ABSTRACT

CHERRY, NATALIE ANNE. Ecological Diversity of *Bartonella*. (Under the direction of Edward B. Breitschwerdt and Sam L. Jones.)

The *Bartonella* genus is composed of over 22 species or subspecies of gram-negative, fastidious, facultative intracellular bacteria that have been identified as the cause of, or associated with, numerous clinical manifestations in humans and dogs. *Bartonella* species have also been detected in a wide range of other mammalian species, including ruminants, horses, rodents, and sea mammals. Individual *Bartonella* spp. have co-evolved with specific mammalian hosts, such as *B. bacilliformis* and *B. quintana* with humans, *B. henselae* with domestic cats, *B. bovis* with cattle, and presumably, *B. vinsonii* subsp. *berkhoffii* with canids. When an organism infects a host without causing clinical pathology, the host species is considered a reservoir, a mammalian niche that allows the pathogen to survive, propagate, and facilitate dispersal to other hosts. The adaptation of *Bartonella* spp. to a broad range of mammalian hosts, as well as vectors which transmit these bacteria, is representative of the complexity of the ecological diversity of members of the *Bartonella* genus.

To further define this ecological diversity and implications of this diversity to human and veterinary medicine, a detailed review of the literature is provided relative to the host, vector, physiological, geographic, and genetic ecology. Furthermore, three studies were conducted to determine the prevalence of *Bartonella* in various mammalian populations, including cattle, dogs, humans, and horses.

The first study investigated the prevalence of *B. bovis* in cattle, the natural reservoir

host of this *Bartonella* sp., from North Carolina, with emphasis on development of new PCR primers to enhance detection of *B. bovis* in these ruminants (Cherry et al., 2009). Results of this study revealed a molecular prevalence of *Bartonella* in over 80% of cattle tested and reported the first detection of *B. henselae* in cattle. Next, a study was conducted to determine if *Bartonella* infection was the cause or a co-factor in illness in a veterinarian/dog breeder in Virginia and her sick Doberman pinschers (Cherry et al., 2011a). Evidence of *Bartonella* infection and/or exposure was detected by serological, molecular, and/ or microbiological methods in the breeder and five of her dogs. These results, together with the clinical manifestations and environmental factors described, indicated *Bartonella* spp. likely played a primary or secondary role in the breeder and dogs' illnesses. Lastly, a study was performed to identify *Bartonella* infection in four horse populations, healthy adults (49), sick foals (15), adults with musculoskeletal manifestations (26), and adults presenting with colic (96), by serological, molecular, and microbiological methods which is the first *Bartonella* prevalence study conducted in horses to our knowledge. *Bartonella* DNA was detected in the healthy (1/49), sick foal (3/15), and musculoskeletal disease (5/26) populations, though there was no evidence of *Bartonella* infection in any horses within the colic population. By statistical analysis, *Bartonella* infection was most frequent in sick foals and adult horses with musculoskeletal manifestations, indicating further studies are warranted to determine the pathogenic potential of bartonellosis in equids.

Development of detection methods for diagnosis of *Bartonella* infection and epidemiological evaluation of *Bartonella* prevalence in mammalian and vector populations

has demonstrated the ubiquitous nature of *Bartonella* spp. Without these developments,

researchers, clinicians, veterinarians, and the general public would not be aware of the

ecological diversity, pathogenic potential, or risk factors of *Bartonella* infection in humans or

animals. Further investigation is warranted into the mechanisms of transmission from vectors

to mammals, the means by which *Bartonella* spp. survive and thrive in these vectors and

hosts, and the implications of these factors relative to public health.

Ecological Diversity of *Bartonella*

by
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A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Comparative Biomedical Sciences

Raleigh, North Carolina

2012

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DEDICATION

This dissertation is dedicated to my parents, Elwood and DiAnna Cherry, and my entire

family for their support, encouragement, and belief in me.

BIOGRAPHY

Natalie received her Bachelor of Science in Biology from North Carolina State University.

ACKNOWLEDGMENTS

My sincerest gratitude goes out to all of my committee members for their kindness, support, and encouragement throughout my PhD career at NC State. I would like to thank Dr. Ed Breitschwerdt, my mentor and advisor, whose unwavering support has allowed me to grow as a student, a scientist, and a person. I would also like to thank my other committee members, Dr. Sam Jones, my co-advisor, Dr. Ricardo Maggi, and Dr. Jennifer Davis, for their advice, encouragement, technical expertise, and clinical knowledge.

Thank you to the former and current faculty and staff of the Intracellular Pathogens Research Laboratory and Vector Borne Disease Diagnostic Laboratory for their belief in me and technical assistance, and for an enjoyable experience while working on my degree, including Dr. Pedro Diniz, Dr. Belen Cadenas, Dr. Nandhakumar Balakrishnan, Dr. Patricia Mascarelli, Julie Bradley, and Barbara Hegarty. I was very blessed to also have the support of the graduate students in the CBS program, including Mrudula Varanat, Jingjing Li, Rita Simoes, and Elizabeth Pultorak. An additional thank you to Dr. Shila Nordone, Melissa D'Annibale-Tolhurst, and Mitsu Suyemoto for their expertise and encouragement.

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LIST OF ABBREVIATIONS

PCR – polymerase chain reaction

CSD – Cat Scratch Disease

BAPGM – *Bartonella* alpha-Proteobacteria growth medium

T4SS – Type IV secretion system

BEP – *Bartonella* effector protein

BadA – *Bartonella* adhesin protein A

TAA – trimeric autotransporter adhesin

HPC – hematopoietic progenitor cells

TLR – toll-like receptor

LPS – lipopolysaccharide

PFGE – pulse-field gel electrophoresis

MLTS – multi-locus type sequencing

Vomp – variably-expressed outer membrane proteins

BrpA – *Bartonella* repeat protein A

Chapter 1. Ecological Diversity of *Bartonella*

Ecological Diversity of *Bartonella*

Introduction

Microbial ecology is the study of the relationships of microorganisms with each other and their environment. The genus *Bartonella* belongs to the class alpha-Proteobacteria and is comprised of pleomorphic, gram-negative, fastidious, facultative intracellular bacteria which are primarily vector-borne. Research over the past two decades has elucidated the vast ecological diversity of this genus with respect to host, vector, geographic, and genetic ecology.

Over 22 species, subspecies, or *Bartonella* with *Candidatus* status have been identified worldwide in a variety of hosts, including humans and domestic and wild animals, including dogs, cats, deer, horses, cows, marine mammals, feral swine, and numerous rodent species (Breitschwerdt and Kordick, 2000; Boulouis et al., 2005; Maggi et al., 2005, 2008; Chomel et al., 2006; Beard et al., 2010). Many *Bartonella* species have been shown to cause, or be associated with, clinical pathology in some of these hosts.

The symbiotic relationships of *Bartonella* species with their respective mammalian hosts is complex. This is due to their promiscuous, fastidious nature and the numerous clinical manifestations they can induce upon infection. Therefore, the study of *Bartonella* species in an experimental setting, *in vitro* and *in vivo*, does not typically mimic natural infection. Advancements in serological, molecular, and microbiological techniques has enabled better understanding of the impact of *Bartonella* on human and animal health and further exploration of therapeutic strategies in treatment of patients with bartonellosis.

Host Ecology

With the exception of *B*. *bacilliformis* and *B*. *quintana*, which are classified as "specialists" because they generally only utilize humans as reservoirs, most *Bartonella* species are referred to as "generalists" because they are able to infect and persist in numerous animal hosts. Although *B*. *quintana* has been categorized as a "specialist", there is evidence to support the possibility that this *Bartonella* sp. may have adapted the means to utilize another mammalian species, the domestic cat, as a reservoir host (Drancourt et al., 1996; La et al., 2005; Breitschwerdt et al, 2007a). For example, *B*. *henselae* is classified as a "generalist" because though the cat is considered the primary reservoir of this species, *B*. *henselae* DNA has been identified in many other mammalian species (Breitschwerdt and Kordick, 2000; Maggi et al., 2005, 2008; Valentine et al., 2007; Harms et al., 2008; Jones et al., 2008; Chomel et al., 2009; Beard et al., 2010; Breitschwerdt et al., 2010a).

In contrast to other vector-borne diseases which are typically limited to one host, many *Bartonella* species have the ability to be transmitted from a reservoir host to an accidental host. Interestingly, the reservoir host of one *Bartonella* sp. may be infected with other *Bartonella* spp., resulting in its utilization by *Bartonella* as an accidental host as well as a reservoir. *Bartonella* infection may at times be readily cleared by these hosts, but it has demonstrated the ability to evade some host immune systems (Vermi et al., 2006; Popa et al., 2007). In cases where the bacteria are able to accomplish this, chronic infection may result, leading to pathophysiological complications in accidental hosts by inadvertent transmission. *Bartonella* spp. have been characterized as "stealth" pathogens for their ability to evade mammalian host innate immune systems and to infect cells, such as erythrocytes, in these

hosts resulting in chronic bacteremia and further evasion of the adaptive immune system (Merrell and Falkow, 2004). This strategy is useful for *Bartonella* as a vector-borne blood pathogen because in order for a particular bacterium to be successful, it must evolve with a primary mammalian source that is able to sustain the bacteria, essentially forming a "commensal" relationship with its host (Dale and Moran, 2006). The establishment of a primary reservoir host for *Bartonella* spp. is key because it allows for persistent infection at a high level of bacteremia for a prolonged period of time in a host, permitting uptake by blood-feeding arthropods and subsequent transmission to other hosts.

Reservoir hosts

Humans

Humans are the known reservoir for two species of *Bartonella*, *B. bacilliformis* and *B. quintana*. *B. bacilliformis* is the causative agent of Carrion's disease, characterized by massive hemolytic anemia, which is found primarily in the Andes region of Peru, Columbia, and Ecuador (Ihler, 1996). It was the only member of the genus *Bartonella* until 1993, when the *Rochalimaea* and *Bartonella* genera were merged (Brenner et al., 1993). Alberto Barton first identified this bacteria in erythrocytes in 1905, and Towsend first documented the sandfly, *Lutzomyia verrucarum*, as the natural vector of *B. bacilliformis* in 1913. Before the etiologic agent of this human bartonellosis agent was identified, there is evidence that pre-Columbian natives of South America were affected by disease caused by *B. bacilliformis* (Alexander, 1995; Minnick and Battisti, 2009). Even today, cases of Carrion's disease are

identified in the Andes region of South America (Gray et al., 1990; Cooper et al., 1997; Ellis et al., 1999a; Kosek et al., 2000; Maco et al., 2004), despite medical advances.

Bartonella quintana is the causative agent of trench fever which is transmitted by the human body louse (Pediculus humanus humanus). Trench fever, also known as five day fever characterized by recurrent, cyclical fever, headaches, dizziness, and leg pain, was first documented in soldiers during World War I with an estimate of more than one million affected individuals at that time (Maurin and Raoult, 1996). Cases were again reported during World War II (Kostrzewski, 1949).

Interestingly, it appears that the clinical manifestations of B. quintana infection are quite variable, presumably due to individual host responses. McNee et al. documented two different clinical presentations in individuals with trench fever; one group presented with classic characteristics of trench fever, whereas the other group presented with shorter initial illnesses but with frequent relapses (McNee et al., 1916). Other pathophysiological consequences of B. quintana infection in humans include chronic bacteremia (Kostrzewski, 1949; Foucault et al., 2002; Foucault et al., 2003; Capo et al., 2003), endocarditis (Houpikian and Raoult, 2005), bacillary angiomatosis (Koehler et al., 1992; Santos et al., 2000; Sala et al., 2005), and lymphadenopathy (Raoult et al., 1994; Drancourt et al., 1996). B. quintana has re-emerged as a human pathogen in recent years with risk factors including homelessness, low socioeconomic status, alcoholism, body lice infestation, and, potentially, exposure to cats (Spach et al., 1995; Jackson et al., 1996; Koehler et al., 1997; Spack and Koehler, 1998; Brouqui et al., 1999; La Scola and Raoult, 1999; Foucault et al., 2006).

Cats

The domestic cat is the known reservoir host of *B. henselae*, the agent of CSD, but cats can also be infected with *B. quintana* (Drancourt et al., 1996; Parrot et al., 1997; Scully et al., 1998; La et al., 2005; Breitschwerdt et al., 2007a) and potentially serve as reservoirs for *B. clarridgeiae* (Clarridge et al., 1995; Kordick et al., 1999) and *B. koehlerae* (Droz et al., 1999; Rolain et al., 2003a; Avidor et al., 2004). A high prevalence of chronic bacteremia in reservoir populations allows *Bartonella* spp. to be transmitted more effectively from host to host, as demonstrated in cat populations worldwide.

B. henselae was first isolated from cats in the early 1990s (Regnery et al., 1992a; Koehler et al., 1994). Since that time, further research, including epidemiological data and experimental infection and transmission studies, have proven the cat as a competent mammalian reservoir host of *B. henselae*. A study by Koehler et al. in 1994 demonstrated cats were able to remain *Bartonella* bacteremic for months at a time, and the authors also isolated *B. henselae* from 41% of 61 cats in the San Francisco Bay region of California. Another study in northern California documented *B. henselae* bacteremia in 39.5% of cats with an overall seroreactivity of 81% against *B. henselae* antigens (La Scola et al., 2002); additionally, the *B. henselae* antibody titers were higher in the bacteremic cats than in the nonbacteremic ones. Authors of this study also evaluated the population of cats tested for risk factors for *B. henselae* infection, and they found stray or impounded cats, younger cats, and flea-infested cats were more likely than pet cats to be bacteremic.

A study in France documented 53% of stray cats as bacteremic with either *B. henselae* or *B. clarridgeiae* (Heller et al., 1997), and shortly thereafter, *B. henselae*

seroprevalence in pet and feral cats in the United Kingdom was reported as 40.6 and 41.8%, respectively (Barnes et al., 2000). More recently, *B. henselae* or *B. clarridgeiae* DNA was amplified from 47.8% of cats from Alabama and Virginia and from 59.8% of the fleas collected from those cats (Lappin et al., 2006).

Since *Bartonella henselae* can be transmitted from cats to humans by bite or scratch, potentially causing clinical illness in the non-reservoir human host, other groups have used molecular methods to test for *Bartonella* DNA in cat samples other than blood. Recently, Kim et al. documented PCR evidence of *B. henselae* from more than 40% of the saliva, nails, and blood samples of feral cats in Korea (Kim et al., 2009). In the US, blood and tissue samples from feral cats were tested for *Bartonella* DNA, and either *B. henselae* or *B. clarridgeiae* was amplified from 31.4% of skin biopsies, 17.6% of both gingival and claw bed swabs, and 56.9% of blood samples (Lappin and Hawley, 2009).

Historically, studies involving cats experimentally-infected with *B. henselae* have yielded comparable results in regard to bacteremia but more contradictory results in clinical presentation. Two studies documented no signs of clinical illness in cats inoculated with *B. henselae* (Regnery et al., 1996; Abbott et al., 1997), similar to cats naturally-infected with *B. henselae* (Regnery et al., 1992a; Kordick et al., 1995). Other studies resulted in febrile, self-limiting clinical illness in experimentally-infected cats. One study demonstrated bacteremia in young cats via intravenous inoculation of *B. henselae* as early as 2 weeks post-infection, persisting up to 32 weeks (Guptill et al., 1997). *B. henselae*-inoculated cats developed fever and anorexia, though these symptoms were typically self-limiting, lasting only 2-7 days. Following euthanization, lesions were demonstrated in organs of the cats by histopathology,

even though they recovered from initial infection and were outwardly healthy. Though this experiment does not mimic the natural route of infection of cats with *B. henselae* by cat fleas, these results show that cats are able to recover from initial infection and remain clinically healthy while sustaining a high level of bacteremia, as these authors concluded. Another study by Guptill et al. in 1998 sought to determine if *B. henselae* infection in female cats resulted in perinatal transmission and/or reproductive effects. These authors found that bacteremic mother cats inoculated with *B. henselae* intradermally did not transfer the bacteria to their offspring but did report reproductive failure, including delayed conception and failure to conceive.

Kordick et al. inoculated specific-pathogen free cats with *B. henselae* and/or *B. clarridgeiae* in 1999 by blood transfusion which produced similar results to the previous study, including acute, self-limiting illness a few days post-*B. henselae* or *B. clarridgeiae* inoculation and histopathological lesions in major organs. Authors of another study documented similar but more severe clinical illness in cats inoculated intradermally with a known pathogenic strain of *B. henselae* (O'Reilly et al., 1999). These authors concluded the variable results between the studies may have resulted from hereditary components, inoculation routes, or, more likely, virulence of the *B. henselae* strain used as the inoculum. Inoculation route is a critical variable when evaluating pathogenesis of a bacteria in a mammalian host experimentally, and studies delivering *B. henselae* intradermally have been the most effective, yielding a larger percentage of cats infected as well as a higher level of *B. henselae* bacteremia (Abbott et al., 1997; O'Reilly et al., 1999). This is consistent

considering *B. henselae* transmission to cats is essentially intradermal through the bite of cat fleas.

B. clarridgeiae was first isolated from the pet cat of an HIV-positive patient (Clarridge et al., 1995). The worldwide distribution of *B. clarridgeiae* isolates is inconsistent, ranging from no greater than 10% in some studies (southeastern United States, Japan, Taiwan) up to 17 to 36% in some regions (France, Philippines, the Netherlands, Thailand). The high prevalence of *B. clarridgeiae* bacteremia in some regions provides evidence to support cats as a reservoir host for this *Bartonella* sp.

B. koehlerae is a relatively newly-recognized *Bartonella* species, and one difficulty researchers have encountered while seeking to further characterize this *Bartonella* species is its fastidious nature (Droz et al., 1999; Avidor et al., 2004). *B. koehlerae* has only been isolated from one cat in Israel (Avidor et al., 2004), one cat in France (Rolain et al., 2003a), and two cats in California (Droz et al., 1999). Experimental inoculation of cats with *B. koehlerae* isolated from one of the cats from California resulted in an average 74 day bacteremia in 4 cats with a specific antibody response to *B. koehlerae* antigens, and they were asymptomatic (Yamamoto et al., 2002). The kitten from which *B. koehlerae* was isolated in France was suspected of inducing CSD in its owner (Rolain et al., 2003a). Collectively, these findings implicate cats as a likely reservoir for *B. koehlerae*.

Given the high prevalence of *Bartonella henselae* bacteremia in domestic cats worldwide, most often without clinical implications, it is clear that the *B. henselae*, its vector, the cat flea (*Ctenophalides felis*), and the domestic cat have adapted a strong evolutionary

relationship. Additionally, there is evidence to support the domestic cat as a reservoir host for *B. clarridgeiae* and *B. koehlerae*.

Canids

Canids, including both domestic dogs and wild canids, such as the coyote, have been implicated as reservoir hosts for *Bartonella vinsonii* subsp. *berkhoffii*, though domestic dogs can develop pathophysiological consequences from *B. vinsonii* subsp. *berkhoffii* infection. Evidence to support the domestic dog as a host reservoir for this *Bartonella* sp. includes the ability of *B. vinsonii* subsp. *berkhoffii* to cause persistent, prolonged bacteremia in dogs (Kordick and Breitschwerdt, 1998; Breitschwerdt and Kordick, 2000).

Coyotes (*Canis latrans*) are thought to be a key reservoir host for *B. vinsonii* subsp. *berkhoffii* in the United States. Chang et al. demonstrated that 35% of coyotes from California were seroreactive to *B. vinsonii* subsp. *berkhoffii* antigens (Chang et al., 1999). A year later, the same group reported a 76% seroprevalence of this *Bartonella* sp. in coyotes tested from California, and molecular detection of *B. vinsonii* subsp. *berkhoffii* was documented in 28% from this population (Chang et al., 2000). *B. vinsonii* subsp. *berkhoffii* prevalence is typically higher in wild canids than domestic dogs, and a likely reason for this is that domestic dogs are less likely to have frequent vector exposure, thereby decreasing their risk for *Bartonella* infection.

In Thailand, 38% of sick dogs had antibodies to *B. vinsonii* subsp. *berkhoffii*, though no *Bartonella* DNA was PCR-amplified from any of these dogs (Suksawat et al., 2001). Seropositivity was also documented in 38% of dogs from Morocco (Henn et al., 2006), with

a higher seroprevalence in stray dog populations. Lower *B. vinsonii* subsp. *berkhoffii* seroprevalence has been reported in other locations, including Israel where only 10% of sick dogs were seroreactive to *B. vinsonii* subsp. *berkhoffii* antigens (Baneth et al., 1998). Studies in dogs from Greece and Brazil documented only 5.3 and 1.5% *B. vinsonii* subsp. *berkhoffii* seroreactivity, respectively (Mylonakis et al., 2004; et al., 2007). Seroreactivity to *B. vinsonii* subsp. *berkhoffii* antigens has also been documented in 8.7% of US military working dogs (Honadel et al., 2001).

To date, four genotypes of *B. vinsonii* subsp. *berkhoffii* have been described, designated as genotypes I, II, III, and IV based on sequences of the 16S-23S ribosomal RNA intergenic spacer region of the *Bartonella* genome as well as the *pap*31 gene, a bacteriophage-associated heme-binding protein (Maggi et al., 2006). Each of the *B. vinsonii* subsp. genotypes have been identified in the following mammals in various geographic locations: genotype I from a human, dogs, and coyotes in the United States, genotype II from dogs, coyotes, humans, and a cat in the United States, genotype III from gray foxes in the United States and a human with endocarditis in Europe, and genotype IV from a dog with endocarditis in Canada (Kordick et al., 1996; Kordick and Breitschwerdt, 1998; Breitschwerdt et al., 1999; Chang et al., 2000; Roux et al., 2000; Hoar et al., 2003; Maggi et al., 2006; Cadenas et al., 2008; Varanat et al., 2009; Cherry et al., 2011a).

B. vinsonii subsp. *berkhoffii* was first isolated from a domestic dog with endocarditis (Breitschwerdt et al., 1995) but has been established as the cause of, or associated with, other clinical manifestations such as myocarditis, cardiac arrhythmias, anemia/thrombocytopenia, arthropathy, epistaxis, and neurological and neurocognitive abnormalities in dogs

(Breitschwerdt et al., 1999; Tuttle et al., 2003; Breitschwerdt et al., 2004; MacDonald et al., 2004; Smarick et al., 2004; Breitschwerdt et al., 2005; Pesavento et al., 2005; Sykes et al., 2006; Cockwill et al., 2007; Cadenas et al., 2008; Diniz et al., 2009a; Breitschwerdt et al., 2010b; Cherry et al., 2011a; de Caprariis, 2011). Granulomatous inflammatory diseases (Pappalardo et al., 2000) and vasoproliferative disorders, such as peliosis hepatis and bacillary angiomatosis, have also been described in dogs with evidence of *B*. *vinsonii* subsp. *berkhoffii* infection (Kitchell et al., 2000; Yager et al., 2010). Recently, *B*. *vinsonii* subsp. *berkhoffii* was isolated or PCR-amplified from idiopathic pericardial, pleural, or abdominal effusions (Cherry et al., 2009) and from joint and seroma fluids (Diniz et al., 2009a).

Factors such as immunosuppression and co-infection could facilitate presentation or exacerbation of clinical symptoms in dogs. In the first case of *B*. *vinsonii* subsp. *berkhoffii* isolation from the dog with endocarditis (Breitschwerdt et al., 1995), the patient had been administered long-term treatment of corticosteroids after a diagnosis of systemic lupus erythematosus (SLE), potentially contributing to endocarditis pathology and subsequent isolation of this *Bartonella* sp., as suggested by the authors of this study and described in two cases of *Bartonella* infection in humans (Turner et al., 2005; Vikram et al., 2007).

In the context of co-infection, numerous studies have reported evidence of co-infection of sick domestic dogs in the United States with *B*. *vinsonii* subsp. *berkhoffii* and another *Bartonella* sp. or another vector-borne organism. One study found molecular evidence of *Bartonella* infection in 28% of dogs diagnosed with infective endocarditis at the UCD School of Veterinary Medicine (MacDonald et al., 2004), including three dogs in which *B*. *vinsonii* subsp. *berkhoffii* was the causative agent, and all the dogs seroreactive to

Bartonella antigens were also seroreactive to *Anaplasma phagocytophilum* antigens. In 1997, Pappalardo et al. documented a 3.6% seroprevalence of *B. vinsonii* subsp. *berkhoffii* among sick dogs from North Carolina and Virginia, with a strong correlation between serological evidence of *B. vinsonii* subsp. *berkhoffii* and either seroreactivity to *Ehrlichia canis* (36%) or *Babesia canis* (57.1%) antigens. A study published in 1999 documented co-infection of sick Walker hounds in North Carolina with *Bartonella* and at least one other vector-borne organism by PCR (Kordick et al., 1999). In addition, 93% of the dogs tested in that study were seroreactive to *B. vinsonii* subsp. *berkhoffii* antigens. Diniz et al. also documented co-infection of two ill dogs with *B. henselae* and *B. vinsonii* subsp. *berkhoffii*, which may have exacerbated clinical symptoms (Diniz et al., 2009a). Recently, molecular evidence of *Bartonella* infection was reported in a prevalence study of sick dogs in the United States, documenting detection of *Bartonella* spp. in 9.2% of those dogs by PCR of blood or BAPGM blood enrichment culture (Perez et al., 2011). A subset of the dogs were infected with *B. vinsonii* subsp. *berkhoffii*, with an additional nine cases of co-infection with *B. vinsonii* subsp. *berkhoffii* and another *Bartonella* sp. Detection of antinuclear antibodies (ANA), an indication of SLE, has been reported in dogs seroreactive to *B. vinsonii* subsp. *berkhoffii*, *Rickettsia rickettsii*, and *Ehrlichia canis* antigens. The host immune response to microbial infection could have led to the development of ANA in these dogs (Smith et al., 2004). This work is supported by other studies reviewed by Pisetsky in 2008 which have documented cross reactivity between genetic sequence motifs of bacterial and mammalian DNA, potentially skewing ANA diagnostic results. Furthermore, it has been shown that immunization of mice with bacterial DNA can induce production of anti-DNA

autoantibodies, possibly initiating a robust immunologic response and autoimmune disease in other hosts.

Cattle

B. bovis (formerly *B. weissii*), *B. chomelii*, *B. schoenbuchensis*, and *B. henselae* are the species to date that have been identified in cattle (Brocklesby, 1970; Chang et al., 2000b; Breitschwerdt et al., 2001; Bermond et al., 2002; Rolain et al., 2003b; Maillard et al., 2004; Kelly et al., 2004; Cherry et al., 2009; Tsai et al., 2011). *B. bovis* was first isolated from cats and classified as *B. weissii* but was renamed *B. bovis* by Bermond et al. in 2002. *Bartonella* infection has been documented in a number of wild and domestic ruminants, and research over the past decade implicates cattle as the natural reservoir hosts of *B. bovis*.

B. bovis infection in cattle was first described in the literature in 1934 (Donatien and Lestoquard, 1934) and since that time, *B. bovis* has been documented in cattle around the world, including North America (Chang et al., 2000b; Breitschwerdt et al., 2001; Maillard et al., 2004; Cherry et al., 2009), Africa (Kelly et al., 2004), and Europe (Brocklesby et al., 1970; Bermond et al., 2002; Tsai et al, 2011). In 2000, Chang et al. reported isolation of *Bartonella* from 49% of beef and dairy cattle from Oklahoma and California (Chang et al., 2000b). By PCR/RFLP analysis, these isolates were most closely related to *B. weissii*. A high prevalence was also reported in beef cattle from North Carolina in 2009, when *B. bovis* was PCR-amplified from the blood of 82.4% of cattle (Cherry et al., 2009).

The high prevalence of bacteremia in clinically healthy populations of cattle indicate that *B. bovis* has successfully co-evolved with cattle as its primary host, but the chronic

nature of *Bartonella* infection may eventually lead to pathology, as demonstrated in cats infected with *B. henselae* (Breitschwerdt and Kordick, 2000). Though cattle are the reservoir hosts of *B. bovis*, a study by Maillard et al. in 2007 defined *B. bovis* as the cause of endocarditis in two adult cows in France.

Rodents

Recent studies have supported the hypothesis that individual *Bartonella* spp. have co-evolved with specific rodent species as reservoir hosts (Telfer et al., 2007a, 2007b), and this finding is important to human medicine because studies have documented detection of rodent-adapted species in human patients with clinical illness. *B. washoensis*, with the presumed reservoir host as the California ground squirrel (*Spermophilus beecheyi*), was isolated in a human patient with cardiac disease in 2003 (Kosoy et al., 2003). This finding suggests that rodents may serve as potential reservoirs for human infection, especially in humans with extensive wildlife contact. In a study by Telfer et al., the prevalence of four *Bartonella* species, *B. birtlesii*, *B. doshiae*, *B. taylorii*, and *B. grahamii*, in two rodent populations, bank voles (*Clethrionomys glareolus*) and wood mice (*Apodemus sylvaticus*), was assessed to determine the population dynamics of these two rodent populations relative to *Bartonella* spp. prevalence (Telfer et al., 2007b). The authors found that the prevalence of *B. doshiae* and *B. taylorii* was higher in wood mice, whereas *B. birtlesii* was more prevalent in bank voles. Support for bank voles as a reservoir host for *B. birtlesii* in that study was demonstrated by the finding that the detection of *B. birtlesii* in wood mice was directly related to the density of bank voles. Bai et al. published a study in 2008 which documented

evaluation of temporal and spatial patterns of *Bartonella* bacteremia in 20 colonies of black-tailed prairie dogs (*Cynomys ludovicianus*) from 2003 to 2005 and reported a *Bartonella* spp. prevalence of 23.1%. Though there was no indication that *Bartonella* prevalence was based on black-tailed prairie dog population density, the authors suggetsed that this does not rule out the potential for these rodents as reservoir hosts for *Bartonella* and thus, significance to public human health.

Studies in other parts of the world have documented a higher prevalence of *Bartonella* bacteremia in rodent populations. A study of rodent populations in the UK found 62.2% of rodents to be *Bartonella* bacteremic (Birtles et al., 1994), and another study in the southeastern United States reported a 42.2% *Bartonella* prevalence (Kosoy et al., 1997). Birtles et al. also documented *Bartonella* bacteremia in 64% of woodland rodents in 2001.

Documentation of *Bartonella* bacteremia in rodents is not only important as a public health concern, but studies conducted in rodent populations allow researchers to gain a better understanding of the ecological diversity of the *Bartonella* genus. Due to the high prevalence of *Bartonella* spp. in rodents and identification of specific rodent-adapted *Bartonella* spp., population dynamic studies can broaden the knowledge of *Bartonella* spp. persistence in specific reservoir hosts.

Accidental hosts

Inadvertent transmission of known or novel *Bartonella* spp. from reservoir hosts, such as wild or domestic animals, to non-reservoir, or accidental, hosts, such as domestic animals or humans, may be more common and represent an underestimated risk for

Bartonella infection. The ability of vector-borne organisms to survive and propagate within specific mammalian reservoir hosts while maintaining the ability to be effectively transmitted by vectors to accidental hosts is an important factor in human and veterinary medicine in the context of *Bartonella* infection as a re-emerging pathogen. The diversity of mammalian hosts *Bartonella* spp. can infect demonstrates this bacterium's ability to continue to evolve, increase in virulence, and spread to new environments.

Humans

Six *Bartonella* species, including *B. bacilliformis* and *B. quintana* which utilize humans as reservoir hosts, have been identified as the cause of clinical pathology in humans, but there is evidence to suggest that up to 13 *Bartonella* spp. can utilize humans as hosts (Boulouis et al., 2006; Chomel et al., 2006; Florin et al., 2008). In reservoir hosts of *Bartonella* species, these bacteria typically persist in the host without pathophysiological consequences, though in accidental hosts, chronic bacteremia can result in clinical illness with a wide range of symptoms. *Bartonella* infection has historically been recognized as the cause of disease in immunocompromised individuals, but it is now known that bartonellosis can cause disease in immunocompetent humans as well (Lucey et al., 1992; Kumasaka et al., 2001; Breitschwerdt et al., 2007b, 2008). Though the means of transmission of various *Bartonella* spp. to humans has not been well-established, risk factors for *Bartonella* infection in humans include frequent, close contact with animals and their bodily fluids, as is the case with veterinary professionals, extensive arthropod exposure, and animal bites and scratches

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(Zangwill et al., 1992; Breitschwerdt et al., 2007b, 2008; Oliveira et al., 2010; Lin et al., 2011).

B. henselae was first documented as the cause of CSD in 1992 by Regnery et al. and was granted recognition as the etiologic agent CSD in the mid-1990s (Regnery and Tappero, 1995; Jerris and Regnery, 1996), though this disease entity has been recognized for the past century. *B. henselae* transmission from a cat to a human can occur by bite or scratch, with an estimated 24,000 cases in the United States annually (Jackson et al., 1993). *B. henselae* is not only able to merely be transmitted by the cat but also to evade the human immune system to survive. Clinical pathology in CSD patients is typically characterized by self-limiting lymphadenopathy and generalized flu-like symptoms, but in 5-10% of CSD cases (Anderson and Neuman, 1997), *B. henselae* infection can cause chronic bacteremia leading to atypical manifestations of CSD, including endocarditis (Raoult et al., 1997; Fournier et al., 2001a; Houpikian and Raoult, 2005), encephalopathy (Armengol and Hendley, 1999), granulomatous hepatitis and nephritis (Kahr et al., 2000), bacillary angiomatosis and peliosis hepatis (Koehler et al., 1997), and musculoskeletal manifestations such as myalgia (Maman et al., 2007), osteomyelitis (Robson et al., 1999) and arthropathy (Hayem et al., 1996; Giladi et al., 2005; Maman et al., 2007).

B. vinsonii subsp. *berkhoffii*, which was originally isolated from a dog w/ endocarditis (Breitschwerdt et al., 1995), has been identified as a new *Bartonella* sp. potentially pathogenic to humans. Of the four genotypes of *B. vinsonii* subsp. *berkhoffii*, genotype II is most frequently detected in humans (Maggi et al., 2006). Roux et al. documented the isolation of *B. vinsonii* subsp. *berkhoffii* (genotype unspecified) from a patient with

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endocarditis, veterinary workers, and a wildlife biologist in 2000. More recently, *B. vinsonii* subsp. *berkhoffii* was detected or isolated in immunocompetent individuals in the United States with symptoms including arthritis, fatigue, and neurological/neurocognitive abnormalities (Breitschwerdt et al., 2007b, 2008). In 2009, *B. vinsonii* subsp. *berkhoffii* genotype II was isolated from the blood of a boy with an epitheliod hemangioendothelioma liver tumor and also detected in the tumor tissue (Breitschwerdt et al., 2009a). Furthermore, studies have implicated direct transmission of *Bartonella* sp. from dogs to humans (Keret et al., 1998; Tsukahara et al., 1998; Kerkhoff et al., 1999a; Chen et al., 2007; Kim et al., 2009).

       B. koehlerae, which is presumed to utilize the domestic cat as its reservoir host, was identified as the cause of culture-negative aortic valve endocarditis in a human in 2004 (Avidor et al., 2004). In 2010, *B. koehlerae* was detected in human blood and BAPGM blood enrichment cultures from eight immunocompetent human patients with symptoms such as fatigue, joint and muscle pain, and headaches (Breitschwerdt et al., 2010b). Serological results in several studies have also suggested *B. clarridgeiae* as a potential zoonotic *Bartonella* sp. with documentation of lymphadenopathy, fever, and, in one case, a chest-wall abscess associated with *B. clarridgeiae* seroreactivity in human patients (Kordick et al., 1997; Margileth and Baehren, 1998; Sander et al., 2000; Laudisoit et al., 2011). *Bartonella melophagi* utilizes sheep as its reservoir host, and isolation of this species from two women with clinical symptoms including pericarditis, fatigue, muscle weakness, myalgia, joint pain, headaches, and neurocognitive abnormalities was documented by Maggi et al. in 2009.

       Other *Bartonella* spp., such as those that utilize rodents as reservoir hosts, including *B. vinsonii* subsp. *arupensis*, *B. alsatica*, and *B. elizabethae*, have been detected in humans

and associated with clinical manifestations such as endocarditis. In 2005, Fenollar et al.

reported diagnosis of *B. vinsonii* subsp. *arupensis*, with the white-footed mouse as its

reservoir host, as the cause of endocarditis in a human based on serology and PCR. *B.*

alsatica, which utilizes the rabbit as its reservoir, and *B. elizabethae*, a rat-adapted *Bartonella*

species, have both been isolated from the valves of patients with endocarditis in France and

the United States, respectively (Daly et al., 1993; Raoult et al., 2006). *Candidatus* B.

washoensis, for which the ground squirrel is the primary reservoir, was also associated with a

case of cardiac disease in Nevada in 2003 (Kosoy et al., 2003). *B. vinsonii* subsp. *arupensis*

was isolated from a rancher with neurological symptoms in Montana (Welch et al., 1999)

who had frequent opportunity for arthropod vector exposure, and the area where he worked

was known for a large population of voles which are known to harbor *Bartonella* spp.;

though these observations were made, no direct link was established between *B. vinsonii*

subsp. *arupensis* from this patient and arthropod or rodent exposure. *B. grahamii* utilizes the

vole as its reservoir host and has been associated with ocular/retinal manifestations in

humans (Kerkhoff et al, 1999b; Serratrice, 2003). *B. rochalimae* is a newly-recognized

species of *Bartonella* for which the fox is the proposed reservoir, and in 2007, Eremeeva et

al. isolated this species from a woman with bacteremia, fever, and splenomegaly. In

Thailand, *B. tamiae* was isolated from three ill patients who experienced symptoms including

fever, fatigue, and headaches (Kosoy et al., 2008). Though no reservoir has been established

for *B. tamiae*, rodents have been proposed as potential reservoir hosts (Kosoy et al., 2010).

Candidatus Bartonella volans is another recently-recognized *Bartonella* sp. that was first

identified in a southern flying squirrel, *Glaucomys volans*, in the United States. A DNA

sequence most closely related to *B. volans* was recently identified in an 86 year-old man with memory loss, encephalopathy, and recent-onset arthritis (Breitschwerdt et al., 2009b).

These studies together indicate that *Bartonella* spp. are important zoonotic pathogens, many which have only recently been identified in humans, and that clinicians should be aware of the potential for *Bartonella* infection and the clinical signs associated with bartonellosis. People with frequent exposure to wild or domestic animals and their ectoparasites, such as veterinary professionals or wildlife biologists, may be at an increased risk for *Bartonella* infection. Further studies are warranted to define modes of transmission, risk factors, as well as treatment regimens in the context of human bartonellosis.

Cats

Though cats are the primary reservoir host for *B. henselae*, and presumably *B. clarridgeiae* and *B. koehlerae*, two other species, *B. vinsonii* subsp. *berkhoffii* and *B. quintana* that utilize dogs and humans as reservoirs, respectively, have also been detected in cats. *B. vinsonii* subsp. *berkhoffi*i genotype II DNA was recently amplified from the BAPGM blood culture of a cat with recurrent osteomyelitis (Varanat et al., 2009, 2011). *B. quintana* has been detected by PCR-amplification from the dental pulp of a domestic cat (La et al., 2005) as well as cat fleas (Rolain et al., 2003c). A serosurvey conducted by Baneth et al. in 1996 reported cats from Israel and North Carolina showing seroreactivity to only *B. quintana* antigens. Regnery et al. published results from an experimental infection study demonstrating two cats infected with *B. quintana* did not become bacteremic or develop apparent clinical signs but did seroconvert (Regnery et al., 1996). Since that time, putative *B. quintana*

transmission by domestic cat to humans has been suggested (Drancourt et al, 1996; Parrot et al., 1997; Scully et al., 1998; Breitschwerdt et al., 2007a), suggesting the domestic cat could be a potential reservoir host for *B*. *quintana*.

Domestic dogs

Canids have been suggested as the primary reservoir host for *B*. *vinsonii* subsp. *berkhoffii*, but numerous other *Bartonella* species have been identified in domestic dogs. *B*. *henselae*, *B*. *quintana*, *B*. *elizabethae*, *B*. *clarridgeiae*, *B*. *washoensis* (Chomel et al., 2006), *B*. *rochalimae* (Diniz et al., 2009b; Henn et al., 2009) and sequences similar to *B*. *volans* have been identified in dogs (Cherry et al., 2011a; Perez et al., 2011), providing evidence that other *Bartonella* species besides *B*. *vinsonii* subsp. *berkhoffii* are able to utilize dogs as hosts.

The route of transmission of *Bartonella* spp. to dogs has not been established, though evidence of cat-adapted *Bartonella* spp., such as *B*. *henselae* and *B*. *clarridgeiae*, infection in dogs suggests cats may play a role in transmission of these organisms to dogs via bite or scratch. Alternatively, the cat flea may play a role in the transmission of these *Bartonella* spp. to dogs, as this flea species has a ubiquitous geographic distribution and will feed on other mammalian hosts than cats. Recent prevalence studies have demonstrated these *Bartonella* spp. are widely distributed in domestic dog populations worldwide. In 2009, Inoue et al. documented isolation of *B*. *clarridgeiae* from 1 stray dog and 64 pet dogs in Thailand. Kim et al. documented isolation of *B*. *clarridgeiae* from nine dogs in Korea, and *B*. *henselae* DNA was detected in blood, saliva, and nail samples, at 16.6%, 18.5%, and 29.6%, respectively, from dogs in this study (Kim et al., 2009). Interestingly, two dogs were co-

infected with *B. henselae* and *B. clarridgeiae*, and the authors of this study suggested dogs may serve as secondary reservoirs for *B. henselae* and/or *B. clarridgeiae*. *B. henselae* and *B. clarridgeiae* have been associated with hepatic disease in dogs (Gillespie et al., 2003), and *B. clarridgeiae* was diagnosed as the causative agent of aortic valve endocarditis in a dog (Chomel et al., 2001).

Though evidence of exposure to or infection with *B. henselae* in domestic dogs has been frequently documented, the first isolation of this *Bartonella* sp. was from a dog in Gabon, Africa in 2004 (Gundi et al., 2004). Associated manifestations of *B. henselae* infection in dogs include peliosis hepatitis, granulomatous lymphadenitis, endocarditis, polyarthritis, and idiopathic effusions (Mexas et al., 2002; Gillespie et al., 2003; Goodman and Breitschwerdt, 2005; Cherry et al., 2009). Interestingly, many studies to date have documented a higher seroprevalence of *B. henselae* in dogs than *B. vinsonii* subsp. *berkhoffii*. For example, a serosurvey of dogs in the southeastern United States revealed 10% seroreactivity to *B. henselae* antigens in healthy dogs and 28% in sick dogs (Solano-Gallego et al., 2004). Studies in Spain, Italy, and Sardinia have documented 17%, 6%, and 28% *B. henselae* seroprevalence, respectively (Solano-Gallego et al., 2006; Di Francesco et al., 2007; Pinna et al., 2007). *B. henselae* seroprevalence surveys in dogs from Hawaii (Demers et al., 1995), Japan (Tsukahara et al., 1998), Zimbabwe (Kelly et al., 2004), and the United Kingdom (Barnes et al., 2000) have varied between 3% and 14%. Henn et al. demonstrated a *Bartonella* spp. seroprevalence of 2.99% in sick dogs in northern California (Henn et al., 2005). Of the *Bartonella* seroreactive dogs, 35.3% were reactive to *B. henselae* antigens only

and 33.3% to *B. clarridgeiae* antigens only, but only 2% were seroreactive to *B. vinsonii* subsp. *berkhoffii* antigens only.

Perez et al. identified *Bartonella* in 9.2% of dogs from the US by either PCR or BAPGM enrichment culture. *B. henselae*, *B. vinsonii* subsp. *berkhoffii*, *B. koehlerae*, *Candidatus* B. volans*, and *B. bovis* DNA were detected, and cases of co-infection were also reported. Duncan et al. also reported molecular detection of multiple *Bartonella* spp. in dogs in 2007, documenting PCR-amplification of *B. henselae*, *B. vinsonii* subsp. *berkhoffii*, *B. quintana*, and *B. bovis* DNA (Duncan et al., 2007, 2008).

Other *Bartonella* spp., including *B. rochalimae*, *B. washoensis*, *B. quintana*, and DNA sequences most closely resembling *Candidatus* Bartonella volans, have been identified in dogs. In 2003, Chomel et al. documented isolation of *B. washoensis* from a dog with mitral valve endocarditis. *B. rochalimae* was also identified as the cause of infective endocarditis in a dog from California (Henn et al., 2009) and detected in a dog from Greece by PCR (Diniz et al., 2009b). Isolation of a *Bartonella* sp. most closely related to *Candidatus* B. volans was documented in two dogs from the United States (Perez et al., 2011), and another study documented evidence of another *B. volans*-like species from a BAPGM blood enrichment culture from a dog in Virginia (Cherry et al., 2011a).

B. quintana, which has historically been considered a "specialist", utilizing only humans as its host, was first documented to infect dogs in 2006 by Kelly et al. Authors of this study demonstrated presence of *B. quintana* DNA in the blood of one dog from the United States and presumably in a valvular lesion of a dog from New Zealand.

Dogs have been suggested as a probable reservoir for *B. vinsonii* subsp. *berkhoffii*, but research has shown that many other *Bartonella* spp. are able to utilize the domestic canid as a host. The implications of this are uncertain, but veterinarians should be aware of the increasing body of knowledge surrounding the risk that dogs can be a reservoir for *Bartonella* infection and bacteremic dogs can manifest a spectrum of clinical signs. It must be noted that most prevalence studies and case reports of *Bartonella* infection in dogs with species other than *B. vinsonii* subsp. *berkhoffii* have targeted sick dog populations, suggesting that domestic dogs are most likely accidental hosts of other *Bartonella* spp. They apparently develop clinical pathology from infections with other reservoir-adapted *Bartonella* spp., such as rodent-adapted *B. washoensis*, *B. rochalimae*, and *Candidatus* B. volans, and human-adapted *B. quintana*.

Horses

Equids are not presumed as a reservoir host for any *Bartonella* spp., but recent studies have demonstrated *Bartonella* infection in horses (Jones et al., 2008; Johnson et al., 2009; Cherry et al., 2011b, Identification). Jones et al. reported the first detection and isolation of *B. henselae* from two horses, one with chronic arthropathy and the other with presumptive vasculitis (Jones et al., 2008). In 2009, Johnson et al. reported abortion of an equine fetus due to *B. henselae* infection confirmed by molecular, histologic, immunohistochemical, and ultrastructural methods. In 2011, Cherry et al. detected this *Bartonella* sp. in a 2 year-old mare from Germany with high-grade hemolytic anemia. Unpublished data by Cherry et al. identified *B. henselae*, *B. vinsonii* subsp. *berkhoffii* genotypes I and III, and a *Bartonella* sp.

most closely related to the rodent-adapted species *Candidatus* B. volans in horses. Collectively, these studies indicate that horses are also accidental hosts for *Bartonella* spp. and are susceptible to *Bartonella* infection.

Summary

The *Bartonella* genus is comprised of at least 22 species, subspecies, or *Candidatus* species that have been identified to date. For most of these species, a reservoir host has been identified, including humans for *B. bacilliformis* and *B. quintana*, cats for *B. henselae* and potentially, *B. clarridgeiae* and *B. koehlerae*, presumably canids for *B. vinsonii* subsp. *berkhoffii*, and various rodent species for species such as *B. grahamii*, *B. taylorii*, *B. birtlesii*, and *B. doshiae*. Individual *Bartonella* spp. have evolved the means to adapt to specific mammals and utilize them as reservoir hosts but also evolved the ability to infect non-reservoir mammalian hosts, potentially leading to chronic bacteremia and clinical illness. Epidemiological data and case reports suggest the implications of this are far-reaching, and future studies are warranted to explore the mechanisms of transmission from reservoir hosts to accidental hosts and how this information can be used to further understanding of the ecological diversity of this genus of bacteria.

Vector Ecology

As is the case with other members of the class alpha-Proteobacteria, such as *Rickettsia*, *Anaplasma*, and *Ehrlichia*, *Bartonella* spp. have adapted to specific vectors in order to persist and be transmitted to mammalian hosts. For example, the sandfly (*Lutzomyia*

verrucarum) is the sole vector for *B. bacilliformis*, the human body louse (*Pediculus humanus humanus*) is the primary vector for *B. quintana*, and the cat flea (*Ctenocephalides felis*) is the primary vector for *B. henselae*. The acquisition of these *Bartonella* spp. by their respective blood-feeding vectors, multiplication within these vectors, and transmission to mammalian hosts have been extensively explored to provide definitive proof of vector competence and ability of vectors to transmit *Bartonella* spp. to hosts.

Sandflies

The sandfly species *Lutzomyia verrucarum* responsible for *B. bacilliformis* transmission is only found in the Andes region of South America, specifically Peru, Columbia, and Ecuador, and it was first implicated as the vector transmitting the agent of this disease in 1913 (Townsend, 1913). Experimental studies in monkeys to identify the vector for *B. bacilliformis* were conducted in the early 1900s (Noguchi, 1928; Noguchi et al., 1929; Battistini, 1929, 1931). Direct transmission of this *Bartonella* sp. by *Lutzyomia* species to monkeys after feeding was documented by Battistini (Battistini, 1929, 1931), though the means of replication and survival of *B. bacilliformis* within the sandfly is unclear. A study by Hertig in 1942 demonstrated organisms morphologically similar to *B. bacilliformis* on the proboscis and in the mid-gut, intestines, and feces of sandflies after feeding upon infected humans. Though transmission of *B. bacilliformis* to humans by *Lutzyomia verrucarum* has been established, further research has implicated other members of the *Lutzomyia* genus, such as *Lutzomyia peruensis* (Ellis et al., 1999a) and *L. columbiana* (Gamarra, 1964; Alexander, 1995), as potential vectors. *Bartonella grahamii* has also been identified in *L.*

peruensis from Peru (Ellis et al., 1999a).

Lice

The human body louse (*Pediculus humanus humanus*) was first suspected as the vector of *B. quintana* when it was found that humans developed symptoms after lice fed upon them (Swift, 1920). Further investigation revealed that the louse fecal matter itself was infective (Swift, 1920; Bruce, 1921), though the causative agent, then presumed to be a Rickettsial species, was not transmitted to louse offspring transovarially (Bruce, 1921). The transmission of *B. quintana* to humans by the human body louse is a three stage cycle: 1) adult louse becomes infected via a bloodmeal, 2) viable *B. quintana* organisms multiply and are maintained in the louse gut, and 3) humans become infected by louse bite or through an open wound contaminated with louse feces containing viable *B. quintana*.

In 1960, research on *B. quintana* revealed that this bacterium replicates within the stomach of the louse, followed by attachment to luminal epithelial cells (Weyer, 1960). Vinson et al. confirmed these findings in 1969 when intrarectal inoculation of *B. quintana* into body lice revealed viable infectious organisms in the louse gut lumen. The authors were also able to visualize *B. quintana* in feces collected from lice feeding on an infected patient. More recent studies confirmed these earlier observations, demonstrating that *B. quintana* does remain in the intestinal lumen of the louse, is excreted in its feces throughout its lifespan, and is not transmitted transovarially, as is the case with *B. bacilliformis* (Fournier et al., 2001b). Furthermore, logarithmic growth of *B. quintana* in the louse midgut was documented where 2×10^3 bacteria from the midgut of each louse were detected on day 3 and

increased up to 1.3×10^8 bacteria by day 17 (Seki et al., 2007). Furthermore, up to 1×10^7 bacteria were detected in louse fecal matter on day 15 in that study.

In addition to the human body louse, a more recent study demonstrated *B. quintana* in head lice (*Pediculus humanus capitis*) from children in Nepal (Sasaki et al., 2006). Other *Bartonella* species have been identified in several lice genera such as the spined rat louse (*Polyplax* spp.) (Reeves et al., 2006; Tsai et al., 2010) and the sucking louse (*Hoplopleura* spp. and *Neohaematopinus sciuri*) (Durden et al., 2004; Reeves et al., 2006; Nelder et al., 2009). *Bartonella* species from either or both of these lice genera include *B. rattimassiliensis*, *B. tribocorum*, *B. phoceensis*, and *B. henselae*. Interestingly, *B. henselae* was recently detected in seal lice (*Echinophtirius horridus*) collected from a harbor seal (*Phoca vitulina*) in the Netherlands (Morick et al., 2009). The significance of *Bartonella* species in lice infesting sea mammals and rodents reamins unclear, but the implications of these findings warrant further investigation.

Fleas

The domestic cat is the natural reservoir for *B. henselae* with the cat flea (*Ctenocephalides felis*) as its primary vector. The cat flea has been demonstrated experimentally to be a competent vector for transmission of this *Bartonella* species (Chomel et al., 1996) when fleas collected from *B. henselae* bacteremic cats were placed on five specific pathogen free (SPF) kittens, and four of those five kittens became bacteremic within two weeks. Furthermore, another experimental study revealed that not only is *C. felis* able to acquire *B. henselae* directly from a bloodmeal on a bacteremic cat, but these bacteria are able

to remain in the flea gut for up to 9 days and replicate within the gut, similar to *B. bacilliformis* in sandflies and *B. quintana* in lice (Higgins et al., 1996). In this study, *B. henselae* was detected in the feces, and viable isolates were obtained on rabbit blood agar plates. In 1998, Foil et al. demonstrated transmission of *B. henselae* to cats by intradermal inoculation of infectious flea feces. Another study was conducted using a streptomycin-resistant strain of *B. henselae* which demonstrated 1.8×10^3 CFU/mg of the bacteria in flea feces after 2 hours with a decrease in colony count after 72 hours (Finkelstein et al., 2002). In summary, these studies document replication of *B. henselae* in the flea gut and its viability in the feces as well as direct transmission of *B. henselae* to SPF cats via intradermal inoculation of infectious flea feces or incubation with bacteremic fleas.

Though the vector competence of the cat flea in the transmission of *B. henselae* has been established, molecular detection of *B. quintana*, *B. koehlerae*, and *B. clarridgeiae* in cat fleas has also been documented (Rolain et al., 2003c). These findings suggest that the cat flea may be capable of transmission of other *Bartonella* spp. to mammalian hosts.

Biting flies

The potential role of Hippoboscidae flies, specifically *Lipoptena*, *Hippobosca*, *Melophagus*, and *Stomoxys* spp., as vectors of *Bartonella* spp. to wild and domestic ruminants has been suggested in the past few years, as *Bartonella* DNA has been detected in these flies in numerous countries. In 2004, *Bartonella* DNA was first identified in North American biting flies collected from cattle barns and feedlots in California (Chung et al., 2004); *B. bovis* DNA was amplified from two horn flies (*Haematobia* spp.), and *B. henselae*

DNA was amplified from a stable fly (*Stomoxys* sp.) in that study. Within the same year, *Bartonella* DNA was also detected in deer flies (*Lipoptena cervi*), horse flies (*Hippobosca equina*), and sheep keds (*Melophagus ovinus*) collected in France (Halos et al., 2004), and *B. schoenbuchensis* was isolated from deer flies collected from roe deer and red deer in Germany (Dehio et al., 2004a). Furthermore, Dehio et al., demonstrated localization of *B. schoenbuchensis* within the lumen of the midgut of deer keds. In 2006, Reeves et al. identified *B. henselae* and *B. schoenbuchensis* DNA in deer keds (*Lipoptena mazamae*) collected from white-tailed deer in the southeastern United States, and *B. schoenbuchensis* DNA was amplified from deer flies in Massachusetts in 2008 (Matsumoto, 2008). Blood-feeding vectors may uptake bacteria during feeding, but detection of bacteria in vector tissues gives evidence of a vector's role in the natural bacterial transmission cycle.

Ticks

The role of ticks in the transmission of *Bartonella* species has been an ongoing debate for years. A review by Billeter et al. in 2008 summarized the numerous tick species harboring *Bartonella* DNA, including *Dermacentor*, *Haemaphylsalis*, *Ixodes*, and *Rhipicephalus*. Though clinical, epidemiological, and molecular data supports the potential for tick transmission (Breitschwerdt and Kordick, 2000; Chang et al., 2001; Sanogo et al., 2003; Skotarczak and Adamska, 2005; Billeter et al., 2008; Angelakis et al., 2010), DNA detection alone does not provide evidence of direct bacterial transmission to animals or humans or prove ticks to be competent vectors for *Bartonella* species (Telford and Wormser, 2010). A recent study by Reis et al. in 2011 proved vector competence of *Ixodes ricinus* in a

murine model, demonstrating acquistion of *Bartonella* by ticks from an infected mouse and subsequent transmission to a naïve mouse by these ticks and localization of these bacteria within adult ticks. In this study, a 4-week old OF1 female mouse was inoculated intravenously with *B. birtlesii*. Following *B. birtlesii* infection, *I. ricinus* larvae and nymphs were allowed to feed on the infected mouse. After molting of larvae, the nymphs were allowed to feed on three naïve 4-week old OF1 female mice. Within seven days, *B. birtlesii* DNA was detected in the blood of the tick infested mice by PCR and sequencing. After six days of blood culture, these bacteria were visualized by immunofluorescence, demonstrating presence as well as viability of the bacteria. Adult ticks, fed on the *B. birtlesii*-infected mouse at the nymphal stage, were fed on a membrane feeder with uninfected blood for 8 days, and these ticks effectively transmitted the bacteria to the uninfected blood, demonstrated by presence of *Bartonella* DNA by PCR from blood on days 3 through 8. This data proves that *I. ricinu*s ticks are capable of acquiring *B. birtlesii* from an infected host and retaining the bacteria transstadially. In contrast to *B. quintana* in the louse (Weyer et al., 1960; Fournier et al., 2001b; Seki et al., 2007) and *B. henselae* in the cat flea (Higgins et al., 1996), this experimental study supports the hypothesis that *B. birtlesii* is most likely transmitted from ticks to a mammalian host through salivary secretions rather than contaminated feces. This study provides definitive proof that ticks are capable of acquiring, retaining, and effectively transmitting *B. birtlesii*.

Billeter et al. demonstrated invasion and replication of seven *Bartonella* species and three *Candidatus* Bartonella species in an *Amblyomma americanum* tick cell line in 2009. The authors of this study were able to visualize *Bartonella* within the cells by electron or

light microscopy, documented cytopathic effects following infection, including cellular vacuolization and cell lysis, as well as amplification of *B. henselae* within the cells by quantitative real time PCR. Recently, *B. vinsonii* subsp. *berkhoffii* DNA was detected in *Rhipicephalus sanguineus* ticks post-capillary tube feeding as well as in the feces of these ticks (Billeter et al., 2012). These results demonstrate that *B. vinsonii* subsp. *berkhoffii* is capable of infecting this tick species, potentially leading to subsequnt infection of mammalian hosts through inoculation of tick feces through broken skin. Though this can not be extrapolated to all species of ticks, *Bartonella* spp., or mammalian hosts, this data does provide evidence warranting consideration of *Bartonella* infection in patients with tick exposure.

Summary

Vector-borne organisms, such as members of the *Bartonella* genus, must adapt to the lifestyle of a specific vector and its physiological environment for survival and propagation. These adaptations vary drastically depending upon the microorganism. *B. bacilliformis*, *B. quintana*, and *B. henselae* have evolved not only to be carried by the sandfly, body louse, and cat flea, respectively, but have developed the ability to survive and replicate in the guts of these arthropods after a blood meal. This is a key component to increase the likelihood of transmission to a mammalian host through skin broken by scratching or through an open wound. *B. henselae* facilitates its transmission to its natural reservoir host, the domestic cat, and its accidental human host by introduction of bacteremic flea feces via scratch or bite (Finkelstein et al., 2002). Identification of *Bartonella* DNA in a blood-feeding vector does

not confirm competence or constitute a direct threat to animal or human global health; however, the promiscuity of *Bartonella* species in their ability to adapt to a variety of arthropods is unparalleled. Reports have demonstrated a broader range of *Bartonella* species identified in arthropods than in mammalian hosts (Abbot et al., 2007; Brinkerhoff et al., 2010; Tsai et al., 2011). Evidence from these studies suggests that *Bartonella*, similar to the *Wolbachia*, may have co-evolved with vector species in their exploitation of mammalian hosts (Charlat et al., 2003; Tsai et al., 2011).

Virulence factors and pathogenesis

Historically, *Bartonella* spp. were thought to be primarily intraerythrocytic bacteria, residing in red blood cells within a host, but further research has revealed that intracellular microorganisms can invade other cell types with distinct pathogenic means of intracellular infection and survival. The ability of species of the same genus to evolve unique pathogenic mechanisms independently of one another to achieve the same goal, i.e. host cell invasion, is known as adaptive radiation. The pathogenic mechanisms of *Bartonella* are demonstrated by the development of the T4SS in *B. quintana*, *B. henselae*, and *B. tribocorum* (Seubert et al., 2003; Saenz et al., 2007; Dehio, 2008), compared to the motility-mediated invasion of erythrocytes by *B. bacilliformis* (Engel et al., 2011). T4SSs are pathogenicity factors often used by human bacterial pathogens such as *Helicobacter pylori*, *Legionella pneumophila*, *Brucella melitensis*, and *Bordetella pertussis* (Christie et al., 2005). Three distinct T4SSs, VirB/VirD4, Vbh, and Trw (Seubert et al., 2003; Schulein et al., 2005; Schmid et al., 2006; Nystedt et al., 2008; Rhomberg et al., 2009; Scheidegger et al., 2009; Selbach et al., 2009;

Vayssier-Taussat et al., 2010), have evolved among *Bartonella* species, leading to invasion of mammalian host cells, is one example of adaptive radiation within the *Bartonella* genus. The VirB T4SS modulates cellular processes by translocation of bacterial effector proteins primarily in endothelial cells, whereas the Trw T4SS facilitates erythrocyte invasion by binding to the surface of erythrocytes with pilus subunits. Both of these T4SS mechanisms have been demonstrated in animal models (Schulein et al., 2001; Schulein and Dehio, 2002).

Erythrocytes

The intracellular, hemotropic nature of *Bartonella* spp. is a key characteristic which allows the transmission of these bacteria from blood-feeding arthropods to mammalian hosts. The importance of localization of *Bartonella* spp. within mammalian red blood cells enables efficient vector transmission, protection from the host immune response, facilitation of vascular dispersion throughout tissues of the host, and potential evasion of antimicrobial efficacy (Jacomo et al., 2002; Rolain et al., 2002).

Nearly 100% of host red blood cells may become infected in patients with *B. bacilliformis* infection. In 1944, Weinman suggested that this likely contributes to phagocytosis of *Bartonella*-infected erythrocytes by the reticuloendothelial system, leading to hemolysis and subsequent severe anemia (Weinman, 1944, Infectious anemias). It is presumed that the flagella-mediated motility of *B. bacilliformis* is essential for its pathogenesis and ability to disperse throughout its human host, demonstrated by lack of bacterial binding to erythrocytes by non-motile or killed *B. bacilliformis* (Walker and Winkler, 1981; Benson et al., 1986). In 1993, Scherer et al. also demonstrated that the

presence of antiflagellin antibodies impaired the motility of *B. bacilliformis in vitro*. Another potential factor contributing to invasion of erythrocytes by *B. bacilliformis* is an extracellular protein called deformin which causes deformation and subsequent invaginations of erythrocyte membranes *in vitro* that can facilitate entry into these cells (Mernaugh and Ihler, 1992; Xu et al., 1995); however, the significance of this protein *in vivo* has not been determined.

Other studies have demonstrated erythrocytic invasion of *B. henselae*, *B. quintana*, and *B. tribocorum*, which, in contrast to *B. bacilliformis*, do not lead to massive hemolytic anemia but rather persist within erythrocytes, causing chronic bacteremia. The Trw T4SS produces multi-variant pilus subunits which are essential for invasion of erythrocytes. This T4SS was first described as a pathogenicity factor for *B. henselae* and was further confirmed in a rat model of *B. tribocorum* infection which demonstrated that the Trw T4SS was essential for intraerythrocytic invasion (Seubert et al., 2003). Seubert et al. detailed the necessity of the Trw T4SS in establishment of chronic intraerythrocytic bacteremia in mammalian hosts, describing the essential role of multi-variant copies of genes encoding pilin (TrwL) and pilus-associated component (TrwJ). Evidence has shown that the VirB/VirD4 T4SS also facilitates colonization of erythrocytes by *Bartonella* in a rat model (Schulein and Dehio, 2002). They showed *B. tribocorum* was unable to infect erythrocytes upon deletion of the VirB or VirD4 genes. It seems plausible that the Trw T4SS is required for erythrocyte invasion but not essential for initial endothelial cell infection or subversion of host cell function through production of effector proteins, as in the VirB/VirD4 T4SS (Dehio et al., 2004b; Schroder et al., 2005). The Trw T4SS is present in many *Bartonella* spp.,

except *B. clarridgeiae* and ruminant-associated *Bartonella* spp., indicating that those *Bartonella* spp. that have developed this type of T4SS have the ability to adapt to a larger range of novel mammalian hosts (Dehio, 2008).

In 2001, Schulein et al. demonstrated invasion and persistence of *B. tribocorum* expressing green fluorescent protein in experimentally-infected rats by flow cytometry, conventional blood culture, and confocal microscopy. Another study showed that *B. tribocorum* was not detectable in blood of the rats until 4-5 days post-inoculation (Dehio, 2001), indicating colonization of an unidentified niche prior to erythrocyte invasion, though it is likely that *Bartonella* localizes to endothelial cells before the bacteremic phase occurs (Dehio, 2005). In the *B. tribocorum*-rat infection model, a periodic release of bacteria into the bloodstream of the rats occurred approximately every five days, which is consistent with the five-day intervals of relapsing bacteremia and fever seen in patients with *B. quintana* infection (Maurin and Raoult, 1996). Once *B. tribocorum* invades erythrocytes, it replicates within a membrane-bound compartment until a maximum density is reached, where it remains for the lifespan of the red cell (Schulein et al., 2001). Similar to other experimental animal models (Boulouis et al., 2001; Pappalardo et al., 2001; Yamamoto, 2002; Yamamoto et al., 2003), *B. tribocorum* bacteremia dropped below the limit of detection by 10 weeks, though longer-lasting bacteremia has been demonstrated in cats infected with cat-adapted *B. henselae* or *B. clarridgeiae* (Kordick and Breitschwerdt, 1997; Yamamoto, 2002; Yamamoto et al., 2003; Arvend et al., 2008). Boulouis et al. demonstrated bacteremia in female BALB/c mice 8 days post-inoculation with *B. birtlesii*, with peak bacteremia on day 14 and clearance of the bacteria by 10 weeks post-infection (Boulouis et al., 2001).

Vayssier-Taussat et al. demonstrated that the Trw T4SS of *Bartonella* mediates host-specific adhesion to erythrocytes in 2010. The authors of this study found that expression of Trw of the rat-adapted species *B. tribocorum* in *B. henselae* and *B. quintana* enabled these two *Bartonella* species to infect erythrocytes of rats. This demonstrates the mediation of host-specific erythrocyte infection by Trw of *Bartonella*.

Endothelial cells

The chronic phase of *B. bacilliformis* infection, known as verruga peruana, is characterized by distinct dermal lesions resulting from endothelial cell proliferation. Inflammatory cells, such as plasma cells and lymphocytes, can be visualized around the proliferating vessels (Arias-Stella, 1986), demonstrated by Warthin-Starry stain and electron microscopy (Recaverren and Lumbreras, 1972). This is also characteristic of *B. henselae* and *B. quintana* (Montgomery and Garcia, 1997; Karem et al., 2000). The ability of *Bartonella* spp. to cause pathological angiogenesis is unique among bacterial pathogens, and lesions are characterized by proliferating endothelial cells and mixed populations of macrophages/monocytes and polymorphonuclear neutrophils containing bacteria within the foci (Kostianovsky and Greco, 1994; Manders, 1996). Factors that may contribute to stimulation of angiogenesis in endothelial cells include direct proliferation of endothelial cells (Conley et al., 1994), inhibition of endothelial cell apoptosis (Kirby and Nekorchuk, 2002), and *Bartonella*-induced secretion of vasoproliferative cytokines such as vasculoproliferative growth factor (VEGF) and hypoxia-inducible factor (HIF-1) (Kempf et al., 2001; Resto-Ruiz et al., 2002; Pugh and Ratcliffe, 2003).

Most studies to date investigating mechanisms of vasoproliferation caused by *Bartonella* spp. have utilized *in vitro* models of human umbilical vein endothelial cells (HUVECs), demonstrating uptake of bacteria into cells via an actin-dependent process (Brouqui and Raoult, 1996; Dehio et al., 1997; Verma et al., 2000). In *B. bacilliformis*, uptake is assisted by enzymes that catalyze the hydrolysis of guanosine triphosphate, GTPases Rho, Rac, and CDC42, and is presumably present in other *Bartonella* spp., as this mechanism is utilized by other intracellular pathogens (Dramsi and Cossart, 1998). *Bartonella* can enter HUVECs by an "invasome"-mediated mechanism (Dehio et al., 1997). In this mechanism, a bacterial aggregate forms on the surface of the endothelial cell followed by internalization by an actin-dependent process. It has also been demonstrated that *B. henselae* and *B. quintana* can prevent endothelial cell apoptosis of HUVECs *in vitro* by suppressing caspase activation in early apoptotic events and DNA fragmentation in late apoptotic events (Kirby and Nekorchuk, 2002), though *B. vinsonii* and *B. elizabethae* did not show any anti-apoptic activity. This is consistent with naturally-occurring disease, as neither of these *Bartonella* spp. have been shown to produce vasoproliferative lesions.

Seven BEPs are translocated by the VirB/VirD4 T4SS and are required for invasion, suppression of apoptotic activity, and pro-inflammatory activation of endothelial cells leading to vasoproliferation (Kempf et al., 2001). The deletion of genes encoding these BEPs resulted in abrogation of these activities (Schulein et al, 2005). Given this evidence, it is hypothesized that these seven BEPs are the key effector molecules necessary for *Bartonella* to successfully colonize the endothelium of a mammalian host.

BadA, a member of the TAAs formerly known as type IV pili, is another factor involved in mediation of binding of *Bartonella* to extracellular matrix proteins and endothelial cells that is responsible for inhibition of endothelial cell apoptosis and induction of secretion of VEGF and HIF-1 (Reiss et al., 2004; Kempf et al., 2005). Reiss et al. also demonstrated the expression of BadA in naturally-occurring *Bartonella* infection among humans and rodents (Reiss et al., 2004). Collectively, these results provide evidence that BadA is likely a key pathogenicity factor involved in the pathogenesis of vasoproliferative disorders such as bacillary angiomatosis and bacillary peliosis.

Epithelial cells

The invasion of epithelial cells by *Bartonella* spp. and the role these cells may play in induction of angiogenic factors, i.e. secretion of VEGF and HIF-1, leading to vasoproliferative disorders, such as bacillary angiomatosis, has been investigated (Relman et al., 1990; Koehler et al., 1992; Tompkins and Steigbigel, 1993). In 1995, Batterman et al. demonstrated expression of pili, similar to type IV pili of other bacterial pathogens, from a clinical isolate of *B. henselae*. It was able to more readily adhere to and invade epithelial cells (Hep-2 cells) in comparison to a *B. henselae* isolate with less pili and *B. quintana* and *E. coli* without pili, as demonstrated by electron microscopy and gentamicin protection assay (Batterman et al., 1995).

Other investigators have sought to determine the significance of adhesin protein expression in *Bartonella* isolates relative to invasion of epithelial cells. In 2006, Schulte et al. revealed that *B. quintana* not expressing the TAA BadA was unable to stimulate secretion of

VEGF in cocultured THP-1 and HeLa 229 epithelial cells. More recently, Kaiser et al.

demonstrated activation of HIF-1 as well as VEGF and IL-8 secretion by the head domain of

B. *henselae* BadA in cultured HeLa 229 epithelial cells (Kaiser et al., 2008). These results

indicate that the stimulation of angiogenic factors by *Bartonella* from epithelial cells may

play a crucial role in development of vasoproliferative disorders in *Bartonella*-infected

patients.

Other cell types

Invasion of other mammalian cell types by *Bartonella* spp. has been demonstrated *in*

vitro, including CD34+ HPCs (Mandle et al., 2005), erythroblast cells (Rolain et al., 2003d),

dendritic cells (Vermi et al., 2006), and microglial cells (Munana et al., 2001). Recent work

by Mandle et al. in 2005 showed that *B*. *henselae* was able to successfully invade human

CD34+ HPCs *in vitro*, suggesting these cell types may serve as a physiological niche for

Bartonella within a host. In this study, the authors demonstrated that these HPCs were able to

internalize *B*. *henselae* and support intracellular replication of the bacteria by gentamicin

protection assay, electron microscopy, and confocal laser scanning microscopy. Furthermore,

infection of these cells with *B*. *henselae* did not affect erythroid differentiation of the HPCs,

as evidenced by fluorescence-activated cells sorting (FACS) analysis. Another *in vitro* study

performed by Rolain et al. in 2003 revealed *B*. *quintana* was able to invade erythroblasts,

nucleated precursor cells within the bone marrow that develop into erythrocytes. *B*. *quintana*

has also been demonstrated within erythroblasts and mature erythrocytes of a homeless man

with trench fever (Foucault et al., 2004). Similar to HPCs, if *Bartonella* spp. are able to

successfully invade these cells prior to full erythrocytic development *in vivo*, this would enable the bacteria to persist within the host indefinitely.

Dendritic cells play a key role in the primary immune response as antigen-presenting cells (Banchereau et al., 2000; Steinman, 2003). These cells activate other immune cells, such as B cells and T cells, in the lymph nodes after exposure to pathogens, leading to initiation of the adaptive immune response (Sozzani et al., 1998; Rescigno et al., 2000; Lanzavecchia and Sallusto, 2001). Subsequent activation of pattern recognition receptors by microbial pathogens, part of TLR activation, activates production of pro-inflammatory cytokines IL-6 and TNF-α and the anti-inflammatory cytokine IL-10 after uptake of *B. henselae* by dendritic cells (Vermi et al., 2006). Interestingly, it seems that *B. henselae* LPS does not stimulate the activation of TLR4, but another component of *Bartonella* may activate immune cells (Zahringer et al., 2004). Furthermore, Popa et al. demonstrated that the LPS of *B. quintana* is actually an antagonist of TLR4, as it did not elicit a pro-inflammatory immune response when incubated with human monocytes *in vitro* but rather inhibited production of IL-1β, TNF-α, and IL-6 by monocytes exposed to *E. coli* LPS (Popa et al., 2007). Though the mechanisms utilized by *Bartonella* to suppress the pro-inflammatory response are not clearly defined, these results suggest that *Bartonella* spp., at least in the case of *B. quintana*, have adapted means to evade the host immune response, allowing the bacteria to persist in its mammalian host.

Summary

Numerous studies have demonstrated the ubiquitous nature of *Bartonella* spp. in cellular niches *in vitro* and mechanisms these bacteria have acquired to assist in cellular invasion (BadA) and intracellular persistence (production of IL-10 and inhibition of IL-1β and TNF-α production). The non-hemolytic, intracellular localization of *B. henselae* and *B. quintana* is essential for survival within a mammalian host, bacterial uptake, and subsequent transmission of *Bartonella* from one host to another.

Geographic distribution and genetic diversity

Bartonella species have a worldwide distribution (Breitschwerdt and Kordick, 2000), and individual species have evolved means to inhabit specific mammalian hosts and survive within distinct blood-feeding arthropods for transmission to these hosts. *B. bacilliformis*, is a prime example of a *Bartonella* sp. that is limited by the geographic location of the sandfly that is only found in the Andes region of South America. Genetic divergence of other *Bartonella* spp. from *B. bacilliformis* has enabled adaptation to new vectors, hosts, and geographic environments. The distribution of vector-borne pathogens are greatly influenced by the movement of humans throughout natural habitats of other animal species. This movement directly affects the ecosystem and the distribution of these pathogens and has assisted them in invading previously unoccupied niches.

Relman addressed the complexity of defining the factors that influence the emergence and re-emergence of infectious diseases, using *B. quintana* as an example and its variability in disease presentation over time (Relman, 1995). Whether the evolution of detection

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methods, molecular characterization, and genomic analysis of *Bartonella* spp. has attracted attention to these bacterial pathogens or if these bacteria have themselves evolved mechanisms to occupy new host niches, contributing to previously unrecognized disease manifestations, is difficult to determine. However, numerous epidemiological and genomic studies have demonstrated that individual *Bartonella* sp. have likely acquired mechanisms over time to inhabit novel hosts, contributing to chronic bacteremia and persistence within those hosts.

Molecular and serologic prevalence studies have been conducted globally to determine *Bartonella* spp. infection/exposure in mammals, demonstrating a ubiquitous geographic distribution. Numerous prevalence studies have been performed to determine distribution of *B. henselae* in domestic cats worldwide due to the clinical relevance of transmission from cats to humans. Table 1 gives a summary of prevalence of *B. henselae* detected by molecular or serological methods in domestic cats (pets and strays), and this collective data is indicative of the global distribution of other *Bartonella* spp. in many mammalian hosts.

The geographic distribution of the four characterized *B. vinsonii* subsp. *berkhoffii* genotypes has demonstrated that individual subspecies may be localized to specific geographic locations, though the factors influencing this distribution, i.e. environment-, host-, or vector-related, remain elusive. The four *B. vinsonii* subsp. *berkhoffii* genotypes have been identified in the following mammalian species and geographic locations: genotype I in dogs and coyotes in the US (Breitschwerdt et al., 1995; Chang et al., 2000; Maggi et al., 2006; Breitschwerdt et al., 2007b; Duncan et al., 2007), genotype II in dogs, coyotes, and humans

in the US (Maggi et al., 2006; Breitschwerdt et al., 2007b; Duncan et al., 2007), genotype III

in gray foxes in the US, one human patient with endocarditis in Europe, and a military

working dog from Germany who was with endocarditis 18 months after arriving in the US

(Roux et al., 2000; Maggi et al., 2006; Cadenas et al., 2008), and genotype IV in two dogs

with endocarditis, one in Canada and one in the US (Maggi et al., 2006; Cockwill et al.,

2007). More epidemiological and prevalence studies should be conducted to determine

worldwide distribution of each of these distinct *B. vinsonii* subsp. *berkhoffii* genotypes. As

Maggi et al. stated in 2006, "Strain typing may also help to better define reservoir potential,

carriership patterns, modes of transmission, and the relative pathogenicity of each of the *B.

vinsonii berkhoffii* types". Once distribution of each of the genotypes is defined, researchers

can discern the variables that define that distribution.

In order to understand the ecological diversity of the *Bartonella* genus, molecular

techniques have been useful in identifying *Bartonella* species and strains. Several genetic

changes have led to the ability of this genus to adapt, survive, and thrive in diverse hosts,

vectors, and geographic locations. Phylogenetic analyses of the sequenced genomes of

Bartonella spp. have broadened our understanding of the relationships and evolution of these

bacteria.

Genes within *Bartonella* spp., such as the 16S ribosomal RNA, 16S-23S ribosomal

RNA intergenic spacer (ITS) region, citrate synthase (*gltA*), and heat-shock protein (*groEL*)

genes, have been used to study the lineage diversity within these species. In 2001, Houpikian

and Raoult reported use of each of these genes to identify clusters of *Bartonella* spp. relative

to host specification and geographic distribution. By phylogenetic analysis and development

of parsimony trees from each of those genes sequenced, six distinct evolutionary clusters were identified: 1) *B. bacilliformis*, 2) *B. clarridgeiae*, 3) *B. henselae*, *B. koehlerae*, and *B. quintana*, 4) *B. vinsonii* subspecies *berkhoffii* and *vinsonii*, 5) three "New World rodent"-adapted *Bartonella* spp., and 6) "Old World rodent"-adapted species, including *B. grahamii*, *B. elizabethae*, and *B. tribocorum*. New World rodents include mice (*Phylottis peruviana*), cotton rats (*Sigmodon hispidus*), and rice rats (*Oryzomys palustris*), whereas Old World rodents include rat species *Rattus rattus* and *Rattus norvegicus*, the mouse species *Mus musculus*, and voles (*Clethrionomys glareolus*) (Ellis et al., 1999b). Clustering of New World and Old World rodent-adapted *Bartonella* spp. suggests a common *Bartonella* sp. which utilized rodents as reservoir hosts may have diverged and evolved independently following the continental drift (Houpikian and Raoult, 2001). Whereas geographic distribution of *B. bacilliformis* is limited by the specific geographic location of its sandfly vector, as previously mentioned, this data would support the probability that *Bartonella* spp. adapted to Old and New World rodents are limited in their distribution by the geographic locations of the reservoir hosts. (Birtles et al., 1994; Birtles et al., 1995; Hofmeister et al., 1998; Welch et al., 1999; Telfer et al, 2007a, 2007b)

A study by Arvand et al. in 2006 identified presence of four genetic variants within individual *B. henselae* isolates, consisting of three primary clinical isolates and two reference strains. High passage isolates contained no genetic variants within single colonies by PFGE; however, all three primary clinical isolates displayed genetic variance within each of the isolates as determined by PFGE and confirmed by PCR and MLTS. As periodic subculturing decreases virulence, this may often be due to a decrease in genetic heterogeneity within

individual *B. henselae* isolates. The authors hypothesized that genetic variation within one strain of *B. henselae* allows this *Bartonella* sp. to evade elimination by host immune responses.

In 2007 Arvand et al. sought to determine if genetic variance coincides with virulence of distinct *B. henselae* strains from North America, Australia, and Europe by MLTS. Though 14 distinct sequence types (STs) were identified among human and feline isolates, 66% of the isolates recovered from ill human patients with bartonellosis on each of the continents were of the ST1 genotype, and 27.2% of the feline isolates were of the ST7 genotype. Interestingly, the ST7 genotype was not identified in any cases of human disease, indicating genetic variance among *B. henselae* populations is consistent with increased virulence in humans. Furthermore, Iredell et al. used MLTS and PFGE to analyze sequence types from human and feline isolates of *B. henselae* and found the sequence types from human clinical isolates was not random. Inheritances of several genes could not be reconciled, indicating probability of horizontal gene transfer and evolution of genetic variation among *B. henselae* genotypes (Iredell et al., 2003). In 2008, Viezens and Arvand reported presence of two distinct 16S rRNA sequences within individual *B. henselae* isolates, suggesting another potential mechanism of host immune system evasion by this *Bartonella* species supported by a similar finding of genomic variations of *B. henselae* isolates from naturally-infected cats (Kabeya et al., 2002). Additionally, Berghoff et al. reported genetic variation in 58.8% of *B. henselae* isolates analyzed by PFGE (Berghoff et al., 2007).

In 2004, Alsmark et al. conducted a study to identify genetic differences between *B. quintana* (1,581,384 basepairs) and *B. henselae* (1,931,047 base pairs) by genomic analysis.

Genomes of *Bartonella* spp. are composed of a singular circular chromosome, but results from this study revealed distinct differences between *B. quintana* and *B. henselae*, including significant genome degradation in *B. quintana*, including loss of a prophage-encoding gene within its genome, and genomic islands within the *B. henselae* genome not found in the *B. quintana* genome which code for filamentous hemagglutinin, a key factor in host cell attachment. In contrast, two gene segments were present in *B. quintana* not found in *B. henselae*, one adjacent to a tRNA gene for prophage lysozyme and another next to a tRNA gene for putative toxin/hemolysin secretion. The authors hypothesized that the genome reduction of *B. quintana*, presumably a genomic derivative of *B. henselae*, has led to its specialist nature.

Evolution of pathogenicity factors, such as the previously described T4SS and the TAAs, of *Bartonella* spp. have been evaluated to determine how these species have evolved to occupy new host niches. T4SSs are absent from the genome of *B. bacilliformis*, the ancestral *Bartonella* species from which all other *Bartonella* spp. are thought to have evolved. Radial speciation is presumed to have resulted in horizontal acquisition of T4SSs, leading to the ability of *Bartonella* spp. to colonize host cells without causing massive hemolytic anemia in mammalian hosts (Schulein and Dehio, 2002; Seubert et al., 2003; Schroder and Dehio, 2005; Saenz et al., 2007). Though the mechanisms *Bartonella* spp. have evolved to confer host adaptability through T4SSs have not been defined, development of theT4SSs of *Bartonella* spp. has enabled these bacteria to translocate bacterial effector proteins to endothelial cells (VirB/VirD4 and Vbh) and facilitate interaction with host

erythrocytes (Trw), resulting in a broader host range and reduced virulence for persistence within these hosts (Dehio, 2008).

Adhesion of *Bartonella* to the extracellular matrix of host cells is mediated by adhesin proteins (Ljungh and Wadstrom, 1995). The TAAs of the *Bartonella* genus belong to the BadA family of *B. henselae* (Riess et al., 2004), the Vomp family of *B. quintana* (Zhang et al., 2004), and the BrpA family of *B. vinsonii* subsp. *arupensis* (Gilmore et al., 2005). These TAA families of proteins are quite variable in size, at 340 kDa, 100 kDa, and 382 kDa, respectively. The Vomp proteins are essential virulence components of *B. quintana* (MacKichan et al., 2008). Kaiser et al. demonstrated in 2008 that the head domain of BadA is critical for binding to endothelial cells and collagen but not fibronectin (Kaiser et al., 2008). Repetitive DNA sequences in stalk domains of TAAs allow recombination events to occur, facilitating evasion of host immune responses by *Bartonella* (Linke et al., 2006) due to "antigenic variation" or "phase variation" of the pathogen, both established mechanisms for persistence by various microbial pathogens (Robertson and Beyer, 1992, Genetic variation).

Summary

The geographic distribution of *Bartonella* spp. in a variety of mammalian hosts worldwide is likely due to a combination of variables, including anthropogenic factors, vector-related distribution, as well as evolution of pathogenicity factors. The extent to which each of these components contributes to *Bartonella* spp. distribution cannot be linearly defined and is likely a combination of each of these factors as well as other unknown environmental variables. By combining historical accounts of *Bartonella* epidemics with

serological and molecular prevalence studies and new methods of genetic characterization, researchers have been able to elucidate previously unknown factors contributing to the geographic distribution and genetic evolution of the *Bartonella* genus. Future studies are warranted to determine how variables relative to dispersal and pathogenesis of the *Bartonella* spp. play a role in its ecological diversity.

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Table 1. Geographic locations, populations tested, detection methods, and prevalence of *Bartonella henselae* in domestic cats worldwide.

Geographic Locations	Pets/Strays	Detection method	Prevalence (%)	References
North America (US, Canada)	Pets	PCR	21.4 – 24	Chomel et al., 1995
				Guptill et al., 2004
		Serology	17.8 – 75	Jameson et al., 1995
				Baneth et al., 1996
				Leighton et al., 2001
				Guptill et al., 2004
				Nutter et al., 2004
				Pearce et al, 2006
	Strays	PCR	61.3	Chomel et al., 1995
		Serology	33.6 – 93	Luria et al., 2004
				Nutter et al., 2004
South America (Brazil, Chile)	Pets	Serology	71	Zaror et al., 2002
	Strays	PCR	10.63	Staggemeier et al., 2010
Asia (Japan, China, Indonesia, Korea, Philippines, Thailand)	Pets	PCR	5.8 – 33.3	Maruyama et al., 2000
				Inoue et al, 2009
				Kim et al, 2009
				Yuan et al, 2011
		Serology	8.8 – 15.1	Ueno et al., 1995
				Maruyama et al., 1998
				Maruyama et al., 2003
	Strays	PCR	16.3 – 68.4	Chomel et al., 1999
				Marston et al., 1999
				Inoue et al, 2009
				Kim et al, 2009
				Yuan et al, 2011
		Serology	47.5 – 68	Chomel et al., 1999
				Marston et al., 1999
				Nasirudeen & Thong, 1999
Northern and Central Europe (Czech Republic, UK, Denmark, Norway, Poland, Sweden, Austria, Switzerland)	Pets	PCR	0 – 18.2	Birtles et al., 2002
				Chomel et al., 2002
				Laycock et al., 2001
				Engvall et al., 2003
				Melter et al, 2003
		Serology	1 – 40.6	Allerberger et al., 1995
				Glaus et al., 1997
				Barnes and Bell, 2000
				Bergh et al., 2002
				Hjelm et al., 2002
	Strays	PCR	18 – 66	Melter et al., 2003
				Engbaek & Lawson, 2004
		Serology	41.8	Barnes and Bell, 2000
Australia and New Zealand	Pets	PCR	13.2 – 17	Branley et al., 1996
				Joseph et al., 1997
	Strays	PCR	40	Ng and Yates, 1997

Table 1 (continued).

Geographic Locations	Pets/Strays	Detection method	Prevalence (%)	References
Africa (Zimbabwe, South Africa, Trinidad)	Pets	PCR	3.2 – 8	Kelly et al., 1996
				Kelly et al., 1998
	Strays	PCR	15.5	Pretorius et al., 1999
		Serology	21	Rampersad et al., 2005
Middle East (Israel, Jordan, Egypt)	Pets	Serology	12 – 39.5	Childs et al., 1995
				Baneth et al., 1996
				Al-Majali, 2004
Southern Europe (Portugal, Italy, Turkey)	Pets	PCR	9.7 – 13.6	Cabassi et al., 2002
				Celebi et al, 2009
		Serology	6.8 – 13	Childs et al., 1995
				Celebi et al, 2009
	Strays	PCR	9.8 – 23	Fabbi et al., 2004a
				Fabbi et al., 2004b
				Celebi et al, 2009
		Serology	27.5 – 39	Fabbi et al., 2004a
				Fabbi et al., 2004b
				Celebi et al, 2009
Germany, France, the Netherlands	Pets	PCR	7.1 – 75	Sander et al., 1997
				Arvand et al., 2001
				Gurfield et al., 2001
		Serology	15 – 56	Bergmans et al., 1997
				Haimerl et al., 1999
	Strays	PCR	18.7 – 70	Bergmans et al., 1997
				Heller et al., 1997
				Arvand et al., 2001
				La Scola et al., 2002
		Serology	50	Bergmans et al., 1997

Chapter 2. PCR detection of *Bartonella bovis* and *Bartonella henselae* in the blood of beef cattle

Manuscript as published in Veterinary Microbiology.

Cherry, N.A., Maggi, R.G., Cannedy, A.L., Breitschwerdt, E.B., 2009. PCR detection of *Bartonella bovis* and *Bartonella henselae* in the blood of beef cattle. Vet Microbiol. 135(3-4):308-312.

PCR Detection of *Bartonella bovis* and *Bartonella henselae* in the blood of beef cattle

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Abstract

Although an organism primarily associated with non-clinical bacteremia in domestic cattle and wild ruminants, *Bartonella bovis* was recently defined as a cause of bovine endocarditis. The purpose of this study was to develop a *B. bovis* species-specific PCR assay that could be used to confirm the molecular prevalence of *Bartonella* spp. infection. Blood samples from 142 cattle were tested by conventional PCR targeting the *Bartonella* 16S–23S intergenic spacer (ITS) region. Overall, *Bartonella* DNA was detected in 82.4% (117/142) of the cattle using either *Bartonella* genus primers or *B. bovis* species-specific primers. Based upon size, 115 of the 117 *Bartonella* genus ITS PCR amplicons were consistent with *B. bovis* infection, which was confirmed by PCR using *B. bovis* species-specific primers and by sequencing three randomly selected, appropriately sized *Bartonella* genus PCR amplicons. By DNA sequencing, *Bartonella henselae* was confirmed as the two remaining amplicons, showing sequence similarity to *B. henselae* URBHLIE 9 (AF312496) and *B. henselae* Houston 1 (NC_005956), respectively. Following pre-enrichment blood culture of 12 samples in *Bartonella* alpha-Proteobacteria growth medium (BAPGM) *B. henselae* infection was found in another three cows. Four of the five cows infected with *B. henselae* were co-infected with *B. bovis*. To our knowledge this study describes the first detection of *B. henselae* in any large ruminant species in the world and supports the need for further investigation of prevalence and pathogenic potential of *B. henselae* and *B. bovis* in cattle.

Introduction

To date, at least 22 species or subspecies of the genus *Bartonella* have been characterized, however, only two species, *Bartonella bovis* (previously described as *Bartonella weissii*) and *Bartonella chomelii*, have been found to infect cattle (Chang et al., 2000; Breitschwerdt et al., 2001; Maillard et al., 2004; Kelly et al., 2005). Bartonellae are small intracellular, gram-negative bacteria, and all species are thought to be transmitted by blood-sucking arthropods (Maurin and Raoult, 1996; Chomel, 2000; Chang et al., 2001; Halos et al., 2004; Alexander, 2005; Skotarczak and Adamska, 2005). In 2004, *Bartonella* DNA was amplified for the first time in North American flies collected from beef and dairy cattle barns and feedlots in California (Chung et al., 2004). In that study, a sequence identical to *B. bovis* was obtained from two horn flies (*Haematobia* spp.) collected from a beef cattle barn. A definite route of transmission of *B. bovis* to cattle has yet to be determined, but it has been suggested that ticks may be a potential vector (Chang et al., 2001; Skotarczak and Adamska, 2005). The detection of *Bartonella* spp. in biting flies and ticks, common ectoparasites of ruminants, supports their potential role for *B. bovis* transmission to cattle (Chung et al., 2004; Halos et al., 2004). Detection of *Bartonella* spp. infection in beef cattle is of potential importance because these highly prevalent and previously unknown intravascular bacteria may play an unrecognized role in complex disease expression (Merrell and Falkow, 2004). Although an organism primarily associated with non-clinical bacteremia in domestic cattle and wild ruminants in North America (Chang et al., 2000; Breitschwerdt et al., 2001; Maillard et al., 2004), domestic cattle in Europe (Bermond et al., 2002; Maillard et

al., 2004, 2006) and in Africa (Kelly et al., 2005), *B. bovis* was recently defined as a cause of bovine endocarditis (Maillard et al., 2007). The extent to which *B. bovis* may interact with or complicate the diagnosis of other infectious and noninfectious diseases of cattle is unknown. The high prevalence of *B. bovis* infection found in many cattle populations studied to date indicates that this organism shares a strong evolutionary adaptation with ruminant species; however, it is possible that chronic infection with these intravascular bacteria contributes to pathology during states of stress, nutritional deprivation or exposure to toxins (Breitschwerdt and Kordick, 2000).

In this study, *B. bovis* infection was identified in 82.4% of North Carolina beef cattle. Using IFA serology, a previous study from North Carolina found 36 of 38 cows had antibodies (>1:32) to *B. bovis* (*B. weissii*) antigens (Breitschwerdt et al., 2001). In a study from California, *Bartonella* bacteremia was documented by blood culture isolation in 81–96% of beef cattle (Chang et al., 2000). In that same study, only 17% of the dairy cattle population from the same region was found to be *Bartonella* bacteremic. In contrast to the California results, our laboratory has experienced difficulty in obtaining *B. bovis* isolates by either direct plating (Breitschwerdt et al., 2001) or by using BAPGM enrichment culture of stored-frozen samples as described in this study. The reason(s) for this discrepancy remains unclear.

A rapid, efficient, and cost effective means to detect *Bartonella* infection in ruminants would facilitate future clinical, pathological and epidemiological studies. Therefore, the purpose of this study was to generate *B. bovis*-specific ITS primers, to compare the sensitivity of these

primers to *Bartonella*-genus-specific primers, and to determine themolecular prevalence of *Bartonella* spp. infection in several beef cattle herds. The 16S–23S intergenic spacer region was targeted because this PCR assay provides sensitive and specific detection of *Bartonella* spp. DNA in biological samples (Roux and Raoult, 1995; Minnick and Barbian, 1997; Jensen et al., 2000; Houpikian and Raoult, 2001; Maillard et al., 2004; Garcı´a-Esteban et al., 2008). In conjunction with this study, co-infection with *B. henselae* and *B. bovis* was found in four cows and one cow was infected solely with B. henselae, which represents the first report of infection with this *Bartonella* sp. in ruminants.

2. Materials and methods

2.1. Study population

From February to November 2001, 142 EDTA anticoagulated blood samples were collected from three separate beef cattle herds located in the Piedmont region of North Carolina, specifically Durham, Wake and Sampson counties. There were no specific exclusion criteria that limited entry into the study, therefore age, sex, breed, health status, and farm management practices were not controlled.

2.2. DNA extraction

DNA was extracted from 200 µl of frozen EDTA-blood samples using a previously described extraction technique (Diniz et al., 2007).

2.3. *Bartonella* ITS (16S–23S ribosomal RNA intergenic spacer) genus PCR amplification

Bartonella genus screening was performed as previously described (Maggi and Breitschwerdt, 2005a). PCR screening of *Bartonella* ITS region was performed in all 142 samples using oligonucleotides 325s: 5'-CTT CAG ATG ATG ATC CCA AGC CTT CTG GCG-3' and 1100as: 5'-GAA CCG ACG ACC CCC TGC TTG CAA AGC A-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), 0.25 µl of 30 µM of each forward and reverse primer (IDT® DNA Technology), 8 µl of molecular-grade water, and 5 µl of DNA from each sample were tested. PCR negative controls were prepared using 5 µl of DNA from blood of a healthy, specific pathogen-free dog. Positive controls for PCR were prepared by serial dilution (using dog blood DNA) of *Bartonella* genomic DNA (down to 0.002 pg/µl stock) from *B*. *henselae*. PCR was performed in an Eppendorf Mastercycler EPgradient® under the following conditions: a single hot-start cycle at 95ºC for 2 min followed by 55 cycles of denaturing at 94ºC for 15 s, annealing at 66ºC for 15 s, and extension at 72ºC for 18 s. Amplification was completed by an additional cycle at 72ºC for 1

min, and products were analyzed by 2% agarose gel electrophoresis with detection using ethidium bromide under ultraviolet light.

2.4. *Bartonella bovis* ITS (16S–23S ribosomal RNA intergenic spacer) PCR amplification

All 142 samples were also tested using newly designed *B. bovis* species-specific primers that incorporated the same PCR conditions described above and the same reverse primer, but the new assay incorporated a forward primer that is specific for the amplification of *B. bovis* (*B. bovisf*: 5'-GGA GCG TTT AAA AAA ACA AAC CAA AAG CG-3').

2.5. *Bartonella* species pre-enrichment culture

One milliliter of blood, previously frozen for several years, from 10 *B. bovis* and the 2 *B. henselae* PCR positive cattle were inoculated into liquid *Bartonella* alpha-Proteobacteria growth medium (BAPGM) and incubated at 35ºC in 5% CO_2, water-saturated atmosphere as previously described (Maggi et al., 2005b; Maggi and Breitschwerdt, 2005c; Duncan et al., 2007). Following a 7-day incubation period, a 1 ml sample from the liquid culture was sub-inoculated onto BAPGM/blood–agar plates and incubated for 14 days under the same conditions. DNA from 200 µl of BAPGM liquid cultures were extracted using a commercially available MagAttract DNA blood kit (BioRobot M48, Qiagen, Chatsworth, CA, USA) and tested for *Bartonella* DNA as above.

2.6. Cloning and sequencing of ITS region amplicons

PCR products were cloned into plasmid pGEM-T Easy Vector System (Promega, Madison, WI, USA) and the recombinants selected based on white/blue screening, EcoRI digestion and 2% agarose gel electrophoresis. After plasmid purification using the Qiagen Miniprep procedure (Qiagen, Valencia, CA, USA), insertions were sequenced at Davis Sequencing (Davis, CA, USA). Chromatogram evaluation and sequence alignment were performed using ContigExpress and AlignX softwares (Vector NTI Suite 10.1, Invitrogen Corp., Carlsbad, CA, USA). Bacteria species and strains were defined by comparing similarities with other sequences deposited in GenBank prior to May 2008 using the Basic Local Alignment Search Tool (Blast version 2.0) (Diniz et al., 2007).

3. Results

3.1. *Bartonella* genus ITS PCR

Using *Bartonella* ITS genus primers, DNA was amplified from 115/142 (81%) North Carolina beef cattle blood samples. For all but two samples, PCR amplicons matched the expected size for *B*. *bovis* (408 bp). Sequencing of three randomly selected ITS amplicons showed a 98.8% homology with *B*. *bovis*, accession number AY116638. Sequences obtained from the two ITS positive PCR samples that did not match the expected *B*. *bovis* amplicon

size were 100 and 99.8% similar to *B*. *henselae* URBHLIE 9 (AF312496), and 99.8 and 99.7% similar to *B*. *henselae* Houston 1 (NC_005956), respectively.

3.2. *Bartonella bovis* ITS PCR

Using *B*. *bovis*-specific primers, 111 of the 142 samples (78.2%) were PCR-positive. Two samples, originally PCR negative using *Bartonella* genus ITS primers, were subsequently amplified using the *B*. *bovis* primer set, whereas four *Bartonella* ITS genus PCR positive samples which contained an amplicon size matching *B*. *bovis*, were PCR negative using *B*. *bovis* primers. Differences in primer sensitivity may be responsible for the lower limit of detection using *Bartonella* genus primers (two gene copies per PCR reaction) as compared to five gene copies per reaction for the *B*. *bovis*-specific primers (results not shown). Combining results from both primer sets, 115/142 (81%) beef cattle blood samples contained *B*. *bovis* DNA. There was 95.7% agreement among the two PCR assays which proved to be only marginally different by statistical analysis. A Kappa value of 0.866 confirmed the comparable diagnostic efficiency of the *Bartonella* genus ITS primers and the newly designed *B*. *bovis*-specific primers (Table 1). The two *B*. *henselae* PCR positive original blood samples, using *Bartonella* genus ITS primers, were PCR negative for *Bartonella* using *B*. *bovis*-specific ITS primers.

3.3. Blood pre-enrichment

A subset of 12 stored-frozen blood samples that were *B. bovis* (10) or *B. henselae* (2) PCR positive using *Bartonella* genus ITS primers were selected for BAPGM culture. Following incubation in liquid BAPGM for 7 days, DNA was amplified from 5/12 samples using *Bartonella* ITS genus primers, of which 3 contained PCR amplicons matching the size expected for *B. henselae* (604 bp). By DNA sequencing, one of these amplicons was 99.8% similar to *B. henselae* URBHLIE 9 (AF312496), and 99.7% similar to *B. henselae* Houston 1 (NC_005956). The 2 remaining PCR positive BAPGM cultures contained an amplicon of the size that matches *B. bovis* (408 bp). Prior to culture, one of these *B. bovis* enrichment positive samples was *B. bovis* PCR positive, whereas the other was *B. henselae* PCR positive. Of the 5 *B. henselae*-infected cows, 4 were concurrently infected with *B. bovis*. No subculture isolates were obtained.

4. Discussion

The purpose of this study was to determine the molecular prevalence of *Bartonella* spp. among three beef cattle herds located in the Piedmont region of North Carolina and to validate a *B. bovis* species-specific PCR assay. As previous studies have documented that ruminants are the primary reservoir hosts for *B. bovis* (Chang et al., 2000; Breitschwerdt et al., 2001; Bermond et al., 2002; Maillard et al., 2004; Kelly et al., 2005), the development of a PCR assay that would specifically confirm infection with this *Bartonella* species in cattle

blood samples was the next logical step. Access to banked bovine blood obtained from three beef cattle herds in North Carolina provided an opportunity to examine the regional *B. bovis* molecular prevalence and to test the comparative efficacy of a newly designed *B. bovis*-specific PCR assay. Using the 325 s forward primer and conditions for a previously described ITS PCR assay (Maggi and Breitschwerdt, 2005a), a new *B. bovis*-specific reverse primer was designed. When results of the two PCR assays were compared, there was 95.7% agreement in the detection of *B. bovis* DNA. Since there was nearly identical disagreement in the number of PCR positive results missed by each assay (four vs. two samples), the discrepancy among assays was most likely related to low template DNA concentrations or minor variations in primer annealing efficiency. Alternatively, a significantly larger quantity of *B. bovis* DNA in a blood sample could result in selective amplification of this species with *Bartonella* genus ITS primers, which could hinder PCR detection of *B. henselae*. Although there was no statistical difference among the two assays, testing solely with *B. bovis*-specific primers would have resulted in failure to identify the five *B. henselae* infected cows, a *Bartonella* species described for the first time in cattle in this study. Therefore, when testing bovine samples we would propose the use of *Bartonella* genus primers as an initial screening assay and *B. bovis*-specific primers for species-specific confirmation. This approach would also allow for the detection and characterization of non-*B. bovis Bartonella* species or potential co-infection with more than one *Bartonella* sp. in bovine blood samples.

Based upon BAPGM pre-enrichment culture results using a subset of samples, single or co-infection with *B. henselae* may not be uncommon in North Carolina beef cattle. Because *B*.

bovis DNA was amplified from 10 original blood samples, but from only one pre-enrichment blood culture, it may be concluded that BAPGM does not support the growth of *B. bovis* or that *B. bovis* in the stored-frozen samples was no longer viable. As BAPGM facilitated the growth and PCR detection of *B. henselae* in 3/12 samples tested, this media does not appear to support the growth of *B. bovis*.

Interestingly, *B. bovis*, the primary *Bartonella* species infecting cattle throughout the world, was initially isolated from a small number of cats, described at that time as *B. weissii*, which may reflect transmission from a reservoir adapted bovine host to a non-reservoir adapted feline host via an as yet undetermined vector (Chomel et al., 1995). The detection of *B. henselae* in cattle in this study along with the previous identification of *B. henselae* in a biting fly collected among cattle in California (Chung et al., 2004) suggests that flies may facilitate the transmission of *B. henselae* to cattle. Assuming that flies are the primary vectors for *B. bovis* transmission, fly control practices may explain previously described differences in the prevalence among beef and dairy cattle (Chung et al., 2004).

It is clear that certain *Bartonella* species, for example *B. henselae* in cats, *B. vinsonii* subsp. *berkhoffii* in canines and *B. bovis* in cattle, are very well adapted on an evolutionary basis to induce chronic, non-clinical intravascular and endothelial infections in reservoir-adapted hosts (Breitschwerdt and Kordick, 2000; Breitschwerdt, 2008). However, for reasons that remain less than clear, these same bacteria induce severe adverse health conditions, such as endocarditis, in reservoir-adapted hosts (Reef and McGuirk, 1996; Malik et al., 1999;

Chomel et al., 2003; Andrews and Williams, 2004; Maillard et al., 2007). Quantitative PCR, immunohistochemistry and fluorescent in situ hybridization assays may prove useful to determine if *B. bovis* is pathogenic or can serve as a co-factor in various bovine diseases. The role of *B. bovis* and *B. henselae* in the pathogenesis of bovine diseases should be considered in future studies.

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Table 1. Comparative efficiency of *Bartonella*-genus primers and *B. bovis*-specific primers for the detection of *B. bovis*.

	Bartonella-genus primers		Total
	Positive	Negative	
B. bovis-specific primers			
Positive	109	2	111
Negative	4[a]	25	29
Total	113	27	140

Kappa value of agreement: 0.866; S.E.ss: 0.053; 95% CI: 0.762< κ < 0.971.
[a] The only two *B. henselae* positive amplicons were removed from the table for statistical analysis.

Chapter 3. Ecological diversity of *Bartonella* species infection among dogs and their owner in Virginia

Ecological diversity of *Bartonella* species among dogs and their owner in Virginia

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Abstract

Bartonella species comprise a genus of gram-negative, fastidious, intracellular bacteria that have been implicated in association with an increasing spectrum of disease manifestations in dogs and human patients. In this study, chronic canine and human disease, for which causation was not diagnostically defined, were reported by the breeder of a kennel of Doberman pinschers. In addition to other diagnostic tests, serology, polymerase chain reaction, and enrichment blood culture were used to assess the prevalence of *Bartonella* sp. infection in the dogs and their owner. From five dogs, *Bartonella vinsonii* subsp. *berkhoffii* genotype I, multiple *Bartonella henselae* strains, and a species most similar to *Candidatus* B. volans, a rodent-associated *Bartonella* sp., were amplified and sequenced from biopsy tissues, cerebrospinal fluid, or blood enrichment cultures. The owner was bacteremic with *B. vinsonii* subsp. *berkhoffii* genotype I, the same subsp. and genotype detected in one of her dogs. These results further emphasize the ecological complexity of *Bartonella* sp. transmission in nature.

Introduction

Bartonella species are gram-negative, fastidious intracellular bacteria of the family alpha-Proteobacteria, which are transmitted by animal bites or scratches, by blood transfusion and by blood-sucking arthropods, such as sandflies, fleas, body lice, and potentially by ticks, keds, and other arthropods (Carithers 1985, Lucey et al. 1992, Kordick and Breitschwerdt

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1997, Chang et al. 2002, Rolain et al. 2003, Dehio et al. 2004). Within the genus *Bartonella*, over 26 species or subspecies, some of which have *Candidatus* status, have been characterized. These intraerythrocytic, endotheliotropic bacteria have been implicated in association with an increasing spectrum of disease manifestations in both dogs (Breitschwerdt et al. 1995, 1999, Pappalardo et al. 2000b, Kitchell et al. 2000, Yager et al. 2010) and human patients (Roux et al. 2000, Breitschwerdt et al. 2009a, 2009b, 2010a, 2010b, Breitschwerdt and Maggi 2009). Pathophysiological abnormalities associated with Bartonella infection in dogs or humans include lymphadenopathy, granulomatous inflammatory diseases, arthropathy, endocarditis, neurological signs, including seizures, sensory or motor neuropathy, and neurocognitive abnormalities, and vasoproliferative lesions in immunocompromised patients, including bacillary angiomatosis and peliosis hepatis (Breitschwerdt et al. 1995, 2009a, 2009b, Breitschwerdt and Maggi 2009, Chomel et al. 2009b, Kitchell et al. 2000, Yager et al. 2010).

In conjunction with the recent identification of numerous, previously unknown *Bartonella* species, there has been a concurrent recognition of novel reservoir hosts and the identification of an expanding spectrum of transmissioncompetent arthropod vectors (Chang et al. 2000, 2002, Dehio et al. 2004, Chomel et al. 2009a, Minnick and Battisti, 2009). Although the extent to which persistent infection with various *Bartonella* sp. can occur in pet dogs is poorly understood, natural infection with *Bartonella vinsonii* subsp. *berkhoffii* can span months in duration (Kordick and Breitschwerdt 1998). After experimental infection, *B*.

vinsonii subsp. *berkhoffii* was associated with suppression of humoral and cell-mediated immunity (Pappalardo et al. 2000a, 2001).

Sequencing of the 16S-23S intergenic spacer (ITS) region has identified four *B. vinsonii* subsp. *berkhoffii* genotypes (Kordick and Breitschwerdt 1998, Chang et al. 2000, Maggi et al. 2006, Breitschwerdt et al. 2007, Duncan et al. 2007a). A specific vector that transmits genotypes of this subspecies among wild and domestic canines has not been determined, though ticks are currently considered a likely vectorial candidate (Breitschwerdt and Kordick 2000, Billeter et al. 2008, Angelakis et al. 2010, Telford and Wormser 2010). Risk factors associated with the transmission of most *Bartonella* sp. to animals or human patients remain poorly understood. Although flea and tick exposure and residing in rural environments are defined risk factors associated with *Bartonella henselae* and *B. vinsonii* subsp. *berkhoffii* exposure in dogs (Pappalardo et al. 1997, Henn et al. 2005), other studies have suggested that animal contact may represent an important risk factor for exposure and/or infection with these *Bartonella* sp. in immunocompetent patients (Kumasaka et al. 2001, Breitschwerdt et al. 2007). Several studies have implicated direct transmission of *Bartonella* sp. from dogs to humans (Keret et al. 1998, Tsukahara et al. 1998, Kerkhoff et al. 1999, Chen et al. 2007). Although bacterial viability in saliva has not been confirmed, *Bartonella* DNA has been sequenced from dog saliva (Duncan et al. 2007a, Kim et al. 2009). Due to frequent, close contact between companion animals and their owners, pets may serve as a source of human infection, as natural reservoirs for arthropod transmission or as environmental sentinels for

the detection of these bacteria (Kordick and Breitschwerdt 1998, Breitschwerdt and Kordick 2000).

The overall objective of this study was to determine whether infection with a vector-borne organism was contributing to chronic illness in a kennel of dogs or their owner. When testing for *Anaplasma*, *Babesia*, *Ehrlichia*, and *Rickettsia* sp. proved negative or inconclusive, the molecular prevalence of *Bartonella* sp. was determined. Five dogs were infected with *B. vinsonii* subsp. *berkhoffii* genotype I, multiple *B. henselae* strains, and a species most similar to *Candidatus* B. volans. *B. vinsonii* subsp. *berkhoffii* genotype I was isolated by blood culture from the owner using *Bartonella* alpha-Proteobacteria growth medium (BAPGM).

Materials and Methods

Dogs and kennel owner

A veterinarian, who moved from New York to Virginia in 1977 at 20 years of age, resided in Floyd, Roanoke, and Montgomery counties from 1981 to the present time. This individual maintained a kennel of Doberman pinschers as household pets. Most of the dogs were also used in a breeding program, from which puppies were sold or maintained as personal pets. Historically, all Doberman pinschers bred by this veterinarian between 1978 and 1995 were considered healthy, with no illnesses such as polyarthritis, lymphadenopathy, dermal lesions, or neurological manifestations occurring during this time frame. Shortly after relocation of

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her household and kennel in 1995, the dogs experienced a heavy tick infestation. Ectoparasite control measures were instituted and neither tick or flea infestations were subsequently considered problematic; however, during the ensuing years the veterinarian reported a spectrum of chronic, progressive, and unexplained medical problems in all of the adult dogs that she maintained as personal pets or breeding animals. In her professional opinion, the number, type, and severity of these problems were unprecedented and were in contrast to her experiences as a breeder. Because transmission of a vector-borne pathogen was suspected, because prior diagnostic testing was negative or inconclusive, and because antibiotic and other treatments were ineffective, the kennel owner requested requested testing for vector-borne pathogens by the North Carolina State University College of Veterinary Medicine Vector Borne Diseases Diagnostic Laboratory (NCSU-CVMVBDDL). In collaboration with the owner and faculty at the Virginia-Maryland Regional College of Veterinary Medicine (VMR-CVM), serological and molecular testing for other vector-borne pathogens was performed between 1997 and 2010.

Historically, all dogs lived predominantly indoors and slept in the house with the owner. When the dogs were outdoors, they were confined to a large fenced enclosure that included an open field with access to surrounding wooded areas. Dogs had direct or indirect contact with numerous wild animals that frequented the enclosure, including rabbits, deer, moles, voles, wild turkeys, and gray squirrels. Dogs were also observed ingesting wild animal feces. In April 2006, the kennel owner became acutely ill and subsequently has experienced chronic disease manifestations up to the present time.

Serology-based assays

Serological testing was performed using immunofluorescent assays (IFA) for detection of antibodies against *Rickettsia rickettsii*, *Ehrlichia canis*, and *Babesia canis* as previously described (Kordick et al. 1999). The starting dilution was 1:16, with endpoint titers defined as the last dilution at which brightly stained organisms could be detected by fluorescence. An ELISA-based test kit (SNAP 3Dx or SNAP 4Dx; IDEXX Laboratories, Inc., Westbrook, ME) was also used to test for *Anaplasma* sp., *Borrelia burgdorferi*, and *Ehrlichia* sp. antibodies. *B. vinsonii* subsp. *berkhoffii* and *B. henselae* antibodies were determined following traditional IFA practices with fluorescein-conjugated goat anti-dog or human IgG. *B. vinsonii* subsp. *berkhoffii* genotype I (isolate 93-CO-1 from the NCSUIPRL, ATCC #51672) and *B. henselae* (strain Houston 1, ATCC #49882) were passed from agar-grown cultures of each organism into DH82 (a continuous canine histiocytic cell line) cultures. Heavily infected cell cultures were spotted onto 30- well Teflon-coated slides, air-dried, acetone-fixed, and stored frozen. Serum samples were diluted in phosphate-buffered saline containing normal goat serum, Tween-20, and powdered nonfat dry milk to block nonspecific antigen binding sites. Patient sera were screened at dilutions of 1:16 to 1:64. All sera that remained reactive at a titer of 1:64 were further tested with twofold dilutions out to a final dilution of 1:8192. A standard cut-off titer of 1:64 was defined as being seropositive.

BAPGM diagnostic platform

The BAPGM diagnostic platform incorporates DNA amplification of *Bartonella* sp. in three independent steps, as described previously (Maggi et al. 2005, Duncan et al. 2007b). Two milliliters of aseptically obtained blood, serum, or cerebrospinal fluid (CSF) samples was inoculated into 10mL of BAPGM and cultured for 7 days (Duncan et al. 2007b). An un-inoculated BAPGM culture flask was processed simultaneously and in an identical manner with each batch of patient samples tested.

Tissue processing and DNA extraction

For fresh, frozen, or paraffin-embedded tissues, and blood samples, DNA was extracted for polymerase chain reaction (PCR) testing using the QIAmp mini kit and protocol provided by Qiagen (Valencia, CA). Cross contamination during necropsy collection or processing of paraffin-embedded tissues has previously been described (Varanat et al. 2009a); therefore, embedded samples were cut from the blocks using a new sterile scalpel blade, and for each processed block, a new paper work surface was used to obtain tissues from the block. All DNA samples were eluted in nuclease-free water and stored at - 20ºC until use.

Bartonella *genus ITS region PCR*

PCR screening for *Bartonella* DNA from blood, serum, fluids, tissues, and BAPGM cultures was performed using two primer sets in two individual PCRs targeting the ITS region located between the 16S and 23S rRNA genes, using oligonucleotides 325s-1100as as described

previously (Duncan et al. 2007b) and 438s-1000as (438s: 5'-GGT TTT CCG GTT TAT CCC GGA GGG C-3' and 1000as: 5'-CTG AGC TAC GGC CCC TAA ATC AGG-3'). The annealing temperature for primer amplification was 68.5ºC with 55 cycles. The detection limit for these PCR assays is 2.5 genomic copies per reaction. A *B. henselae* Houston 1 strain type was used as a positive control throughout the study. All PCR and BAPGM culture-negative controls remained negative throughout the study.

Sequencing of ITS region amplicons

All PCR products were sequenced directly or after cloning as previously described (Diniz et al. 2007). Bacterial species and strain were defined by comparing similarities with other sequenced bacteria deposited in the GenBank database using the Basic Local Alignment Search Tool (Blast version 2.0).

Results

The veterinarian first sought consultation with the NCSUCVM- VBDDL in 1998 because Dogs 1 and 2 had developed illnesses of unknown cause. Dogs 3–5 became ill after the breeder relocated to a new home within the same county in 2001. Dogs 1–2 died during the course of this investigation, whereas dogs 3–5 continue to live with the owner. The case designation, year of birth, sex, date of onset of illness, sample date providing the DNA evidence of *Bartonella* infection, predominant clinical abnormalities, and diagnoses for the

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dogs and owner are summarized in Table 1. Because testing by commercial laboratories had identified antibodies to *R. rickettsii* antigens, our initial diagnostic efforts focused on spotted fever group (SFG) *Rickettsia* serology and PCR testing. When these efforts generated no consistent indication of exposure to or infection with rickettsiae, diagnostic efforts were redirected to assess the prevalence of other vector-borne pathogens and *Bartonella* sp. Patient samples were tested retrospectively and prospectively.

Based upon PCR amplification and DNA sequencing, active infection with a *Bartonella* sp. was confirmed in blood, cerebrospinal fluid, or tissue biopsy samples derived from the owner and from Dogs 1 to 5. At no time point were any of the five dogs seroreactive to *B. henselae* or *B. vinsonii* subsp. *berkhoffii* antigens, whereas the owner was minimally seroreactive to *B. henselae* and *B. vinsonii* subsp. *berkhoffii* antigens.

Dog 1

In 1997, upper small intestinal biopsies were obtained endoscopically at VMR-CVM from Dog 1 due to suspected inflammatory bowel disease. Idiopathic polyarthritis was subsequently diagnosed in 1998, and in 2000, an acanthomatous epulis was biopsied. In 2007, the dog developed hypothyroidism and aspiration pneumonia secondary to megaesophagus, which persisted until 2009 when the dog was euthanized due to recurrent bouts of aspiration pneumonia. Retrospective *Bartonella* PCR testing in 2010 was performed on DNA extracted from formalin-fixed, paraffin-embedded tissue samples of the intestinal

biopsy taken in 1997. The ITS sequence obtained from this amplicon was 99.5% (210/211 bp) similar to *B. henselae* (DQ529247); however, the strain could not be confirmed due to the short length of the clean, readable sequence. *Bartonella* DNA was not amplified from the acanthomatous epulis sample obtained in 2000.

Dog 2

Dog 2 was healthy until 1998, when cutaneous draining lesions and thrombocytopenia were documented. In September, the dog was reportedly seroreactive to rickettsial antigens with an endpoint titer of 1:64; however, repeat testing in October 1998 at the NCSU-VBDDL revealed no *R. rickettsii* seroreactivity. Retrospective PCR testing for SFG *Rickettsia* and *Bartonella* DNA using stored blood from October 1998 was also negative as well as blood and lymph node samples collected in July 2000. In 2007, *B. henselae* DNA was amplified and sequenced from surgically obtained frozen liver tissue removed at VMR-CVM in July of that year to investigate biochemical and sonographic evidence of a hepatopathy. The ITS amplicon was 99.4% similar (626/630 bp) to *B. henselae* strain SA2 (AF369529), excluding a 15 bp insertion at the 3' end. It was also 99.5% homologous (602/605 bp) with a cat isolate obtained in our laboratory in 1995 (Isolate 95FO93), which contained the same 15 bp insertion at the 3' end. At the time of the liver biopsy, a cutaneous histiocytic sarcoma was also surgically removed. By retrospective *Bartonella* ITS PCR in 2010, two bands were amplified from the paraffin-embedded histiocytic sarcoma (Fig. 1). Each band was excised and successfully sequenced following gel extraction. One band was 99.8% homologous

(487/488 bp) with *B. henselae* Cal-1 (AF369527), whereas the other band was 99.2% homologous (514/518 bp) with *B. henselae* strain SA2 (AF369529). Despite treatment with azithromycin and morbofloxacin for 4 weeks, Dog 2 died in 2007 due to complications associated with glomerulonephritis.

Dog 3

Bartonella PCR was performed retrospectively using a surgically resected paraffin-embedded fibrosarcoma removed from the ventral thorax in 2005. An amplicon was 99.8% (446/ 447 bp) similar to *B. henselae* strain Houston 1 (NC005956). In early 2005, following a trip to Texas, both Dog 3 and Dog 4 developed dermal lesions on their muzzles, presumed to be histiocytomas. Shortly after this time, both dogs developed lesions on hocks, which were diagnosed as hamartomas. Samples from dermal lesions from Dog 3 were not available for *Bartonella* PCR. In January 2007, Dog 3 was referred to NCSU-VTH for shifting leg lameness, painful joints, and lymphadenopathy. Testing at this time revealed no seroreactivity to *R. rickettsii*, *B. henselae*, or *B. vinsonii* subsp. *berkhoffii* in serum, and blood was PCR negative for SFG *Rickettsia* and *Bartonella* DNA. BAPGM enrichment blood culture was repeated in December 2007, and a *Bartonella* sequence, not previously obtained in our laboratory or found in the GenBank database, was amplified. This ITS sequence had an 18 bp deletion at the 3' end. After excluding this deletion, the remaining sequence was most similar (93% homology, 432/465 bp) to Candidatus *Bartonella volans* (EU294521), previously isolated from Southern flying squirrels. The sequence was 99% similar (482/487

bp) to a sequence obtained in our laboratory from a sea otter in 2008 (Carrasco et al. 2008). In February 2008, the dog was referred to the NCSU-VTH Neurology Service due to neck pain, ataxia, and hindlimb paresis. By magnetic resonance imaging there was evidence of cervical spondylomyelopathy at C5-C6 and the caudal cervical paraspinal muscles were hyperintense, suggestive of myositis or denervation atrophy. Cytological and protein analyses of cisternal cerebrospinal fluid were normal; however, *B. vinsonii* subsp. *berkhoffii* genotype I was PCR-amplified and sequenced from the CSF fluid sample. The CSF sequence was 99.8% similar (587/588 bp) to *B. vinsonii* subsp. *berkhoffii* genotype I (AF167988). The dog was not seroreactive to *R. rickettsii*, *B. henselae* or *B. vinsonii* subsp. *berkhoffii* antigens. PCR for SFG *Rickettsia* DNA was negative in blood and CSF samples. After treatment with azithromycin and rifampin for 6 weeks, neurological signs resolved and the dog has remained healthy.

Dog 4

In early 2005, Dog 4 developed dermal lesions on her muzzle, presumably histiocytomas, similar to those seen on Dog 3. Later in 2005, following surgical removal of a cutaneous mass, Dog 4 was found to have a cutaneous follicular hamartoma. DNA was extracted from a stored paraffin-embedded hamartoma tissue sample, and ITS PCR generated an amplicon that was 99.8% (487/488 bp) similar to *B. henselae* SA2 (AF369529). In August 2006, the dog developed tremors and twitching of the distal extremities of undetermined cause. Between September and December 2006, splenomegaly accompanied by mild to moderate

thromobocytopenia and mild anemia were repeatedly documented. When incessant chewing at the elbows and aberrant behavior (inattention and staring at the elbows) developed, Dog 4 was treated with doxycycline for 6 weeks. In December 2006, PCR from blood was negative for SFG *Rickettsia* and *Bartonella* sp. Serum was not provided. Serum obtained in December 2007 was reactive by IFA testing to *E. canis* antigens (titer 1:256) but was negative for *Ehrlichia* by SNAP 4DX testing and weakly SNAP positive to *Anaplasma* antigens (*A. phagocytophilum* or *A. platys*). Repeat testing in January 2008 yielded only seroreactivity to *Anaplasma* by SNAP 4DX testing, most likely reflecting seroconversion to *A. phagocytophilum*. There was no serological or PCR evidence of infection with *Bartonella* or SFG *Rickettsia* between 2006 and 2009.

Dog 5

In 2005, Dog 5 was found to have an idiopathic peripheral neuropathy at VMR-CVM, in conjunction with coincident seroreactivity to *B. burgdorferi*. In August 2006, a large, firm, moveable dorsal thoracic mass containing 12mL of serosanguineous fluid was aspirated. Cytological findings included a large number of macrophages (68%) with accompanying erythrophagocytosis, nontoxic neutrophils (17%), lymphocytes (14%), rare eosinophils (1%), and no visualized organisms. A complete blood count was normal and aerobic, anaerobic, and fungal cultures of the aspirated fluid were negative. Due to continued drainage, the mass was surgically excised in October and the histopathological diagnosis was pyogranulomatous panniculitis. Repeat aerobic, anerobic, fungal cultures, and atypical mycobacterial cultures

were negative. The dog was not seroreactive to *B. henselae*, *B. vinsonii* subsp. *berkhoffii*, or *R. rickettsii* antigens. After administration of doxycycline for 6 weeks, there was no additional palpable pathology in the dorsal thoracic region. Retrospective testing of surgically obtained paraffin-embedded tissue from the mass was PCR positive for *B. henselae* (Fig. 2). The ITS sequence was 99.6% (516/518 bp) similar to *B. henselae* SA2 (AF369529).

Owner

Beginning in April 2006, the female veterinarian, who bred and owned the Doberman pinschers described in this investigation (Dogs 1–5), developed progressive neurological disabilities, experienced chronic joint and muscle pain, and was found to have a sensory-motor neuropathy. Five months after the onset of illness, she was barely able to walk or use her hands and arms. In addition to occupational exposure in her practice, this veterinarian reported frequent close contact with her dogs, including unprotected exposure to their saliva and infrequent minor scratch or bite wounds. In September 2006, after a 5-month history of progressive illness, the veterinarian was found to have Lyme disease, after which she was treated with doxycycline for 4 weeks. As there was no resolution of symptoms after doxycycline treatment, she was subsequently treated with 4 weeks of intravenous ceftriaxone, which resulted in overall symptomatic improvement; however, she was classified as neurologically disabled and continued to experience symptoms, including continuous pain, swelling and edema in her extremities, chest pain, and dermal lumps and

plaques. In December 2007, the veterinarian was entered into an Institutional Review Board (IRB)-approved research study (NCSU IRB #164-08-05) designed to assess the occupational risk of *Bartonella* sp. infection in animal health professionals. Using *Bartonella* ITS primers, *B*. *vinsonii* subsp. *berkhoffii* genotype I was amplified from a BAPGM enrichment blood culture. The amplicon was 100% homologous (659/659 bp) with *B*. *vinsonii* subsp. *berkhoffii* genotype I (AF167988). By IFA testing, seroreactivity to *B*. *vinsonii* subsp. *berkhoffii* and *B*. *henselae* antigens was not detected. *B*. *vinsonii* subsp. *berkhoffii* genotype I DNA was again amplified following direct extraction of a serum sample obtained in January 2008. ITS PCR from the blood sample was also positive, but the amplicon was not successfully sequenced. After enrichment in BAPGM for 14 days, *B*. *vinsonii* subsp. *berkhoffii* genotype I was sequenced from an agar plate subculture isolate. Sequences of PCR products obtained from the serum and the subculture isolate, which was obtained weeks later, were 100% homologous with *B*. *vinsonii* subsp. *berkhoffii* genotype I (AF167988). At this time, the patientwas seroreactive to *B*. *henselae* and *B*. *vinsonii* subsp. *berkhoffii* genotypes I, II, and III at titers of 1:32, 1:16, 1:64, and 1:64, respectively. There was no postantibiotic PCR evidence of *Bartonella* infection in February 2008, when blood and serum were again tested using the BAPGM platform; however, similar antibody titers to those obtained from samples in January 2008 were obtained to the respective Bartonella antigens.

Discussion

In this study, we describe molecular evidence of *Bartonella* sp. infection in an owner and five adult dogs maintained in her household. From an ecological perspective, numerous domestic and wild animals have coevolved with various *Bartonella* sp. and with the vectors that transmit these bacteria among animal populations. Vector preference generally confines transmission of a given *Bartonella* sp. among defined animal populations, such as cats, cows, coyotes, foxes, or rodents; however, inadvertent transmission to incidental hosts, such as pet dogs or humans, can occur with resulting pathology.

All five *Bartonella* PCR-positive dogs were infected with various ITS strains of *B. henselae*, which most likely reflects exposure to multiple strains of this species or, alternatively, changes in the 16S-23S ITS region may have occurred during in vivo infection of the host (Diniz et al. 2007, Oliveira et al. 2010). Precautions were taken to prevent DNA contamination or carryover during sample processing. Contamination of tissue samples with *Bartonella* DNA can occur during necropsy or histological tissue processing (Varanat et al. 2009a); therefore, in this study, sterile scalpel blades were used to manually cut patient tissues from individual paraffin blocks. All BAPGM enrichment cultures and PCR procedures were conducted under stringent conditions to avoid PCR amplicon contamination, and all negative controls remained negative throughout the course of this study.

Due to current limitations in knowledge regarding *Bartonella* sp. vector transmission, the long time span of this study, and the intermittent and uncontrolled timing of diagnostic testing of these dogs and their owner, it is not possible to determine the source of *Bartonella* infections or mode(s) of transmission. For Dog 2, three distinct ITS strains of *B. henselae* were sequenced from liver or the histiocytic tumor tissue. One strain was most similar to *B. henselae* Cal-1, the second strain to *B. henselae* SA2, and the third was an SA2-like strain. In contrast, Dog 3 was infected with *B. henselae*, *B. vinsonii* subsp. *berkhoffii* genotype I, and a novel *B. volans*–like strain at different time points between 2005 and 2008. For the owner and Dog 3, enrichment culture in BAPGM was required before PCR was able to confirm *B. vinsonii* subsp. *berkhoffii* genotype I infection in the owner and *B. henselae* strain Houston 1 infection in the dog, respectively. Previous studies have supported enhanced diagnostic sensitivity associated with this liquid enrichment culture approach in dogs and human patients (Maggi et al. 2005, Duncan et al. 2007b, Kosoy et al. 2008, 2010, Breitschwerdt et al. 2010a, 2010b).

Both the veterinarian and Dog 3 may have been infected by a common environmental source of *B. vinsonii* subsp. *berkhoffii*, as the same ITS genotype sequence was amplified from the dog's cerebrospinal fluid and the veterinarian's BAPGM blood culture and subculture isolate. Several recent studies support an increased likelihood of *Bartonella* exposure and/or infection in immunocompetent people with extensive animal and arthropod contact (Kumasaka et al. 2001, Breitschwerdt et al. 2007, 2008). *B. vinsonii* subsp. *berkhoffii* genotype I bacteremia and seroconversion were recently reported in a veterinarian after

experiencing a needle stick, which occurred while aspirating a cutaneous histiocytic tumor in a dog who subsequently was found to be infected with *B. vinsonii* subsp. *berkhoffii* (Oliveira et al. 2010). In addition, *Bartonella* sp. DNA has been detected in dog saliva (Duncan et al. 2007a, Kim et al. 2009), and direct bite transmission of *Bartonella* sp. from dogs to humans has been suggested (Keret et al. 1998, Tsukahara et al. 1998, Kerkhoff et al. 1999, Chen et al. 2007). In light of evolving information, the possibility of zoonotic transmission of *Bartonella* sp. from dog to owner in this study cannot be discounted due to their close interactions. As of yet, no definitive mode of transmission for any *Bartonella* sp. to a dog has been established (Billeter et al. 2008). Regardless of the mode(s) of transmission, *B. vinsonii* subsp. *berkhoffii* has become an important, emerging zoonotic pathogen. Disease development may be more likely when dogs become immunocompromised (Yager et al. 2010) or when opportunistic infection becomes established in a nonreservoir host such as a cat, dog, or human (Breitschwerdt et al. 2009b, 2010, Breitschwerdt and Maggi, 2009, Varanat et al. 2009b).

Based upon the large number of rodent-adapted *Bartonella* sp. found in various urban and rural ecosystems throughout the world, including several rodent species that are frequently sold as pets (Inoue et al. 2009), it is likely that rodent-reservoir *Bartonella* sp. infection of dogs and people is a more frequent occurrence than is currently appreciated (Kosoy et al. 2008, 2010). A high prevalence of *Bartonella* bacteremia (42.2%) has been reported in rodent surveys from the southeastern United States (Kosoy et al. 1997); therefore, dogs in frequent contact with rodents, as was the case in this study, may become infected with a variety of rodent *Bartonella* species, for which the pathogenic potential is currently

unknown. Previously, *Bartonella washoensis* and *Bartonella elizabethae*, with the California ground squirrel (*Spermophilus beecheyi*) and the rat (*Rattus* sp.) as the associated reservoirs, respectively, have been shown to infect dogs and people (Mexas et al. 2002, Chomel et al. 2003, Marie´ et al. 2006). In addition, we have described infection with a novel Bartonella strain, most closely related to *Candidatus* Bartonella volans, in an 86-year-old farmer who experienced several postoperative complications, including Candida albicans esophagitis, hallucinations, seizures, and encephalopathy after surgery for a fractured femur (Breitschwerdt et al. 2009a). That strain was similar (98.9%, 460/465 bp) but not identical to the BAPGM enrichment blood culture *Bartonella* sequence detected in Dog 3. Currently, the reservoir(s), mode of transmission, and putative arthropod vector(s) for these novel *B*. *volans*–like strains are unknown.

As noted in other studies, there were discrepant results between *Bartonella* PCR results and seroreactivity to the *Bartonella* test antigens in the current study. A study from our laboratory documented *Bartonella* infection in dogs that lacked a detectable humoral immune response by IFA testing (Duncan et al. 2007b). When testing human sera, varying antigenic expression between *B*. *henselae* strains also appears to contribute to false-negative *B*. *henselae* IFA results (Drancourt et al. 1996). In addition, several case reports have demonstrated detection and/or isolation of *Bartonella* in seronegative human patients (Raoult et al. 1994, Drancourt et al. 1996, Brouqui et al. 1999, Ehrenborg et al. 2000).

On an evolutionary basis, *Bartonella* sp. are highly adapted, vector-transmitted, erythrocytic, and endotheliotropic bacteria; therefore, satisfying Koch's postulates for disease causation has been challenging in both human and veterinary medicine (Jacomo et al. 2002, Chomel et al. 2009a). Prospective studies are needed to better define the duration of bacteremia and the humoral response after natural infection and to determine if *Bartonella* species play a role in dog and human patients with chronic disease manifestations, including polyarthritis, glomerulonephritis, and peripheral neuropathies. Intermittent sampling for over a decade did not allow us to establish the onset or modes of *Bartonella* sp. transmission, but DNA of an intriguing number of *Bartonella* sp. and strains were detected in tissues, CSF, and blood enrichment cultures over time. Our goal in reporting these results is not to infer disease causation for the owner or for her dogs but to further emphasize the ecological complexity of the genus *Bartonella* in nature.

Acknowledgements

Disclosure statement

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 is the Scientific Technical Advisor and Laboratory Director for Galaxy Dx. The remaining authors have no potential conflicts of interest.

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Table 1. Signalment, year of illness onset and *Bartonella* detection, primary clinical abnormalities, *Bartonella* species identified by DNA sequencing, and the clinical status at the conclusion of the study for five Doberman pinscher dogs kennel and the kennel owner

Case number	Year of birth	Sex	Year of illness onset	Year of initial Bartonella PCR-positive sample	Clinical abnormalities	Bartonella species identified	Clinical status
1	1994	F	1997	1997	P, S, I	*B. henselae*	Deceased (2009)
2	1996	F	1998	2007	D, G	*B. henselae*	Deceased (2007)
3	2000	M	2001	2005	P, L, D, E, N, H	*B. volans*–like *Bvb* genotype I *B. henselae*	Persistent dermal lesions of face and head
4	2003	F	2005	2005	L, D, N, H	*B. henselae*	Asymptomatic
5	2005	F	2005	2006	D, N, PN	*B. henselae*	Asymptomatic
Owner	1950	F	2006	2007	S, D, N, C	*Bvb* genotype I	Persistent pain and neurological complications

Clinical abnormalities: F, fever; P, polyarthritis; S, swollen joints/extremities; I, inflammatory bowel disease; L, lymphadenopathy; D, dermal lesions; E, epididymitis; G, glomerulonephritis; N, neurological manifestations; H, hamartoma; PN, panniculitis; C, chest pain.
Bvb, *Bartonella vinsonii* subsp. *berkhoffii*; PCR, polymerase chain reaction.
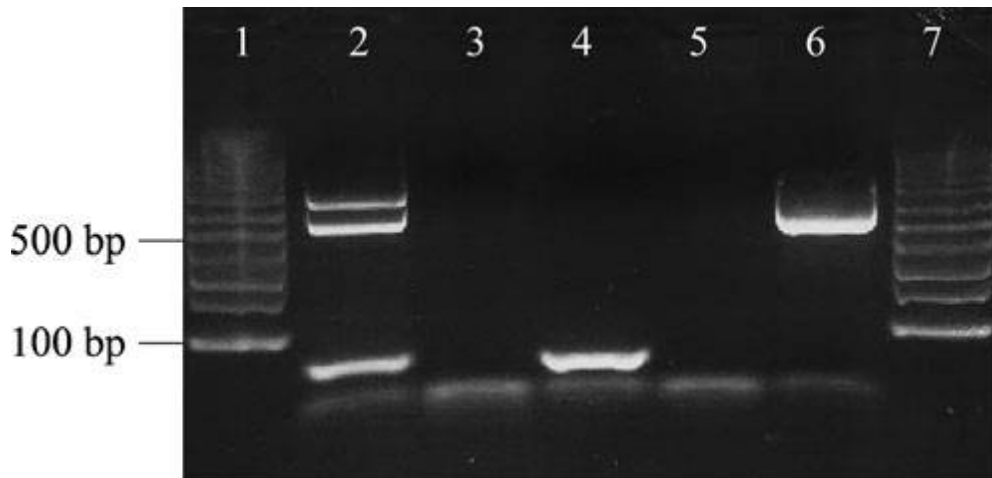
FIG. 1. Agarose gel electrophoresis illustrating *Bartonella* genus intergenic spacer (ITS) polymerase chain reaction (PCR) amplicons (lane 2) obtained from the paraffin-embedded histocytic sarcoma from Dog 2. By sequencing, the top band contained *Bartonella henselae* Cal-1 DNA and the bottom band contained *B. henselae* SA2 DNA. Lanes 3 and 4: PCR-negative tissue extractions. Lane 5: PCR-negative control (specific pathogen-free dog DNA). Lane 6: PCR-positive control (*B. henselae* Houston 1 strain). Lanes 1 and 7: 1-Kb molecular marker.
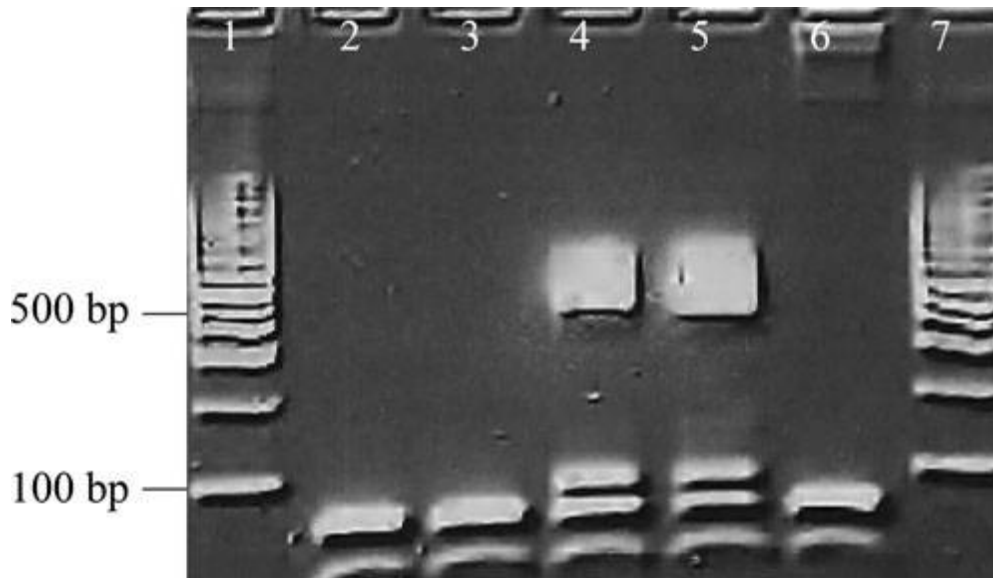
FIG. 2. Agarose gel electrophoresis illustrating a *Bartonella* genus ITS PCR amplicon (lane 4) obtained from the paraffin-embedded panniculitis lesion from Dog 5. DNA sequencing of the amplicon confirmed *B. henselae* (SA2 strain). Lanes 2 and 3: PCR-negative tissue extractions. Lane 5: PCR-positive control (*B. henselae* Houston 1 strain). Lane 6: PCR-negative control (specific pathogen-free dog DNA). Lanes 1 and 7: 1-Kb molecular marker.

Chapter 4. Serological and molecular prevalence of *Bartonella* spp. infection in healthy and sick horses and foals from the southeastern United States

Serological and molecular prevalence of *Bartonella* spp. infection in healthy and sick horses and foals from the southeastern United States

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Short title: Prevalence of *Bartonella* in horses

Keywords: *Bartonella vinsonii*; lameness; co-infection; foals

Abbreviations: *Bartonella* alpha-Proteobacteria Growth Medium (BAPGM)

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Acknowledgements

Abstract

Background: *Bartonella* species have been identified in numerous animal species and are known to cause, or are associated with, numerous clinical manifestations in dogs and human patients; however, no studies have been published to date that have sought to determine exposure and infection prevalences in horses.

Objective: To determine the *Bartonella* serological and molecular prevalences in healthy and sick horses and sick foals within the southeastern United States.

Animals: Forty-nine healthy horses, 15 sick foals, 26 horses with musculoskeletal manifestations, and 96 horses with colic were tested for *Bartonella*.

Methods: IFA serology and PCR pre- and post-BAPGM (*Bartonella* alpha-Proteobacteria Growth Medium) enrichment blood culture were performed. Fisher's exact test was used for statistical analysis.

Results: *Bartonella* antibodies were detected in only one horse with colic. Three *Bartonella* species, *B. henselae*, *B. vinsonii* subsp. *berkhoffii* (genotypes I and III), and a *Bartonella* species with closest homology to *Candidatus* Bartonella volans, were PCR-amplified and sequenced from pre- and/or post-enrichment blood culture samples from 1/49 healthy horses, 3/15 sick foals, 5/26 horses with musculoskeletal manifestations and 0/96 colicing horses. Statistically, *Bartonella* infection was most frequent in adult horses with musculoskeletal manifestations and in sick foals.

Conclusions and clinical importance: To our knowledge, this is the first study designed to assess *Bartonella* prevalences in horses. As three *Bartonella* species were identified and there was an association between *Bartonella* infection and illness in two equine populations,

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further investigation into the pathogenic potential of *Bartonella* infection in horses is warranted.

Bartonella spp. are fastidious, intracellular, gram-negative aerobic bacteria with a worldwide distribution.[1] Transmission occurs via bites, scratches, needle sticks, blood-feeding arthropods, and, for some *Bartonella* spp., by transplacental transmission.[2-5] At least 26 different species or subspecies of this highly-adaptive intravascular bacterium have been isolated from numerous domestic and wild animals.[1,6-9] Members of the genus *Bartonella* are gaining recognition as pathogens of substantial clinical importance across several animal species; however, there is limited data in regard to pathogenic potential of *Bartonella* spp. infection in horses.

Bartonella henselae bacteremia was first described in two horses in 2008 when the organism was isolated from a 7 year-old mare with presumptive vasculitis and was PCR-amplified and sequenced from the blood of an 11-year-old gelding with chronic arthropathy.[9] Subsequently, Johnson et al. reported *B. henselae* infection in an aborted equine fetus. In that study, molecular, histological, and immunohistochemical techniques were used to confirm *B. henselae* in the liver, lung, and kidney of the fetus.[10] More recently, our laboratory, in collaboration with the Laboratory for Clinical Diagnostik ZeckLab, documented *B. henselae* infection in a 2-year-old mare from Germany with hemolytic anemia.[11]

Though *B. henselae* is the primary, if not sole, causative agent of Cat Scratch Disease, this *Bartonella* species has been associated with diverse and often non-specific disease manifestations in animals and people. Endocarditis, granulomatous inflammation and vasoproliferative lesions, including bacillary angiomatosis and peliosis hepatis, are distinct pathologies that occur in dogs and human patients infected with *Bartonella* spp., and *Bartonella* species have also been implicated in association with neurological and arthritic symptoms in dogs and people, as recently reviewed by Breitschwerdt et al., in 2010.[12] *Bartonella henselae* and *Bartonella vinsonii* subsp. *berkhoffii* are two of the better characterized *Bartonella* species that cause illness in animals and human patients. The cat flea (*Ctenocephalides felis*) is the primary vector for *B. henselae* transmission among cats,[13] whereas ticks, lice, and biting flies are potential vectors for transmission of other *Bartonella* spp. to hosts.[3,14-15]

Numerous arthropod-borne viral infections, such as equine infectious anemia and West Nile virus, and bacterial infections, such as Lyme disease and equine anaplasmosis, constitute important, and potentially life-threatening, equine diseases; however, the potential pathophysiological consequences of *Bartonella* sp. infection have not been thoroughly investigated in horses. Therefore, this study was designed to determine the serological and molecular prevalence of *Bartonella* species infection in healthy and sick horse populations within the southeastern United States. Serology was attempted to establish exposure rates and BAPGM enrichment blood culture was used to amplify and sequence *Bartonella* spp. DNA from healthy horses, sick foals, and horses with musculoskeletal disease or colic.

Materials and Methods

Study populations

Healthy horses from North Carolina and Virginia and three populations of sick horses examined at the North Carolina State University Veterinary Teaching Hospital were tested for serological, microbiological, or molecular evidence of *Bartonella* infection. Group I consisted of 49 healthy adult horses with no concurrent clinical signs of illness (age range = 2-25 years, median age = 11 years). Group II was composed of 15 sick foals (age range = 1-90 days, median age = 2 days) among which the predominant symptoms included diarrhea, possible sepsis, and/or general failure to thrive. Group III consisted of 26 adult horses with lameness attributable to musculoskeletal manifestations, including laminitis, arthritis, and joint swelling (age range = 1.5-30 years, median age = 15 years). Group IV consisted of 96 horses that presented with signs of colic (age range = 1-27 years, median age = 9 years). Each of these four groups were specifically chosen at the beginning of the study as populations to test for *Bartonella*. With the exception of the majority of samples from horses in Group IV, which had been stored at -80º C and were tested retrospectively, most samples from horses in Groups I, II, and III were collected prospectively for testing.

Serological testing

For detection of *Bartonella* antibodies, an indirect fluorescent antibody assay (IFA) was performed using *B*. *henselae* (Houston 1 strain) and *B*. *vinsonii* subsp. *berkhoffii* genotype I organisms grown in DH82 cells as antigens as previously described.[16] Serum was available from 42 of 49 horses in Group I, 7 of 15 foals in Group II, 25 or 26 horses in Group III, and

from all 96 horses in Group IV. Serum (kindly provided by Dr. Bruno Chomel) from a horse experimentally-infected with *B. henselae* (SA2 strain, isolate designation 2008EO-1) was used as an IFA positive control. Starting dilution was 1:16, with endpoint titers defined as the last dilution at which brightly stained organisms could be detected by fluorescence. The cut-off titer for seroreactivity was arbitrarily defined as 1:64.

Bartonella alpha-Proteobacteria Growth Medium (BAPGM) Enrichment Blood Culture

BAPGM enrichment blood culture was performed as previously described[17,18] with slight modifications, using 2 mL of aseptically-collected EDTA or ACD anti-coagulated blood inoculated into 10 mL BAPGM and incubated for 14 to 21 days at 35ºC with 5% CO_2. At 7, 14, and, for some cases 21, days post-enrichment culture, 500 µL of BAPGM blood culture was sub-inoculated onto 10% sheep blood agar plates and incubated using identical culture conditions. At the same time points, DNA was extracted from 200 µL of culture followed by PCR amplification using *Bartonella* primers, as described below. For 8 of the 15 sick foals, previously culture-negative in the NCSU-VTH Clinical Microbiology Laboratory using the BD BACTEC™[a] system, DNA was extracted and BAPGM enrichment cultures (2 mL from the BACTEC™ bottle into 10ml of BAPGM) were established.

Bartonella PCR Testing

For amplification of *Bartonella* spp. DNA from blood, serum, and BAPGM subcultures, PCR was performed targeting the 16S-23S ribosomal RNA intergenic spacer (ITS) region. Primers and PCR conditions were used as previously described.[16,19] PCR amplicons were

sequenced directly or following cloning using pGEM-T Easy Vector System[a] as previously described.[20]

Statistical Analysis

Fisher's exact test was used to determine if there was a statistical difference in the prevalence of *Bartonella* infection among the groups. The prevalence of infection in the healthy adult horse population (Group I) was compared to Groups II-IV, consisting of foals or horses with specific disease manifestations. *Bartonella* prevalence was also compared between each of the sick populations (Groups II-IV). A p-value of 0.05 or less was considered statistically significant.

Results

B. henselae antibodies were not detected in 7 foals or 163 horses for which serum was available. One colic horse had a 1:64 titer to *B. vinsonii* subsp. *berkhoffii* genotype I antigens; however, *Bartonella* DNA was not amplified from blood or enrichment blood culture.

Bartonella spp. DNA was PCR-amplified from blood, BACTEC™ blood culture samples, or post-enrichment BAPGM blood cultures from 9 of 186 horses (4.8%). One of 49 (2.0%) healthy horses (Horse 1), 3 of 15 (20%) sick foals (Horses 2-4), and 5 of 26 (19.2%) horses with musculoskeletal disease manifestations (Horses 5-9) were infected with a *Bartonella* sp. *Bartonella* DNA was not amplified from blood or BAPGM enrichment cultures from the 96

colic cases. With the exception of Horses 1, 3, and 6 from which *Bartonella* DNA was
amplified from blood, enrichment blood culture was necessary to achieve a positive
Bartonella PCR in the remainder of the infected foals or horses. For Horse 6, *B. vinsonii*
subsp. *berkhoffii* genotype I was sequenced from an enrichment blood culture and *B.*
henselae Houston-1 DNA was amplifed from an extracted blood sample 15 months later. No
isolates were obtained following subculture onto sheep blood agar plates.

DNA sequences obtained directly from the blood of Horse 1 (9-year-old Arabian mare with
no clinical signs of illness) and from the 21-day BAPGM blood enrichment culture of Horse
2 (3-month-old Arabian/Quarter Horse crossbreed colt with septic arthritis caused by
Salmonella sp. of the right tibiotarsal and left stifle joints) were most similar to *B. henselae*
San Antonio-2 strain (GenBank Accession# AF369529). *B. henselae* Houston-1 strain
(GenBank Accession# BA016SRB) DNA was amplified directly from the blood of both
Horse 3 (30-hour-old Quarter Horse colt with Staphylococcal septicemia and enterocolitis
caused by *Clostridium perfringens*) and Horse 6 (11-year-old Quarter Horse mare with
idiopathic polysynovitis of the left stifle, right carpal, and right hock joints). Horse 5 (14-
year-old Quarter Horse mare with chronic laminitis) was infected with a *B. henselae* Fizz
strain (GenBank Accession# AF369526) or a *B. henselae* Cal-1 strain (GenBank Accession#
AF369527), as available sequence from the 14-day BAPGM blood enrichment culture did
not differentiate among these two strains. *B. vinsonii* subsp. *berkhoffii* genotype III DNA
(GenBank Accession# DQ059764) was amplified from the 21-day BAPGM blood
enrichment culture of Horse 4 (12-hour-old Holsteiner colt with meconium

impaction),whereas *B. vinsonii* subsp. *berkhoffii* genotype I DNA (GenBank Accession#

AF167988) was amplified and sequenced from the 14-day BAPGM blood enrichment culture

of Horse 6, the 7-day BAPGM blood enrichment culture of Horse 7 (22-year-old Quarter

Horse mare with laminitis and arthritis), and the 14-day BAPGM blood enrichment culture of

Horse 8 (30-year-old Arabian mare with arthritis). The three *B. vinsonii* subsp. *berkhoffii*

genotype I sequences (457 bp) were 98.9 to 99.3% identical.

A distinct *Bartonella* sequence was amplified from the 7-day BAPGM blood enrichment

culture of Horse 9 (15-year-old Quarter Horse crossbreed mare with laminitis). The

sequence was most similar to *Candidatus* Bartonella volans, an isolate obtained from a

southern flying squirrel, *Glaucomys volans* (GenBank Accession# EU294521), and

Bartonella DNA amplified from a squirrel flea *Orchopeas howardi* (GenBank Accession#

DQ336386). Sequences from Horse 9, the flying squirrel isolate, and the squirrel flea all

contained a 17 base pair deletion at the 3' end. In addition, the Horse 9 sequence was similar

to the following GenBank sequences: 1) 98.6% identity (357/362 bp) with an amplicon from

the blood of an 86 year-old man with encephalopathy, memory loss, and recent onset

arthritis,[21] 2) 98.9% identity (360/364 bp) with an amplicon from a post-enrichment blood

culture from a dog with a 2-year history that included muzzle histiocytomas, hock

hamartomas, shifting leg lameness, painful joints, and lymphadenopathy,[16] and 3) 98.9%

identity (360/364 bp) with an amplicon obtained in our laboratory from a sea otter with

vegetative valvular endocarditis.[c]

There was a significantly higher prevalence of *Bartonella* infection in sick foals (p = 0.037) and in horses with musculoskeletal disease (p = 0.017) as compared to the healthy horse population. Also, *Bartonella* prevalence was significantly higher in the sick foals (p = 0.002) and horses with musculoskeletal manifestations (p = 0.0003) compared to horses with colic.

Discussion

In this study, horses with clinical manifestations of musculoskeletal disease (i.e. laminitis, arthritis, joint swelling) and sick foals were statistically more likely to be infected with a *Bartonella* spp. than healthy or colicing horses. Lameness and polyarthritis have been reported in dogs[22,23] and human patients[24,25] infected with *Bartonella* spp. Similar to three previous reports,[9-11] *B. henselae* was the species infecting a healthy horse, two sick foals, and two horses with musculoskeletal manifestations.

In conjunction with a previous report describing *B. henselae* infection in an aborted equine fetus, it does appear that foals can be infected *in utero,* during the perinatal period, or shortly after birth.[10] As *B. henselae* causes more serious disease manifestations or unusual vasoproliferative lesions such as bacillary angiomatosis and peliosis hepatis in immunocompromised humans,[26] it is possible that the pathogenicity in foals or aged horses would be greater than infection with the same strain in a healthy immunocompetent horse. If the foals were infected by transplacental transmission, one might expect to detect *Bartonella* maternal antibodies, unless the mare was anergic. Alternatively, if these foals were infected shortly after birth, perhaps by vector transmission (i.e. biting flies), then antibodies might not

have achieved a level detectable by IFA testing at the time of disease onset. Unfortunately, convalescent serum samples were not obtained as a component of this prevalence study to determine if seroconversion occurred.

Three horses with musculoskeletal manifestations were infected with *B*. *vinsonii* subsp. *berkhoffii* genotype I, a genotype that was originally isolated from a dog from North Carolina with endocarditis.[27] *Bartonella vinsonii* subsp. *berkhoffii* genotype III DNA was amplified from a 21-day BAPGM enrichment blood culture from a foal with meconium impaction. *Bartonella henselae* DNA was also amplified and sequenced from the blood of Horse 6 in March 2009, which was previously found to be infected with *B*. *vinsonii* subsp. *berkhoffii* genotype I in November 2007, suggesting that co-infection or sequential infection with more than one *Bartonella* sp. can occur in horses, as has been reported for dogs and people.[28,29] Although the role of *B*. *henselae* or other *Bartonella* spp. in the pathogenesis of equine arthritic diseases is unknown, the increased prevalence of infection in lame horses suggests that veterinarians should consider *Bartonella* spp. as potential pathogens when assessing horses with laminitis, arthritis, and/or joint swelling.

In recent years, *B*. *vinsonii* subsp. *berkhoffii* has been identified as an important zoonotic pathogen.[12] Based upon specific insertions and deletions in the 16S-23S ITS region, there are four characterized *B*. *vinsonii* subsp. *berkhoffii* genotypes, designated genotypes I, II, III, and IV. *B*. *vinsonii* subsp. *berkhoffii* genotype I has been isolated from coyotes and dogs and has been detected in human blood, dog saliva, and, more recently, from a feral pig in

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North Carolina.[25,27,30-33] *B. vinsonii* subsp. *berkhoffii* genotype III has been isolated from gray foxes in the United States, detected in a human patient with endocarditis in Europe, and isolated from a military working dog that originated from Germany and developed endocarditis after transport to the United States.[32,34,35] *B. vinsonii* subsp. *berkhoffii* infection in dogs can cause chronic bacteremia, possibly lasting for months.[36] The mode(s) of transmission for *B. vinsonii* subsp. *berkhoffii* to any animal species is currently unknown. Given the wide range of hosts that this subspecies can infect and the potential for chronic bacteremia, further investigation is warranted to determine the mode(s) of transmission for dogs, horses, humans, and other animals. Because the three horses infected with *B. vinsonii* subsp. *berkhoffii* genotype I shared the same pasture in the Piedmont region of North Carolina for many years, transmission by a common insect vector seems plausible. Samples from horses in all four groups were obtained at various times of the year, so it is not possible to speculate whether seasonal differences in vector activity contributed to detection of *Bartonella*. Furthermore, bartonellosis does not typically present as an acute infection with specific clinical symptoms, so it is often not possible to determine when a patient originally became infected with a *Bartonella* sp. Risk factors that have been established for *B. henselae* and *B. vinsonii* subsp. *berkhoffii* infection in dogs include flea and tick exposure as well as residing in rural and, in particular, farm environments,[23,37] which obviously applies to most horses. Clinical, epidemiological, and PCR data from questing ticks supports the possibility of tick transmission,[1,3] though this data does not provide definitive evidence of tick transmission of *B. vinsonii* subsp. *berkhoffii* to animals and humans;[15] however, tick transmission of *Bartonella birtlesii* was recently confirmed in a rodent model.[14]

Also in this study, DNA most closely related to *Candidatus* B. volans and to a DNA sequence obtained from a squirrel flea was amplified and sequenced from a BAPGM blood culture from a horse with laminitis. Numerous *Bartonella* spp. utilize rodents as reservoir hosts, including *B. washoensis* in California ground squirrels (*Spermophilus beecheyi*) and *B. elizabethae* in the rat (*Rattus* sp.). A study by Kosoy and colleagues in 1997 found 42.2% of rodents within the southeastern United States were *Bartonella* bacteremic.[38] More recently, infection with the same rodent-associated *Bartonella* spp. was identified in dogs and in febrile human patients in Thailand.[39] Importantly, the *Bartonella* sp. identified in Horse 9, or a phylogenetically closely-related bacterial cluster, has now been found in the blood of a dog from Virginia,[16] a human being from Maryland,[21] and a sea otter from Alaska.[c] Horses are frequently exposed to rodents, and potentially to the ectoparasites that feed on rodents, thus rodent exposure should be investigated as a risk factor for infection with rodent-adapted *Bartonella* spp.

Based upon the IFA assays used in this study, serology was not useful in establishing prior exposure to or infection with a *Bartonella* sp. in foals or adult horses. A poor correlation between PCR results and IFA seroreactivity has been previously documented in dogs and humans with bartonellosis. In a study by Duncan and colleagues, 50% of *B. henselae* bacteremic dogs had not seroconverted to *B. henselae* antigens, despite a history of chronic illnesses.[18] Discrepant results have also been reported in *Bartonella* seronegative human patients from which a *Bartonella* sp. was detected by PCR and/or isolated.[40-42] False negative *B. henselae* IFA results in human patients can result from antigenic variation among

B. henselae strains used for IFA testing.[42] Alternatively, these horses may have been anergic, resulting in false negative IFA results, as has been suggested in human patients with *Bartonella* bacteremia.[43]

Identification of infection with a *Bartonella* species is difficult to achieve by serology, PCR amplification, or by isolating the bacteria. With the exception of three samples from which *Bartonella* DNA was amplified directly from blood, BAPGM enrichment culture for incubation periods of 7, 14, or 21 days was necessary to facilitate the growth and successful PCR amplification of *Bartonella* sp. DNA from 7 of 9 horses. BAPGM is currently the most efficient means to grow *Bartonella* to a level of PCR detectability from dogs, horses, or human patients.[17,18] Similar diagnostic trends were documented in studies that tested blood samples from sick dogs and human patients.[18,43] It should also be noted that while *Bartonella* DNA was PCR-amplified directly from blood samples from two horses, the 7, 14, and 21-day BAPGM enrichment culture PCR results were negative. This is most likely due to decreased viability of the bacteria in these particular samples or a "dilution effect" when viable bacteria are inoculated into a large volume of BAPGM enrichment media, which represents a novel nutritional environment requiring bacterial adaptation.[18] Additionally, if either of these two horses were administered antibiotics during the time period the samples were taken, this may have accounted for inhibition of *Bartonella* growth in the enrichment media. Unfortunately, for most of the horses we do not have this information.

Regarding storage of samples prior to processing, there was variability between the four groups. Either samples were stored at 4ºC for 1-2 days (fresh samples) before inoculation into BAPGM enrichment media or samples were taken from -80ºC for processing. The majority of samples processed from healthy adult horses in Group I (47/49), sick foals in Group II (15/15), and adult horses with musculoskeletal disease in Group III (22/26) were fresh, where the potential loss of bacterial viability was a non-issue, as demonstrated previously.[44] For samples used for testing Group IV adult horses with colic, samples from only 8 of 96 horses were fresh, and the remaining samples had been stored at -80ºC for a prolonged period of time, some as long as 10 years. Though the freezing process would not likely affect detection of *Bartonella* DNA in blood or antibodies in serum, bacterial viability may have been compromised, making it more difficult to grow the organisms in liquid enrichment culture which was necessary for detection of *Bartonella* sp. infection in most of the horses in this study. Alternatively, Brenner et al. demonstrated in 1997 that freezing EDTA anti-coagulated blood samples from *B. henselae* bacteremic cats for 26 days at -65ºC yielded the largest number of *B. henselae* colonies upon plating compared to plating blood immediately after samples were drawn or plating after 24 hours when the blood samples had been kept at 25ºC.[45] Due to the intracellular nature of *Bartonella*,[46] the authors appropriately postulated that this was likely due to necessary lysis of host cells for release of bacteria for plate isolation and/or inactivation of a growth inhibitor during freezing and thawing, stating "it is clear that viability, if anything, is enhanced by a single frozen storage of blood specimens". Furthermore, the authors of this study also demonstrated there was no significant difference in mean colony counts when blood was frozen for 1 day compared to

30 days, supporting the notion that as long as a blood sample is deep frozen, the length of time should have little effect on bacterial viability. La Scola and Raoult reported similar results in 1999, demonstrating a higher rate of isolation of *B. quintana* from bacteremic human patients when blood was frozen prior to inoculation.[47] Additionally, we have documented growth of *Bartonella* spp. from cattle blood in BAPGM enrichment media that had been frozen for up to seven years.[48] Whether or not the extended time period horse samples from the colic group were stored affected our current results is not possible to determine, but it is worth noting when interpreting the results for this group. Although three *Bartonella* species and several strains were identified by PCR from blood or post-BAPGM liquid enrichment culture samples, no isolates were obtained after sub-inoculation onto sheep blood agar plates from these horses. This remains a common microbiological limitation when working with *Bartonella* species, and future studies are necessary to identify a reliable means to obtain agar plate isolates from dogs, horses, and human patients. In addition, testing a larger population of foals for *Bartonella* spp. would have been optimal, and having paired blood and serum samples for all the foals would have given a more complete picture rather than cases where BAPGM enrichment cultures were inoculated with post-incubation microbiological blood cultures.

In conclusion, *Bartonella* infection may be associated with musculoskeletal pathology in adult horses and various types of illness in foals. Also, two *Bartonella* species were identified within these horse populations that have not been previously reported as a cause of infection in horses to our knowledge. Serology, as used in this study, did not prove useful in

identifying horses or foals that had been exposed to or were infected with a *Bartonella* sp. Although the pathogenic potential of *Bartonella* infection in horses is yet to be determined, this data supports the need for further investigation into the possibility that these organisms contribute to disease in foals with underdeveloped immune systems and in adult horses with laminitis or chronic joint disease.

Footnotes

[a] Becton, Dickinson and Company

[b] Promega, Madison, WI, USA

[c]Carrasco, SE, Chomel, BB, Mazet, JA, et al. Investigation of *Bartonella* spp. in Northern Sea Otters (*Enhydra lutris kenyoni*) with Vegetative Valvular Endocarditis in Alaska. Poster #280, 5th International Meeting on Rickettsiae and Rickettsial Diseases, Marseille, 2008.

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Chapter 5. Summary and conclusions

The *Bartonella* genus, originally comprised of only one species, *B. bacilliformis*, has emerged over the past two decades to include over 22 identified species or subspecies, many of which have pathogenic potential in animals and humans. The time and effort given by researchers to expand current knowledge relative to the ecological diversity of *Bartonella* spp. has led to identification of these bacteria in numerous mammalian species. The ecological diversity of the *Bartonella* genus is multi-faceted, and advances in technology have enabled us to understand how these bacteria are able to survive and replicate within arthropod vectors, be transmitted by these vectors, establish infection and chronic bacteremia within mammalian hosts, and determine risk factors for infection. Once a *Bartonella* sp. is transmitted to a mammalian host, by arthropod vector or otherwise, it is capable of infecting numerous cell types, including erythrocytes, endothelial, epithelial, progenitor, and dendritic cells, as demonstrated *in vitro* and/or *in vivo*. The intracellular persistence and survival of *Bartonella* allows evasion of the host immune response, dispersal to other target cells and organs within a host, preservation for efficient vector transmission, and potential evasion of microbial efficacy.

The first step in effectively evaluating the importance of *Bartonella* infection is to determine its prevalence in nature. Though *in vitro* studies are important to evaluate clinical pathology in a host, results from a laboratory setting are not able to mimic naturally-occurring disease. Using optimized diagnostic techniques, such as PCR and BAPGM, we sought to determine the prevalence of *Bartonella* in a beef cattle population, a kennel setting, and healthy and sick horse populations.

Upon testing of blood samples from a healthy beef cattle population in North Carolina, we provided further evidence supporting the role of cattle as a natural reservoir host for *B. bovis*, documenting detection of this *Bartonella* sp. in over 80% of cattle tested. Furthermore, we reported detection of *B. henselae*, a cat-adapted *Bartonella* sp., in cattle for the first time. The implications of this finding are particularly relevant in the context of the ecology of *Bartonella*, as this suggests there are likely unidentified modes of transmission of *Bartonella* to mammalian hosts, i.e. arthropod vectors which feed on cattle.

Horses, similar to cattle, are prone to frequent vector exposure and contact with wildlife, each possible risk factors for *Bartonella* infection in both equine and cattle populations. From testing of healthy adult horses, sick foals, adult horses with musculoskeletal disease, and adult horses with colic, we found sick foals and adult horses with musculoskeletal manifestations were statistically more likely to be infected with a *Bartonella* sp. This indicates that further studies are warranted to determine if *Bartonella* may be a yet undefined infectious microbial pathogen causing illness in horses. Two strains of *B. henselae*, *B. vinsonii* subsp. *berkhoffii* genotypes I and III, and a *Bartonella* species most closely related to *Candidatus* B. volans were the species identified in horses in this study. Though previous studies have identified *B. henselae* infection in horses, the documentation of *B. vinsonii* subsp. *berkhoffii* and the rodent-adapted species *B. volans* in horses are novel findings.

Our report of a number of *Bartonella* species identified in dogs from a kennel in Virginia as well as their owner is an exceptional example of the broad ecological diversity of *Bartonella* species. This study demonstrated the promiscuous nature of *Bartonella* and ability

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of these species to adapt to different mammalian hosts as well as the heterogeneity of *Bartonella* spp. in an individual population and geographic location. In addition, evidence of co-infection with two *Bartonella* spp. was identified in our study, hypothesized to exacerbate clinical symptoms in mammalian hosts. We sequenced two strains of *B. henselae* DNA from the same blood sample, which is consistent with previous findings that mixed populations of *B. henselae* can be identified in one clinical *Bartonella* isolate. This finding supports the hypothesis that this is a mechanism evolved by *B. henselae* to evade the host immune response. Interestingly, *B. vinsonii* subsp. *berkhoffii* genotype I was isolated from the owner by BAPGM enrichment culture and sequenced from the cerebrospinal fluid of Dog 3. There are two possible explanations for this finding: either both the dog and its owner were infected by the same environmental source or there was transmission of this *Bartonella* sp. from the dog to the owner, as there has been evidence to support *Bartonella* transmission from dogs to humans.

Collectively, the three studies we performed demonstrate the ecological diversity of *Bartonella* spp. in reservoir and accidental hosts as well as clinical manifestations associated with *Bartonella* infection in these hosts. Further studies are warranted to investigate the ecology of *Bartonella* spp. and how the diversity of the *Bartonella* genus may represent risk factors for infection in both humans and animals.