table 2). Of the Bartonella bacteremic patients in group 2, only 3 patients (23.1%) had positive specimens from more than 1 day. No patient had a positive result for all three specimen dates.

Discussion

In order to enhance detection of Bartonella spp. in immunocompetent patients, serial testing of multiple specimens collected during a 1-week period should be considered. In this study, our analysis was restricted to a subpopulation of individuals who had been tested previously for Bartonella spp. through the IPRL (8); therefore, individuals were not randomized into sampling groups prior to entry into the study, and our results might be subject to unintentional selection bias. For example, patients tested multiple times for Bartonella
bacteremia might have been more likely to test positive due to more severe symptoms. Due to the limited sample size within our group 1 population, we were unable to control for potential confounders, such as a discrepancy of disease severity or exposure between groups that might have affected the observed association between bacteremia and serial specimen collection. While there was no difference between our two groups for self-reported demographics, disease severity, exposure, or disease symptom variables, as shown in Table 1, due to the univariate nature of our analysis, we acknowledge that unmeasured confounders might exist that could potentially distort our observed association. Additionally, our retrospective analysis relied on previously obtained data, and no projection can be accurately made as to the true proportion of positive versus negative individuals or the exact increase in sensitivity obtained within this overall study population. To confirm the association obtained from our observational data, experimental studies are needed to evaluate the effect of serial testing on the diagnostic sensitivity of *Bartonella* testing in individuals or animals with equal known infection rates. Diagnostic testing for *Bartonella* spp. using the BAPGM platform has been shown to be highly specific because amplicon identity is confirmed by DNA sequencing; however, sensitivity is currently unclear due to the low levels of bacteria found in human patient samples (2, 8, 10). *Bartonella* bacteremia is more readily documented in a primary reservoir species, such as cats or rodents, and might occur less frequently or to a much lower level in accidental hosts, such as humans. For example, in humans, the average bacterial levels in blood are 1 to 10 genome copies/μl, compared to the 105 to 106 copies/μl often found in cats (2). In a recent study of 192 patients, the BAPGM enrichment blood culture-PCR detected *Bartonella* spp. in an additional 34.7% of patients compared to PCR on
extracted blood alone (8). Overall, in that study, 46 patients (23.9%) were infected with *Bartonella*. Serial sampling over a 1-week period in the current study appears to have further increased the diagnostic sensitivity of the BAPGM platform within the study population.

*Bartonella* bacteremia appears to exist in a cyclical nature within its natural reservoir hosts (17, 20, 21, 26). In the *Bartonella tribocorum* rat model of *Bartonella* infection, high numbers of bacteria are detectable in the blood following the initial infection, until a level of 8 to 15 bacteria per erythrocyte is reached. Bacteremia then declines and drops below a detectable level (20, 21). While the location of *Bartonella* during this nonbacteremic phase of infection is currently unknown, endothelial cells and bone marrow have been hypothesized as primary niches in both incidental and reservoir hosts (17, 20, 27, 28). Bacteria have been observed to release from the primary niche and reinvade circulating erythrocytes to create peaks of bacteremia at intervals of 3 to 6 days (17, 20). Several other observations support the persistence of *Bartonella* in a cellular/acellular compartment, with subsequent seeding into circulation (17, 29, 30). In a cat naturally infected with *B. henselae*, high levels of bacteria initially demonstrated in the blood by culture gradually declined to undetectable levels over a 5-month period. Bacteremia was again documented 2 months later, and the cat became cyclically culture negative at 2-month intervals (29). Although the pattern of bacteremia was variable among individual animals, similar results were found during experimental *B. henselae* transmission studies involving specific-pathogen-free (SPF) cats (19, 30).

Unfortunately, the unpredictable nature of bacteremia within the host might result in an inaccurate microbiological diagnosis even if the patient is tested three times during a 1-week period.
Bartonellosis is an emerging infectious disease, with 17 *Bartonella* spp. having been associated with an expanding spectrum of human pathology (2, 8). Due to the zoonotic potential of *Bartonella* spp. as human pathogens, the medical relevance of this genus is undergoing rapid redefinition (2). Although epidemiologic studies are needed to establish causation for many nonspecific *Bartonella*-associated symptoms, enhanced diagnostic detection of *Bartonella* is necessary to accurately generate data for epidemiological investigations and to properly define disease pathology, determine effective treatment options, and assess microbiological outcomes for patients. Obtaining three sequential specimens during a 1-week period appears to enhance detection of *Bartonella* bacteremia in human patients and should be considered as a diagnostic approach when bartonellosis is suspected.

**Acknowledgments**

We thank Julie Bradley for coordinating specimen submission for this study and Tonya Lee for help in preparing the manuscript.

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In conjunction with Sushama Sontakke and North Carolina State University, Edward B. Breitschwerdt holds U.S. patent no. 7,115,385, Media and Methods for Cultivation of Microorganisms, which was issued 3 October 2006; he is the chief scientific officer for Galaxy Diagnostics, a company that provides diagnostic testing for the detection of *Bartonella* species infection in animals and in human patient samples. Ricardo G. Maggi has led efforts to optimize the BAPGM platform and is the scientific technical advisor and
laboratory director for Galaxy Diagnostics. The other authors have no conflicts of interest to declare.
Table 1. Demographic characteristics of 91 individuals tested for *Bartonella* spp. using the BAPGM platform, by collection group.

<table>
<thead>
<tr>
<th></th>
<th>Group 1</th>
<th>Group 2</th>
<th>P-value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 Collection Period in 1 wk&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3 Collection Periods in 1 wk</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>25 (45.5)</td>
<td>14 (38.9)</td>
<td>0.53</td>
</tr>
<tr>
<td>Female</td>
<td>30 (54.5)</td>
<td>22 (61.1)</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>42.0 [30-53]&lt;sup&gt;c&lt;/sup&gt;</td>
<td>38.0 [28-50]&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.40</td>
</tr>
<tr>
<td>Length of symptoms (months)</td>
<td>24 [0-60]&lt;sup&gt;f&lt;/sup&gt;</td>
<td>3 [0-42]&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.48</td>
</tr>
<tr>
<td>Chronically Ill (self-reported)</td>
<td>22 (53.6)</td>
<td>17 (54.8)</td>
<td>0.92</td>
</tr>
<tr>
<td>Animal Contact</td>
<td>39 (70.9)</td>
<td>31 (86.1)</td>
<td>0.21</td>
</tr>
<tr>
<td>Tick Exposure</td>
<td>38 (69.1)</td>
<td>29 (80.6)</td>
<td>0.41</td>
</tr>
<tr>
<td>Flea Exposure</td>
<td>38 (69.1)</td>
<td>28 (77.8)</td>
<td>0.39</td>
</tr>
<tr>
<td>Prior antibiotic therapy</td>
<td>21 (38.2)</td>
<td>16 (44.4)</td>
<td>0.41</td>
</tr>
<tr>
<td>Balance Problems</td>
<td>20 (36.4)</td>
<td>16 (44.4)</td>
<td>0.43</td>
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<td>Shortness of breath</td>
<td>15 (27.3)</td>
<td>14 (38.9)</td>
<td>0.31</td>
</tr>
<tr>
<td>Blurred Vision</td>
<td>19 (34.5)</td>
<td>13 (36.1)</td>
<td>0.32</td>
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<td>17 (30.9)</td>
<td>14 (38.9)</td>
<td>0.39</td>
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<td>Depression</td>
<td>16 (29.1)</td>
<td>14 (41.7)</td>
<td>0.29</td>
</tr>
<tr>
<td>Bowel/Bladder dysfunction</td>
<td>14 (25.5)</td>
<td>11 (30.5)</td>
<td>0.40</td>
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<td>Difficulty Remembering</td>
<td>22 (40.0)</td>
<td>18 (50.0)</td>
<td>0.38</td>
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<td>Insomnia</td>
<td>20 (36.4)</td>
<td>16 (44.4)</td>
<td>0.40</td>
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<td>Disorientation</td>
<td>8 (14.5)</td>
<td>7 (19.4)</td>
<td>0.39</td>
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<td>Fatigue</td>
<td>32 (58.2)</td>
<td>23 (63.9)</td>
<td>0.38</td>
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<td>26 (47.3)</td>
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<td>0.18</td>
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<td>17 (47.2)</td>
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<td>Joint Pain</td>
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<td>Muscle Pain</td>
<td>20 (36.4)</td>
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<td>0.36</td>
</tr>
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<td>20 (36.4)</td>
<td>17 (47.2)</td>
<td>0.36</td>
</tr>
<tr>
<td>Paralysis</td>
<td>4 (7.3)</td>
<td>2 (5.6)</td>
<td>0.38</td>
</tr>
<tr>
<td>Sleeplessness</td>
<td>27 (49.1)</td>
<td>20 (55.6)</td>
<td>0.41</td>
</tr>
<tr>
<td>Syncope</td>
<td>8 (14.5)</td>
<td>6 (16.7)</td>
<td>0.41</td>
</tr>
<tr>
<td>Tremors/Shakes</td>
<td>9 (16.4)</td>
<td>12 (33.3)</td>
<td>0.12</td>
</tr>
<tr>
<td>Weight Loss</td>
<td>4 (7.3)</td>
<td>11 (30.6)</td>
<td>0.01&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Previous consultation with:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neurologist</td>
<td>15 (27.3)</td>
<td>16 (44.4)</td>
<td>0.18</td>
</tr>
<tr>
<td>Infectious disease doctor</td>
<td>11 (20.0)</td>
<td>13 (36.1)</td>
<td>0.16</td>
</tr>
<tr>
<td>Rheumatologist</td>
<td>6 (10.9)</td>
<td>10 (27.8)</td>
<td>0.08</td>
</tr>
<tr>
<td>Endocrinologist</td>
<td>7 (12.7)</td>
<td>7 (19.4)</td>
<td>0.34</td>
</tr>
<tr>
<td>Psychiatrist</td>
<td>1 (1.8)</td>
<td>2 (5.6)</td>
<td>0.25</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values shown are n (%) unless otherwise specified.<br><sup>b</sup> The Fisher exact test was used when the cell size was <5. The nonparametric Mann-Whitney U test was used for nonnormally distributed continuous variables.<br><sup>c</sup> Continuous variables are reported as medians (interquartile ranges).<br><sup>d</sup> Statistically significant difference
Table 2. *Bartonella* PCR amplification results from blood, serum, and enrichment blood culture processed using the BAPGM platform by collection group.

<table>
<thead>
<tr>
<th></th>
<th>Group 1 1 Collection Period in 1 wk</th>
<th>Group 2 3 Collection Periods in 1 wk</th>
<th>(P)-value(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Blood</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Direct Extraction</td>
<td>3 (5.4)</td>
<td>3 (8.3)</td>
<td>0.67</td>
</tr>
<tr>
<td>BAPGM enrichment</td>
<td>3 (5.4)</td>
<td>7 (19.4)</td>
<td>0.04(^c)</td>
</tr>
<tr>
<td>Subculture Isolates</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>-(^d)</td>
</tr>
<tr>
<td>Total</td>
<td>5 (9.1)</td>
<td>10 (27.8)</td>
<td>0.02(^c)</td>
</tr>
<tr>
<td><strong>Serum</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Direct Extraction</td>
<td>4 (7.3)</td>
<td>4 (11.1)</td>
<td>0.71</td>
</tr>
<tr>
<td>BAPGM enrichment</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>-</td>
</tr>
<tr>
<td>Subculture Isolates</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>4 (7.3)</td>
<td>4 (11.1)</td>
<td>0.71</td>
</tr>
<tr>
<td><strong>Blood &amp; Serum Combined</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Direct Extraction</td>
<td>6 (10.9)</td>
<td>7 (19.4)</td>
<td>0.35</td>
</tr>
<tr>
<td>BAPGM enrichment</td>
<td>3 (5.4)</td>
<td>7 (19.4)</td>
<td>0.04(^c)</td>
</tr>
<tr>
<td>Subculture Isolates</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>7 (12.7)</td>
<td>12 (33.3)</td>
<td>0.02(^c)</td>
</tr>
</tbody>
</table>

\(^a\) Numbers do not sum to the total because an individual could be positive at more than one stage of the BAPGM platform.

\(^b\) The Fisher exact test was used when the cell size was <5.

\(^c\) Statistically significant difference.

\(^d\) —-, no statistics were calculated for zero cell comparisons.
Table 3. Detected species of *Bartonella* following the BAPGM platform by group.

<table>
<thead>
<tr>
<th>Species</th>
<th>Group 1 1 Collection Period in 1 wk</th>
<th>Group 2 3 Collection Periods in 1 wk</th>
<th>P-value^b</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. henselae</em></td>
<td>2 (3.6)</td>
<td>4 (11.1)</td>
<td>0.21</td>
</tr>
<tr>
<td><em>B. koehlerae</em></td>
<td>5 (9.1)</td>
<td>10 (27.8)</td>
<td>0.02</td>
</tr>
<tr>
<td><em>B. vinsonii</em> subsp. berkoffii</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>-c</td>
</tr>
<tr>
<td>Total</td>
<td>7 (12.7)</td>
<td>12 (33.3)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

^a Numbers do not sum to the total because an individual could be positive for more than one species of Bartonella.

^b The Fisher exact test was used when the cell size was <5.

^c —, no statistics were calculated for zero cell comparisons.
References


CHAPTER 6.
PREVALENCE OF *BARTONELLA* SPP. IN CANINE CUTANEOUS HISTIOCYTOMA
Prevalence of *Bartonella* spp. in Canine Cutaneous Histiocytoma

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Abstract

Canine cutaneous histiocytoma (CCH) is a common, benign neoplastic proliferation of histiocytes of Langerhans cell (LC) origin that often ulcerate, become secondarily infected, and regress spontaneously. Bartonella is a fastidious genera of intracellular pathogens that can be transmitted through arthropod bites and epidermal animal scratches and has been previously identified in the cytoplasm of histiocytes within granulomatous lesions and in skin biopsies of inflammatory pustules and papules. Based upon the established oncogenic properties of Bartonella, we hypothesized that Bartonella spp. can be molecularly detected in CCH and can be localized within skin neoplasms using indirect immunofluorescence (IIF).

Paraffin embedded surgical biopsies from dogs with CCH and non-neoplastic skin adjacent to osteosarcomas (control group due to wide surgical margins) were retrieved from NCSU-CVM pathology. DNA was extracted and the 16S-23S rRNA intergenic transcribed spacer (ITS) region, pap31 and gltA genes were amplified using Bartonella-specific primers. IIF was performed using a B. henselae monoclonal or B. spp. polyclonal as primary antibodies and Cy3 goat anti-mouse IgG secondary antibody to localize Bartonella in tissues of PCR positive dogs. Bartonella spp. was amplified from 1/17 (5.8%) control tissues and 4/29 (13.8%) CCH tissues (p=0.63). Bartonella was identified in 2/4 (50.0%) CCH tissues using IIF. Bartonella spp. are unlikely to cause CCH. Though Bartonella can be visualized in CCH using IIF, cellular localization of Bartonella within the skin has reduced sensitivity due to low organism load, a limitation well-supported by previous attempts by our laboratory to localize the bacterium in various tissues and lesions.
Introduction

Skin tumors comprise the most common type of neoplasm in dogs, and account for nearly 30% of canine tumors (Monteiro et al. 2011). Canine cutaneous histiocytoma (CCH) is a common, benign tumor, consisting of proliferation of intraepidermal dendritic antigen-presenting cells, also called Langerhans cells, and is most prevalent in young dogs (<4 yrs old), though histiocytomas occur in dogs of all ages (Goldschmidt & Hendrick, 2002; Fulmer & Mauldin, 2007). Tumors often arise on the head, but may appear anywhere on the body (Gross et al. 1992). Lesions typically undergo spontaneous immune-mediated regression within 1 to 2 months and no treatment is typically necessary (Wu et al. 2004); regression is characterized by lymphocyte infiltration upon histopathologic examination (Fulmer & Mauldin, 2007). The presence of multiple tumors is considered rare, and reports of recurrence of histiocytomas are uncommon (Monteiro et al. 2011). Predisposed breeds include boxers, bulldogs, Scottish terriers, Doberman pinschers, and cocker spaniels (Monteiro et al. 2011; Goldschmidt & Hendrick, 2002). Dogs diagnosed with cutaneous histiocytoma have an excellent prognosis, with lesions very rarely metastasizing to the lymph nodes (Monteiro et al. 2011). While these tumors are able to spontaneously regress without medical treatment, ulceration, itching, and secondary infections are often problems that may require medical or surgical interventions.

Previous research has identified a high prevalence of *Bartonella* spp. DNA in tissues containing histiocytic inflammation or neoplasia, such as fibrohistiocytic nodules (FHN), a histiocytic disease of the spleen, and granulomatous lesions in the skin (Varanat et al. 2011; Keynan et al. 2007; Lin, et al. 2006). *Bartonella* spp. have previously been identified in the
cytoplasm of histiocytes located within granulomatous lesions and in skin biopsies containing inflammatory pustules and papules in patients with Cat Scratch Disease (CSD); additionally, animal scratches and arthropod bites through the skin represent the primary exposure route for *Bartonella* infection, suggesting a potential role in the development of CCH (Lin et al. 2006; Avidor et al. 2001; Breitschwerdt et al. 2010; Boulouis & Chomel, 2004; Chomel et al. 2006; Kipar et al. 1998). Because CCH consists of an abnormal proliferation of histiocytes within the skin, and because *Bartonella* chronically infect macrophages and histiocytic cells *in vitro*, and inhibit apoptosis (Kempf VA, 2005), we hypothesized that these bacteria may induce the proliferation and formation of a histiocytoma. No clinical or epidemiological studies have been performed to evaluate a potential association between *Bartonella* spp. infection and histiocytic tumors involving the skin. Therefore, we conducted a retrospective cross-sectional study to evaluate the association between *Bartonella* spp. and CCH, using formalin fixed paraffin embedded (FFPE) tissues, as compared to histologically normal skin.

Conventional methods of *Bartonella* spp. detection in FFPE tissues has relied on molecular methods such as polymerase chain reaction (PCR). However, due to potential DNA carryover and contamination of *Bartonella* from infected into uninfected FFPE tissues within animal necropsy rooms, intracellular localization of the bacterium within these tissues was necessary to determine if *Bartonella* spp. contributes to the pathogenesis of these canine skin diseases (Varanat et al. 2009). The cellular localization of *Bartonella* spp. within PCR positive tissues would more clearly implicate these bacteria in the development of CCH. Based upon the established oncogenic properties of *Bartonella* spp., we hypothesize that *Bartonella* spp. can
induce certain histiocytic skin tumors, specifically CCH, in dogs, and can be cellurally localized within skin neoplasms using localization techniques.

**Methods**

**Sample collection**

Using the NCSU-CVM Pathology Data Base, paraffin embedded surgical biopsy samples from two groups of dogs were retrieved from pathology archive storage facilities. Group I included marginal sections of skin from dogs with surgically amputated osteosarcoma (n=17), which was used as a control group to represent ‘non-diseased’ canine skin. Group II included skin biopsies from dogs with CCH (n=29). Archival tissues used in this study were collected between 2009-2012. Skin tissue samples were independently reviewed by a pathologist to confirm the histopathological diagnosis and to determine any/the extent of regression occurring in cases of CCH. Cases of CCH undergoing high levels of regression were excluded from analysis. Following DNA extraction, all paraffin-embedded splenic tissues were tested by PCR for the presence of *Bartonella* sp. DNA.

**DNA extraction**

For each specimen, two 30μm sections of tissue were excised from the paraffin-embedded tissues using a microtome. A negative control paraffin block, containing no tissue, was cut in between each tissue block and processed in an identical manner as samples to determine if any DNA carryover was occurring through the use of the microtome. Tissues were processed in small batches and the work surface was thoroughly cleaned using ethanol and DNAse between each tissue block to avoid *Bartonella* spp. DNA carry over between the samples.
DNA was extracted using QIAamp FFPE Tissue Kit (Qiagen, Valencia, CA) following manufacturer’s instructions. Elution buffer was used as a reagent control with each set of DNA extractions. DNA concentrations and purity were determined using a spectrophotometer (Nanodrop, Wilmington, DE). Extracted DNA was stored at -20°C.

**Polymerase Chain Reaction**

Samples were tested for the presence of *Bartonella* sp. DNA, using multiple sets of primers, as listed in Table 1. *Bartonella* genus primers targeting the 16S-23S rRNA intergenic transcribed spaced region (ITS 325s-1100as, ITS 425s-1100as), pap31 (1s-688as) gene, and glta (781s-1137as) gene were used. *Bartonella* species-specific primers targeting the 16s-23s rRNA ITS region of *B. koehlerae* (1s-1125as) and *B. vinsonii* subsp. *berkhoffii* (p1-p2) were also used. Reaction conditions used for each PCR are given in Table 2. A 114 region of the *Bartonella* 16S-23S rRNA ITS region was amplified using real-time PCR and primers ITS 325s-438as. Reaction mixtures contained 12.5 μl of either MyTaq HS Red Mix or SsoAdvanced SYBR Green Supermix (Takara Bio USA Inc, Madison, WI), 7 μl of molecular grade water, 0.25 μl each of forward and reverse primers (100 μM), and 5 μl of template DNA. Amplified products were analyzed on a 2% agarose gel stained with ethidium bromide. DNA extracted from the blood of a healthy dog was used as a negative control. Positive controls included 0.01 pg/μl of *B. henselae* DNA, 0.01 pg/μl of *B. koehlerae* DNA, or 0.01 pg/μl of *B. vinsonii* subsp. *berkhoffii* DNA. Amplified products were analyzed on a 2% agarose gel stained with ethidium bromide. All PCR positive amplicons were sequenced directly to establish the species, strain or genotype.
Immunohistochemistry

Immunohistochemistry was performed on 5μm sections from paraffin embedded tissues on poly-L-lysine coated slides. Slides were deparaffinized and rehydrated using graded ethanol washings, and rinsed in 1X PBS and 0.02% Tween 20 (PBST). Following deparaffinization and rehydration, slides were subject to a heat induced epitope retrieval; briefly, slides were immersed in Sodium Citrate buffer (pH 6.0) and placed in a Decloaking chamber at 115 °C for 30 seconds. After blocking overnight with 10% normal goat serum (NGS) in 1X PBST, the slides were incubated at 4°C for 1 hour with 1:50 dilution of mouse monoclonal antibody against B. henselae (Abcam, Cambridge, MA) in 1X PBST containing 1% NGS. After incubation with primary antibody, immunodetection was carried out using a secondary goat anti-mouse immunoglobulin linked to a fluorophore (Cy-3 Conjugate; Jackson Laboratories, Bar Harbor, ME) diluted (1:1000) in 1X PBST containing 1% NGS at 4°C for 20 minutes in the dark. Tissues sections were counterstained with DAPI prior to coverslipping.

Immunohistochemistry was also performed separately using polyclonal B. henselae Houston-1 antibodies obtained from human patient serum. The serum was shown to have a B. henselae Houston-1 titer of 1:512 through IFA and was further diluted 1:100 in 1X PBST containing 1% NGS for tissue incubation. After incubation with this primary polyclonal antibody, immunodetection was carried out using a secondary goat anti-human immunoglobulin linked to a fluorophore (Cy-3 Conjugate; Jackson Laboratories, Bar Harbor, ME) diluted (1:1000) in 1X PBST containing 1% NGS at 4°C for 20 minutes in the dark, and counterstained with DAPI prior to coverslipping. Slides were analyzed on an
epifluorescent microscope at 20x for 20 fields, or the entire tissue, and then further analyzed on a Zeiss LSM 710 confocal microscope.

**Statistical analysis**

Statistical analysis was performed using SAS/STAT 9.2 software (SAS Institute, Cary, North Carolina). The prevalence of *Bartonella* sp. DNA was compared between and among the study groups using a Fishers exact test. The level of significance was set at *p*<0.05.

**Results**

**Study animals**

A total of 46 dogs made up the study population. Group I was comprised of 17 dogs, while Group II was comprised of 29 dogs. Signalment information was missing for 2 dogs (4.4%). Nearly half of dogs were castrated males (n=22; 47.8%), while 16 (34.8%) dogs were spayed females, 5 (10.9%) were intact males, and 1 (2.2%) dog was an intact female. The proportion of intact animals (*p*=0.38) or males vs females (*p*=0.76) did not vary by group. The median age of dogs in group I was 108 months (IQR: 96-120) and was greater than the median age in Group II (48; IQR: 24-78) (*p*<0.001). Approximately one-quarter (n=7; 25.9%) of Group II dogs were listed as mix breeds, compared to 0% (n=0) of dogs in Group I (*p*=0.03).

**PCR analysis, Sequencing, and Visualization**

All extraction, PCR negative controls, including the blank paraffin block controls, tested negative throughout this study. The prevalence of *Bartonella* spp. in Group I was 5.8% (n=1/17). The amplified product from this dog was sequenced as *B. vinsonii* subsp.
berkhoffii Genotype II. The prevalence of *Bartonella* spp. in Group II was 13.8% (n=4/29). The amplified products from these four dogs were all sequenced as *B. henselae* SA2. There was no difference in the prevalence of *Bartonella* spp. between Group I and Group II (p=0.63). PCR positive results, by primer set and gene target, can be found in Table 3. Bacteria were visualized by IIF using our primary monoclonal antibody in 2/4 (50%) of tissues in Group II (Figure 1). Bacterial visualization by IIF using the polyclonal primary antibody was unsuccessful due to a large amount of non-specific binding to our tissue (Figure 2).

**Discussion**

We did not detect a significantly increased molecular prevalence of *Bartonella* spp. in CCH (13.8%) compared to control skin (5.8%) (p = 0.63). Additionally, using a *B. henselae* commercial monoclonal antibody, we were only able to visualize *Bartonella* spp. in 50% of PCR positive tissues in Group II (n=2/4). Due to the small tissue size that is frequently obtained in CCH tissue blocks, the entire tissue section was examined under a fluorescent microscope to observe signal. In tissues that stained positive for *Bartonella*, signal was observed in only several fields, with no more than one signal per field. A major limitation of the microscopic visualization of *Bartonella* is the low sensitivity, particular when few organisms are located in the tissues. Organism load varies by tissue type (Breitschwerdt et al., 2010), and may exist at very low copy number within the skin. Visualization of *Bartonella* may be more easily detectable in heavily infected tissues, such as heart valves obtained from dogs or humans with *Bartonella* endocarditis, compared to the skin (Sander A, 1999; Breitschwerdt EB, 2010). Due to low organism load, examination of a 5 μm section
may not provide enough tissue to accurately depict organism presence/load within the skin or other low organism load tissues. Thicker tissue sections examined on a confocal microscope should help to increase the sensitivity of bacterial visualization, as previously used to detect *B. henselae* in infected human striae (Maggi RG, 2013).

As is true for the direct visualization of *Bartonella* in skin tissues, PCR amplification of *Bartonella* from paraffin-embedded diagnostic biopsy skin samples remains technically difficult. PCR has remained an effective diagnostic tool for organism rich samples, including *Bartonella* endocarditis heart biopsies. However, the sensitivity of PCR is poorly characterized when targeting *Bartonella* in low-organism load tissues, including the skin. Additionally, the skin contains high host DNA concentrations that may interfere with DNA amplification of the *Bartonella* target. While this should only result in non-differential misclassification of positive and negative cases in both Group I and Group II, as both group samples contained skin biopsies, the true prevalence of *Bartonella* spp. within our groups may be underestimated. Previous studies have shown that cross-contamination with *Bartonella* spp. DNA in necropsy or histopathology processing laboratories may occur from the transfer of infected blood or fluids from one necropsy/biopsy case to the next (Varanat et al., 2009), and additionally, transfer of *Bartonella* DNA may occur through the use of contaminated microtomes. While we were unable to control for potential contamination in necropsy/histopathology processing rooms, our microtome was cleaned with DNase and ethanol between each block processing. Additionally, a negative control block was cut in between each sample block to ensure cross-contamination did not occur during the tissue cutting process.
Though we were unable to detect a higher molecular of *Bartonella* in CCH compared to controls, *Bartonella* has been associated with a variety of other inflammatory/or vasoproliferative manifestations within the skin (Chian et al., 2002). *Bartonella* induced Cat Scratch Disease, Bacillary Angiomatosis (BA), and verruga peruana typically manifest within the skin as papules or angiomatous lesions consisting of a diffuse inflammatory cell infiltrate comprised of various cells, such as neutrophils, histiocytes, scattered eosinophils, or plasma cells (Carithers HA, 1985; Spach & Koehler, 1998; Schwartz et al., 1997; Cockerell CJ, 1995). Near these lesions or papules, clusters of bacteria are often observed, and the lesion resolves with the progressive clearing of these bacteria in CSD patients (Chian et al., 2002). Indeed, following vector transmission or direct inoculation, *Bartonella* spp. are known to trigger an acute and chronic inflammatory reaction that is seen in many of these skin conditions. Canine cutaneous histiocytoma is often seen with marked inflammatory infiltrate, which may suggest that CCH is representative of an immune response to an unknown stimulus, inducing dermal invasion and local proliferation of immune cells (Kipar et al., 1998). It is through this hypothesis that the role of *Bartonella* in CCH provides biological plausibility, given *Bartonella*’s exposure route through the skin and the bacterium’s inflammatory properties. However, the progressive infiltrate seen in CCH is often accompanied by visible degeneration in tumor cells, which suggests that the inflammatory infiltrate represents an immune mediated anti-tumor host response, and not a response to a bacterial stimulus; this is supported through the ability of CCH to spontaneously regress (Cockerell et al., 1979). CCH has also been shown to be of monoclonal origin through X-linked clonality testing of tumors in dogs, which also argues in favor of an oncogenic origin.
and against an immune stimulatory origin (Delcour et al., 2013). Furthermore, the visualization of *Bartonella* within our CCH skin tissues revealed no clusters of bacteria anywhere within the tumor; only scattered independent Bartonellae were observed in positive tissues. Historically, *Bartonella* has been observed as distinct clusters surrounding inflammatory lesions within the skin, and therefore the individual Bartonellae observed in our study may represent incidental, unrelated infection. It is apparent that the oncogenic origins of CCH are quite different from the other *Bartonella*-induced inflammatory and vasoproliferative lesions of the skin, and it is therefore unsurprising that *Bartonella* is not present in CCH as it is in other inflammatory processes in the skin.

Though it is unlikely that *Bartonella* spp. plays a role in the development of CCH, *Bartonella* has been detected in a myriad of oncogenic processes, including inflammatory breast cancer, epithelioid hemangioendothelioma (EHE), hemangiopericytoma, splenic hemangiosarcoma, and splenic fibrohistiocytic nodules (Varanat et al., 2011; Fernandez et al., 2012; Breitschwerdt et al., 2009). One striking characteristic that the majority of these processes have in common is the high degree of angiogenic activity or vascularization associated with each tumor’s progression. Histology has provided evidence of a high degree of angiogenesis and vascularization in inflammatory breast cancer, EHE, and hemangiopericytoma (Gaur et al., 2012; Van Der Auwera et al., 2004), and though the etiology of canine hemangiosarcoma is unknown, it is thought that hypoxia, inflammation, and intense angiogenesis play a critical role in its development (Varanat et al., 2013). Previous studies that describe the molecular pathogenesis of *Bartonella* have clearly implicated this bacterium as a cause or co-factor in angiogenesis, and the molecular
mechanism by which *Bartonella* participates in angiogenesis has been previous described (Koehler & Tappero, 1993; Pulliainen & Dehio, 2012; Dehio C, 2005; Minnick et al., 2003; Cerimele et al., 2003; Kempf et al., 2001; Kempf et al., 2005; Maeno et al., 1999). CCH does not have an angiogenic etiology, and the cause of CCH in dogs is currently unknown (Kipar et al., 1998; Goldschmidt & Hendrick, 2002; Wu et al., 2004). Though we did not find an association between *Bartonella* and CCH, our data lends insight into the specificity of *Bartonella* infection in different tumor types and the potential role of the bacterium as a co-factor in the progression of highly vascularized tumors. The consistent detection of *Bartonella* in independent reports and studies of various cancers with an angiogenic etiology or high degree of vascularization, and absence in those without, raises the hypothesis that the bacterium may act as a contributor to the tumor microenvironment and regulate angiogenesis for tumor progression.

It is now well recognized that inflammation, specifically leukocyte infiltration, precedes the early stages of tumor progression to create a favorable environment for the development of cancer (Lorusso et al., 2008). Chronic inflammation, consisting of infiltrating macrophages, neutrophils, monocytes, and eosinophils, initiated through *Bartonella* infection, would create a stromal environment that is favorable for tumor growth and promotes the progression of oncogenic lesions. Furthermore, the formation of a tumor-associated angiogenic vasculature is essential for tumor progression (Weis et al., 2011), and it is possible that *Bartonella* spp. promote the formation of tumor-associated vessels that provide oxygen and nutrients for tumor growth. *Bartonella* induced pathological angiogenesis has been shown to produce tumor-like lesions that are packed with lobes of
immature capillaries that are lined with a swollen endothelium. Following colonization of the vascular endothelium, *Bartonella* is able to directly activate HIF-1α, a signal of hypoxia and a principle regulator of angiogenesis, which can stimulate expression of VEGF to increase oxygen delivery, via endothelial cells, to generate new blood vessels to supply areas with additional oxygen (Dehio C, 2005; Kempf et al., 2005). Studies have shown that in the absence of sufficient vascularization, most tumors cannot exceed a few mm³ in volume and subsequently remain clinically benign (Folkman J, 1995). *Bartonella* spp. may facilitate the ‘angiogenic switch’, determined through the bacterium’s signals from within the stroma, inflammatory infiltrate, and appearance of hypoxia, to ensure exponential tumor growth.

Though we did not test dogs with angiogenic or highly vascularized skin tumors in our study, future studies should aim to elucidate the differences in prevalence of *Bartonella* spp. in tumors of varying angiogenesis/vascularization.

Due to recent improvements in the diagnostic documentation of *Bartonella* spp., the bacterium has been identified in association with a myriad of oncogenic clinical manifestations in humans and animals. *Bartonella* spp. have been described as ‘versatile pathogens’ for the wide range of hosts the bacteria can infect and the plasticity of lifestyle within a particular host; this can result in a lack of consistency in detection of the bacterium in a specific oncogenic disorder. As such, it remains difficult to conduct population-based studies to elucidate *Bartonella* as a cause or co-factor of one specific disease. As a predominately ‘generalist’ genera of stealth pathogens, it is unplausible that the bacterium is the primary cause of each clinical manifestation described in every published report. However, through co-infections, co-morbid conditions, and an aging immunescenescenct
population, *Bartonella* spp. may contribute to the previously programmed course of disease and act as a ‘tipping point’ to accelerate the progression or severity of disease. It is therefore important to evaluate the compilations of case reports that have provided evidence of persistent infection with *Bartonella* spp. collectively in order to identify characteristics of tumorigenesis that are frequently detected with *Bartonella* spp. Though we did not detect *Bartonella* spp. in dogs with CCH, our negative results lend insight into the specificity of the bacterium’s role in cancer development and should be used to develop hypotheses related to the types of tumors *Bartonella* spp. may play a role in.

**Acknowledgements:** The authors would like to thank the histopathology personnel who facilitated sample collection for testing purposes and Tonya Lee for editorial assistance.

**Conflict of Interest:** In conjunction with Dr. Sushama Sontakke and North Carolina State University, Dr. Breitschwerdt holds U.S. Patent No. 7,115,385; Media and Methods for cultivation of microorganisms, which was issued October 3, 2006. He is the chief scientific officer for Galaxy Diagnostics, a newly formed company that provides diagnostic testing for the detection of Bartonella species infection in animals and in human patient samples. Dr. Ricardo Maggi has lead efforts to optimize the BAPGM platform and is the Scientific Technical Advisor and Laboratory Director for Galaxy Diagnostics.
References


Table 1. *Bartonella* PCR primers used in this study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>DNA Sequence 5’-3’</th>
<th>Target Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bspp 325s</td>
<td>CTT CAG ATG ATC ATC CCA AGC CTT CTG GCG</td>
<td><em>B</em> spp. ITS</td>
</tr>
<tr>
<td>Bspp 425s</td>
<td>CCG GGG AAG GTI TTC CGG TTT ATC C</td>
<td>Bspp. ITS</td>
</tr>
<tr>
<td>Bspp 438as</td>
<td>GCC CTC CGG GAT RAA YYR GWA AAC C</td>
<td>Bspp. ITS</td>
</tr>
<tr>
<td>Bspp 1110as</td>
<td>GAA CCG ACC ACC CCC TGC TTG CAA AGC A</td>
<td><em>B</em> spp. ITS</td>
</tr>
<tr>
<td>Pap31 1s</td>
<td>GAC TTC TGT TAT CGC CTT GAT TT</td>
<td><em>B</em> spp. pap31</td>
</tr>
<tr>
<td>Pap31 688as</td>
<td>CAC CAC CAG CAA MAT AAG GCA T</td>
<td><em>B</em> spp. pap31</td>
</tr>
<tr>
<td>Bkoehl-1s</td>
<td>CTT CTA AAA TAT CGC TTC TAA AAA TTG GCA TGC</td>
<td><em>B. koehlerae</em> ITS</td>
</tr>
<tr>
<td>Bkoehl-1125as</td>
<td>GCC TTT TTT GGT GAC AAG CAC TTC TCT</td>
<td><em>B. koehlerae</em> ITS</td>
</tr>
<tr>
<td>BVB p2s</td>
<td>TGC TTA ACC CAC TGT TGA GAA ACT CC</td>
<td><em>B. vinsonii</em> subsp <em>berkhoffii</em> ITS</td>
</tr>
<tr>
<td>BVB p1as</td>
<td>GAA AGC GCT AAC CCC TAA ACC GAT T</td>
<td><em>B. vinsonii</em> subsp <em>berkhoffii</em> ITS</td>
</tr>
<tr>
<td>gltA 781s</td>
<td>GGG GAC CAG CTC ATG GTG G</td>
<td><em>B</em> spp. gltA</td>
</tr>
<tr>
<td>gltA 1137as</td>
<td>AAT GCA AAA GAA CAG TAA ACA</td>
<td>Bspp. gltA</td>
</tr>
</tbody>
</table>

Table 2. PCR conditions used in this study for the amplification of *Bartonella* spp. target genes. All the reactions were performed using an Eppendorf Mastercycler epgradient (Eppendorf, Westbury, NY).

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Reaction Conditions</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bartonella</em> spp. ITS</td>
<td>95°C x 3 min, 94°C x 15 sec, 66°C x 15 sec, 72°C x 18 sec, 72°C 30 sec</td>
<td>55</td>
</tr>
<tr>
<td><em>Bartonella</em> spp. pap31</td>
<td>95°C x 3 min, 94°C x 15 sec, 62°C x 15 sec, 72°C x 18 sec, 72°C 30 sec</td>
<td>55</td>
</tr>
<tr>
<td><em>Bartonella</em> spp. gltA</td>
<td>95°C x 5 min, 95°C x 1 min, 56°C x 1 min, 72°C x 1 min, 72°C x 10 min</td>
<td>40</td>
</tr>
<tr>
<td><em>Bartonella</em> spp. ITS real-time</td>
<td>95°C x 2 min, 94°C x 10 sec, 66°C x 10 sec, 72°C x 10 sec, 95°C x 30 sec</td>
<td>55</td>
</tr>
</tbody>
</table>

Table 3. PCR results for Group I and Group II by gene target and primer set.

<table>
<thead>
<tr>
<th></th>
<th>qPCR* 325s-438as</th>
<th>*ITS 325s-1100as</th>
<th>*ITS 425s-1100as</th>
<th>pap31 1s-688as</th>
<th>gltA 781s-1137as</th>
<th><em>B. koehlerae</em> 1s-1125as</th>
<th><em>Bvb</em> p2s-p1as</th>
</tr>
</thead>
</table>

*Bartonella* spp. ITS gene target
Figure 1. Representative images of *Bartonella henselae* in tissues using indirect immunofluorescence and a commercial *B. henselae* monoclonal primary mouse antibody. Goat anti-mouse Cy-3 conjugated secondary antibody also used. Fields shown as 63x magnification on Zeiss LSM 710. Upper Left: Positive control rat lung injected with *B. henselae*. Upper Right: Positive control rat lung injected with *B. henselae* treated with secondary antibody only. Lower Left: *B. henselae* PCR positive CCH tissue showing positive staining. Lower Right: *B. henselae* PCR positive CCH tissue treated with secondary antibody only. Blue: Nuclei stained with DAPI; Red: *B. henselae*
Figure 2. Representative images of *Bartonella henselae* in tissues using indirect immunofluorescence and patient serum with polyclonal antibodies against *B. henselae Houston-1*. Fields shown as 63x magnification on Zeiss LSM 710. Upper Left: Positive control rat lung injected with *B. henselae*. Upper Right: Positive control rat lung injected with *B. henselae* treated with secondary antibody only. Middle Left: *B. henselae* PCR positive CCH tissue showing positive staining. Middle Right: *B. henselae* PCR positive CCH tissue treated with secondary antibody only. Lower Left: PCR negative CCH tissue showing positive staining. Middle Right: PCR negative CCH tissue treated with secondary antibody only. Blue: Nuclei stained with DAPI; Red: *B. henselae*;
CHAPTER 7.

CONCLUSIONS AND FUTURE DIRECTIONS

Excerpts as submitted for publication in Confronting Emerging Zoonoses: The One Health Paradigm.

Conclusions and future directions

Zoonotic disease represents a constant threat to humans, domestic animals, and wildlife. Transmission of pathogens into human populations and non-reservoir hosts from other animal species occurs from changes in the interactions between host, vector, and environment. The expansion of vector borne diseases in humans and a variety of mammalian hosts and arthropod vectors draws attention to the need for enhanced diagnostic techniques for documenting infection in hosts, effective vector control, and treatment of individuals with associated diseases. Key challenges in combating zoonotic infections have to do with the non-specificity and similarity of symptoms caused by a variety of infections. This highlights the need for continued development of high-quality laboratory based diagnostics so as to facilitate the rapid identification of infections for proper surveillance and appropriate treatment. Use of the C6 peptide in the SNAP DX test kit has resulted in a highly sensitive and specific commercial enzyme immunoassay used for in-house detection of antibodies against *B. burgdorferi* in blood, serum, or plasma. Veterinarians can use this in-house test kit to determine if a dog has been exposed to 4 vector-borne pathogens. Our research found that veterinarians’ perception of the risk of borreliosis in North Carolina was consistent with recent scientific reports pertaining to geographic expansion of borreliosis in the state. As knowledge of the epidemiologic features of borreliosis in North Carolina continues to evolve, veterinarians should promote routine screening of dogs for *Borrelia burgdorferi* exposure as a simple, inexpensive form of surveillance that can be used to better educate their clients on the threat of transmission of borreliosis in transitional geographic regions. Future studies should continue to evaluate the seroprevalence of *B. burgdorferi* in dogs to better understand
the changing epidemiology of borreliosis throughout the country. Increased use of rapid, accurate diagnostic tests will afford researchers and clinicians with the ability to conduct more reliable epidemiologic and treatment-related studies and will more accurately characterize clinical outcomes associated with *Borrelia* infection.

Rapid changes in test sensitivity for the detection of *Bartonella* spp., in conjunction with enhanced understanding of the microbial ecology of these bacteria, have resulted in the expansion of the *Bartonella* genus from two species known to exist prior to the 1990s, to 30-40 *Bartonella* species that are currently reported in the literature (Kosoy et al., 2012). The use of polymerase chain reaction (PCR) has given researchers and microbiologists the ability to rapidly detect and identify the DNA of *Bartonella* spp. in a variety of blood, effusion, and tissue specimens. Our studies have indicated that a combinatorial approach that independently tests blood, serum, and enrichment culture by PCR can improve the diagnostic documentation of *Bartonella* spp. bacteremia in animals and humans. Due to the potential for relapsing bacteremia associated with *Bartonella* spp. infected humans, as occurs in cats and rodents, we found that serial testing of blood samples from a three-day collection period further enhances the diagnostic sensitivity of *Bartonella* spp. detection in bacteremic patients. However, it still remains difficult to detect *Bartonella* in tissue and blood samples from non-reservoir hosts (incidental infections). Though combinatorial and serial testing approaches increase diagnostic sensitivity of *Bartonella* testing, it is imperative to maintain a high degree of specificity during testing, and specificity is inherently decreased using these testing methods; as the number of diagnostic tests performed increases, so does the likelihood of obtaining a false positive result through human error. Improvements in culture media
could further improve the detection of *Bartonella* in various samples, without decreasing the specificity of the overall PCR platform through the addition of multiple tests that could increase the probability of obtaining a result in error. Microbiologist should continue the challenging effort to improve diagnostic testing by more sensitive PCR assays, which is unlikely, or methods that increase or concentrate the bacteria prior to PCR testing. In the interim, microbiologists and diagnostians should establish *a priori* diagnostic criteria in terms of the number of tissue sections/blood draws that will be tested and the number of gene targets/primer sets to be utilized, based upon the animal species and tissue type that will be processed. A standard, but flexible, case definition, in addition to *a priori* strategies pertaining to the number of tissue sections samples tested and the number of PCR targets utilized, should be created in order to maintain a consistent level diagnostic sensitivity and specificity within laboratories around the world.

A variety of animals have been identified as reservoirs for different *Bartonella* species. For example, recent reports suggest that *B. henselae* may be a multi-host and multi-vector pathogen, rather than a bacterium that is limited to cats and their fleas. Substantial antigenic variation among *B. henselae* strains may allow the bacterium to easily exploit new hosts and vectors, (Pedersen et al., 2005), but also appears to contribute to virulence differences among these strains. Differences in *Bartonella* spp. outer membrane proteins, presence of flagella in some *Bartonella* sp., and varying other virulence factors across/within species interact with the host immune response and in part determine the subsequent spectrum of disease expression that may occur following pathogen transmission. We found that antibody response, determined through IFA testing, does not accurately reflect exposure
or current infection with a specific *Bartonella* spp.. Therefore, IFA results do not appear to be reliable for evaluating the epidemiological features of Bartonellosis. The fastidious nature of *Bartonella*, as previously described, creates numerous obstacles in conducting epidemiologic studies to determine associations between infection and disease manifestations. Due to difficulties in diagnostic detection, the prevalence estimate of a *Bartonella* sp. is often low in a given population. The statistical analysis of a low prevalence estimate can result in a violation of the necessary assumptions for the utilization of a specific statistical test; as a result, analysis cannot be conducted or the interpretation of the statistic may be inaccurate. It can be difficult to achieve a high enough bacterial prevalence of a single *Bartonella* sp. in a population to conduct accurate and reliable statistical analysis while controlling for confounders. As such, many investigations result in epidemiologists analyzing potential associations with ‘any *Bartonella* sp. infection’. As there is substantial inherent antigenic variation across and within *Bartonella* species, epidemiologic studies should examine the different bacterial variants, so as to be able to accurately investigate the associations between different pathologies and each specific variant. Until there are additional improvements in *Bartonella* diagnostic test sensitivity, epidemiological studies will remain analytically challenging due to limitations inherent in small sample sizes and low probability events. Therefore, epidemiologic studies may contain great degrees of variability in the estimates of odds of infection risk and disease manifestation associations. Potential associations may be underestimated, have large confidence intervals, or be irreproducible as a result. In one study, we were able to examine disease manifestations associated with *B. henselae* and *B. koehlerae*, specifically. We used the BAPGM enrichment blood culture
platform in a large population of humans to detect a high prevalence of bacteremia, so as to conduct our epidemiologic analysis. Studies designed to evaluate Bartonellosis in a large cohort of humans/animals will allow researchers to differentiate associations among *Bartonella* spp, and preferably strains.

As previously described, a frustrating component in studying the epidemiology of *Bartonella* infection stems from the decreased sensitivity of detection in non-reservoir hosts, including sick dogs and humans. Therefore, detection of the bacterium within an infected individual can be inconsistently detected. *Bartonella* has been detected in humans or animals suffering from a wide spectrum of seemingly unrelated pathological disorders. The inconsistency in detection of these bacteria creates a potentially inaccurate appearance that the presence of *Bartonella* in tissues/blood is random, thereby implicating the bacterium as an unconcerning passerby in disease pathogenesis. This would suggest that *Bartonella* spp. does not cause every disease in the reported literature. Though it remains difficult to detect *Bartonella* spp. in clinical/surveillance samples, study the mechanism of pathogenesis, and conduct epidemiologic investigations, the common features of the pathology found in association with *Bartonella* infection, in addition to the body of literature pertaining to angiogenesis, lends insight into the potential pathological role of *Bartonella* in these diverse disorders. Previous studies that describe the molecular pathogenesis of *Bartonella* have clearly implicated this bacterium as a cause of angiogenesis, and the molecular mechanism by which *Bartonella* participates in angiogenesis has been studied extensively (Koehler & Tappero, 1993; Pulliainen & Dehio, 2012; Dehio C, 2005; Minnick et al., 2003; Cerimele et al., 2003; Kempf et al., 2001; Kempf et al., 2005). In one study described in this thesis, we
detected a very high molecular prevalence of *Bartonella* spp. in patients examined by a single rheumatologist for a spectrum of rheumatologic diseases. Angiogenesis has recently been incriminated as a key player in the pathogenesis of several rheumatic diseases, including rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, systemic sclerosis, systemic lupus erythematosus, and vasculitides, and it has been hypothesized that macrophages are pivotal cells in the promotion of angiogenesis in rheumatic disease (Haywood et al., 2003; Bonnet & Walsh, 2005; Maruotti et al., 2013). *Bartonella* is able to invade and to recruit macrophages through the secretion of MCP-1, which subsequently secrete VEGF, an important angiogenic factor. VEGF is thought to induce endothelial cells to express adhesion molecules, allowing monocytes and lymphocytes to migrate into the extracellular matrix. Additionally, studies have shown that *Bartonella* can induce plasminogen activator, which is thought to be responsible for the degradation of the extracellular matrix in rheumatoid disease (Garcia et al., 1992; Maruotti et al., 2013). Recent research has concentrated on the role of macrophage derived angiogenic factors in rheumatic disease, and it is possible that *Bartonella* induced angiogenic factors act as a driver in the progression of rheumatic disease, which may explain our high prevalence of *Bartonella* spp. DNA and antibody reactivity in our study population of individuals with rheumatic disease. In that study, we also found an association between *B. henselae* and neurologic symptoms and previous consultation with a neurologist. Importantly, this observation lends further support to the large collection of published cases of Neurobartonellosis that are reported in the scientific literature (Breitschwerdt et al., 2013). It is now well known that angiogenic factors, specifically VEGF, play a role in the birth of new neurons (neurogenesis), mitigation
of neural injury (neural protection), but also in the pathogenesis of a variety of neurologic diseases (Greenberg & Jin, 2005). Observations have shown that chronic overexpression of VEGF can result in breakdown of the blood brain barrier (BBB) (Argaw et al., 2008; Dobrogowska et al. 1998; Zhang et al., 2000). Studies in animals have shown that a breakdown in the BBB results in transcriptional changes in the neurovasculature that lead to neuron dysfunction and degeneration (Shlosberg et al., 2010). In Chapter 6 of my thesis, I did not find a higher prevalence of Bartonella spp. in CCH (a tumor of non-angiogenic etiology) compared to control skin tumors. Though it is unlikely that Bartonella spp. plays a role in the development of CCH, Bartonella spp. DNA has been amplified from a myriad of oncogenic processes, including inflammatory breast cancer, epithelioid hemangioendothelioma (EHE), hemangiopericytoma, splenic hemangiosarcoma, and splenic fibrohistiocytic nodules (Varanat et al., 2011; Fernandez et al., 2012; Breitschwerdt et al., 2009). A majority of these cancers have a high degree of angiogenic activity or vascularization associated with each tumor’s progression. Bartonella spp. may be far more ubiquitous in humans than has been historically realized. If true, these ‘resident’ bacteria could turn-on the ‘angiogenic switch’, determined by bacterial-induced inflammatory infiltrate and HIF-1α signaling, to stimulate expression of VEGF to increase oxygen delivery, via endothelial cells, to generate new blood vessels to supply areas with additional oxygen and increase vascularization and exponential tumor growth (Dehio C, 2005; Kempf et al., 2005). As described above, angiogenesis or angiogenic factors play an important and potentially central role in a variety of pathological processes, ranging from cancer, to rheumatic disease, to neurologic disease. Due to the wide breadth of important processes that
angiogenic factors are able to affect, it’s perhaps unsurprising that *Bartonella* can be found in association with a large range of pathologies, and it is possible that this bacterium facilitates the progression of all these pathologies through its angiogenic properties. And because angiogenic factors can be induced by a variety of other disease processes and infectious agents that are unrelated to *Bartonella*, it would also be unsurprising that *Bartonella* are not always detected in these disorders. The ability of *Bartonella* spp. to infect a myriad of cell types, including endothelial cells, erythrocytes, macrophages, dendritic cells, CD4+ progenitor cells, potentially resulting in dissemination to anywhere in the body, would also implicate the bacterium as an influence in a spectrum of VEGF-related disorders (Breitschwerdt et al., 2004). Future studies should be conducted to better elucidate the role of *Bartonella* or *Bartonella*-induced-angiogenesis in different pathological disorders of animals and human patients.

Prior to 1990, *Bartonella* infection had never been reported in human or animal species in North America. The AIDS epidemic and subsequent recognition of *Bartonella*-induced vasoproliferative disorders in immunocompromised individuals has helped to unveil *Bartonella henselae* as a complex, stealth pathogen of substantial medical importance. In turn, attention has been drawn on the varied importance of the *Bartonella* genus in medical microbiology. The difficulties in studying *Bartonella* spp. risk factors, pathology, and potential treatment options lie in the bacterium’s generalist and versatile host affiliation and its ability to induce a spectrum of disease expression in a variety of species that ranges from self-limiting and benign to chronic and complex. *Bartonella*'s strain variability in combination with the nuances of a specific host response (influenced by various nutritional,
genetic and other medically relevant factors) creates an unpredictable pattern of disease expression within or across infected species. As such, it remains difficult to reproduce epidemiological study results and to date an accurate and medically applicable animal model of *Bartonella* infection has not been established. Furthermore, as a fastidious organism, it remains technically difficult to detect and diagnose cases of naturally occurring Bartonellosis within animal and human patient populations; misclassification of true cases of disease as false negatives limits the ability of researchers to conduct controlled and insightful epidemiologic studies that will better elucidate risk factors for infection or subtle manifestations of disease. To combat the difficulties in studying this complex pathogen, a One Health approach that combines clinical, microbiological, and pathological observations across animal genera and species has proved necessary to further elucidate the ability of *Bartonella* spp. to induce disease processes in humans and animals. A broad scale analysis of *Bartonella* literature has observed that similar pathologies following *Bartonella* infection can be seen across multiple mammalian species. The DNA of *Bartonella* species has been detected in both humans and dogs with clinically similar and histolopathologically confirmed vasoproliferative lesions; granulomatosus inflammation has occurred in naturally infected cats, dogs, and humans. And most notably, *Bartonella* induced culture-negative endocarditis has been observed across various mammalian species, including humans, dogs, cats, and cattle (Breitschwerdt et al., 2013). These observations have proved crucially insightful to researchers and medical professionals that have previously struggled to attribute disease causation to *Bartonella* spp.. The importance of a comparative examination of the biological and pathological behavior of *Bartonella* and other stealth pathogens across different
animal/arthropod species cannot be understated. As humans, pets, domestic animals, and wildlife are expanding their number of overlapping habitats, a close integration of veterinary, medical, and public health professionals is a necessary framework of disease investigation for pathogens shared between humans and animals, with *Bartonella* spp. as only an example.
References


APPENDIX
APPENDIX

Lyme Disease in North Carolina
A Survey of Veterinarians
NCSU College of Veterinary Medicine

1. How many cases of canine Lyme disease have you diagnosed in the last year?
   ☐ 1-5
   ☐ 6-25
   ☐ 26-50
   ☐ Greater than 50
   ☐ None

2. Do your clients reside in (Check all that apply):
   ☐ Rural environments
   ☐ Urban environments
   ☐ Suburban environments

3. Do you believe Lyme disease is endemic in North Carolina?
   ☐ No
   ☐ Yes

4. Do you believe that any dog has acquired Lyme disease in North Carolina?
   ☐ No
   ☐ Yes

5. Do you use the SNAP 3 DX or SNAP 4 DX results when confirming a clinical diagnosis of Lyme disease in dogs?
   ☐ No
   ☐ Yes

6. What percentage of your dog patient population has a history of tick attachment?
   ☐ <10%
   ☐ 11-25%
   ☐ 26-50%
   ☐ 51-75%
   ☐ 76-100%
7. Why do you test dogs for Lyme disease? (Check all that apply):
☐ I only test sick dogs for suspicion of Lyme disease
☐ I test healthy dogs for routine surveillance for Lyme disease transmission
☐ I only test for Lyme disease at owner's request
☐ I do not test for Lyme disease
☐ Other __________________

8. If you obtained a positive SNAP test result for Lyme disease in a pet dog, do you believe that your client is at-risk for acquiring *B. burgdorferi* in North Carolina?
☐ No
☐ Yes
☐ I have not had a SNAP positive dog in my practice

9. Have you identified *B. burgdorferi* SNAP positive dogs in your practice that you believe acquired infection in a Lyme disease endemic state outside of North Carolina?
☐ No
☐ Yes

10. Of the dogs you diagnose with Lyme disease, what proportion do you believe were exposed to *B. burgdorferi* inside the state of NC and what proportion were exposed outside the state. Pick which answer best describes your belief:
☐ Essentially all were exposed in NC
☐ The majority (>50%) was exposed in NC
☐ About half were exposed in NC and half elsewhere
☐ Only a few were exposed in NC
☐ None were exposed in NC

11. Which best describes your vaccination practices against Lyme disease:
☐ I routinely recommend dogs in my practice to be vaccinated.
☐ I recommend vaccination for dogs with unusual risk factors (ex. travel to highly endemic area like Connecticut to go deer hunting).
☐ I only vaccinate against Lyme disease if my client requests it.
☐ I do not recommend vaccination

12. What percentage of your clients follows your recommendations for the use of flea and tick preventative products?
☐ <10%
☐ 11-25%
☐ 26-50%
☐ 51-75%
☐ 76-100%
13. If you test healthy dogs for exposure to *B. burgdorferi* with a C6 SNAP test, what proportion are positive?
☐ Do not or very rarely test healthy dogs
☐ Dogs are rarely positive
☐ Dogs are frequently positive
☐ The majority (>50%) of dogs are positive
☐ Nearly all dogs are positive

14. If you test dogs you suspect may have Lyme disease for exposure *B. burgdorferi* with a C6 SNAP test, what proportion are positive?
☐ Do not or very rarely test dogs
☐ Dogs are very rarely positive
☐ Dogs are frequently positive
☐ The majority (>50%) of dogs are positive
☐ Nearly all dogs are positive